Biomarkers of the Tumor Microenvironment

Basic Studies and Practical Applications

Lars A. Akslen
Randolph S. Watnick
Editors

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The editors would like to dedicate this book to the memory of the late Judah Folkman, who, through his visionary research and mentoring, inspired the concept that the tumor microenvironment is a critical component of tumor biology.

The editors would also like to express their deepest appreciation to their families for continuous patience and support. Dr. Akslen, specifically, thanks his wife, Åse, daughter, Heidi, and son, Andreas. Dr. Watnick thanks his wife, Jing, daughter, Audrey, and son, Eytan.

Foreword

Professor Lars A. Akslen and Professor Randolph S. Watnick, two well-known experts in the field of the tumor microenvironment, have commissioned multiple authors to assemble a very comprehensive, multifaceted view of the structure and function of the different tumor components. As elegantly outlined in the prologue, Prof. Robert Weinberg reminds all of us that a tumor is not just made up of somewhat disorganized clusters of cancer cells but that these cells are intimately mixed with multiple types of stromal cells of distinct lineages. One now must consider a tumor as a complex organ in which all cells interact through either direct contact or, more often, through their extracellular matrix and a wealth of growth factors and cytokines. A "community effect" can be established in a tumor to promote its growth and its refractoriness to conventional and targeted therapeutics. This community effect was originally discovered by Sir John Gurdon in the early 1990s to explain how mesodermal cells in *Xenopus* embryos, with several differentiation potentials, will adopt a unique fate in one particular region. These cells through reciprocal interactions will reach an equilibrium leading to one particular differentiation program, such as muscle or cartilage. However, in tumors, the complexity of the microenvironment will lead to a more plastic, somewhat stochastic, evolution in stromal and cancer cells. Cancer pathologists have been able to categorize a number of these features for many years. Today, molecular pathologists could revisit these complex organizations to include the epithelial-to-mesenchymal transition spectrum of these cancer cells—rather than grading—and incorporate the differentiation status of the distinct stromal cell components based on bona fide biomarkers. More than a thousand new clinical trials have been launched following the recent favorable clinical outcomes in a limited number of patients treated with targeted therapeutics and in quite larger number of patients treated by targeting immune checkpoints. These clinical trials are aimed at exploring the potential of combinations of chemotherapeutic agents, small-molecule inhibitors, and antibodies, in multiple modalities. Nonetheless, it is clear that we still do not have biomarkers that can predict responses to antibodies directed against immune checkpoints; indeed, the prevalence of immune infiltrate (hot versus cold tumors) is not yet sufficient to assess whether a tumor is likely to regress through activated cytotoxic T-cells. Even the distribution

of drugs and antibodies in tumors remains a real issue, due to the biomechanics of the microenvironment. Soft matter physics could help to unravel how to defeat anisotropy, stiffness, and high pressure in the tumor microenvironment, three important aspects that prevent the uniform diffusion of drugs and hypoxia. This book provides a strong background for such an enterprise, with the ultimate goal of guiding therapeutic intervention. Milestones for this important mission can be easily listed based on the excellent reviews included in this book.

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Preface

The theory that tumors require a permissive environment or "tissue predisposition" to grow, both in the primary site and following dissemination to distant organs, was first recognized by Ernst Fuchs and then formally postulated by Stephen Paget in 1889 as the "seed and soil" hypothesis. This seminal concept on tumor progression was based on the analogy that tumor cells were *seeds* and needed the proper *soil* to grow. However, the study of cancer for the next hundred years focused primarily on characterizing the morphologic and molecular aberrations unique to tumor cells. This field of study yielded remarkable insights into intrinsic tumor cell biology with dozens of oncogenes and tumor suppressors discovered. In contrast, very few papers dealt with the "tumor microenvironment" until the 1990s.

That being said, the understanding of tumors has transitioned from being groups of tumor cells that grow in an uncontrolled fashion to miniature tissues or complex "organs" with a vasculature and stroma consisting of both resident and bone marrow-derived cells. This conceptual shift has been extended by the fact that tumor cells interact with their microenvironment and, in so doing, affect and are affected by the reciprocal intercellular signaling. These signaling events were recognized as critical by Judah Folkman, who postulated in the late 1960s that tumor growth and spread were linked to the ability to induce angiogenesis. In so doing, he helped foster the notion that the tumor microenvironment was not a passive bystander but an active collaborator in tumor progression. Today, many pathologists use microvessel density or vascular proliferation as biomarkers for determining the aggressiveness of tumors. Moreover, the first identified angiogenic factor, VEGF, and its growth promoting receptor, VEGFR2, are targets for anticancer therapeutic agents used widely in the treatment of cancer patients.

Subsequently, many different cell types within the tumor microenvironment have been identified and characterized as having growth promoting and inhibiting roles in tumor progression. Among the most prominent cell types that affect tumor growth are immune cells. Harold Dvorak famously described tumors as "wounds that do not heal." This seemingly simple statement has profound implications, not only on how tumors are studied and perceived but also how they are now treated. Advancements in immunotherapy have identified novel therapeutic targets that

blunt the adaptive immune response and prevent the immune system from attacking tumors despite the presentation of mutant antigens. Immune checkpoint inhibitors that block PD-1, PD-L1, and CTLA-4 have been approved for multiple indications and have shown potent and durable responses in a subset of patients. Therapeutic agents targeting CD47, an inflammatory checkpoint protein that signals to macrophages not to phagocytose seemingly damaged cells, are in clinical trials.

It is now recognized that evasion of immune and inflammatory cells is not mediated solely by the expression of checkpoint inhibitors on the surface of tumor cells. Myeloid derived cells, including monocytes and macrophages, are potent suppressors of immune and inflammatory cells, as are cancer-associated fibroblasts (CAFs). Notably, these cells stimulate angiogenesis by upregulating the expression of VEGF, bFGF, IL8, and other pro-angiogenic factors as directed by tumor cell signaling. Such cells might also repress the expression of thrombospondin-1, a potent anti-angiogenic, anti-inflammatory, and immunomodulatory protein. Recent findings have highlighted that angiogenesis and immune evasion are co-regulated, further underscoring the complexity and significance of these interactions between tumor cells and the microenvironment.

This book contains 22 chapters that highlight the multidimensional and complex nature of the tumor microenvironment and its role in tumor progression, biomarker development, and therapeutic targeting. It is divided into two main parts; the first part deals with basic mechanisms and biomarkers of the tumor microenvironment according to its various components, and the second part gives some examples of organ-directed biomarker studies and clinical applications. Following a masterful overview with integrated perspectives on this field by Robert Weinberg, Watnick (Chap. 1) sets the stage and focus on the importance of microenvironmental context in the regulation of tumor angiogenesis. It is argued that paracrine signaling should be considered in the search for tumor progression drivers and novel targets along with companion biomarkers of potential clinical importance. This field should be complementary to the one-sided search for mutations and aberrant signaling in tumor cells. In Chap. 2, Grzelak et al. outline how tumor cells interact with specific endothelium in various organs to prepare for dissemination. Further, they describe the interactions between disseminated tumor cells and the endothelium post extravasation, and how this influences the balance between tumor dormancy and active outgrowth at the secondary site, introducing the niche concept. In Chap. 3, Akslen describes how tissue-based markers of angiogenesis and vascular invasion can be defined and applied in studies of human cancers and how their aggressive behavior can be graded by such markers. In particular, the potential value of microvascular proliferation is discussed in the context of prognostication and response prediction. Novel angiogenesis markers, such as Nestin-Ki67, are mentioned.

In Chap. 4, Zeltz et al. outline the dual functions of the tumor stroma as supportive or inhibitory with respect to cancer progression. Especially, the role of the insoluble extracellular matrix (ECM) is discussed, and how its components influence matrix remodeling, tumor metastasis, and even tumor heterogeneity. In Chap. 5, Östman describes the paracrine interactions between mesenchymal cells and epithelial or endothelial cells, with particular reference to the PDGF family of growth factors and receptors. By combining experimental and clinical studies, the PDGF

signaling systems appear as critical regulators of tumor growth, metastasis, and drug efficacy. In Chap. 6, Chang and Dudley focus on the exciting field of adipocyte-derived factors in the tumor microenvironment. They postulate that the presence of adipocytes and various adipokines as well as associated inflammatory cytokines might significantly influence tumor development and progression, through both paracrine (local) and endocrine (systemic) regulation. In Chap. 7, Sulciner and coworkers elaborate further on the function of lipid signaling and the dual role of inflammation in cancer. The complex interactions between various classes of immune cells and how these appear to be regulated by fatty acid-derived lipid mediators such as prostaglandin E2 are discussed.

In Chap. 8, Chellappa et al. describe the repertoire of innate and adaptive immune cells and their dual role with respect to tumor development and progression. In addition, the authors discuss mechanisms of immune evasion and how the potency of CTL function in malignant tumors is generally compromised. In Chap. 9, Brekken and Wnuk-Lipinska discuss the regulation and relationship of epithelial plasticity (EMT programs) and immune escape mechanisms. The authors focus on molecules that can drive the immunosuppressive state in the tumor microenvironment and potentially serve as biomarkers for poor prognosis. In Chap. 10, Corthay and Haraldsen comment on a range of inflammatory biomarkers in cancer such as cytokines and interleukins converging on STAT3 signaling. In particular, the authors discuss in more detail the biology of IL-33, the most recently identified member of the IL-1-family. In Chap. 11, Van Den Eynden et al. describe some tissue-based biomarkers of the immune response in solid tumors, such as tumor infiltrating lymphocytes (TILs) and tertiary lymphoid structures (TLSs), and how these can be recorded in human tumor tissues.

In Chap. 12, Johnson and coworkers outline the role of blood platelets in the tumor microenvironment and how tumors can co-opt the normal functions of platelets in order to advance tumor progression and metastasis. In particular, the authors focus on how platelets drive multiple aspects of tumor growth and progression. In Chap. 13, El Rayes et al. discuss how tumor progression and metastasis are influenced by the bone marrow. Thus, bone marrow-derived cells constitute a significant fraction of the primary tumor microenvironment and processes such as angiogenesis as well as metastasis and growth in distant sites. In Chap. 14, Li and Bielenberg comment on the role of Neuropilin 1 and Neuropilin 2 in cancer progression. These cell surface receptors are known to drive diverse processes including neuronal guidance, vasculogenesis, lymphangiogenesis, immunity, smooth muscle tone, epithelial cell migration and branching, and epithelial-to-mesenchymal transition. In Chap. 15, Davidsen and coworkers discuss the role of the Axl receptor tyrosine kinase in tumor cell plasticity and tumor progress. In particular, the authors focus on the relationship between EMT programs, immune evasive phenotypes, and drug resistance, and how this suggests a potential for anti-Axl combination therapy in a range of aggressive cancers.

In the second part of the book, chapters focus more on organ-related studies and clinical translation. In Chap. 16, Wik and Akslen comment on gene expression signatures of the tumor microenvironment in breast cancer. The authors argue that composite signatures or profiles as biomarkers and clinical tools might capture and reflect some of the complexity in human tumors that are not immediately available

by using individual markers related to tumor cells or even the microenvironment. In Chap. 17, Kim and coworkers discuss the use of noninvasive magnetic resonance imaging (MRI) and spectroscopy (MRS) as dynamic biomarkers for tumor characterization and monitoring during treatment and follow-up. Importantly, contrast-enhanced MRI methods are used to evaluate tumor vascularization and vascular function by measuring the kinetics and distribution of intravenously administered contrast agents. In Chap. 18, Lin and LaBarge argue for a dominant role of the microenvironment in tumor progression. The authors explore the influence of tissue architecture on drug response, by focusing on applications and analytic approaches used for functional cell-based exploration of combinatorial microenvironments using microarray technology. In Chap. 19, Azeem and coworkers discuss the establishment of novel prostate cancer models, their applications, and their critical role in understanding disease progression and therapeutic strategies.

In Chap. 20, Leiss et al. comment on the specific features of tumor-host interactions in malignant gliomas. These regulations are shaped by the structural organization of the CNS and involve multiple cell types, extracellular matrix components, and host cell-derived soluble factors that are unique to the CNS. In Chap. 21, Straume and Schuster discuss the importance of the tumor microenvironment in progress of cutaneous melanoma to the metastatic stage. The dual nature of the melanoma microenvironment, both stimulatory and inhibitory, as well as the immune regulation are major focuses. In Chap. 22, Jebsen and coworkers outline the three most active pathways to therapy development: mutation-driven drug development, immunomodulatory therapy, and evolution of conventional chemo- and radiotherapy. They argue that all of these therapeutic modalities require more precise biomarkers, not only to increase precision and enhance efficiency but also to avoid unnecessary toxicity for the patient, and costs for the society.

It would be impossible to provide an exhaustive and thorough analysis of this rapidly expanding field in a single volume. Still, we hope that readers find this book useful. Studies on the complex and fascinating regulation of the tumor microenvironments and niches at primary and distant sites are increasing, and they present many exciting possibilities for novel treatment targets and companion biomarkers. However, there are many challenges in contemporary medical oncology and precision medicine, and this calls for more knowledge in basic sciences and innovative approaches in translational work with special focus on trial design and follow-up of the patients.

Finally, we thank Springer Publishing for allowing us the opportunity to work together on this project. As longtime collaborators and friends who share a common passion for unlocking the mysteries and therapeutic potential of the tumor microenvironment it has been truly awe inspiring to curate the remarkable advances made in this field. As is the case with any undertaking of this size and complexity, we, the editors, could not have successfully completed it without the help of several important contributors. We would like to thank the staff at Springer Publishing, as well as our own staff, for their assistance and valuable advice.

Bergen, Norway — Lars A. Akslen
Boston, MA, USA — Randolph S. Watnick

The Tumor Microenvironment: A Prologue

Until about two decades ago, a powerful reductionist paradigm held sway of cancer research: those interested in studying the mechanisms of cancer pathogenesis embraced the notion that the biology of tumors could be understood by analyzing the biology of the constituent cancer cells. Moreover, the biology and pathophysiology of individual cancer cells could be understood, in turn, by studying their genomes, more specifically cancer-associated somatically mutated genomes. This was, to be sure, a powerful paradigm, in that it enabled two decades of discovery of the genetic determinants of cancer pathogenesis, including oncogenes, tumor suppressor genes, and yet others involved in DNA repair and apoptosis.

As useful as this paradigm was, it overlooked an important aspect of cancer pathogenesis: tumors are histologically complex structures composed of multiple distinct cell types, a reality recognized by pathologists for more than a century. Accordingly, beginning in the 1990s, it became increasingly clear that tumors were functionally far more complex than aggregates of cancer cells. Thus, as tumors develop, it became obvious that neoplastic cells rely on recruited host cells for various types of cell-physiologic support. The latter cells had been termed stroma by the pathologists. More detailed characterization of the stroma revealed that it consists, at least in the case of common carcinomas, of a diverse collection of cells, virtually all of which are of mesenchymal origin. Included in the stroma are cells that often form its bulk, including fibroblasts and myofibroblasts, the latter often termed carcinoma-associated fibroblasts (CAFs). Interwoven among these stromal connective tissue cells are a variety of cells of hematopoietic origin, including endothelial cells, various subsets of lymphocytes, macrophages, and occasional granulocytes.

As carcinomas develop and progress to higher grades of malignancy, the stroma usually changes in lockstep, becoming increasingly "reactive" and thus assuming a biological state that exists only transiently in the wound sites within normal tissues that are in the midst of healing. Indeed, such reactive stroma increasingly resembles "wounds that do not heal." The coordinated changes of neoplastic cells together with adjacent stroma provided, on its own, a clear indication that the two groups of cells intercommunicate, doing so via processes that are often termed heterotypic signaling, i.e., communication between distinct types of cells.

In principle, this signaling might be unidirectional, in that, as an example, the neoplastic cells within a carcinoma might release signals that recruited a diverse array of stromal cells to the growing tumor and thereafter orchestrated their behavior. In truth, however, the heterotypic signaling is bidirectional, in that recruited stromal cells release signals that impinge reciprocally on the carcinoma cells that previously recruited them. Hence, the co-evolution of neoplastic cells and the co-opted host cells is enabled by bidirectional signaling. Importantly, while the neoplastic cells undergo both genetic and epigenetic evolution, the evidence to date indicates that the recruited stromal cells – which together form the "tumor microenvironment" – undergo phenotypic changes that are not driven by somatic mutations.

In fact, the histopathological appearance of islands of tumor cells is often strongly influenced by the signals that these cells receive from the tumor-associated stroma. Most prominent among the phenotypic changes experienced by carcinoma cells is the activation of a latent cell-biological program termed the epithelial-mesenchymal transition (EMT), which is normally operative during early embryogenesis, where it programs the interconversions of cell types that are destined to form distinct tissues and organs; in addition, the EMT program also operates transiently during wound healing. In the case of carcinomas, the EMT-inducing signals received by carcinoma cells from their microenvironment drive the acquisition of a complex spectrum of cell-biological changes involving the shedding of preexisting epithelial traits (inherited from normal cells-of-origin) and the acquisition of mesenchymal traits, such as motility, invasiveness, an elevated resistance to various types of therapeutic intervention, and an ability to disseminate to anatomical sites distant from the primary tumor. The resulting secondary tumor colonies – metastases – are responsible for 90% of cancer-associated mortality.

The grim reality is that the heterotypic interactions between neoplastic cells and their stromal microenvironment are extremely complex. Each of the participating cell types release a complex mixture of heterotypic signals that impinge upon and influence multiple other cell types. This multi-body problem dwarfs in its complexity the 3-body problem that has thwarted the attempts by physicists to describe such interactions mathematically. As a consequence, we come to realize that the study of tumor microenvironments, which is already a highly active area of research, is still in its infancy, given the complexity of the cell-cell signaling networks that operate within the tumor-associated stroma and between this stroma and the nearby cancer cells. The present volume lays out some of the more salient of these interactions. As complex as they are, they still represent a beginning, since the complexity of the signaling networks vastly outstrips at present our ability to understand them in their entirety, i.e., to understand how multiple heterotypic interactions conspire to create the complex biology of high-grade malignancies. Still, what is presented in this volume represents an interesting and exciting beginning!

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Part I
Basic Mechanisms and Biomarkers

Chapter 1
The Role of the Tumor Microenvironment in Regulating Angiogenesis

Randolph S. Watnick

Abstract The tumor microenvironment plays a crucial role in cancer development and progression. Paracrine signaling between tumor cells and the nonneoplastic, genetically normal, cells that make up the microenvironment is a critical component influencing the progression of tumors from the in situ stage to metastatic disease. Despite the importance of these paracrine signaling mechanisms and factors, the vast majority of academic research and development in the pharmaceutical industry is still targeted toward mutations and aberrant signaling pathways within tumor cells. As a result, the intercellular signaling between tumor cells and the microenvironment has not been as extensively studied with regard to the regulation of angiogenesis. In this chapter we define the key players in the regulation of angiogenesis and examine how their expression is regulated in the microenvironment. The resulting analysis presents observations that at first glance may seem paradoxical. However, these nuances serve to underscore the complexity of interactions and the need to better delineate and define the environmental context underlying these mechanisms.

Keywords Microenvironment • Angiogenesis • Cancer

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Introduction

In the earliest stages of cancer, epithelial tumors (carcinomas) are physically confined within the region of the tissue from where they arise. These early lesions (carcinomas in situ) are separated from the tissue parenchyma by the basement membrane [1]. Opposite the basement membrane are a myriad of cells consisting of fibroblasts, myofibroblasts, immune/inflammatory cells, and endothelial cells [2]. In addition to these cell types are the extracellular matrix proteins which they secrete and to which they, and tumor cells, attach [2].

In order for tumors to progress to a clinically relevant and potentially lethal disease, they must acquire the capacity to escape the environment of origin (the epithelial compartment), invade the local parenchyma, and disseminate systemically. To enable this process, tumor cells must degrade the basement membrane that separates the epithelial compartment from the parenchyma. Invasion of the tissue parenchyma by the tumor, or conversely invasion of stromal cells into the tumor, initiates a phase of tumor progression in which tumor growth becomes dependent on non-cell autonomous processes regulated by paracrine and juxtacrine signaling interactions between the tumor and its microenvironment (Fig. 1.1) [3–5].

In other words, in order to expand in size beyond the diffusion limit of oxygen in tissue, a new vasculature must form in and around the tumor. The ingrowth of this

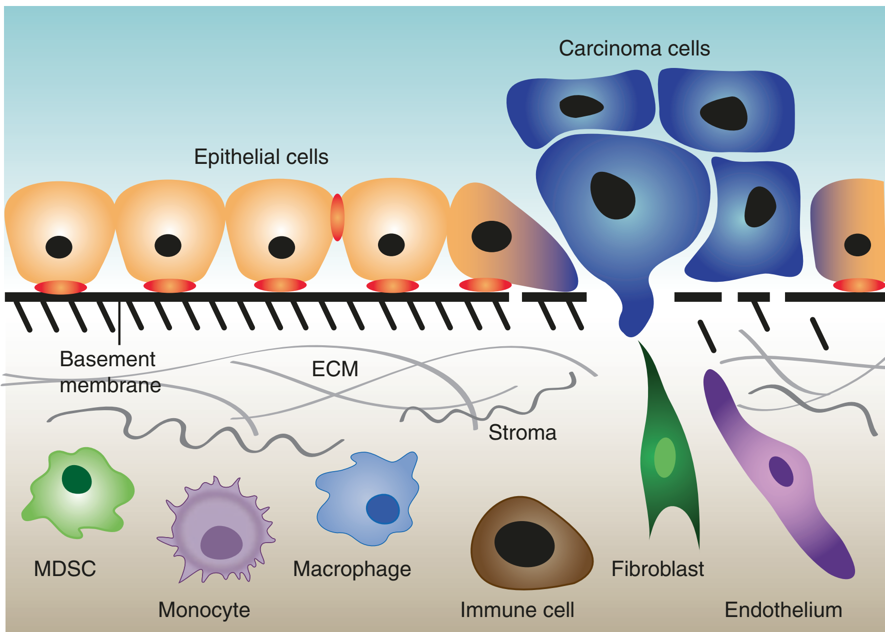

Fig. 1.1 Schematic diagram of tissue architecture with regard to the spatial distribution of cellular and extracellular components

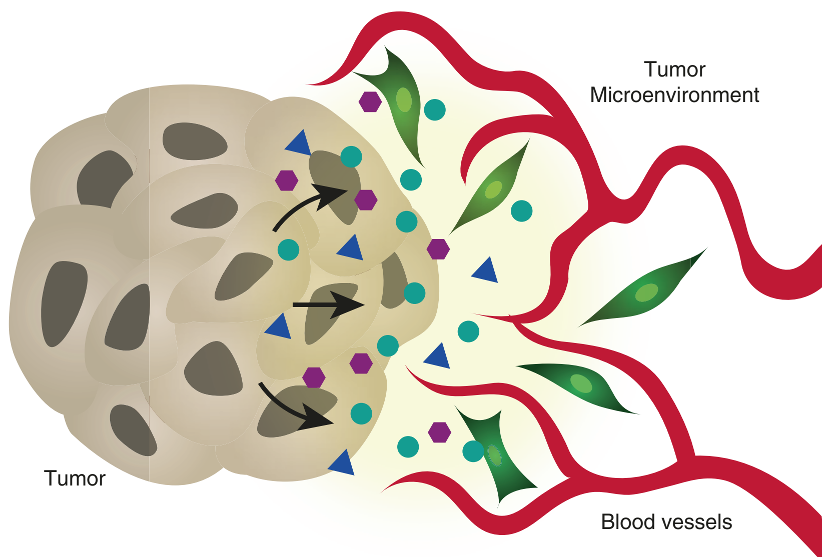

Fig. 1.2 Schematic diagram of the interaction between the tumor and microenvironment as mediated by tumor-secreted factors paracrine-acting factors that modulate angiogenesis

vasculature is fueled by soluble and matrix-bound growth factors and enzymes produced and secreted by both the tumor cells and the stromal cells comprising the microenvironment (Fig. 1.2) [6]. Strong evidence exists indicating that the stromal cells play as central a role in matrix remodeling, invasion, and metastasis as the tumor cells themselves [7–9]. Critical to this process is the observation that carcinoma cells are manipulated and co-opt the surrounding stromal cells to enhance tumor growth [10]. Specifically, tumor-stromal paracrine signaling pathways have been demonstrated to play a major role in the tumorigenesis and subsequent outgrowth of tumors in multiple sites [11–13]. For example, stromal fibroblasts from prostate tumors are able to stimulate tumor formation of immortal but non-transformed prostate epithelial cells when the mixture is injected orthotopically into nude mice [10].

As stated above, tumor angiogenesis is intricately linked to signaling between the tumor and microenvironment. In normal tissue architecture, the epithelial compartment is not vascularized as it is generally only a few cell layers in thickness, thus allowing oxygen to diffuse across the basement membrane and nourish both layers. However, when squamous tumors grow in an uncontrolled manner or glandular tumors form and the lumen of epithelial-lined ducts fills with tumor cells, the cells in the center of the mass eventually become hypoxic due to the distance from existing blood vessels [14]. Thus, in order for carcinoma cells to gain access to blood vessels, the basement membrane must be degraded, allowing the blood vessels to grow into the epithelial compartment. In order for this to happen, tumor cells must

secrete pro-angiogenic growth factors, turn off or reduce production of anti-angiogenic factors, and recapitulate this process in the microenvironment by secreting paracrine signals that act on the stromal cells to induce a pro-angiogenic environment. Though a myriad of pro- and anti-angiogenic factors have been discovered and studied, the initial effort in understanding their regulation was examined in a cell autonomous fashion, with most of the attention paid to vascular endothelial growth factor (VEGF) [15–19] and thrombospondin-1 (Tsp-1) [20, 21], two of the major positive and negative regulators of angiogenesis. However, as the importance of the tumor microenvironment became more apparent, the study of the regulation of angiogenic factors in stromal cells also increased.

Not only is the regulation of angiogenesis in the tumor microenvironment critical to primary tumor growth but also for metastatic dissemination and growth in distant organs. It is well established that tumors arising in different sites preferentially metastasize to specific organs [22]. For example, prostate cancer metastasizes preferentially to the bone and liver, while breast cancer metastasizes to the brain, bone, and lung [23]. The ability of a tumor cell to survive and proliferate in a metastatic environment ultimately relies on its ability to augment the angiogenic output of its microenvironment.

The tumor microenvironment can grossly be categorized into two types of cells: (1) resident tissue cells that are present prior to tumor development and (2) infiltrating cells that are recruited to the tumor from the circulation or bone marrow. The former is mainly comprised of fibroblasts and endothelial cells, while the latter is comprised of immune/inflammatory cells, which include B and T cells, neutrophils, mast cells, dendritic cells, and macrophages. In this chapter we will explore the roles of these different cell types, as well as the growth factors and extracellular matrix proteins that contribute to tumor progression.

Cell Signaling Mechanisms and Factors Influencing Stromal Angiogenesis

In order to understand how resident and infiltrating cells contribute to tumor angiogenesis, it is first necessary to delineate and describe the major angiogenic factors that stimulate and inhibit vessel ingrowth.

VEGF

Vascular endothelial growth factor (VEGF) stimulates endothelial cell migration and proliferation. It was first identified via its ability to increase vascular permeability and thus was initially called vascular permeability factor or VPF [24, 25]. The regulation of VEGF in tumor cells has been exhaustively studied. Signal transduction pathways leading from receptor tyrosine kinases or oncogenic Ras and PI3 kinase

via the MAPK or Akt pathway lead to increased transcription of VEGF and its subsequent secretion into the extracellular matrix [17]. However, carcinoma cells also secrete proteins into the extracellular space, which do not act directly on endothelial cells but rather modulate VEGF production and secretion by stromal cells in the microenvironment, such as TGF-β, PDGF, and bFGF [26, 27].

Stromal VEGF expression was first demonstrated to be regulated by carcinoma cells in a transgenic mouse model in which GFP, driven by the VEGF promoter, was inserted into the mouse genome [28]. In this model, activation of the VEGF promoter results in the expression of GFP. Examination of tumor xenografts in these VEGF-GFP mice revealed, via fluorescence microscopy, that stromal fibroblasts had infiltrated the tumor fluoresced green, indicating that the VEGF promoter had been activated. Strikingly, in normal tissues there were no fluorescent cells, indicating that VEGF expression is not required for normal tissue homeostasis. These results indicated that tumors secrete factors that act on cells in the microenvironment to stimulate VEGF expression.

However, while it was clear from these experiments that VEGF expression was being stimulated, it was not evident whether this stimulation was required for tumor growth, supportive of tumor growth, or merely a physiological reaction to local tumor growth. The evidence that stromally produced VEGF was critical for tumor growth was obtained from studies designed to test the efficacy of a human specific anti-VEGF antibody, bevacizumab (Avastin). In these experiments, human tumor cells were injected into immunocompromised mice, which were subsequently treated with the human-specific VEGF antibody [29]. While the antibody was able to dramatically inhibit tumor growth, the tumors still grew. The authors of the study hypothesized that the continued growth of the treated tumors was due to a residual angiogenic stimulus driven by VEGF produced and secreted from the murine tumor microenvironment, which could not be inhibited by the human-specific antibody. To test this hypothesis, human tumor xenografts were treated with human-specific VEGF antibodies as well as a soluble version of the murine VEGFR1 (mFlt) fused to IgG, which acts as a decoy receptor for VEGF [30]. This combination treatment resulted in the complete blockage of tumor growth, demonstrating the importance of the contribution stromal-produced VEGF.

Basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor (bFGF, FGF2) is another potent pro-angiogenic growth factor [31–33]. One interesting oddity about bFGF is despite the presence of high-affinity cell surface receptors [34] and the myriad of observations that bFGF stimulates endothelial cell proliferation and angiogenesis in vivo and in vitro, the protein lacks a signal sequence to direct its secretion [35, 36]. The paracrine regulation of bFGF in stromal cells and subsequent effect on tumor angiogenesis has been confounded by its ability to potently stimulate tumor cell proliferation through FGFR signaling via both autocrine and paracrine signaling [37–39]. Nevertheless,

bFGF expression in the stroma of lung adenocarcinoma patients inversely correlates with disease progression and overall survival [40]. Additionally, bFGF production is stimulated by stem cell factor (SCF) and TGF-β in inflammatory cells, including macrophages, mast cells, and neutrophils [41]. The role of these cells in tumor angiogenesis will be detailed later in this chapter.

TGF-β

One growth factor with perhaps the most paradoxical role in tumor growth and angiogenesis is TGF-β. While TGF-β has potent pro-angiogenic activity in vivo [42], its in vitro effects on endothelial cells are in diametric opposition as it is actually growth inhibitory in this context [43, 44]. These seemingly incongruous activities were resolved by the discovery that TGF-β stimulates the expression of VEGF in stromal fibroblasts, indicating that the pro-angiogenic effects of TGF-β were mediated by the induction of VEGF in the tumor microenvironment [26, 45]. Additionally, TFG-β also dramatically stimulates the expression of bFGF in fibroblasts [46]. These results suggest that low levels of tumor-secreted TGF-β stimulate tumor-associated fibroblasts to express VEGF and bFGF thereby stimulating angiogenesis. Conversely, higher levels of TGF-β may act directly on endothelial cells inhibiting their proliferation and thus having an anti-angiogenic effect.

Adding to the paradox of TGF-β's role in tumor angiogenesis is its ability to stimulate the expression of the anti-angiogenic protein Tsp-1 (which will be discussed in detail later in the chapter) and which then activates TGF-β from its latent form [47–51]. TGF-β is activated by two discrete processes: via proteases that cleave the latency-associated peptide and via undergoing a conformational change that exposes the receptor-binding region. Tsp-1 activates TGF-β via the latter mechanism. Moreover, TGF-β expression in fibroblasts is induced by hypoxia, which is most often a result of a lack of tumor vascularization [52].

PDGF

Another growth factor that possesses both pro- or anti-angiogenic characteristics is PDGF. In 1991, Goldsmith et al. demonstrated that PDGF was able to potently stimulate bFGF in lung fibroblasts [46]. Additionally, in response to the results achieved with the human-specific VEGF antibody described above, it was demonstrated that stromal VEGF expression was stimulated by tumor-derived PDGF [53]. In that context, inhibition of PDGF activity via a soluble version of PDGFR was able to block the stimulation of VEGF in the microenvironment and inhibit angiogenesis. Moreover, another member of the PDGF family, PDGF B, is also able to upregulate VEGF expression in vascular smooth muscle cells [26]. These data indicated that tumor-derived PDGF is a potent inducer of VEGF expression in the microenvironment.

The most logical conclusion to be drawn from the above study is that PDGF promotes angiogenesis via induction of stromal VEGF. However, somewhat analogous to TGF-β, the activities of PDGF are not as straightforward as these results would indicate. In addition to stimulating VEGF and bFGF, PDGF also stimulates Tsp-1 expression [54]. PDGF stimulation of Tsp-1 in fibroblasts is mediated by the Raf-MAPK pathway in a manner analogous to the stimulation of Tsp-1 by serum [55]. Intriguingly, the PDGF-mediated stimulation of VEGF is also mediated by the Raf-MAPK pathway [56]. Thus, whether PDGF acts as an anti-angiogenic factor or a pro-angiogenic factor is most likely dependent on orthogonal signals that act to inhibit or stimulate VEGF or Tsp-1.

Hormones and Nuclear Receptors

The studies described above indicate that two of the most potent inducers of stromal VEGF and, consequently, angiogenesis also possess the seemingly counterproductive ability to stimulate Tsp-1. These divergent events downstream from TGF-β and PDGF ligation to their receptors indicate that tumor-derived TGF-β and PDGF expression should have no net effect on angiogenesis. That being said, it has also been demonstrated that inhibition of PDGF activity inhibits tumor angiogenesis [53]. Also, as described above, despite its ability to stimulate Tsp-1, TGF-β is a potent stimulator of angiogenesis. One potential explanation for the observed pro-angiogenic activities of these two proteins is that the expression of Tsp-1 in the microenvironment is suppressed by an independent signaling mechanism. This suppression of Tsp-1 would result in the stimulation of only the pro-angiogenic factors VEGF and bFGF by these two growth factors and thus resolve the seemingly paradoxical observations.

Two candidates for such a Tsp-1-repressing factor are the hormones estrogen and androgen, which have both been demonstrated to repress Tsp-1 expression [57, 58]. While these hormones both repress Tsp-1 expression, the mechanisms utilized are different. Estrogen inhibition of Tsp-1 is mediated by activation of ERK1/2 and JNK [57]. Additionally, Tsp-1 repression by estrogen is mediated via inhibition of both transcription and protein secretion. Conversely, androgen-mediated repression of Tsp-1 is solely mediated by inhibition of transcription, through an androgen-responsive element in the Tsp-1 promoter [58].

While hormone-mediated effects on tumor growth have been largely studied through their actions on hormone-responsive tumor cells, it has also been demonstrated that estrogen can induce angiogenesis on a systemic level [59]. This study revealed that estrogen receptor (ER)-positive stromal cells stimulate angiogenesis and promote tumor growth in response to estrogen even when the tumor cells were ER negative.

It has also been demonstrated that the peroxisome proliferator-activated receptors (PPAR), another nuclear receptor family, can regulate both VEGF and Tsp-1 expression. Specifically, it has been demonstrated that tumor cells injected into PPAR$\alpha^{-/-}$ mice remained dormant for a prolonged period of time [60]. Moreover, the dormancy of these tumors was due to increased Tsp-1 expression in the host

stroma. Surprisingly, it was later determined that fenofibrate and WY14643, two agonists of PPARα, also stimulated the expression of Tsp-1 [61]. These seemingly discordant results suggest that in the absence of PPARα, another member or the PPAR family, perhaps, may compensate and stimulate the expression of Tsp-1. Of note, PPARγ also stimulates the expression of CD36 [62], a receptor for Tsp-1. In keeping with these observations, it was demonstrated that the PPARγ agonists rosiglitazone and pioglitazone inhibit bFGF and VEGF-mediated angiogenesis [63].

Matrix Metalloproteases

The ability of tumors to invade locally, across the basement membrane, is critical for tumor growth and ultimately metastasis. One critical step in tumor invasion and migration is the remodeling of the extracellular matrix by extracellular proteases. Some of the major players in this field are the matrix metalloproteases or MMPs. For example, an experiment in which MCF7 breast cancer cells and fibroblasts were co-injected into mice resulted in the significant acceleration of tumor growth [64]. Moreover, in a parallel experiment in which the fibroblasts ectopically expressed TIMP-2 (tissue inhibitor of metalloprotease 2), an inhibitor of MMP activity, the tumor-stimulating activity was abrogated [65]. Analogously, administration of a broad-spectrum MMP inhibitor, batimastat, also abrogated the ability of fibroblasts to stimulate tumor formation by MCF7 cells [65].

The matrix remodeling carried out by MMPs not only facilitates tumor cell migration into the surrounding microenvironment but also stimulates the migration of endothelial cells into the tumor by facilitating the formation of the leading edge of new blood vessels. MMPs also liberate growth factors, such as VEGF and bFGF that are otherwise sequestered in the ECM. The ability of MMPs to stimulate angiogenesis was established in an elegant genetic experiment in which tumor-prone RIP-TAG2 mice were crossed with various matrix protease knockout mice [66]. By crossing the RIP-TAG mice with MMP2 knockout mice, the authors demonstrated that tumor growth was impaired but not due to any defect in angiogenesis [66]. Conversely, MMP9$^{-/-}$ RIP-TAG mice displayed inhibited tumor growth and defective angiogenesis [66]. In addition to cleaving matrix proteins, MMP9 also cleaves the latency-associated peptide from TGF-β, converting it to the active form and thereby stimulating tumor growth in a mammary tumor model [67].

Thrombospondin-1

While much of the attention in the field of angiogenesis has been paid to the identification and characterization of pro-angiogenic factors, the studies detailing the role of one of the most potent anti-angiogenic proteins, Tsp-1, should not be overlooked. Thrombospondin-1 (Tsp-1) is an endogenous anti-angiogenic protein that functions

via a multimodal approach: it binds to cell surface receptors CD36 and CD47 on the endothelial cell surface and renders the cell insensitive to both VEGF and bFGF, as well as inducing caspase-dependent apoptosis mediated by downstream signaling from CD36 [68–71]. Tsp-1 also binds to MMP9 and functionally inactivates it [66, 72]. In tumor cells, Tsp-1 expression is repressed via a signal transduction cascade emanating from PI3 kinase via Rho GTPase to ROCK to Myc, which represses Tsp-1 in a phosphorylation-dependent manner [73]. This pathway has been shown to be active in several human breast cancer cell lines in which Tsp-1 expression was virtually silenced [73]. Furthermore, in a majority of the surveyed breast cancer cell lines, the pathway previously described [74] was demonstrated to be responsible for the silencing. Thus, this pathway represents the first biochemical elucidation of a cell-autonomous "angiogenic switch."

While the expression of VEGF in the tumor-associated stroma is widely accepted to have a positive correlation with tumor progression [28, 75, 76], the role of thrombospondin-1 (Tsp-1) expression in the tumor-associated stroma is unclear. Tsp-1 expression by epithelial tumor cells is observed infrequently, and ectopic expression of Tsp-1 is inhibitory to tumor growth [20, 73, 77]. Stromal Tsp-1, meanwhile, has been correlated with a desmoplastic response and increased invasiveness in a subset of breast cancers [75, 78, 79], while it has been demonstrated to be inhibitory to early-stage breast cancers [80]. Expression of Tsp-1 by stromal fibroblasts has been shown to inhibit tumor formation and growth [81]. Intriguingly, the same report demonstrated that tumors that arose in an environment high in Tsp-1 eventually overcame the inhibitory effects of this protein by increasing their production of VEGF. Thus, the complex interrelationship between these two proteins and their relative expression levels in the tumor-associated stroma can play a key role in the induction and maintenance of the angiogenic phenotype in human tumors.

The work described above demonstrated that VEGF expression in the stroma is a critical component in tumor-mediated angiogenesis. Conversely, Tsp-1 expression in the tumor-associated stroma can be a potent inhibitor of tumor angiogenesis and growth. The question that arises then is how do tumors stimulate the expression of VEGF in the stroma while concomitantly repressing the expression of Tsp-1?

Nonprotein Mediators of Angiogenesis

The vast majority of studies of angiogenesis focus on the roles of cytokines and growth factors. However, one largely understudied signaling mechanism is lipid and phospholipid signaling. Significantly, two landmark studies demonstrated that these molecules could regulate the expression of Tsp-1. Two independent studies showed that generation of phospholipids and the resultant signaling pathways potently repress Tsp-1 expression in stromal fibroblasts. The first demonstrated that platelet-mediated generation of phospholipids, specifically sphingosine-1-phosphate (S1P), downregulated Tsp-1 expression in dermal fibroblasts by activating the G_i-protein-coupled S1P receptors [82].

The second study also implicated S1P as a repressor of Tsp-1 by demonstrating that secretion of a low molecular weight molecule (<3kD) was upregulated in Ras-transformed cells and that it repressed Tsp-1in dermal fibroblasts in an S1P-dependent manner [83]. These two reports indicate that tumor cells augment the angiogenic output of their microenvironment by secreting factors that repress Tsp-1 in the surrounding stromal cells.

Carcinoma-Associated Fibroblasts

Tumor progression is intricately regulated by the interactions with fibroblasts present in the tumor microenvironment [84]. Fibroblasts present in the tumor microenvironment are referred to as carcinoma-associated fibroblasts (CAFs) [10]. Based on genetic analysis, CAFs are very similar, virtually identical, to activated fibroblasts found in the stroma of damaged or wounded tissue [85–88]. Specifically, both express smooth muscle actin, EGF [89, 90], HGF [91–95], IGF-I, and IGF-2 [96–98] as well as matrix metalloproteases (MMPs) [65, 99–108].

Strikingly, underscoring the importance of their contribution to tumor progression, some carcinomas are comprised of up to 90% fibroblasts [109]. While the paracrine signaling mechanisms that convert normal fibroblasts to CAFs have not been completely delineated, in vitro studies have demonstrated that TGF-β can induce CAF-like properties in normal fibroblasts [110]. Moreover, human carcinoma cells have been observed to convert normal fibroblasts into CAFs in a mouse xenograft model [111]. The CAF phenotype is also very stable as they can be cultured in the absence of carcinoma cells in culture until they undergo senescence [112].

When carcinomas progress to the invasive state, the basement membrane is degraded, and stromal cells, including CAFs, inflammatory response cells, and newly formed capillaries, come into contact with the tumor cells [113]. CAFs in the stroma of invasive carcinoma continue depositing large amounts of ECM, including tenascin C in some cases [114, 115]. It has been shown that in breast and bladder carcinomas, expression of tenascin C correlates with increased tumor invasiveness [116, 117]. The accumulation of ECM in tumors contributes to increased interstitial fluid pressure that hinders oxygen and nutrient diffusion [118, 119]. Thus, CAF-mediated hypoxia could lead to the expression of HIF-1α and the induction of VEGF, thus providing a mechanism by which CAFs can promote angiogenesis in tumors.

An elegant study utilizing both in vivo and in vitro models demonstrated that injecting human breast cancer cells mixed with CAFs into the mouse mammary gland resulted in tumors that grew faster and were more angiogenic than when tumor cells were mixed with normal fibroblasts [112]. The increase in tumor growth was mediated by the production and secretion of stromal cell-derived factor 1 (SDF1) by CAFs which, in turn, bound to and activated its cognate receptor, CXCR4, on the surface of tumor cells. Moreover, the CAF-secreted SDF1 also stimulated angiogenesis by recruiting endothelial progenitor cells (EPCs) to the tumor.

Another study demonstrating the tumor-promoting activity of CAFs utilized a genetically engineered mouse model in which the Mts1 gene, which stimulates tumor metastasis, was knocked out. When otherwise metastatic breast cancer cells were injected into these mice, they were unable to form metastases [120]. However, when tumor cells were mixed with Mts1-expressing fibroblasts and injected into *Mts1* knockout mice, the metastatic potential of these tumors was partially restored.

Bone Marrow-Derived Cells

In addition to fibroblasts, the tumor microenvironment is made up of several other nonresidents that were not present prior to tumorigenesis but migrated to the tumor. The most prominent among these cells are bone marrow-derived cells: mesenchymal stem cells, macrophages, neutrophils, mast cells, and T cells. These cells migrate in response to the growing tumor mass and by the secretion of discrete growth factors and chemokines produced by the tumor cells, which create a wound-like environment.

Macrophages

By far, the most prevalent nonresident cells present in tumors are tumor-associated macrophages (TAMs) [121]. Activated macrophages, those recruited to sites of inflammation, are generally categorized into two types: M1 and M2 [122–124]. M1 macrophages are effector cells that are able to potently kill microorganisms as well as tumor cells [122]. They also secrete high levels of proinflammatory cytokines [122]. M2 macrophages scavenge debris and stimulate angiogenesis as well as tissue remodeling and repair [122, 125–128]. TAMs are most similar to M2 macrophages.

TAMs have been shown to stimulate the growth and progression of both human and experimental tumor models [129]. TAMs are also preferentially recruited to sites of hypoxia, which in non-tumorigenic contexts is symptomatic of damaged or inflamed tissue [129]. Hypoxia stimulates the activity of the transcription factor HIF-1, which activates the expression of the pro-angiogenic growth factors VEGF, bFGF, TNFα, and CXCL8 [129].

While the TAMs normally stimulate tumor, they can also inhibit tumor growth. For example, CSF has been shown to stimulate production and secretion of metalloelastase by macrophages [130, 131]. Metalloelastase is an extracellular protease that cleaves plasminogen into multiple fragments, one of which is the anti-angiogenic protein angiostatin [132]. Thus, the effects of macrophage recruitment on tumor growth are highly context dependent.

Mast Cells

Mast cells are multifunctional secretory cells, characterized by numerous large electron-dense granules comprised of proteoglycans, predominantly heparin [133]. Mast cells are the progeny of pluripotent bone marrow progenitor cells, which are characterized as positive for CD34, c-kit, and CD13 [134]. In the circulation, mast cells are progenitor-like cells that differentiate/mature after being recruited to a given tissue. Mast cells express and secrete a myriad of proteases, most notably chymases, tryptases, and matrix metalloproteases, which are stored in secretory granules [133]. These proteases, specifically MMP2 and MMP9, are crucial for the ability of mast cells to stimulate tissue repair and remodeling [135]. Additionally, mast cell secretory granules are depots for cytokines and growth factors, including VEGF, bFGF, TNF-α, GM-CSF, SCF, EGF, PDGF, IFN-gamma, multiple interleukins, and chemokines, such as MIP-1 α and MCP-1 [133]. The release of proteases, cytokines, and growth factors stored in the secretory granules of macrophages can be triggered by multiple cytokines, including IL-1, IL-3, GM-CSF, platelet factor 4, IL-8, SCF, (MCP)-1, and MIP-1 alpha [136]. Moreover, mast cells also produce and secrete matrix metalloproteases (MMPs) MMP2 and MMP9, which have been shown to promote angiogenesis by liberating VEGF and bFGF from the extracellular matrix [137, 138]. Interestingly, mast cells have been shown to be recruited to tumors by the pro-angiogenic proteins VEGF, bFGF, and TGF-β [139, 140]. Thus, conditions within a tumor that necessitate the growth of new blood vessels recruit mast cells, which in turn further stimulate angiogenesis.

Experimental evidence for the functional role of mast cells in angiogenesis and tumor growth was provided by an elegant murine genetic model in which Myc expression in β cells was driven via fusion to a mutant form of the estrogen receptor [141]. In this model it was demonstrated that Myc activation by systemic administration of 4-hydroxy tamoxifen induced β-cell tumors that were characterized by blood vessel infiltration accompanied by mast cell recruitment. These findings indicated that mast cells are required for angiogenesis at the onset of tumorigenesis and for maintenance of angiogenesis during tumor growth and progression.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are bone marrow-derived cells that have the ability to differentiate into a myriad of cells of mesenchymal lineage including fibroblasts, osteoblasts, chondrocytes, adipocytes, pericytes, and muscle cells. MSCs are an extremely rare cell type within the bone marrow, comprising between 0.01% and 0.001% of the mononuclear cells [142, 143]. Human MSCs are defined by the expression CD44 adhesion molecule (HCAM), CD73, CD90, CD105 (endoglin), CD106 (VCAM-1), and STRO-1 [144].

MSCs have been shown to be recruited to sites of wounding or inflammation, as well as to tumors [145]. MSCs are recruited to tumors by multiple different growth factors and cytokines, including VEGF, bFGF, IL-8, EGF, HGF, and PDGF as well as CCL2, CCL7, and CXCL12 (SDF-1) [146–150]. Following recruitment to the tumor, MSCs have been shown to secrete VEGF to stimulate angiogenesis [151]. Moreover, in melanoma, a correlation has been demonstrated between MSCs and angiogenesis [152].

In addition to correlation and expression studies, MSCs have been demonstrated to stimulate angiogenesis in in vitro models as well as in murine pancreatic xenografts [153]. In that study, tumors formed after injecting wild-type MSCs had twice as many blood vessels as control tumors. Conversely, tumors in mice injected with MSCs, in which VEGF had been silenced by lentiviral shRNA, had comparable numbers of blood vessels to control tumors [153]. Thus, the ability of MSCs to home to tumors and secrete VEGF stimulates tumor growth via enhanced angiogenesis.

Neutrophils

While TAMs are the most prevalent and common leukocyte present in the tumor microenvironment, neutrophils are the most abundant leukocyte in the circulation in cancer patients [136]. Neutrophil recruitment from the bone marrow is mediated, in part, by the chemokine CXCL12 (SDF-1) as its cognate receptor, CXCR4, is expressed at high levels on the cell surface of neutrophils [154]. There are two types of neutrophils present in the circulation: circulating neutrophils, which, as their name suggests, are freely circulating and are recruited to tumors [155, 156], and marginated neutrophils, which are bound to the endothelium of capillaries [136]. The marginated pool can be mobilized into the circulating pool by cytokines such as IL-6 [157, 158].

Elevated levels of neutrophils are associated with multiple human tumors, including colon, lung, melanoma, myxoid fibrosarcoma, and gastric carcinoma [159–162]. In addition to CXCL12, one of the most potent chemoattractants of neutrophils is CXCL8, which is expressed by both tumor and stromal cells in many types of tumors [159, 163]. Once recruited to tumors, neutrophils stimulate angiogenesis by secreting VEGF and matrix metalloproteases, which release angiogenic growth factors from sequestration in the extracellular matrix [164, 165].

In a genetic murine model of squamous cell skin carcinoma, it was observed that the source of MMP9 in the skin tumors was not from the tumors themselves but from neutrophils. Specifically, it was found that MMP9 produced and secreted by neutrophils was required for the angiogenic switch [138]. These results have since been recapitulated using anti-GR1 antibody-mediated neutrophil ablation in the RIP-TAG2 islet cell tumor model as well as a human ovarian cancer xenograft model in MMP9 deficient mice [166, 167].

Similar to what has been observed with macrophages and mast cells, neutrophils also possess antitumor activity. For example, in 1975 it was observed that neutrophils could kill tumor cells [168]. The original conclusion was that the killing was mediated exclusively by myeloperoxidase. However, it was later shown that neutrophils kill tumor cells via multiple mechanisms including secreting proteases, membrane-perforating agents, reactive oxygen species, and cytokines such as TNFα and IL-1β [169]. Additionally, neutrophils can actually inhibit angiogenesis via two distinct mechanisms, mediated by the same protease—neutrophil elastase. The first mechanism is characterized by neutrophil elastase degradation of VEGF and bFGF [170]. Secondly, neutrophil elastase also cleaves plasminogen into angiostatin, which inhibits VEGF- and bFGF-mediated angiogenesis [171]. These findings underscore the complexity of the role of the tumor microenvironment in tumor angiogenesis and progression and serve as an example that analysis of any cell type of biomarker requires a more complete understanding of the contextual signals within the tumor microenvironment.

Conclusion

Angiogenesis is a complex process that is stimulated by a myriad of growth factors and cytokines and inhibited by an equally diverse cohort of proteins. Accordingly, the regulation of angiogenesis by the tumor microenvironment is an extremely complex phenomenon. The signaling molecules secreted by tumors that act on stromal cells can often have different, and even opposite, activities with respect to the production of pro- and anti-angiogenic factors. Therefore, the composition of the tumor microenvironment as well as the stage of the tumor has profound effects on determining whether the tumor microenvironment is pro-angiogenic or anti-angiogenic. The complex signaling mechanisms described in this chapter provide a myriad of potential and, as yet largely untapped, targets for therapeutic intervention to inhibit tumor growth in patients. Ultimately, the strategy of targeting molecules that mediate processes, such as angiogenesis, via tumor-stromal interactions may prove to be hugely successful as the accounts of genomic instability and mutation in the cells comprising the microenvironment are exceedingly rare. The hope then is that anti-angiogenic therapy targeting the tumor microenvironment will result in lower rates and incidences of acquired resistance than traditional therapeutic strategies.

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Chapter 2
More Than a Barrier: How the Endothelium Instructs Metastasis

Candice Alexandra Grzelak, Andrea Rachel Lim, and Cyrus Michael Ghajar

Abstract For metastasis to occur, a tumor cell must interact with endothelium at many steps and on multiple levels. The first half of this chapter highlights specific ligand–receptor interactions between tumor cells and the endothelium required for successful metastatic dissemination to occur, with an eye on how the specificity of endothelium influences this process in different tissues. The second half of this chapter focuses on interactions between disseminated tumor cells (DTCs) and endothelium post-extravasation. Evidence that a niche comprised by microvasculature is responsible for both maintaining cellular dormancy and facilitating tumor cell outgrowth is presented. By contrasting these studies with the known roles of endothelial-derived signals in development, maintenance of organ homeostasis, wound healing, and in stem cell niches, we describe how endothelium could dictate these opposing cellular responses during metastasis. Elaborating upon the role of endothelium as a regulator of DTC dormancy and outgrowth in multiple tissues—perhaps for multiple cancers—will guide development of therapies to combat and even prevent metastasis.

Keywords Endothelium • Metastasis • Micrometastasis • Breast cancer • Disseminated tumor cell • Circulating tumor cell • Tumor dormancy • Microenvironment • Perivascular niche • Angiocrine • Signaling • Capillary • Intravasation • Extravasation

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Abbreviations

ADAM	A disintegrin and metalloproteinase
Aes	Amino-terminal enhancer of split
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
CXCL12	Chemokine (C-X-C Motif) ligand 12
CXCR7	C-X-C chemokine receptor type 7
Dll4	Delta-like protein 4
DTC	Disseminated tumor cell
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
Eph	Ephrin
HB-EGF	Heparin-binding EGF-like growth factor
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin
IL	Interleukin
L1CAM	L1 cell adhesion molecule
LGALS3BP	Galectin-3-binding protein
LSEC	Liver sinusoidal endothelial cell
MAPK	Mitogen-activated protein kinase
$MENA^{INV}$	Mammalian enabled homologue, invasion
MLCK	Myosin light chain kinase
NO	Nitric oxide
NSC	Neural stem cell
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
POSTN	Periostin
PSGL1	P-selectin glycoprotein ligand 1
PVN	Perivascular niche
SCF	Stem cell factor
sLe^{x}	Tetrasaccharide sialyl Lewis x antigen
SVZ	Subventricular zone
TGF	Transforming growth factor
TMEM	Tumor microenvironment of metastasis
TNFα	Tumor necrosis factor-α
TSP-1	Thrombospondin-1
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	Von Willebrand factor

Introduction

It is now well appreciated that specific tumor types exhibit a predilection for arising in, and disseminating to, specific tissues. But from the advent of tumor biology, there has been debate over the role of target tissue in these patterns. Were distant sites passively or actively involved? James Ewing's theory that metastasis occurs by purely anatomic and mechanical routes [1] certainly argued for the former and contested Stephen Paget's "seed and soil" hypothesis proposed 40 years earlier [2]. Paget had noted that breast cancer patients tended to succumb to metastases within the lung, bone, liver, and uterus. He theorized that tumor cell "seeds," although dispersed in all directions, would grow only if they landed in favorable "soil" (target organ) [2]. It took nearly a century before this hypothesis was validated by experiments conducted by Hart and Fidler [3], suggesting strongly that tissue microenvironments were a key determinant of metastatic success. As it turns out, the microenvironment surrounding endothelium—the perivascular niche (PVN)—is no exception. In fact, it may be the rule.

Dating back to the 1980s, specific molecular interactions between cancer cells and endothelium that facilitate metastatic dissemination have been unraveled. In particular, ligand-receptor interactions between tumor and endothelial cells (ECs) that allow for cancer cells to traverse the endothelial barrier during intravasation and extravasation have been a principle research focus over this period. These interactions, and the role of endothelium as a barrier, will form the basis of the first half of this chapter.

The second half of this chapter will focus on more recent studies identifying paradoxical roles played by ECs in regulating metastatic outgrowth. By juxtaposing these works with earlier studies of development, maintenance of organ homeostasis, wound healing, and stem cell niches, a paradigm of endothelial phenotype differentially regulating tissue growth will emerge. We conclude with a multitude of unanswered questions and emerging themes within this field.

Origins of the Endothelial Cell

Endothelium is identical in function to any of our epithelial tissues: it forms a network of tubes that carry fluid (in this case, blood or lymph) and separates this fluid from surrounding tissue. As with any epithelium, a basement membrane lines the abluminal/basal surface of the endothelium and separates it from resident tissue cells. ECs facilitate nutrient, gas, and waste exchange in all tissues and help maintain blood pressure through dilation and constriction. Different types of ECs are found in the adult, including arterial, venous, microvascular, and lymphatic ECs. Each has a specialized phenotype and function.

However, ECs do not arise from endoderm and ectoderm, as many epithelial cells do. Instead, they are derived from a germ layer in the embryo called the meso-

derm. The mesoderm gives rise to a common precursor cell known as a hemangioblast. Hemangioblasts can differentiate into hematopoetic stem cells (HSCs, stem cells which give rise to all blood cells) or angioblasts. Angioblasts are primitive endothelial precursors defined by expression of c-kit, CD34, CD45, vascular endothelial growth factor receptor (VEGFR)-2, and Lin (reviewed by [4]) and lack markers commonly associated with differentiated endothelial cells such as von Willebrand factor (vWF), E-selectin, and VE-cadherin. Angioblasts arrange into a structure known as the primitive vascular plexus [5], forming the framework of the vascular network present in adults. This process—coalescence of an endothelial network from naïve mesoderm—is called vasculogenesis. Angioblasts that comprise the vascular plexus subsequently undergo a series of remodeling and specification steps to establish the vascular tree (artery, arteriole; capillary; venule, vein; and lymphatics) found in the adult organism.

Endothelial Heterogeneity

ECs that comprise capillaries within different organ systems display phenotypic signatures that ultimately reflect the functionality of the particular organ in which they are located (reviewed elegantly by [6]). There are three common capillary morphologies (Fig. 2.1a):

1. *Continuous*. These capillaries are lined with a continuous EC body and do not have any fenestrae (pores). A complete basement membrane surrounds the capillary. This is the most common type of capillary and is found in the skin, muscle, brain, and lung.
2. *Fenestrated*. Fenestrated capillaries have 50–150 nm diameter pores (called fenestrae) to allow rapid exchange and passage between the blood and tissues. They also have a diaphragm—areas where the glycocalyx (a carbohydrate-rich luminal layer of membrane-bound glycoproteins and proteoglycans that retains endothelial- and plasma-derived factors) bridges fenestrae. A complete basement membrane surrounds the capillary. Fenestrated capillaries are found in the intestine, endocrine glands (e.g., pancreas), and kidney.
3. *Sinusoidal (or discontinuous)*. Sinusoidal capillaries are the largest capillaries, with large intercellular clefts between cells, many fenestrations, no diaphragm, and a discontinuous (or absent) basement membrane. Sinusoidal blood vessels are found in the liver, bone marrow, and spleen.

Although overt phenotypic disparities between microvascular ECs located in different tissues have long been observed, the molecular profiles underlying observed phenotypes are only beginning to be elucidated. A very insightful study aimed to determine the molecular signature (in terms of the transcription factors, adhesion molecules, metabolic profiles, and surface receptors) expressed by tissue-specific (liver, bone marrow, kidney, heart, lung, brain, muscle, spleen, testis) microvascular ECs during organ homeostasis and regeneration [7]. Importantly, similarity between

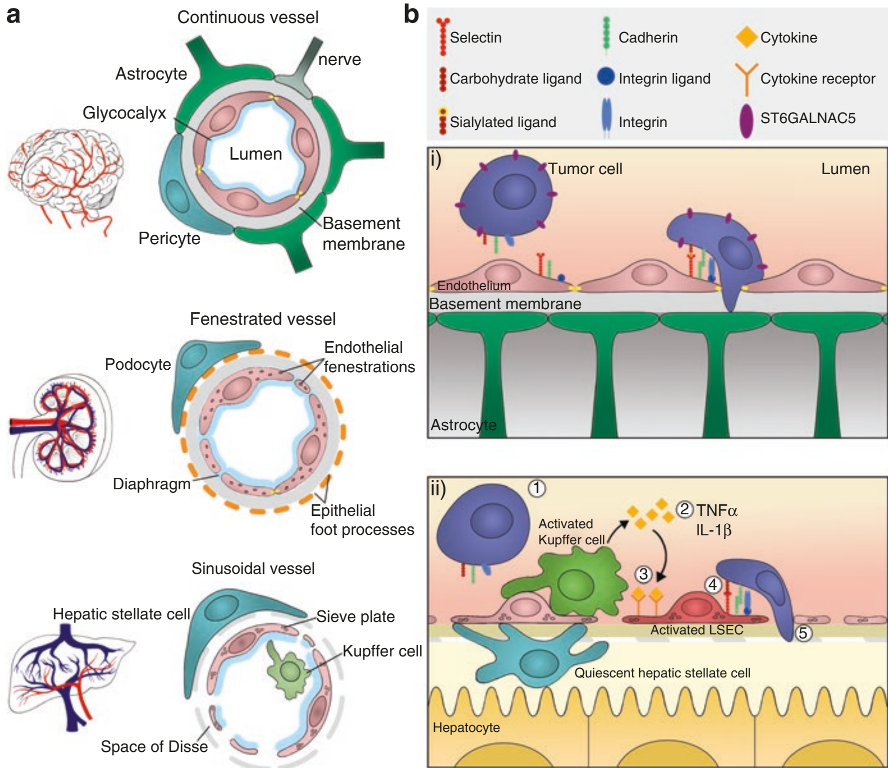

Fig. 2.1 Phenotypic and molecular heterogeneity of endothelium at different metastatic sites. Although the endothelium is a common barrier encountered by tumor cells at all metastatic sites, tissue-specific heterogeneity means the barrier encountered varies between tissues. (**a**) Examples of the differences encountered between continuous capillaries (found in the brain, strongest barrier), fenestrated (found in the kidney), and sinusoidal (found in the liver, slightest barrier) are provided for comparison. In particular, differences include the type of junctions between endothelial cells, presence/absence of a diaphragm, fenestrations and intercellular clefts, basement membrane density, and the associated resident tissue cell types within each perivascular microenvironment. Furthermore, the phenotypic and molecular heterogeneity between resident endothelial cells means that mechanisms coopted by tumor cells to extravasate at various metastatic sites are specialized. (**b**) This panel illustrates how tumor cells may interact with the endothelium encountered within the brain or liver during extravasation. (**b**(*i*)) In the brain, tumor cells express ST6GALNAC5, a sialyltransferase [47]. It is thought ST6GALNAC5 modifies cell adhesion molecules expressed by brain endothelial cells through sialylation, which subsequently permits the tumor cell to selectively interact with the brain endothelium and then transmigrate through the blood-brain barrier. (**b**(*ii*)) In the liver, tumor cell entry into the hepatic sinusoids triggers a rapid pro-inflammatory cascade (*1*) activating local Kupffer cells which secrete TNFα and IL-1β (*2*) This increases E-selectin, ICAM-1, and VCAM-1 expression on LSECs (*3*) facilitating tumor cell adhesion (*4*) and subsequent transmigration through the LSEC barrier [139, 140]

molecular phenotypes closely reflected shared phenotypes of tissue-specific endothelia. For example, liver microvascular endothelium was found to be most similar to the spleen and least similar to the lung, whereas brain microvascular endothelium was most similar to the heart and dissimilar to the bone marrow. Additionally, as with seminal studies performed decades ago showing epithelial specification by stroma [8] (recently reviewed by [9]), stroma (and other microenvironmental cues) also confers tissue-specific profiles/properties to implanted "generic" ECs [7].

In the context of the discussion that follows, it is important to consider endothelial heterogeneity and the likely differences in a tissue's perivascular microenvironment when contemplating how different phenotypes of perivascular tumor cells emerge. Even when the end result is identical, the factors that guide the said phenotype from tissue to tissue are likely to differ.

The Endothelium as a Barrier to Metastasis

The metastatic cascade comprises a number of steps that a tumor cell must accomplish to establish a metastatic lesion at a secondary tissue site. Successful metastatic progression requires that tumors cells escape from a primary site, enter the lymphatic system or bloodstream (intravasation), exit from the circulation (extravasation), survive within a non-native tissue, and colonize secondary tissues. Thus, it is clear that endothelium is a physical barrier that tumor cells must successfully interact with and navigate in order to progress through the cascade (Figs. 2.1b and 2.2). A number of examples of precisely how tumor cells and ECs interact at the molecular level, particularly within the context of intravasation and extravasation, are provided below. Broadly, for tumor-EC interactions to occur, both parties must express complementary ligand-receptor pairs. However, the complexity of potential molecular interactions becomes evident when one considers the array of primary tumor cell origins and the heterogeneity of endothelium across tissues.

Tumor Cell Intravasation

Transendothelial migration is a process used by cancer cells use to traverse the endothelial barrier when entering or exiting the circulation. Typically, vessels found in a tumor mass have been recently remodeled via angiogenesis—i.e., new blood vessels formed from preexisting blood vessels. This indicates that blood vessels in tumors are often immature and leaky due to weak cell–cell junctions, so tumor cells can more easily enter the circulation. Tumor cells, in particular metastatic breast cancer cells, have been found to transmigrate either through EC junctions

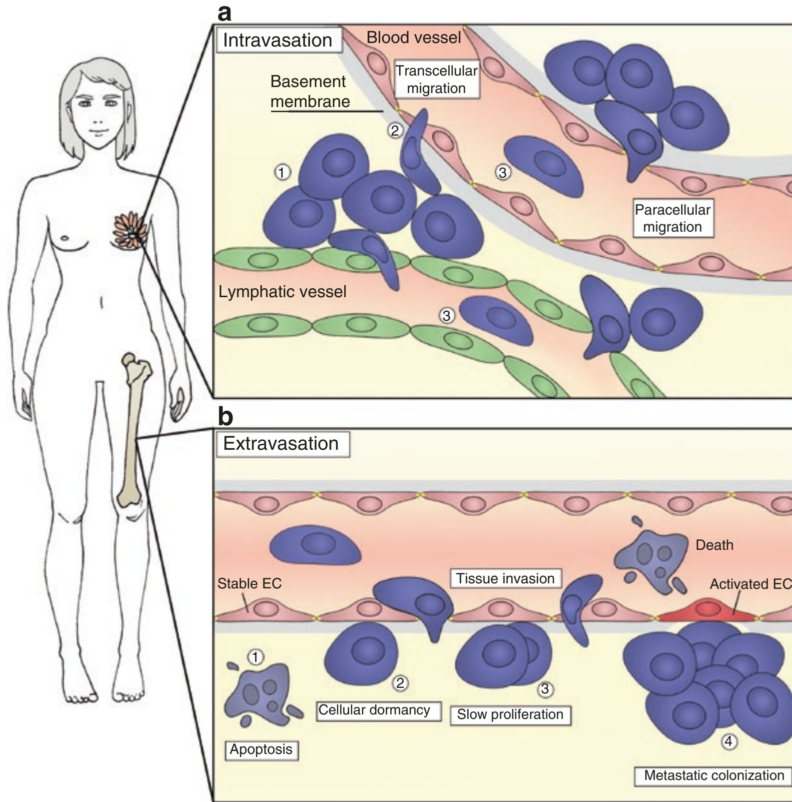

Fig. 2.2 The endothelium has direct contact with tumor cells during all steps of the metastatic cascade. (**a**) Although this example illustrates a patient with breast cancer and dissemination to the bone marrow, the steps involved in metastatic dissemination are similar for all types of primary cancer and secondary sites. The top right panel illustrates a closeup view of the cellular interactions involved in the initial steps of the metastatic cascade up until intravasation. Namely, these are (*1*) epithelial tumor cell escape from neoplastic tissue or tumor, (*2*) invasion through the basement membrane, and (*3*) intravasation and escape into either the circulatory or lymphatic system following direct molecular interaction with endothelial cells. Note the paracellular or transcellular route that the tumor cells can take during the transendothelial migration process. (**b**) During extravasation, tumor cells directly interact with the luminal face of the endothelium and can either undergo a transcellular or paracellular route. There are four possible outcomes for the tumor cell once it has encountered a new tissue microenvironment at a secondary tissue site, which is a perivascular locale: (*1*) the tumor cell encounters an incompatible microenvironment and subsequently dies. (*2*) The tumor cell encounters a perivascular microenvironment containing stable microvascular endothelium and enters into a state of cellular dormancy. (*3*) The tumor cell slowly divides, driven primarily by cell autonomous cues. (*4*) The tumor cell encounters a perivascular microenvironment in which the endothelium is undergoing active remodeling; this supports tumor cell growth and is one of the initial steps leading to metastatic colonization

(paracellular intravasation) or by penetrating the actual EC itself (transcellular intravasation) [10, 11] (Fig. 2.2a).

In paracellular intravasation, varied molecular interactions between tumor cells and ECs lead to the separation of cell junctions between ECs, providing a "path" for the tumor cell to enter the circulation. A number of signaling mechanisms are implicated in mediating paracellular intravasation. In colon cancer, amino-terminal enhancer of split (Aes) expression is lost at the invasive front of the primary tumor *in vivo* [12]. In culture, it was demonstrated that loss of Aes expression in colon cancer cells leads to activation of the Notch signaling pathway, with Jagged1 ligand expressed by ECs and Notch1 receptor by the cancer cells. Aes typically keeps the Notch pathway transcriptionally repressed; however when Aes is lost, the Notch pathway becomes transcriptionally active and promotes transendothelial migration [12].

A second molecular mechanism that cancer cells may exploit in order to intravasate involves upregulated expression of ADAM12 (a disintegrin and metalloproteinase) on the vasculature during breast cancer progression [13]. ADAM12 is a protein capable of converting membrane-anchored ligands and cytokines into soluble, active forms. This is known as "ectodomain shedding." ADAM12 releases active ligands and growth factors such as Sonic Hedgehog [14], Delta-like 1 [15], heparin binding-epidermal growth factor (HB-EGF) [16], and endothelial-specific proteins such as Flk-1, Tie2, vascular cell adhesion molecule (VCAM)-1, and VE-cadherin [13]. Importantly, VE-cadherin is involved in maintaining adherens junctions: protein complexes found at cell–cell junctions. Demonstration that ADAM12 enhances endothelial-derived shedding of VE-cadherin suggests that it facilitates breakdown of EC–EC junctions and is another molecular mechanism cancer cells take advantage of in order to intravasate.

Endothelial and tumor cells also communicate with other cells within their local microenvironment during intravasation. Using two-photon microscopy, researchers identified a particular anatomical structure they called a "tumor microenvironment of metastasis (TMEM)" that formed prior to intravasation of mammary tumor cells in mice [17–19]. This anatomical structure contains mammary tumor cells expressing high levels of mammalian enabled homologue ($MENA^{INV}$; an *inv*asion-specific spliced variant), in direct contact with both macrophages and ECs [17]. Alternative splicing of MENA is associated with increased breast tumor invasiveness through increased sensitivity to epidermal growth factor (EGF) [20–22]. Macrophages supply EGF to the $MENA^{INV}$ high tumor cells. This subsequently enhances the number of tumor cells that intravasate into the circulation [23]. Notably, other mechanisms exist whereby cancer–EC molecular interactions inevitably lead to rearrangement of cell junctions between ECs, allowing cancer cell passage into circulation; the reader is directed to this review for more examples [24]. Crucially, the main concept is that direct molecular interactions and cross talk occurring between cancer cells and endothelium enable intravasation.

Less is known about transcellular intravasation (Fig. 2.2a), which is also termed "emperipolesis"—meaning the presence of an intact cell (tumor cell) within the cytoplasm of another cell (EC). Tumor cell transcellular intravasation shares many parallels with leukocyte transcellular intravasation [25–28], with most mechanistic

insight for tumor cell transcellular intravasation coming from studying endothelial–leukocyte dynamics. Nevertheless, it has been shown that breast tumor cells can be internalized by ECs through a molecular mechanism mediated by myosin light chain kinase (MLCK). Activation of MLCK by Ca^{2+}-calmodulin allows phosphorylation of MLC. The result is contraction of an actomyosin protein complex and creation of a pore-like structure in the EC due to the rearrangement of the endothelial cytoskeleton, which can subsequently envelop the tumor cell [11, 29].

Cancer cell dissemination via the lymphatics is also important in metastatic progression, yet surprisingly little is known about how tumor cells enter the lymph system. Although junctions formed by lymphatic ECs are composed of proteins typically found in tight and adherens junctions, unique junctions are formed. The lymphatic endothelium located at the beginnings of the lymphatics is linked together by "button"-like junctions, providing a leakiness to this section of the lymphatic vessel that allows direct fluid flow without disassociation of cell–cell junctions [30]. Incidentally, this proximal portion of the lymphatics is also where the majority of leukocytes enter the vessels. Conversely, the collecting lymphatics contain "zipper"-like junctions, which provide a continuous barrier between cells without openings [30].

Due to the architecture of the proximal lymphatics, it was long assumed that tumor cell entry into the lymphatic channels was a passive process. However, the molecular mechanisms mediating active tumor cell entry into the lymphatics are currently under investigation. The enzyme 15-lipoxygenase-1, which catalyzes the breakdown of arachidonic acid, is one such mediator of tumor cell intravasation into the lymphatics. Metabolites from this conversion disrupt lymphatic vessels by creating holes in the endothelium through which mammary carcinoma cells can penetrate via bulk invasion [31]. These holes are due to centrifugal migration of lymphatic ECs rather than apoptosis. Lymphatic ECs are seven times more prone to lipoxygenase activity than blood ECs, indicating a specific mechanism for lymphatic infiltration by tumor cells [31]. Similar endothelial disruptions have also been observed with melanoma cells cultured on lymphatic EC monolayers [32].

Additionally, it should be noted that the majority of studies examine intravasation in the context of a primary tumor mass, where tumor cells enter tumor-associated vessels with pathological phenotypes (discussed in section "Remodeling or Activated Endothelium and Metastatic Colonization"). Importantly, precise mechanisms of tumor cell intravasation are likely to differ in neoplastic tissue when tumor cells disseminate via relatively normal, physiological vessels.

Tumor Cell Extravasation

Similar to intravasation, tumor cells also negotiate the endothelial barrier in either a paracellular or transcellular manner upon exit (Fig. 2.2b). The main difference between intravasation and extravasation is that the tumor cell breaches the endothelial barrier from the opposite side in each process. This means that when a tumor cell approaches endothelium following dissociation from the primary tumor, it will

be exposed to basally expressed proteins on the endothelium. Conversely, when the tumor cell approaches from the circulation, it will encounter apically expressed endothelial proteins. Therefore, a tumor cell must express cognate ligand and/or receptor proteins relevant to each process.

Once a tumor cell has entered the circulation, a combination of both mechanical and adhesive factors mediates its arrest within target organ capillaries [33]. Until the mid-2000s, it was still a popular idea that mechanical entrapment alone within smaller capillaries was sufficient to induce tumor cell arrest within microvessels. However, it has since been demonstrated that attachment and stable adhesion to the endothelium via different cell adhesion molecules is a requirement for transendothelial migration [33]. There are five major classes of cell adhesion molecules—selectins, cadherins, the immunoglobulin (IgG) superfamily, mucins, and integrins—that facilitate adhesion.

Initial Attachment: Selectins and Cadherins

Initial attachment of tumor cells to the endothelium is mediated by selectins ("selected lectins"—carbohydrate-recognizing proteins that bind sialylated carbohydrates, mediating heterotypic cell–cell adhesion) and cadherins (calcium-dependent adhesion—mediating homophilic cell–cell adhesion). Importantly, tumor cell attachment to endothelium via selectins can be initiated under high sheer stress, a scenario a tumor cell will more than likely encounter during dissemination via the circulation and subsequent arrival at a secondary site. Selectins expressed by ECs and relevant to metastasis include E- and P-selectin, which bind to a variety of ligands expressed by tumor cells. These include tetrasaccharide sialyl Lewis x (sLe^x) antigen, galectin-3-binding protein (LGALS3BP), Mucin 1, various glycoforms of CD44, P-selectin glycoprotein ligand 1 (PSGL1), and CD24 [24]. Cadherins, such as N-cadherin, can be expressed both by endothelial cells and tumor cells. Cadherin binding is mediated by self-interaction in the presence of calcium.

Stable Attachment: Integrins

Stable adhesion of tumor cells to the endothelium is mediated by integrins, a family of heterodimeric transmembrane receptors that transduce cell–cell and cell–extracellular matrix (ECM) interactions [34]. Integrins "integrate" outside cellular cues in order to dictate cellular responses such as cell adhesion, survival, migration, proliferation, and differentiation (reviewed by [35]). The capacity for integrins to command such a wide range of cellular outputs is in part due to the permutations available in the receptor repertoire. Integrins are composed of 18 α and 8 β subunits that can form at least 24 combinations of heterodimeric receptors, which can subsequently bind a number of ECM ligands [35]. Crucially, unlike selectin-mediated attachment, binding of tumor cells to integrins can only occur: (1) after initial selectin adhesion, due to the slow rate of integrin binding, or (2) under conditions of low sheer stress.

There are a number of relevant examples of how integrin binding between a tumor cell and endothelium dictates the resultant pattern of metastasis observed with different cancer types. For example, it has been shown that expression of the integrin ligand L1 cell adhesion molecule (L1CAM) by breast cancer tumor cells [36] mediates adhesion to its receptor $\alpha_V\beta_3$ integrin expressed on the luminal and abluminal face of lung microvascular endothelium [37]. This interaction mediates breast cancer cell homing and metastasis to the lungs, as well as spreading upon the basal surface of brain endothelium [38]. It has been proposed that the latter is a critical indicator of the capacity of a disseminated tumor cell (DTC) to colonize tissue [38].

Integrin expression by lymphatic endothelium also dictates metastatic outgrowth within the lymph node. Vascular endothelial growth factor (VEGF)-C production within the primary tumor microenvironment systemically promotes lymphangiogenesis—the growth of new lymphatic vessels from preexisting lymphatics [39]. VEGF-C-induced PI3Kα-mediated remodeling induces $\alpha_4\beta_1$ integrin receptor expression on lymphatic endothelium [40]. In turn, this promotes colonization of the lymph node by VCAM-1$^+$ metastatic tumor cells (such as gastric, breast, and renal carcinoma cells) [40].

Paracellular and Transcellular Extravasation

Once a tumor cell has stably adhered to the endothelium, the cell may exit by either a paracellular or transcellular route into the parenchyma (Fig. 2.2b). Currently, which route cancer cells prefer *in vivo* is unknown. In culture, the majority of cells take a paracellular path [41]. Few ligand-receptor pairs that mediate extravasation have been identified. However, one of these pairs is the interaction of endothelial-expressed CD31 (also known as platelet endothelial cell adhesion molecule (PECAM)-1, localized to EC–EC cell junctions) with tumor cell $\alpha_v\beta_3$ integrin [42]. Blocking $\alpha_v\beta_3$ integrin via antibody or RNAi prevents transendothelial migration, even though tumor cell adhesion to the luminal endothelial surface is unaffected [43]. Therefore, CD31/$\alpha_v\beta_3$ ligand-receptor pairing is a mechanism that specifically mediates extravasation.

Furthermore, the extravasation process can differ significantly in the time required for a tumor cell to traverse the endothelium, dependent upon what vascular bed the tumor cell is navigating. Tumor cells take much longer to extravasate into the brain than other organs. For example, breast and lung cancer cells take days to extravasate into the brain [44, 45], whereas lung cancer cells can extravasate into the liver in a matter of hours [45]. Breast tumor cells that successfully form macrometastases within the brain of mice were found to extravasate by day 3, although cells were able to extravasate until day 14 postinjection [46]. Some of the specific mechanisms tumor cells use in order to infiltrate the blood–brain barrier (BBB) are discussed below.

Breaching the Blood–Brain Barrier

It is important to recognize that tumor cells must navigate distinct endothelial barriers depending on the organ (and likely the *part* of the organ) into which they extravasate. As an example, navigating the brain's microvascular endothelium presents a host of unique challenges. Brain capillaries are encapsulated by basement membrane, tight junctions, and astrocyte end feet, requiring tumor cells to co-opt more advanced mechanisms to gain access to the brain parenchyma. One such mechanism employed by tumor cells involves expression of a sialyltransferase, ST6GALNAC5 [47] (Fig. 2.1b(i)). ST6GALNAC5 expression was initially found enriched in "brain-tropic" human breast cancer lines. The authors subsequently tested whether or not this molecule was involved in mediating infiltration of the BBB by gain- and loss-of-function studies in mice. Overexpression of ST6GALNAC5 increased the ability of human breast cancer cells to traverse the BBB. Conversely, depletion of ST6GALNAC5 from "brain-tropic" breast tumor cells diminished extravasation and brain metastases back to basal levels [47]. Thus, unique but still poorly understood mechanisms are required to breach the BBB. Whether specific factors mediate extravasation within, e.g., the lung, bone marrow, and liver is currently undetermined.

Other Cells Involved in Extravasation

Thus far, we have focused on how tumor–EC interactions facilitate breakdown of the endothelial barrier and entry of circulating tumor cells into a tissue. However, other cell types within the circulatory microenvironment can also influence endothelial function and facilitate tumor cell extravasation. Platelets have long been known to play a role in tumor cell dissemination, and tumor cells activate platelets through multiple mechanisms [48–57]. Nevertheless, it was shown recently that activated platelets associated with tumor cells release adenosine triphosphate (ATP), which binds the $P2Y_2$ receptor expressed on ECs. In response, EC–EC junctions relax, and tumor cells enter the parenchyma more easily [58]. There are a number of other cell types that doubtlessly contribute to vascular leakiness. Rather than enumerating every known interaction, below we present the unique concept that a tumor can affect leakiness from afar in order to facilitate metastasis.

Tumor-Derived Factors Influence Vascular Leakiness

Tumors are capable of aiding and abetting extravasation and survival of their disseminated seeds. The "pre-metastatic niche" [59] refers to how the primary tumor can systemically influence the resident cells of a target organ, bias the factors they produce, and stimulate recruitment of pro-metastatic bone marrow-derived cells to the organ to prime it for the subsequent arrival of DTCs. This in turn facilitates the survival of DTCs and supports their expansion into micrometastatic foci. In particular, the recruitment and engraftment of $VEGF1^+$ $CD11b^+$ $CD34^+$ bone marrow-derived hematopoetic progenitor cells in secondary sites precedes DTC arrival, during which time these cells remodel the local microenvironment in order to provide a more

hospitable one for DTCs [59]. More recently, exosomes, cell-derived vesicles secreted both during physiological and pathological conditions, were discovered to dictate metastatic outcome due to their ability to effect a pre-metastatic niche [60]. Since they contain DNA, mRNA, miRNA, and proteins, exosomes facilitate intercellular communication and can result in activation of the recipient cell. Importantly, whether they are uptaken by ECs in a target organ or not, the first change exosomes effect at a secondary site is enhanced vascular permeability [61], which increases the ability of bone marrow-derived cells and tumor cells to enter and ultimately colonize the tissue.

Interchangeable Role of the Perivascular Microenvironment: Fostering Tumor Cell Dormancy and Malignancy

So far, our emphasis has been to understand how tumor cells and the endothelium interact to allow for specific steps (intra- and extravasation) of the metastatic cascade to occur. In this context, the endothelium is a physical barrier the tumor cell must circumnavigate to travel to a new site. Once there, it is becoming increasingly apparent that endothelial-derived cues dictate metastatic outcome. This should not necessarily come as a surprise, given the role the perivascular microenvironment has on organ development [62, 63], homeostasis [64–66], wound healing [66, 67], and in controlling stem cell quiescence and growth [68, 69]. Nevertheless, recent studies have shed light on how the endothelium and the microenvironment surrounding the endothelium, i.e., the PVN, dictate whether a DTC survives, enters a quiescent state known as cellular dormancy, and/or outgrows once it reaches a secondary site [70]. This opens up an exciting avenue for novel metastatic therapies [71], and further extends our view of the EC as an active player within the cellular microenvironment.

The Perivascular Microenvironment

The local microenvironment of a given cell refers to its anatomical location and the specific cell–cell interactions, ECM, and secreted factors that influence its phenotype. Signals that come directly from the microenvironment that result in a change within the cell are referred to as "non-cell autonomous" cues. Conversely, signaling events that can be initiated by the cell itself, without the influence of the local microenvironment, are referred to as "cell autonomous" cues.

A perivascular microenvironment ("peri," around or near) simply refers to the immediate vicinity around vascular endothelium. The intricacies of tissue-specificity will mean that the perivascular microenvironment encountered by a tumor cell that has extravasated into the brain is different from that it would encounter in the liver, for example. Nevertheless, angiocrine (i.e., endothelial-derived [72]) factors and basement membrane proteins deposited by the endothelium and perivascular cells such as pericytes (that wrap around endothelial capillaries to provide structural support) are common components of the PVN. Only recently has it become apparent

that the perivascular microenvironment plays diverse and paradoxical roles in determining cell fate depending on context. This is somewhat of a paradigm shift, given that it was only discovered in 2000 that endothelium actively directs tissue morphogenesis.

Endothelial-Derived Cues Instruct Organogenesis

The first insight that ECs functionally provide instructive cues, extending their role from what was thought to be limited to passive metabolic exchange, related to liver development [63]. Using a liver bud explant culture system, angioblasts were found to physically interact with hepatoblasts (hepatic endodermal cells that give rise to adult hepatic epithelium) prior to the detection of closed vascular structures. When ECs were either absent or inhibited in this system, the consequence was a dramatic defect in hepatic outgrowth [63]. This demonstrated for the first time the requirement for endothelial-derived signals in directing organogenesis. Similar studies in the pancreas confirmed signals from the aorta are necessary to instruct endocrine pancreatic cell differentiation during development [62]. Thus, a paradigm of activated/invasive ECs promoting growth and differentiating ECs fostering differentiation was born.

The Endothelium During Homeostasis and in Wound-Healing

Just as it is important to appreciate how endothelium functions to direct development, understanding how the endothelium functions both in adult homeostasis and wound-healing is necessary to fathom how it could operate in a metastatic setting. The liver provides an interesting case study.

Liver sinusoids are lined by liver sinusoidal ECs (LSECs); reviewed by [73]. LSECs are the only example of mammalian endothelium that combines lack of a diaphragm across their fenestrae with lack of a typical basement membrane. Furthermore, the fenestrations on LSECs are arranged into sieve plates. It is this unique phenotype that allows ideal contact and rapid macromolecule exchange between hepatocytes (liver epithelial cells) and blood while providing the necessary latticework for cellular structure and function [74, 75]. On the luminal side, LSECs are in direct contact with Kupffer cells (resident liver macrophages) that line the sinusoids [76, 77]. On the abluminal side is an extracellular space (“the space of Disse”) where hepatic stellate cells reside in direct contact with and support LSECs [78–80]. An important point to consider is that LSECs are in a prime position to directly contact Kupffer, hepatic stellate cells, and hepatocytes. Given this unique sinusoidal anatomy, hepatocytes have even been found to contact circulating T cells directly by extending cytoplasmic extensions through endothelial fenestrations and into the sinusoid [81].

LSECs and hepatic stellate cells—the equivalent of a liver pericyte—have a dynamic relationship. During liver homeostasis, quiescent LSECs prevent the activation of hepatic stellate cells (via nitric oxide (NO) secretion) and promote reversion of activated hepatic stellate cells to a quiescent phenotype [65]. Reciprocally, hepatocyte- and hepatic stellate cell-derived VEGF help maintain the physiologic phenotype of LSECs [64].

In response to epithelial damage, a liver wound healing response will ensue. This is a cellular reaction comprised of inflammatory, regenerative, and fibrogenic components that intrahepatic cell populations coordinate in an attempt to repair the liver and return to homeostasis (reviewed in detail by [82, 83]). Importantly, fibrogenesis is driven by activation of the hepatic stellate cell—which acquires a myofibroblast-like phenotype and begins secreting excessive ECM [84]. Liver injury is accompanied by activation of LSECs, a process called capillarization. Capillarization sees LSECs take on a more typical vascular phenotype, which is characterized by loss of fenestrae and abnormal/excessive deposition of basement membrane proteins into the PVN. Activated LSECs stop producing NO and can no longer support maintenance of the quiescent hepatic stellate cell phenotype [85]. Furthermore, activated LSECs increase production of fibronectin [86] and endothelin [87], which are drivers of hepatic stellate cell activation.

It is obvious then that within the liver microenvironment, LSECs are central players in maintenance of homeostasis, the wound healing response, and perpetuating fibrosis. In other organs, such as the lung and kidney, ECs and the PVN also direct fibrosis [88, 89]. For example, in the lung, repeated injury suppresses C-X-C chemokine receptor type 7 (CXCR7) expression on lung capillary ECs. This results in the recruitment of perivascular macrophages, which increases Jagged-1 ligand expression on ECs. Subsequently, Jagged-1 ligand binds to the Notch receptor on adjacent perivascular fibroblasts, which enhances fibrosis. Either CXCR7 agonists or knockdown of Jagged-1 in lung ECs skews the downstream cellular response from a fibrotic one to one more directed toward promoting repair [88]. Therefore, the endothelium lies at the crux of tissue repair and fibrosis in multiple organs.

A Perivascular Niche for Stem Cells, Across Tissues

How the PVN can simultaneously regulate the quiescence and growth of a cell is perhaps best illustrated by examining stem cell maintenance. The subventricular zone (SVZ) houses one of two adult neural stem cell (NSC) niches. In the mammalian brain, the subventricular zone [90]. Here, quiescent type B stem cells become activated type B stem cells, which give rise to transit amplifying type C progenitors that subsequently differentiate into type A neuroblasts. Both type B and C cells are found in direct contact with the vasculature; however they each have a unique interaction with the endothelium. Type B cells extend specialized end-feet projections that make stable contact with endothelium. On the other hand, type C cells make more transient endothelial contact, at smaller sites [69]. The nature of the contact

with the endothelium dictates which signaling pathways are activated within type B/C cells, thus influencing whether NSCs remain quiescent (type B) or begin to proliferate/differentiate (type C) [69]. The brain endothelium expresses Jagged-1 and Ephrin B2, ligands that bind to Notch or Ephrin (Eph) receptors, respectively. Both type B and type C cells express these receptors, and importantly, ligand–receptor binding can only occur in the case of direct cell–cell contact. As type B cells are in tight contact with brain endothelium, Notch and Eph receptor signals are transduced simultaneously. This attenuates mitogen-activated protein kinase (MAPK) signaling, preventing growth factor-induced activation of cyclin D and cell cycle progression. It is this combination of Notch/Eph signal transduction that keeps type B cells quiescent. On the other hand, type C NSCs interact with the endothelium transiently, leading to insufficient engagement of Notch and Eph receptors. The result is growth factor-induced activation of the MAPK cascade, cyclin D accumulation, and cell cycle turn over [69]. This is a prime example of how the endothelium expresses specific factors that dictate both quiescence and cell cycle progression dependent upon the nature of the physical interactions between a cell and the endothelium.

Bone marrow stem cells (i.e., HSCs) are also regulated by the PVN. The great majority of HSCs localize to sinusoidal endothelium within bone marrow [91–93]. Stem cell factor (SCF) is a key maintenance factor of the HSC niche [94]. Deletion of SCF from various cellular compartments within bone marrow identified its cellular source [95]. Only deletion of SCF from the endothelial compartment or the Lepr^{+}-perivascular stromal cell compartment led to HSC depletion [95]. This strategy of using cell-specific deletion of SCF allowed the authors to conclude unambiguously that HSCs reside within and are maintained by a PVN and that factors derived from this niche are responsible for maintaining HSCs.

However, EC phenotype is critical to this function. Specifically, angiocrine factors secreted from ECs signaling via an Akt-driven mechanism support self-renewal of long-term HSCs and expansion of hematopoietic stem and progenitor cells (HSPCs) [68]. Conversely, ECs transducing MAPK-driven signals drive HSPC differentiation and expansion [68]. This provides another striking example of how the EC is capable of dictating diverse cellular outcomes (quiescence versus proliferation) within a localized microenvironment, all depending on EC phenotype.

The preceding paragraphs describe examples of perivascular regulation of brain and hematopoietic stem cells. However, it should be noted that stem/progenitor cells in other tissues such as the liver, skin, skeletal muscle, the extramedullary HSC niche in the spleen [96], and also mesenchymal stem cells in the kidney, lung, heart, and liver occupy a PVN. The deduction of the endothelial/perivascular contribution to maintenance of these various stem cell niches is ongoing; nevertheless, it is clear that endothelial-derived signals are integral to controlling both quiescence and cellular growth under a variety of tissue-specific settings. How this paradigm applies to regulation of DTC quiescence and outgrowth is described below.

The Endothelium and Tumor Cell Dormancy

Once a DTC has extravasated into a distant organ, what are the scenarios that can subsequently play out? There are a number of possibilities (Fig. 2.2b). Firstly, if the tumor cell finds that the foreign microenvironment of the secondary organ it has invaded does not provide adequate survival cues, the tumor cell may die. Secondly, the DTC may begin slowly dividing, driven primarily by autonomous cues and emerge years down the road. Thirdly, the cell may encounter a favorable microenvironment—known as a "metastatic niche"—and initiate metastatic outgrowth. And finally, the cell may enter into a state known as cellular dormancy.

Not a great deal is known about how DTCs survive in foreign microenvironments. However, even here the PVN has been implicated, specifically signaling through heterotypic connexin gap junctions formed between ECs and DTCs that facilitate DTC survival [97]. If a DTC survives, what determines whether it falls asleep or whether it grows?

When discussing tumor dormancy, there are two distinctions to be made. There is a state known as "population dormancy"—in which proliferation and death are balanced within a micrometastatic nodule. This ultimately keeps the micrometastatic mass in a static growth state [98]. Escaping this state requires evasion of immune surveillance [99] and induction of the "angiogenic switch" [100]. Thus, it was long assumed that the endothelium's role in regulating metastatic colonization was a passive one; as long as new blood vessels were induced, outgrowth would occur.

However, a second and possibly more frequent type of tumor dormancy also exists that is not restricted by nutrient availability. This state, known as "cellular dormancy," describes a single tumor cell or small cluster of tumor cells that enter G_0 of the cell cycle and are mitotically arrested in secondary sites for an unspecified period [101]. The remaining discussion will deal specifically with this state of dormancy. Cellular dormancy is an amazing phenomenon in that of the ~30% [102] of breast cancer patients who will develop distant metastases, 20% of these clinically disease-free cases will relapse 7–25 years after adjuvant therapy [103]. How do these single cells/small clusters of cells persist for such a long period of time within a tissue? Further, what cues subsequently drive them to reemerge?

The first microenvironment a DTC will encounter upon entering into a new tissue is a perivascular one. Perhaps it is no surprise then that dormant disseminated breast tumor cells reside within a PVN *in vivo*. Dormant DTCs were found in a PVN in all target organs that breast cancer commonly metastasizes including the bone marrow, lung, brain [70], lymph nodes, and liver (Lim et al.; Grzelak et al., unpublished work). Having deduced that the breast cancer dormant niche was a perivascular locale, Ghajar, Bissell, and colleagues commenced to interrogate the perivascular cues that sustain breast cancer cell quiescence. Using a proteomics-based approach, the authors were able to narrow down a candidate list of ECM-derived "dormancy" factors expressed highly within the PVN. Intriguingly, thrombospondin-1 (TSP-1), long known as an anti-angiogenic factor [104], was identified [70]. TSP-1 was expressed both in culture and *in vivo* along the length of vessel "stalks"—stable

regions along the endothelial vessel not undergoing endothelial remodeling. Gain- and loss-of-function studies demonstrated that TSP-1 functioned to prevent breast cancer cell outgrowth. Therefore, TSP-1 is at least one PVN factor, secreted from ECs, that has an apparent role in controlling breast cancer tumor cell dormancy in the lung and bone marrow. Another EC-derived factor shown to suppress tumor cell growth is perlecan, the major heparin sulfate proteoglycan expressed by ECs. Knockdown of perlecan in ECs resulted in ECs that were no longer able to suppress the invasiveness of breast or lung cancer cells. Further, mice injected with lung cancer cells pretreated with EC-conditioned media, rather than EC-conditioned media from perlecan-silenced ECs, had reduced metastatic burden [105]. This indicates perlecan is another PVN-derived factor that may also function to maintain tumor cell dormancy.

Intriguingly, around the same time that TSP-1 was identified as a dormancy factor within the PVN, another research group similarly identified the importance of TSP-1 in controlling metastatic outgrowth in an alternative context [106]. Bone marrow-derived CD11b^{+} Gr1^{+} myeloid cells also express TSP-1 in mice with metastasis-incompetent tumors. These are cells typically recruited to secondary tissue sites and are involved in establishing a pre-metastatic niche. However, the authors proposed that in primary tumors *incapable* of generating metastases, the tumor secretes high levels of prosaposin—a glycoprotein precursor that generates saposins, sphingolipid activator proteins that induce TSP-1 expression [107]. By manipulating the expression of TSP-1 within the bone marrow compartment, the authors demonstrated that bone marrow-derived cells still homed to the lung in the absence of TSP-1 expression, but that depletion of TSP-1 within the bone-marrow-cancer cell niche was necessary for lung colonization [106]. This study further implicates TSP-1 as a dormancy-promoting/anti-metastatic factor.

Remodeling or Activated Endothelium and Metastatic Colonization

Before discussing how endothelium can also aid metastatic colonization, a further appreciation of the heterogeneity displayed by microvascular endothelium—even within the same capillary—must be addressed. It is vital to appreciate that within a capillary, there can be three distinct and functionally specialized positions (and thus niches) an EC may fill. First, an EC may function as a tip cell (a "leader" cell); second, an EC may function as a stalk cell ("trailing," proliferative cells) [108]; the third, a phalanx cell, is an EC composing stabilized, quiescent microvasculature [109]. Tip cells are single ECs that extend filipodial protrusions, are highly polarized, have a distinct molecular profile (platelet-derived growth factor (PDGF)-β^{high} VEGFR2^{high}), do not contain a lumen, and do not proliferate in response to VEGF-A [108]. Instead, a VEGF-A gradient guides endothelial tip cell migration, whereas

stalk cell proliferation is dependent on the concentration of VEGF-A [108]. Remarkably, during angiogenesis ECs compete for the “pole position” in order to be a tip cell. A VEGF-Notch [108, 110–114] autoregulatory feedback loop exquisitely regulates the number of tip cells within a system, as well as which EC will function as the tip. ECs with a VEGFR1^{low} VEGFR2^{high} Delta-like protein 4 (Dll4)^{high} phenotype are at an advantage to overtake the tip cell position, whereas a VEGFR1^{high} VEGFR2^{low} Dll4^{low} cell will function as a stalk cell [115]. This example of endothelial niche competition highlights the molecular heterogeneity of microvascular ECs even within a local microenvironment, which is an important concept when considering how the endothelium can have seemingly contradictory actions in directing dormancy and metastatic outgrowth.

Indeed, TSP-1 expression is lost at neovascular tips—sites of active endothelial remodeling [70]. Instead, the tip cell niche is enriched with factors such as tenascin-C, versican, fibronectin, active transforming growth factor (TGF) β1, and periostin (POSTN), proteins known to facilitate micrometastatic outgrowth within metastatic niches [59, 116–120]. In fact, tip cells were shown to promote metastatic outgrowth through these factors.

Tip cells are essentially an activated EC, and researchers had already begun to make the link that EC activation is involved in the facilitation of metastasis years ago. ECs become activated in response to various inflammatory cues. Pro-inflammatory cytokines (e.g., interleukin (IL)-6, tumor necrosis factor (TNF)-α) induce expression of adhesion molecules on the endothelium (e.g., VCAM-1, intercellular adhesion molecule 1 (ICAM-1), E-selectin—referred to as endothelial “activation” markers) [121]. In turn, this mediates leukocyte recruitment and enables attachment of leukocytes to the endothelial wall in order to facilitate an inflammatory response. When activated, ECs also secrete storage granules called Weibel–Palade bodies [122] which contain vWF [123] and P-selectin [124, 125], further enabling leukocyte recruitment and assisting in an inflammatory response [126].

In these early metastasis studies, the secretion of pro-inflammatory cytokines (e.g., IL-1β, TNFα) was shown to increase metastatic burden of melanoma cells within the lung or liver following endothelial activation (e.g., upregulation of VCAM-1 expression) [127–131] (Fig. 2.1bii). In addition to enhancing inflammatory cell recruitment, activated ECs secrete a pro-tumorigenic set of molecules, decrease quiescence-promoting and anti-inflammatory genes, and consequently increase lung cancer cell growth, invasion, and metastasis [105, 132].

In summary, endothelial-derived factors deposited within distinct endothelial subniches may either prevent or promote tumor cell outgrowth (Fig. 2.3). Namely, stable microvascular endothelium functions as a dormant niche, while endothelial remodeling promotes metastatic outgrowth. Overall, this suggests that disruption of endothelial homeostasis can dictate metastatic outcome and highlights the importance of endothelium in directly regulating metastatic progression. Further investigation into comprehensive sets of tissue-specific angiocrine factors that mediate breast tumor cell dormancy—and dormancy of other tumor types—is underway.

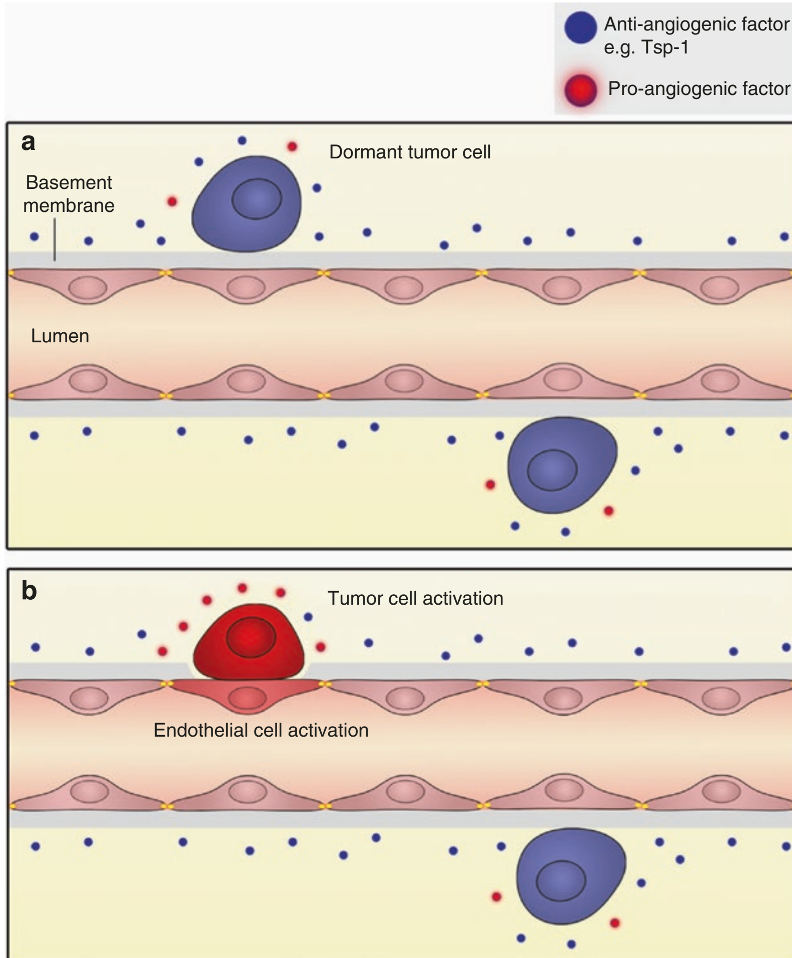

Fig. 2.3 Endothelial subniches can dictate tumor cell dormancy or metastatic outgrowth. This diagram depicts possible ways the endothelium may control tumor cell dormancy or alternatively spur micrometastatic outgrowth [70]. (**a**) Two tumor cells have encountered a compatible foreign microenvironment following extravasation. They are in contact with resident endothelial cells, which have deposited both pro-angiogenic and anti-angiogenic factors within their local microenvironment. However, as the balance favors anti-angiogenic (dormancy-promoting) factors, both tumor cells initially enter into a state of cellular dormancy. (**b**) Cues have led to the activation of endothelium (*top left*), which is in direct contact with a dormant tumor cell. This alters the perivascular microenvironment to be enriched with pro-angiogenic factors (tumor growth promoting). (**c**) We can see that endothelial remodeling is now taking place and that tumor cells are actively proliferating. The tumor cells are surrounded by an abundance of pro-angiogenic/tumor growth-promoting factors. Most likely, resident macrophage and fibroblast activation will also facilitate this process. A myriad of paracrine interactions most likely occur between the endothelium, tumor cells, macrophages, and fibroblasts facilitating micrometastatic outgrowth and ECM remodeling, in a manner somewhat reminiscent of a wound-healing response. Note that the second tumor cell has remained dormant, as the endothelium it is in contact with has remained quiescent/stable, and the perivascular microenvironment remains enriched with anti-angiogenic/"dormancy" factors

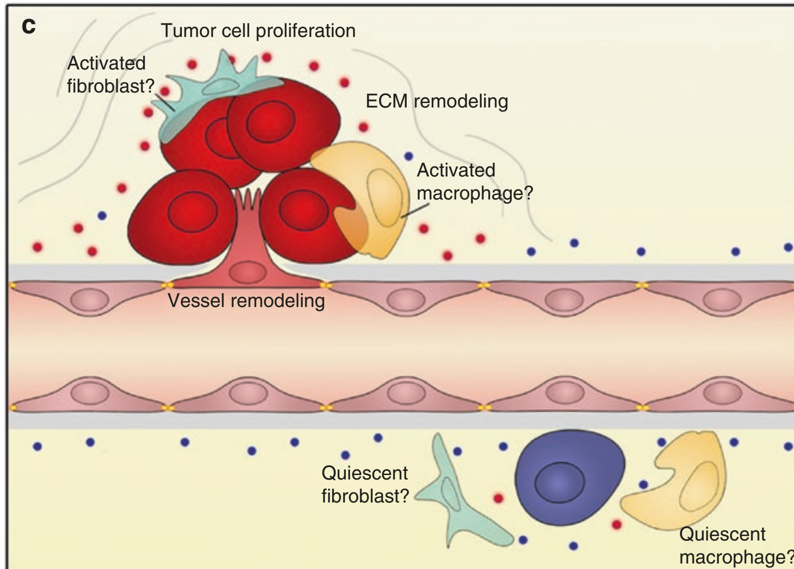

Fig. 2.3 (continued)

Perspectives and Conclusions

This chapter aimed to provide a framework for understanding the role of endothelium as an equipped and active contributor to the metastatic cascade. By addressing endothelial cell origins, angiocrine instruction during organogenesis, and the tissue specificity of endothelia, the reader should appreciate the physiologic role of ECs and the function-specific heterogeneity exhibited by ECs. The second focus of this chapter was the endothelium as a barrier during metastatic progression. This portion of the chapter focused on molecular mechanisms responsible for successful tumor–EC interactions as tumor cells navigate intermediate steps of the metastatic cascade. The third focus of this chapter was the PVN as an active regulator of DTC phenotype. The endothelium is paradoxically involved in maintaining tumor cell dormancy and in directing metastatic colonization in secondary tissues, dependent upon endothelial phenotype (tip vs. phalanx; stable vs. activated) that DTCs interact with. We also drew parallels between endothelial cell functionality and activation state and liver homeostasis and wound healing, as well as the dual role of the PVN in maintaining stem cell quiescence vs. proliferation.

From the evidence presented, it appears that the outcome of metastatic initiation within a tissue is dependent, at least in part, upon the perivascular microenvironment that a tumor cell encounters once it has extravasated into a distant tissue microenvironment. It seems that DTCs that come into contact with quiescent, stable endothelium in a new tissue microenvironment may enter into a state of tumor cell dormancy. This microenvironment is rich in factors that support an anti-angiogenic, quiescent state, e.g., TSP-1. Conversely, tumor cells that encounter a perivascular microenviron-

ment associated with activated endothelium or endothelium undergoing active remodeling (e.g., a tip cell niche) will continue to grow, encountering pro-tumorigenic factors such as TGF-β and POSTN. Does this mean, in effect, that the switch between a dormant and actively growing tumor cell is regulated by the microvascular endothelium?

If this is the case, a number of pertinent questions remain to be answered:

1. Precisely how is the dormancy/growth switch controlled? Is it simply a balance between anti-angiogenic vs. pro-angiogenic factors present in the PVN?
2. Is this trigger the same in different metastatic sites? For example, do the same changes within the PVN dictate the reactivation of dormant breast cancer cells residing within the lung, bone marrow, liver, brain, and lymph node? Further, is the switch different for different types of cancers?
3. Is activation of the endothelium alone sufficient to reawaken a dormant DTC? Or is there a sequence of events (e.g., subsequent macrophage activation, fibroblast activation, ECM remodeling, etc.) similar to processes involved in wound-healing that result in micrometastatic foci formation, and are these steps interchangeable (Fig. 2.3c)?
4. What cue(s) are sufficient to disrupt endothelial homeostasis and cause exit from dormancy? If inflammatory cytokines and lipopolysaccharide are sufficient to activate endothelial cells, does even encountering a cold put former cancer patients at risk? Or are more flagrant and/or chronic insults (e.g., tissue fibrosis [133]) necessary?
5. Is aging sufficient to promote exit from dormancy? For example, aging LSECs undergo capillarization (thus activation) in both mice [134] and humans [135]; is age alone a risk factor for DTC reemergence? If so, is this tissue dependent?
6. And finally, how does the PVN influence immune surveillance? T cells actively survey our bodies, and DTCs that leave the primary site during tumor progression must present actionable antigen(s). However, given that endothelium is a rich source of immune-repellant molecules such as chemokine (C-X-C Motif) ligand 12 (CXCL12) [136], TGF-β [137], and PD-L1 [138], does the PVN constitute an immunotolerant microenvironment and unwittingly protects DTCs?

These and many more questions require exploration before we gain any true insight into precisely how ECs and the PVN may dictate metastatic reemergence in cancer survivors later down the line. Nevertheless, it is obvious that the endothelium exerts a major influence on metastatic outcome and has a much greater role than being a mere conduit for tumor cell dissemination.

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Chapter 3
Tissue-Based Biomarkers of Tumor-Vascular Interactions

Lars A. Akslen

Abstract Studies have indicated that the prognosis of cancer patients might be improved by targeting the tumor-associated vascular system. There is, however, a lack of markers that can predict the clinical response to such antitumor therapy and thereby select patients for optimal management. Whereas microvessel density is known to be an effective prognostic factor, information on response prediction is virtually lacking. In addition to the use of novel endothelial proteins for improved tumor imaging and targeting strategies, the potential practical value of selected histologic markers such as vascular density, microvessel proliferation, and vascular maturation for predictive purposes needs to be validated in future clinical studies.

Keywords Tissue biomarkers • Angiogenesis • Microvessel density • Vascular proliferation • Vascular maturation • Glomeruloid microvascular proliferation • Vascular invasion

Introduction

In 1971, Folkman suggested that the growth of malignant tumors is dependent on the process of angiogenesis and that tumors can be treated by attacking their blood supply [1]. Since then, mechanisms of angiogenesis have been explored [2–5], and multiple cell types and regulatory pathways have been shown to interact in this complex process, e.g., tumor cells, endothelial cells, stromal cells, inflammatory cells, and circulating endothelial progenitor cells from the bone marrow [3, 6, 7]. Studies have

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indicated an effect of anti-angiogenesis treatment on certain human cancers, such as metastatic colorectal carcinoma, breast cancer, and other tumors [8–10]. A few attempts have been made to identify predictors of response to anti-angiogenesis treatment or traditional chemotherapy [11–15]. Identification of predictive factors would be important for individual patients and for cost-effective clinical practice. However, this search has not been convincing [16], in contrast to the reported value of various angiogenesis markers, such as microvessel density, as significant prognostic factors.

Is it possible to classify or grade the vascular response in malignant tumors on a routine basis, so that this information can be used for improved prognostication or response prediction? Histologic grading of tumor-associated angiogenesis was suggested by Brem et al. in 1972 [17] and was later modified by Weidner and Folkman with the introduction of microvessel density (MVD) as a prognostic indicator [18]. Although MVD has later been shown to predict patient prognosis in multiple clinical studies, this marker has some limitations [19]. Hlatky et al. stated that microvessel density is not a simple measure of the angiogenic dependence of tumors, but is rather a reflection of the metabolic burden of supported tumor cells. Also, the authors claimed that there would be no direct relationship between microvessel density and a response to anti-angiogenesis therapy.

Other prognostic features of angiogenesis have been reported such as vascular proliferation [20–23] and vascular maturation status [23, 24]. Also, architectural patterns such as vascular nesting or glomeruloid microvascular proliferation have been focused and studied in relation to the diversity of tumor-associated angiogenesis and to the aggressive features and prognosis in human cancers [25–27].

In addition to markers of tumor-associated angiogenesis, studies have also reported the frequency and impact of vascular invasion, i.e., the ability of tumor cells to enter blood vessels or lymphatic vasculature, and the different influence of these characteristics on tumor progress in various organs [28–30].

Since there is limited data on the prediction of response to anti-angiogenic treatment or standard chemotherapy using histology-based markers of tumor angiogenesis, this needs to be further explored and validated in translational studies of clinical trials, with respect to response prediction in the era of targeted treatment and cost-effective practice.

It should be mentioned, although not reviewed here, that the process of angiogenesis in solid tumors is not only a local process, but systemic aspects have gained increasing attention [5]. Thus, it has been shown that populations of circulating bone marrow-derived endothelial progenitor cells can differentiate into mature endothelial cells and contribute to pathological neovascularization. These cells can be detected in tissue sections by immunohistochemistry. However, the relative contribution of circulating endothelial progenitor cells to tumor neovascularization in humans is not well known in various tumors. Further, the premetastatic niche concept represents an important part of these systemic interactions and regulatory cross talk between primary tumors, bone marrow, and distant tissues that can be influenced to receive or resist metastatic cells. From a diagnostic point of view, circulating cells, e.g., tumor cells, endothelial precursor cells, or other classes of cells, have also received much attention lately as representing a key part of the "liquid biopsy" concept [31]. These diagnostic modalities will likely supplement the tissue-based assessment of primary and metastatic lesions in the future.

Markers of Angiogenesis

Microvessel Density

In 1972, Brem, Cotran, and Folkman suggested criteria for histologic grading of tumor-associated angiogenesis [17], based on the combined assessment of vasoproliferation (number of microvessels within a microscopic field), endothelial cell hyperplasia (number of endothelial cells lining the cross section of a capillary), and endothelial cytology (nuclear changes in proliferating endothelium). In 1988, Srivastava et al. showed in a small study that histologic quantification of microvessels provided significant prognostic information in melanoma [32]. In 1991, Weidner and Folkman reported criteria for microvessel density (MVD) and demonstrated prognostic value in breast cancer [18, 33]. After highlighting the vessels or individual endothelial cells by pan-endothelial markers like factor VIII (von Willebrand factor) or CD31, microvessels are counted in the most active area of the tumors (i.e., hot spots). Subsequently, after these important papers, MVD has been widely studied for prognostication in several types of malignant tumors, like breast cancer [18, 33], endometrial cancer [34], gastrointestinal cancer, lung cancer, malignant melanoma [32, 35], and prostate cancer [36, 37]. MVD has been a significant prognostic factor in a majority of studies reported, although some have been negative [38]. In a large meta-analysis of breast cancer [39], including 43 studies and almost 9000 patients, MVD was a significant but weak prognostic factor. The conclusions implied that other angiogenic markers might potentially add prognostic information and should be studied.

Modifications of this method have been reported, by using Chalkley counts or image analysis and morphometric measurements based on random area selection [40–42]. The Chalkley counts, giving a relative area estimate of immunostained vessels, may increase the reproducibility of counts within a given hot spot [39]. Tissue sampling is important since there is considerable heterogeneity within individual tumors [43]. However, these methods have not increased the practical value of microvessel counts.

Whereas most studies suggest that microvessel density is a significant prognostic factor, data on response prediction are very limited. Paulsen et al. reported in 1997 that clinical response to neoadjuvant doxorubicin monotherapy for locally advanced breast cancer could not be predicted by MVD [11]. Similar conclusions were reached by others [12]. Further, Jubb et al. [13] concluded that MVD, in addition to VEGF and TSP-1 expression, did not correlate with treatment response or patient outcome in the series of metastatic colorectal carcinoma for which the effect of bevacizumab was first shown [8]. In the era of targeted anti-angiogenesis treatment, more data on the predictive value of different tissue-based and other angiogenesis markers is clearly needed.

In a study by Tolaney et al. from 2015 [44], a trial of preoperative bevacizumab treatment followed by a combination of bevacizumab and chemotherapy in HER2-negative breast cancer patients was performed to determine how vessel morphology and function was influenced by bevacizumab. The clinical response appeared to

reflect the process of vascular normalization primarily in patients with high baseline tumor microvessel density, especially among triple-negative breast cancers.

Vascular Proliferation

The proliferation of endothelial cells has not been much studied in human cancers, and its prognostic or predictive importance is not well described in most cancers (Fig. 3.1). A few studies of breast, colorectal, and prostate tumors have reported a vascular proliferation rate ranging from 0.15% to 17% [20–22, 24, 45–47]. Eberhard et al. studied endothelial cell proliferation in six types of human tumors and found a range from 2.0% (prostate) to 9.6% (glioblastomas) within vascular hot spots [24]. Fox et al. showed a mean labeling index for endothelial cell proliferation in breast cancer of 2.2%, being highest in the tumor periphery [45]. Interestingly, there was no correlation between endothelial cell proliferation and microvessel density in any of these studies, similar to what others have reported [47]. In a study of 21 colorectal carcinomas, Vermeulen et al. found an average endothelial proliferation labeling index of 9.9%, compared to 21% in vascular hot spots [21].

In these early studies, there was no information on the importance of vascular proliferation for patient prognosis. In 2006, Stefansson et al. showed for the first time that vascular proliferation (i.e., proliferating microvessel density, pMVD; microvessel proliferation, MVP) was an independent prognostic factor, shown in endometrial cancer, and pMVD was superior to microvessel density by multivariate analysis [23]. The median vascular proliferation index (VPI), i.e., the percentage of

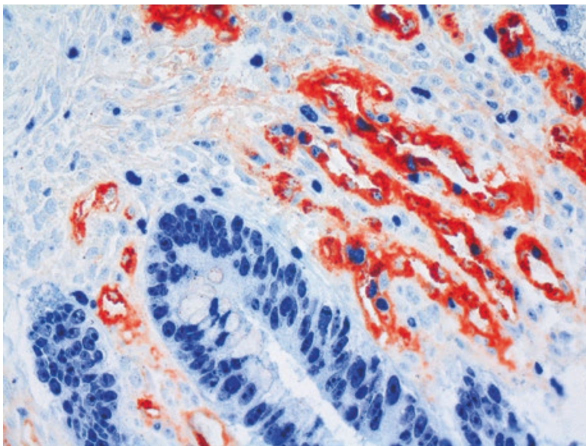

Fig. 3.1 Microvascular proliferation: microvessels in *red* (factor VIII) with some dividing endothelial cells in *blue* (Ki67). Tumor cells (to the *left*) show high degree of proliferation (Ki67-positive nuclei)

microvessels, within hot spot areas, with evidence of proliferating endothelial cells by Ki67 staining, was 3.9%, with a range of 0–21% within the tumor tissue. Microvessel proliferation was found to be increased in cases with the presence of tumor necrosis and with high tumor stage (by FIGO categories). In the same study, vascular proliferation was an independent prognostic factor by multivariate analysis in addition to histologic grade, vascular invasion by tumor cells, and tumor stage.

In studies of breast cancer, using three independent cohorts including 499 patients, Arnes et al. found that median vascular proliferation ranged from 0.95% to 1.95% and was associated with estrogen receptor-negative tumors and reduced patient survival, whereas microvessel density was not significant [48]. It was further shown by Nalwoga et al., in 2 breast cancer cohorts including 431 cases, that vascular proliferation was significantly increased in estrogen receptor-negative cases and in tumors with a basal-like phenotype [49]. The mechanism for such a relationship in breast cancer is not known. It was shown that basal-like and triple-negative cancers were associated with VEGF expression [50], a key regulator of breast cancer angiogenesis [51], and VEGF-driven angiogenesis might contribute to the increased vascular proliferation that we found among basal-like tumors. Increased vascular proliferation in basal-like compared to luminal breast cancer was recently also shown by Kraby et al. [52].

It was reported in 2009 by Gravdal et al. that when combining Ki67 for endothelial proliferation with a marker of immature endothelium, nestin, the prognostic sensitivity was increased [53]. By studying prostate cancer, nestin/Ki67 co-expression, as a marker of vascular proliferation, was four- to fivefold higher in castration-resistant cancers and metastases compared with localized tumors and prostatic hyperplasias. Among localized cancers, high vascular proliferation was a strong and independent predictor of biochemical failure, clinical recurrence, and time to skeletal metastasis by multivariate analysis. In castration-resistant cancers, vascular proliferation was still associated with reduced patient survival. In breast cancer, by nestin/Ki67 co-expression, a median vascular proliferation of 2.7% was found by Krüger et al. [54]. There were significant associations with estrogen receptor-negative tumors as well as basal-like and triple-negative phenotypes. In this study, vascular proliferation was an independent predictor of death from breast cancer.

Interestingly, in a study by Haldorsen et al., microvascular proliferation in endometrial cancers was compared with imaging parameters obtained from preoperative dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and diffusion-weighted imaging (DWI) to explore the relationship between these markers and their potential ability to identify patients with poor outcome [55]. Microvascular proliferation was found to be negatively correlated to tumor blood flow by MRI, possibly reflecting an abnormal and reduced functionality in newly formed tumor-associated vasculature. In this study, vascular proliferation was significantly associated with reduced patient survival.

In a study by Stefansson et al. in 2015, a 32-gene expression signature was found to separate tumors with high versus low microvascular proliferation [56]. This 32-gene signature was associated with high-grade tumor features and reduced

survival by independent cohorts. Interestingly, copy number studies revealed a strong association between microvessel proliferation and 6p21 amplification. VEGF-A is known to be located in the 6p21 chromosomal region [57], and integrated analyses demonstrated significant associations between increased vascular proliferation and VEGF-A mRNA expression, pointing to a possible angiogenesis driver mechanism in endometrial cancer. In a previous study of endometrial cancer, VEGF-A was significantly associated with vascular proliferation and reduced patient survival [23].

Vascular Maturation

The structural integrity and maturation status of blood vessels, i.e., the degree of coverage by cells like pericytes, have been reported [5, 58], and several factors are known to contribute to pericyte recruitment [59, 60]. Reduced maturation might contribute to the atypical structure of vessels in malignant tumors [25, 61]. Also, tumor-associated pericytes are often abnormal when present [62]. Vascular maturation, as estimated by pericyte coverage, appears to be a dynamic process. In prostate cancer, androgen ablation therapy may induce a downregulation of intra-tumoral VEGF followed by selective regression of immature tumor microvessels by apoptosis of endothelial cells not covered by pericytes [63]. These authors suggested that vessel maturation status of individual tumors might predict the efficiency of anti-VEGF tumor treatment. In 2001, Jain proposed that anti-angiogenic therapy might lead to improved maturation and normalization of the tumor vasculature, thereby increasing the efficacy of combined treatment including chemotherapy or radiation [64, 65]. In a clinical study, injection of anti-VEGF was followed by increased maturation of tumor-associated vessels [66], as has also been shown in experimental studies [67, 68]. It was shown that anti-VEGFR2 treatment creates a "normalization window" of the vasculature for increased efficiency of additional radiation treatment by upregulation of angiopoietin-1 (Ang-1) and degradation of the basement membrane by MMP activation [69]. In a trial of preoperative bevacizumab followed by a combination of bevacizumab and chemotherapy in HER2-negative breast cancer, Tolaney et al. reported that the tumor response appeared to reflect vascular normalization, primarily in patients with high tumor microvessel density [44].

Data on human tumors are limited with respect to clinical correlates and outcome. In a previous study of lung cancer [70], better outcome was found for tumors with high vascular maturation. The mean vascular maturation index (VMI) was 46%, and high VMI was associated with low microvessel density and absence of nodal metastases. In contrast, a study of breast cancer showed no prognostic impact of VMI [71]. In both studies, the basement membrane antibody LH39 was used as a maturation marker. Eberhard et al. studied vascular maturation in six human tumor types and found a wide range in pericyte coverage index from 13% (glioblastoma) to 67% (breast cancer) [24], although no clinical or prognostic evaluation was presented. The authors concluded that differences between various tissues in vascu-

lar proliferation and maturation might be of importance for the suitability of anti-angiogenic treatment. In a study of endometrial cancer, Stefansson et al. showed that median pericyte coverage, as estimated by the α-SMA coverage index (SMAI), was 35%, and low SMAI was significantly associated with increased vascular invasion by tumor cells and impaired patient prognosis [23].

In a study of colorectal cancer, semiquantitative and digital-image-analysis-based scoring identified significant associations between low expression of perivascular PDGFR and shorter overall survival. Importantly, perivascular PDGFR-α and PDGFR-β remained independent factors for survival by multivariate analyses [72].

Glomeruloid Microvascular Proliferation

Although tumor vessels frequently have an abnormal structure, both architectural and cytologic atypia might be difficult to assess, and there is no consensus on how to report vascular morphology in a reproducible and efficient way. Some studies have suggested pattern-based angiogenesis markers, such as glomeruloid microvascular proliferations (GMPs) (Fig. 3.2). GMPs, also called "microvascular nests" or "glomeruloid bodies," are focal proliferative buddings of a mixture of vascular cells (primarily multilayered endothelial cells in addition to pericytes and macrophages) that at least superficially resemble renal glomeruli [73–76]. In standard tissue sections, GMPs generally consist of 15–100 cells; one or more vascular lumens are usually present, especially in more mature GMPs, but are not necessary.

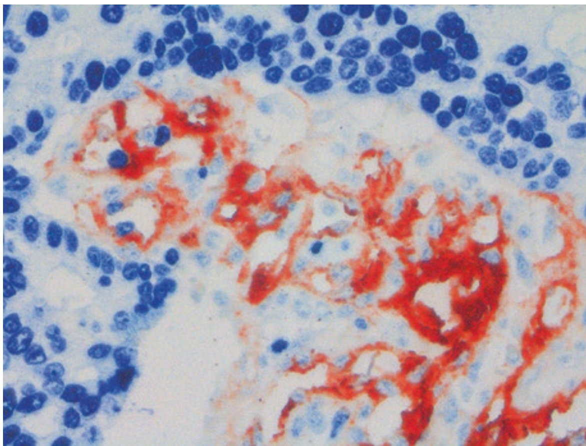

Fig. 3.2 Glomeruloid microvascular proliferation (GMP) (*red* vessels, factor VIII), with a few dividing endothelial cells in *blue* (Ki67), and marked proliferation in tumor cells (Ki67)

GMPs represent a defining histologic feature of glioblastoma multiforme [73, 74] and have been associated with increasing aggressiveness of brain tumors [77, 78]. GMP-like patterns have also been sporadically reported in other tumors, including gastrointestinal carcinomas, thymomas, and different vascular tumors [75, 79–83]. However, until quite recently, human tumors have not been studied systematically.

In animal studies, Dvorak and coworkers induced the formation of "glomeruloid bodies" from preexisting microvessels in mouse skin, through the injection of an adenoviral vector expressing VEGF-A_{164}, indicating that the formation of GMP might represent a VEGF-A-dependent and dysregulated angiogenic response [84]. The formation of new blood vessels through several steps, each with a distinctive morphology, was described in detail; these include *mother vessels* (MOV), *glomeruloid microvascular proliferation* (GMP), and *arteriovenous malformations* (AVM) [25, 75, 76, 85]. The GMP phenotype was dependent on the continued presence of VEGF-A_{164}, and as VEGF-A_{164} expression declined, GMPs underwent apoptosis and progressively devolved into smaller, more normal-appearing microvessels [76]. Thus, the GMP generated in this model required exogenous VEGF-A_{164} for their maintenance as well as for their generation. This finding is likely relevant to GMP in human tumors. All of the tumor types known to form GMP also express VEGF-A. Another human parallel appears to be the POEMS syndrome, where increased VEGF-A levels are associated with glomeruloid vascular proliferations in the skin, i.e., glomeruloid hemangioma [79].

In a study by Straume et al. in 2002 of more than 700 human cancers (breast, endometrial, prostate, melanoma), approximately 20% of the cases were considered GMP positive (range 13–23%). The presence of GMP was significantly related to poor prognosis [27], and this has been confirmed in studies of non-small cell lung cancer [86] and pancreatic cancer [87]. This angiogenic phenotype was found to be a better predictor of outcome than microvessel density [16].

In the series of nodular melanomas, 23% were GMP positive, and the presence of GMP was significantly associated with aggressive tumor features like increasing lesion thickness (a.m. Breslow) and ulceration. In survival analysis, GMP was an independent prognostic factor along with Clark's level of tumor invasion and ulceration, and GMP was of greater value in this regard than microvessel density. To extend these studies, the presence of GMP in relation to the expression of several different angiogenic factors and their receptors in melanoma was evaluated [88]. GMP was associated with increased endothelial cell expression of VEGF receptor-1 (FLT-1), VEGF receptor-2 (KDR), and neuropilin-1. The expression of VEGF-A protein in tumor or endothelial cells was not associated with the presence of GMP, whereas VEGF-A expression was significantly stronger in GMP endothelium compared with non-GMP endothelium within the tumors. There was a significant association between the lack of Tie-2 expression in tumor-associated endothelial cells and the presence of GMP, whereas there was no association with the expression of angiopoietin-1 (Ang-1) [88]. Taken together, these findings indicate that increased expression of VEGF receptors on the endothelium in melanomas was associated with presence of GMP, whereas the opposite was found for Tie-2, a receptor that has

been linked to vessel maturation [10]. Expression of bFGF was decreased in GMP endothelium, and this has been associated with a less mature vasculature [27].

In the initial study [27], 17% of breast carcinomas were GMP positive, and the presence of GMP was related to the ductal histotype, high grade, estrogen receptor negativity, and HER2 expression. Regarding prognosis, GMP was found to be an independent prognostic indicator by multivariate analysis, providing additional information beyond basic variables such as tumor size, histologic grade, and lymph node metastases. Notably, GMP was not correlated with microvessel density which was not prognostic in this patient cohort. These findings indicate that GMP may provide a novel prognostic marker, indicative of a more aggressive vascular phenotype.

Further studies on breast cancer indicated that GMP is associated with multiple markers of aggressive tumors like estrogen receptor negativity and a basal-like phenotype [89], and the GMP vascular phenotype has been associated with the presence of *BRCA1* germline mutations and p53 alterations [90]. *BRCA1*-related breast cancers have a distinct profile on microarray analysis [91] and also a characteristic spectrum of *TP53* mutations [92]. Our data suggest that *BRCA1* mutations might induce a genetic profile of which GMP is an important manifestation and part of the tumor phenotype. Of relevance, BRCA1 protein has been associated with inhibition of VEGF transcription and secretion in breast cancer cells these.

We previously found a significant association between GMP and pathologic expression of p53 protein [90], whereas p53 overexpression was not associated with increased microvessel density. The relationship between p53 and angiogenesis could involve several different mechanisms: (1) p53 is known to suppress the expression of VEGF [94] and interacts with the transcription factor Sp1 [95]; (2) p53 degrades hypoxia-inducible factor-1 [96]; (3) p53 downregulates the expression of bFGF-binding protein [97]; and (4) p53 upregulates thrombospondin-1 expression [98].

In a study of locally advanced breast cancer, treated with standard chemotherapy, Akslen et al. found that the presence of GMP, occurring in 21% of the cases, was significantly associated with high-grade tumors and *TP53* mutations in addition to the basal-like and HER2-positive subtypes of breast cancer as defined by gene expression data [15]. The GMP phenotype was significantly associated with the lack of treatment response and progressive disease, indicating a potential predictive value. In these tumors, GMP was also correlated to a gene expression signature for tumor hypoxia response, pointing to a possible mechanistic relationship. In a study of metastatic melanoma, GMP in primary tumors (25%) or metastatic tissue (12%) did not predict the response to bevacizumab monotherapy, although limited tissue from metastatic lesions could decrease sensitivity [99].

In endometrial cancer, GMPs were found to be significantly associated with increasing histologic grade, diffusely invasive growth pattern, presence of necrosis, vascular invasion, deep myometrial invasion, and high clinical stage [23]. This study also indicated an association between GMP formation and increased vascular proliferation, by factor VIII/Ki67 co-expression. The findings provide further evidence that GMP is an angiogenic marker of high-grade and aggressive tumors.

In prostate cancer, GMP was present in 13% of cases [27] and was associated with high preoperative levels of serum PSA. The GMP phenotype was an independent predictor of time to biochemical failure as determined by multivariate analysis.

In other tumor types, GMP was a significant prognostic factor in a study of non-small cell lung cancer [86]. A total of 25% of these tumors were GMP positive, and the frequency of GMP was not associated with basic factors such as histologic grade or clinical stage. Similar to our findings [27], there was no association between GMP status and microvessel density in these lung cancers. There was no correlation between VEGF-A expression and the frequency of GMP, although this phenotype was more often seen in Ang-1-positive tumors. Multivariate analysis indicated that GMP was a significant and independent prognostic factor, whereas microvessel density was not. Taken together, these data support the initial observation that GMP might be a novel and significant tissue-based angiogenesis marker for potential clinical use.

Other Vascular Patterns

There has been some additional focus on architectural patterns of angiogenesis in malignant tumors [100]. It seems that qualitative features, rather than quantitative metrics of microvessel density and other markers, may provide some prognostic relevance in certain tumor types, like glioblastomas of the brain, and ocular melanomas. Some studies have focused on the distribution pattern of microvessels within tumors. The EDVIN concept ("edge versus inner") suggests that comparing vessel counts at the edge of the tumor with the inner area might give a better picture of the angiogenic activity and patient survival. The prognostic value of EDVIN was shown in studies of breast and colorectal cancers [101].

Quantification of vascular pattern by image analysis has shown increased prognostic impact by the use of syntactic structure analysis [102]. Studies of pheochromocytomas, which are highly vascular tumors of the adrenal medulla, have shown that complex and irregular vascular patterns are associated with malignant behavior [103].

Vascular Immunomarkers

Can certain vascular immunomarkers discriminate between endothelial cells in benign tissues and "activated" tumor-associated endothelium? If so, these markers could be applied in tumor imaging and therapeutic targeting, in addition to response prediction and prognostication. This field is very promising but not well developed, and it is not the primary topic of this review. Chi et al. reported expression differences between endothelial cells from various sites of the vascular system [104]. Also, various proteins are differentially expressed in tumor-associated endothelium [105, 106], and such endothelial markers might provide "zip codes" or "maps" for homing of antitumor peptides like LyP1 [107]. St. Croix et al. showed multiple

novel antigens being expressed selectively in tumor endothelium from colorectal cancers, some of them associated with the cell membrane (TEM1, TEM7, TEM8) or extracellular matrix [108]. In the same setting, studies from our team indicate that when using the marker nestin for immature endothelium, in addition to Ki67 as a proliferation marker, enhanced and significant prognostic information can be obtained from tissue sections [53, 54].

Pan-endothelial markers, such as von Willebrand factor (factor VIII), CD31, and CD34, are frequently used to visualize endothelial cells by immunohistochemistry when estimating microvessel density. Some reports suggest that CD105/endoglin, a TGF-β receptor involved in vascular development and remodeling, might be suitable as a marker of active angiogenesis in malignant tumors, as well as a therapeutic target on tumor-associated vessels [109]. Microvessel density by CD105 was superior and independent as a prognostic factor in breast cancer [110]. Similar results were presented for lung cancer [111] and prostate cancer [112], whereas no advantage of CD105 was found in studies of endometrial cancer [113] and malignant melanoma [114].

VEGF and its receptors may be present on tumor cells and vessels and might represent targets for imaging and treatment [115]. It was shown that activated microvessel density (aMVD), as estimated by VEGF/KDR staining on endothelial cells, was highest in the tumor periphery and superior to standard microvessel density (sMVD) as a prognostic factor evaluated by multivariate survival analysis of non-small cell lung cancer [116].

Expression of bFGF on tumor-associated endothelial cells was inversely associated with lymph node metastases and pathological stage of non-small cell lung cancer [117]. Similar findings, together with a prognostic role, have been found for prostate cancer [118] and malignant melanoma [119]. These findings further support the diversity of tumor-associated vessels.

Other angiogenesis markers have been explored, like the expression of tumor-specific endothelial (TEM) antigens [120–122]. Expression of certain integrins, like αvβ3, has been associated with tumor vasculature [123], and this marker might also be applied for imaging [124] and treatment strategies [125]. The main challenge will be to validate such proteins in future studies. Whether simple histology-based tissue markers will prove effective in comparison with other classes of angiogenic markers, like circulating endothelial cells, remains to be studied. Taken together, the study of vascular markers is important for our understanding of tumor-associated angiogenesis, vascular imaging techniques, and the development of therapeutic modalities. Whether gene expression signatures might capture the complexity of malignant tumors and better reflect their angiogenesis capacity remains to be studied in detail.

Markers of Vascular Invasion

One important hallmark of cancer progression is the ability of tumor cells to migrate into vascular channels, i.e., blood vessels or lymphatic vasculature, as an early step of metastatic spread [126]. In breast tumors, vascular invasion is usually considered

to be lymphatic vessel involvement (LVI) more often than blood vessel invasion (BVI) [29], but there are few studies in this field. Vascular invasion, as observed on standard tissue sections, is associated with increased risk of tumor recurrence, metastasis, and death from disease [29, 127]. Lymphatic invasion is particularly important as a prognostic factor in early stage breast cancer [128, 129]. Gujam et al. highlighted that immunohistochemistry discriminates better between BVI and LVI, and this distinction improves the prognostic value of vascular invasion compared to standard sections [130].

A potentially different impact of blood vessel invasion as compared with lymphatic involvement has not been well established, for example, in relation to the molecular subtypes of breast cancer. This might be due to the lack of firm criteria to separate blood vessel and lymphatic invasion. Usually, CD31 staining for blood vessel endothelium and D2-40 for lymphatic vessels are applied, although overlapping staining patterns exist. Still, D2-40 expression is considered to be specific for lymphatic endothelium. In a study by Klingen et al., blood vessel invasion, present in 15% of the cases, showed strong associations with non-luminal tumors such as the basal-like, triple-negative, and HER2-positive subgroups [30]. In survival analysis, BVI was significantly associated with recurrence-free and breast cancer-specific survival, whereas LVI was not. When adjusting for basic factors, BVI was an independent prognostic marker, indicating that this feature might be recorded in breast cancer diagnostics, although more studies need to confirm these findings. Development of even more specific markers for blood vessels would be desirable in a routine setting to identify patients at a higher risk for early systemic spread. The potential use of such diagnostic approaches for improved therapy among cases with blood vessel invasion should be considered.

We previously reported that basal-like breast cancers appear to have increased angiogenesis with more microvessel proliferation and higher frequency of the glomeruloid microvascular pattern (GMP) when compared with other breast cancer subtypes [48, 49]. These findings suggest a possible relationship between increased angiogenesis and blood vessel invasion among basal-like breast cancers. The relationships between vascular proliferation, immature vessels, and vascular invasion have also been shown in endometrial cancer [23].

Notably, studies of disseminated tumor cells from the bone marrow, as well as expression profiles of primary tumor cells, suggest that hematogenous spread is often an early event in tumor progression [131]. Early systemic dissemination of breast cancer cells is associated with a specific expression signature, and the molecular pathways associated with primary hematogenous spread and lymphatic dissemination appear to be different [132]. The present data suggest that blood vessel invasion by tumor cells is strongly associated with aggressive tumor subtypes (basal-like, triple negative, HER2 positive). Blood vessel invasion has also been related to interval breast cancer presentation compared with screen-detected tumors [30]. Based on such findings, it might be of practical importance to examine the presence of blood vessel invasion in breast cancers.

It has been suggested that the basal-like phenotype of breast cancer may be related to non-lymphatic spread [133], and findings indicate a reduced risk of axil-

lary lymphatic spread in triple-negative breast cancer [134]. Although the presence of metastases in axillary lymph nodes predicts development of distant metastases, 20–30% of patients with node-negative breast cancer develop metastatic spread at distant sites [135]. As mentioned, early systemic dissemination of breast cancer cells is associated with a specific gene expression signature [132].

In a large study of endometrial cancer, 18% of the tumors showed blood vessel invasion, whereas 31% of the tumors revealed lymphatic involvement [28]. Both BVI and LVI were associated with features such as high histologic grade and diffuse tumor growth. Patients without vascular invasion had the best prognosis and those with BVI (with or without LVI) had the worst outcome, whereas patients with LVI had an intermediate survival by univariate analysis. Both BVI and LVI had independent prognostic importance. Such findings support the biological significance of vascular spread through the hematogenic and lymphatic routes in endometrial cancer. The significant correlation found with clinical phenotype indicates that these markers may be relevant for patient management.

In further studies of endometrial cancer, certain gene expression patterns were associated with vascular invasion by tumor cells as examined on standard sections [136]. Thus, a vascular invasion signature of 18 genes was significantly associated with patient survival and clinicopathologic phenotype. Vascular involvement was related to gene sets for epithelial-mesenchymal transition, wound response, endothelial cells, and vascular endothelial growth factor (VEGF) activity. Further, expression of collagen 8 and MMP3 was associated with vascular invasion, and ANGPTL4 and IL-8 showed a relationship to patient survival. These findings indicate that vascular involvement within primary tumors is associated with gene expression profiles related to angiogenesis and epithelial-mesenchymal transition. This 18-gene expression signature was furthermore studied in multiple cohorts of breast cancer and found to associate with aggressive features like high tumor grade, hormone receptor negativity, HER2 positivity, a basal-like phenotype, reduced patient survival, and response to neoadjuvant chemotherapy [137]. The 18-gene vascular invasion signature was associated with several other gene expression profiles related to vascular biology and tumor progression, including the Oncotype DX breast cancer recurrence signature. Taken together, the findings indicate that markers for vascular invasion by tumor cells in the primary tumor, including gene expression patterns, might provide information that indicate an increased risk of metastatic spread.

Concluding Remarks

It has become increasingly evident that some malignant tumors can be treated by attacking their blood supply. At the same time, both experimental and clinical data have demonstrated that tumor-associated angiogenesis is more complex than reflected simply by the number of microvessels on tissue sections. In the era of targeted therapy, companion biomarkers are becoming crucial to increase

treatment efficacy by defining subgroups of patients with high probability of response to the treatment [13, 16], similar to the role of HER2 in breast cancer management. Whereas this is a "hallmark of tailored treatment," such markers have not yet been successfully established in the field of anti-angiogenesis therapy. In the case of anti-VEGF regimens, there is no simple relationship between the presence of the target (VEGF) and treatment response [13], and no reliable association with the "endpoint" of angiogenic stimulation, i.e., microvessel density, has been found. At the same time, there is a relative lack of translational studies of human tumors, and tissue-based angiogenesis markers should therefore be further studied and validated. Markers reflecting the angiogenic response in primary tumors, such as vascular proliferation and vascular maturation status, need to be examined across different tumor types to increase the evidence of their potential utility, especially as predictive factors. The presence of glomeruloid microvascular proliferation (GMP), reflecting some of the increased irregularity and complexity of tumor-associated angiogenesis and a marker of VEGF-driven angiogenesis, should be considered. Furthermore, a refined immunophenotypic profiling of the tumor vasculature might improve the basis and indications for novel imaging techniques and treatment targets. Complementary systemic biomarkers, such as circulating endothelial progenitor cells, are likely to gain increased importance. Different markers might be combined into profiles to obtain a balance between high-technology methods and simpler cost-effective techniques.

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Chapter 4
Role of the Extracellular Matrix in Tumor Stroma: Barrier or Support?

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Abstract Extensive evidence exists to functionally implicate stromal cancer-associated fibroblasts in tumor progression. Data from experimental cancer models has questioned the exclusive tumor-supportive function of the tumor stroma and suggested that the stroma might also act as a barrier to inhibit tumor metastasis. With consideration of this shift in dogma, we discuss the role of a specific part of the tumor stroma, the insoluble extracellular matrix (ECM), in tumor growth and spread. We summarize data from experimental tumor models on the role of fibrillar collagens, the fibronectin EDA splice form, proteoglycans, and the matricellular proteins, periostin and tenascins, which are all major components of the tumor stroma. In addition to the composition of the ECM being able to regulate tumorigenesis via integrin-mediated signaling, recent data indicate that the stiffness of the ECM also significantly impacts tumor growth and progression. These two proper-

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ties add to the complexity of tumor-stroma interactions and have significant implications for gene regulation, matrix remodeling, and tumor metastasis. The role of the tumor stroma is thus extremely complex and highlights the importance of relating findings to tumor-type-, tissue-, and stage-specific effects in addition to considering inter-tumor and intra-tumor heterogeneity. Further work is needed to determine the relative contribution of different ECM proteins to the tumor-supporting and tumor-inhibiting roles of the tumor stroma.

Keywords Tumor microenvironment • Tumor stroma • Extracellular matrix Fibrillar collagen • Tumor growth • Tumor metastasis • Tumor stiffness • Lysyl oxidase • Fibronectin EDA • Periostin • Tenascins • Proteoglycans

Introduction

How one views a solid tumor depends on which "glasses" one uses. One can thus look at a tumor from a pathologist's point of view, from a cell biologist's point of view, or from a molecular biologist's point of view. These different approaches provide different perspectives and information. A pathologist might note different aspects related to encapsulation, vascularization, and the amount of stroma. A cell biologist might distinguish signs of inflammation and degree of vascularization and choose to isolate cells to study their phenotype *in vitro*. A molecular biologist aims to understand the molecular and genetic mechanisms involved in tumor pathogenesis and design experiments accordingly. No matter which "glasses" you have on, developments in the field of tumor cell-tumor stroma interactions highlight the importance of the tumor microenvironment (TME), and it is becoming increasingly clear that one needs to pay close attention to the tumor stroma when analyzing tumors.

With the understanding that the tumor microenvironment influences tumor cell growth, this also has implications for the design and interpretations of *in vitro* experiments. It is becoming obvious that simple 2D *in vitro* coculture experiments are not sufficient to recapitulate the complex interactions that take place in the tumor in situ. Thus, in order to understand the cellular dynamics in the tumor, one needs to create model systems where the 3D aspects as well as multiple cell type aspects are incorporated. In molecular studies, intercellular communication, amount and properties of the extracellular matrix (ECM), and paracrine signaling, which all influence the signaling within cells, have to be taken into consideration when interpreting the data. New innovative strategies to study the influence of ECM in tumorigenesis are needed, e.g. heterospheroids [1, 2] being one recent methodological development with great potential.

When discussing different mechanisms in the tumor microenvironment, it is important to avoid generalizations and always relate the findings to a certain tumor and the specific experimental conditions. The reasons to avoid such generalizations are:

– The TME can vary greatly between different tumors. Part of this heterogeneity is due to the source and nature of the stromal fibroblasts [3].

- The composition of the TME varies with the dynamics in, and stage of, the tumor: initiation, growth, and metastasis phases, all contain a TME with specific characteristics (e.g., differences in amounts of immune cells, fibroblasts activation states, proteolytic activity, and stiffness).
- Matrix stiffness is another critical feature for tumor growth and for tensional homeostasis in the tumor [4, 5]. Matrix stiffness has been shown to be intimately linked to posttranslational modifications of the matrix such as glycation and cross-linking but also to collagen organization and appears to vary between different regions within the tumor [6].
- In addition to the complexity in the assembly and structure of the ECM, the recent findings that tumor-derived exosomes affect cellular interactions in the TME introduce yet another level of complexity. Provocative data have described roles for exosomes in chemoresistance, miRNA-directed effects on gene silencing, and even mediating changes in integrin repertoire affecting metastasis of tumor cells [7, 8].

The function of collagen in the tumor stroma is tightly linked to stromal fibroblasts, which in the solid tumor context are called cancer-associated fibroblasts (CAFs) [3, 9, 10]. CAFs have different roles in the tumor stroma (including paracrine signaling [10] and chemoresistance [11]), which will not be discussed in this chapter. CAFs serve as producers of ECM proteins like fibrillar collagens and act as mechano-sensitive cells performing integrin-mediated reorganization of the matrix, resulting in changes in stromal stiffness. In order for CAFs to take on this contractile function, they need to become activated. A prime signal for CAF activation is TGF-β. Data has demonstrated that integrin αvβ6 on the tumor cells is involved in TGF-β activation (by binding to an RGD sequence in the latency-associated peptide (LAP) of the TGF-β/LAP complex, resulting in increased TGF-β bioavailability). This activation of TGF-β results in CAF activation [12]. Moreover, antibodies to αvβ6 *in vivo* have been shown to reduce growth and metastasis of the 4T1 murine breast cancer cell line [13]. Data in fibrosis and *in vitro* models further suggest that myofibroblasts themselves can play an active role in activating TGF-β, by prestraining the matrix and sensitizing TGF-β to activation [14–16]. Another integrin, αvβ1, has also been shown to directly take part in TGF-β activation of myofibroblasts [17].

At the stage of metastasis, CAFs have been reported to generate migratory paths in the stroma that facilitate collective cell invasion in an integrin-, caveolin-1-, RhoA-, Rab21-, and YAP-dependent manner [18, 19]. Interestingly, two reports have challenged the dogma that the tumor stroma plays a supportive role in tumor growth and metastasis [20, 21]. Both studies take advantage of advanced genetic techniques to ablate stromal cells in experimental models for pancreatic cancer (in a form of genetic stroma-targeting strategy) after the tumors had formed. Contrary to what was expected, the pancreatic tumors became more aggressive in the absence of the stroma. Since the source of CAFs can vary, this does not mean that all fibroblast-targeted therapy approaches are doomed to fail in tumors, but it highlights the complexity of tumor-stroma interactions and points to the potential need to target specific subsets of fibroblasts or even specific signaling pathways in fibroblasts, which are central to the tumor-promoting aspect of the stroma. Another

study suggests that a minor perivascular Gli-positive stem cell population in the lung stroma is the main producer of a fibrotic ECM, and careful analyses of dermal fibroblasts have revealed different origins of reticular and papillary fibroblasts [22–24]. Analogous to these studies demonstrating fibroblast heterogeneity in tissues, different mesenchymal cell populations in the tumor stroma might have different roles. In summary, a global targeting of all CAFs may not be the best therapeutic strategy [3, 25] since both tumor-supportive CAFs and tumor-inhibitory CAFs appear to exist in the tumor stroma. Cell lineage tracing will be critical to unravel these mechanisms and provide useful insight into new CAF-associated therapies for treating tumors.

The Extracellular Matrix of the Tumor Stroma

Figure 4.1 schematically illustrates the structure of the ECM molecules that we describe below.

Fibrillar Collagens in the Stroma

Fibrillar Collagen Types in the Tumor Stroma

The collagen family is composed of 28 trimeric triple-helical proteins [26, 27]. The most abundant collagens are the fibrillar collagens, which together with a subset of fibril-associated collagens with interrupted triple helices (FACIT collagens) are present in interstitial tissues [26]. In interstitial tissues, collagen I dominates with lesser amounts of collagen III being present. Collagen V in some studies has been suggested to constitute less than 5% of interstitial matrices, and collagen XI, under physiological conditions, is present only in specialized matrices [26, 27]. In carcinomas, the fibrillar collagens I/III dominate, and relatively little information is available on the status or roles, if any, of collagens V and XI [28]. The tumor stroma has been likened to a *wound that does not heal*, representing the tumor stroma in a sense as a granulation tissue, which is rich in fibrillar collagens [29, 30]. In the granulation tissue collagen III is replaced with collagen I as the wound heals [31], but in the tumor stroma, the ratio of collagen I and III is determined by tumor type as well as the stage of the tumor and tissue-specific factors.

Cells can adhere to collagen matrices, either directly or indirectly, via proteins bound to collagens. Direct binding occurs via collagen receptors such as the integrins α1β1, α2β1, α10β1, and α11β1 [32, 33]. Indirect binding is mediated via collagen-integrin bridging molecules (COLINBRIs), which typically bind RGD-binding integrins like α5β1, αvβ1, αvβ3, and αvβ5 [32, 34]. Interestingly, the discoidin domain receptors (DDRs) have recently been shown to affect the function of collagen-binding integrins by supporting integrin activation [35–37].

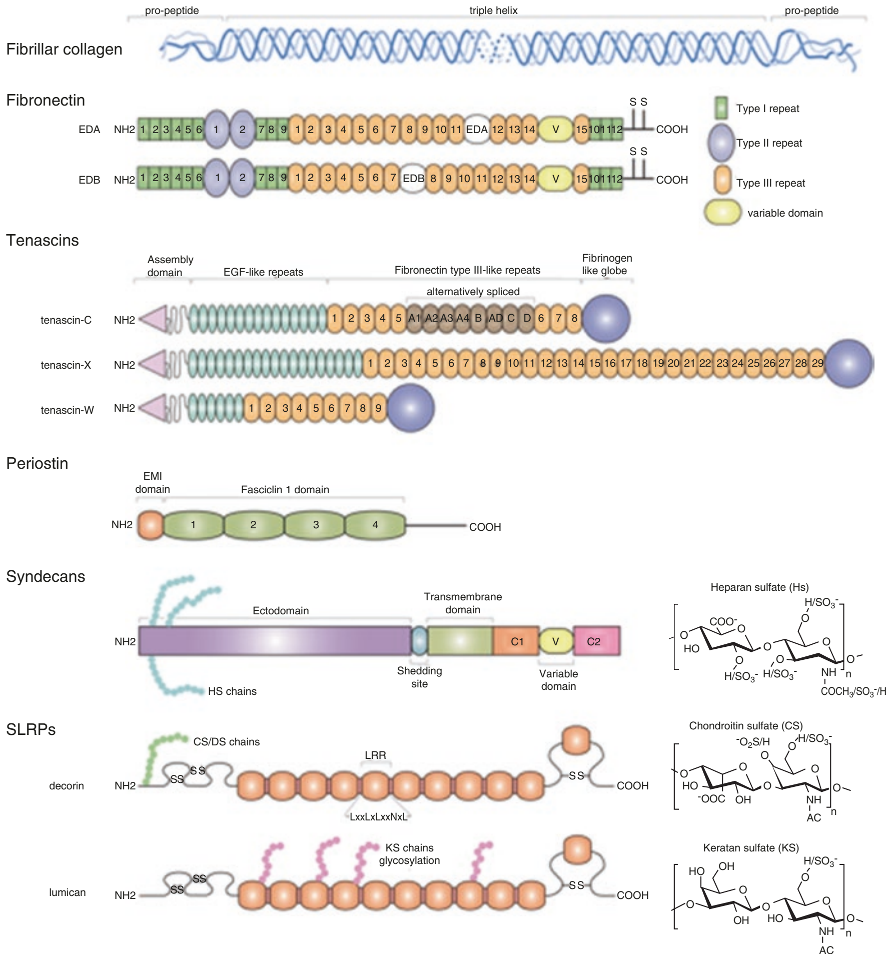

Fig. 4.1 Structure of some major stromal ECM proteins. Fibrillar collagens are composed of three chains that form a triple helix. The pro-peptides are cleaved for collagen assembly into fibrils. Fibronectin presents alternatively spliced domains, EDA, EDB, and the variable domain. Fibronectin dimerizes through two disulfide bonds in the C-terminal part of the protein. Members of the tenascin family display an assembly domain at the N-terminal to form hexamers (tenascin-C) or trimers (tenascin-X). Tenascin-C presents an alternatively spliced region within the fibronectin type III-like repeats. Periostin is composed of an EMI domain and four fasciclin 1 domains. Syndecans is a family of four members that differ by the size of the ectodomain and the variable domain. All syndecans exhibit heparan sulfate (HS) chains, but only syndecan-1 and syndecan-3 have chondroitin sulfate (CS) chains in the ectodomain part close to the transmembrane domain. Small leucine-rich proteoglycans (SLRPs) display a tridimensional "banana" shape structure possible through the presence of two N-terminal and one C-terminal disulfide bonds. SLRPs are composed of leucine-rich repeats (LRR) that contain the LxxLxLxxNxL motif (L, leucine; N, asparagine; x, any amino acid). Decorin could exhibit one chain of chondroitin sulfate (CS) or dermatan sulfate (DS), whereas lumican could exhibit one to four keratan sulfate (KS) chains or polylactosamine chains. Heparan sulfate, chondroitin sulfate, and keratan sulfate chains are composed of repeats of disaccharide units that could be sulfated (SO_3^-) at different locations as indicated in the figure

Due to the critical role of the TME in tumor growth and metastasis, attention must also be given to the role of fibrillar collagens in the tumor stroma. Some of the most provocative studies have addressed the role of collagen composition and processing and posttranslational modifications including cross-linking in regulating stiffness, tumor growth, tumor invasion, and metastasis [38–42].

The ability of fibroblasts to produce and remodel the collagen matrix is in turn affected by interactions with other cell types in the TME such as the tumor cells themselves and different types of inflammatory cells and vascular cells [43]. Cell-mediated collagen remodeling can be mediated by collagen-binding integrins and COLINBRI-binding integrins [34, 44]. The main integrin-collagen receptors for direct binding to the fibril form of fibrillar collagens are α2β1 and α11β1 [45]. They are both efficient in remodeling the collagen matrix, as assessed in floating collagen gel contraction assays [46]. This is a widely used assay to monitor the ability of cells to reorganize a fibrillar collagen I matrix, a process shown to be dependent on β1-integrins [46]. Although *in vitro* experiments have largely failed to demonstrate a direct binding of α1β1 to collagens fibrils, α1β1 has been postulated to bind indirectly to the fibrillar forms of collagens I and III via Fibril Associated Collagens with Interrupted Triple helices (FACIT) collagens [47]. In addition to the direct role of collagen-binding integrins in mechanotransduction to remodel the matrix, a role for matrix metalloproteinases (MMPs) to help and facilitate remodeling of the collagen matrix has also been demonstrated [48, 49]. This aspect is developed in a latter section of this chapter.

In the tumor context, the organization of the collagen matrix has been suggested to serve as an optical biomarker for metastatic propensity [50]. For this purpose, the term "tumor-associated collagen signatures (TACS)" has been introduced: TACS-1 (normal stage), anisotropic, wavy collagen fibrils, similar to normal quiescent tissue; TACS-2 (predisposed stage), prealigned collagen fibrils; TACS-3 (desmoplastic stage), and aligned collagen fibrils [50]. It is important to remember that the tumor ECM is complex, and although collagen might align in specific patterns, cellular interactions during tumor spread might occur via many mechanisms, both collagen-dependent and collagen-independent. As such, the TACS signature may have to be combined with other biomarkers to be clinically useful.

Although there is some tendency to consider the biological effects of all stromal collagens to be equivalent, a recent study suggests that different fibrillar collagens have divergent functions. Whereas collagen I in the tumor stroma, according to the dogma, was considered to be pro-carcinogenic (increased tumor density and stiffness, which promote tumor growth and invasion), another independent study suggests that fibrillar collagen III has opposite effects [51]. In that study using collagen III$^{+/-}$ ($^{-/-}$ mice rarely survive perinatal age), it was demonstrated that mammary carcinomas grown in these mice were larger and more invasive and contained thicker, more organized, linearized, collagen stroma. It is likely that in this model several indirect mechanisms were operative, which need to be elucidated, including characterization of possible changes in integrin repertoire concomitant with collagen ratio switch.

Role of the Stromal Collagens in Tumorigenesis

In the TME, the epithelial-derived carcinoma cells are, to varying degrees, surrounded by basement membrane structures. As cells dedifferentiate and go through epithelial-to-mesenchymal transition (EMT), they are exposed to fibrillar collagens in the tumor stroma. Multiple studies have highlighted the importance of collagens and the matrix metalloproteinases (MMPs) in this process [52–54].

Collagens Affecting Tumor Cell Growth

A number of studies have demonstrated that a collagen matrix promotes tumor growth. In the MMTV-PyMT breast cancer tumor model, crossing the MMTV-PyMT mice with transgenic mice expressing a collagen α1 chain in which the collagenase cleavage site has been mutated resulted in increased breast cancer tumor growth and in increased collagen accumulation at the tumor site [55]. In subsequent experiments, collagen synthesis was blocked by inactivating certain enzyme isoforms, such as the intracellular enzymes prolyl 4-hydroxylase [56] and lysyl hydroxylase [57]. In the stroma, blocking these enzymes resulted in reduced collagen accumulation and reduced collagen stiffness. The result was an attenuation of breast and lung tumor progression and lung metastasis, thus supporting a role of fibrillar collagens in tumor growth. Of note, fibrillar collagens have been shown to induce apoptosis of tumor cells [58]. In one study, MMP-14 was demonstrated to protect invading mammary carcinoma cells from collagen apoptosis once they entered the fibrillar collagen I matrix [59, 60]. Identification of the underlying molecular mechanism for this effect may be a critical step in the further understanding of the role of the TME in tumor progression.

Collagens Affecting Cell Migration

Several studies have also reported MMP-dependent changes in fibril diameter. In one study, MMTV-PyMT mice crossed with mice genetically deficient in MMP-13 demonstrated no effect of MMP-13 depletion on breast tumor progression and lung metastasis [61]. Conversely, another study using a similar model observed a modest increase of lung metastasis in the absence of endogenous MMP-13 activity [62]. In the latter study, monitoring of breast tumors revealed that in the absence of MMP-13, the collagen content was not increased but was comprised of thinner fibrillar collagen fibrils and a different organization of collagen at the tumor-stroma interface [62].

Two interesting explanations from the last study were proposed to explain the effects of the thinner fibrillar collagen structures. First, the normal cleavage of telopeptides from collagen I by MMP-13 may affect lateral fibril growth. Thus, if cleavage is reduced, fibrillar growth would be inhibited [63]. Alternatively, MMP-13 can also cleave collagen III, which acts to regulate fiber diameter, offering another possible mechanism for the observed thinner fibrils in the absence of

MMP-13 [64]. Interestingly, a study of wound healing in zebrafish revealed that increased levels of MMP-9 lead to larger fibril diameter. The authors suggest that this might be due to a switch in synthesis from collagen III to collagen I [65], offering more indications that MMP levels can have unpredictable effects on collagen fibril diameter. Although the effect of MMPs is complex due to multiple targets, the effects on fibril diameter are interesting and warrant further studies in the context of tumor growth and spread. Finally, in a study by Herchenhan et al., lysyl oxidase (LOX) inhibition in artificial tendon cultures also resulted in irregular fibril diameters, suggesting a role for LOX enzymes in regulating fibril diameter [66]. So far, corresponding effects have not been reported in the tumor context, but one might expect similar results in dense tumor matrices. The findings of different collagen fibril diameters might mainly be relevant for tumor cell migration. Previous elegant studies have demonstrated that cells can switch between protease-dependent and protease-independent migration, in 3D matrices, depending on the matrix pore diameter [67, 68].

Collagen Stiffness Regulating Tumor Growth

The stiffness of the tumor stroma has also been recognized as being able to influence tumor growth. Since collagens are major constituents of the tumor stroma, they might also play a major role in this regard. There are different mechanisms that can affect stiffness, including glycation [6]. A landmark paper in this area demonstrated that artificially forced expression of LOX in CAFs in a xenograft breast tumor model increased stiffness of the tumor with increases in β1 integrin/FAK/ERK signaling in tumor cells, resulting in increased tumor growth [4]. It is worth noting that in nonexperimental tumors, LOX is produced by different cell types, not only by CAFs [69]. Moreover, the role of LOX has also received considerable attention in relation to the metastatic niche and tumor metastasis [42, 70, 71]. These studies have demonstrated that LOX is deposited and cross-links the basement membrane collagen IV at future sites of metastasis. In addition to collagens, other important ECM components of the metastatic niche stroma include periostin, fibronectin, EDA, and tenascin-C [72–74].

LOX expression has also been associated with poorer patient prognosis in lung adenocarcinoma [75]. For example, it has been shown that downregulation of LOXL1 in xenograft tumors of non small cell lung cancer lines grown in α11 knockout SCID background reduce tumor growth compared to growth in wild-type SCID mice. The decrease in tumor growth was closely associated with reduced organization and stiffness of fibrillar collagen matrices (Fig. 4.2) [76].

In summary, collagen matrices that are rich in collagen I and comprised of large diameter fibrils seem to be required for optimal support tumor growth and metastasis. Furthermore, stiffer matrices composed of linear fibrils around the tumor can provide routes for invasion. Stromal collagen organization is dependent on (1) CAFs which produce the majority of the matrix, (2) LOX enzymatic activity for matrix cross-linking, and (3) MMPs to facilitate reorganization.

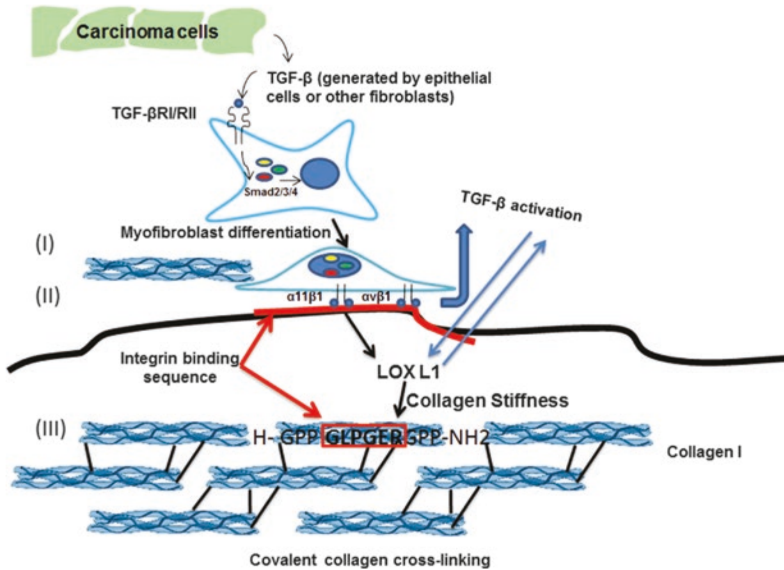

Fig. 4.2 Possible mechanism of tumor cell-stroma interactions in mediating tumorigenicity and metastasis. A number of soluble autocrine and paracrine mechanisms are likely to be involved in directly or indirectly stimulating the growth of the tumor cells. TGF-β plays a major role in tumor-stroma interactions. Excessive TGF-β activity is present in stromal, inflammatory, and cancer cells within a tumor, and the metastatic phenotype can develop when the epithelium overcomes the growth-inhibitory effect of TGF-β. TGF-β signaling induces fibroblast differentiation into contractile myofibroblasts (I). The myofibroblasts express and deposit collagen, express collagen-binding integrins (α11β1) and αv-integrins (αvβ1) that mediate collagen remodeling, and activate latent TGF-β from the matrix (II). Based on microarray differential gene expression analysis, it is possible that LOXL1, a fibrillar collagen cross-linking enzyme belonging to LOX family oxidases (LOXL 1–5), is under the regulation of integrin α11β1. Secreted LOX is responsible for the invasive properties of hypoxic human cancer cells through focal adhesion kinase activity and cell-to-matrix adhesion and is associated with collagen cross-linking and the organization and stiffness of fibrillar collagen matrices (III). MMPs collaborate with LOX to facilitate collagen maturation, and MMPs and LOX regulate the expression and activity of soluble factors such as TGF-β that regulate tumor cell behavior. TGF-β in turn regulates enzymes including LOXs (III), and TGF-β increases levels of factors that evoke inflammation, induce fibrosis, and promote metastasis

Still other experiments using two different experimental model systems that severely restrict production of mouse pancreatic tumor stroma have demonstrated that global obliteration of the stroma can result in tumors becoming more aggressive [20, 21]. One way of interpreting these data is that in desmoplastic pancreatic tumors, the stroma acts as a barrier, the removal of which facilitates tumor cell migration and invasion. In light of these findings, it becomes critical to reconcile the data suggesting that linearized fibrillar collagen acts as a highway for tumor invasion [50, 55] with the multiple studies suggesting that a stiff dense matrix promotes

tumor growth and tumor metastasis [4, 56, 57, 77]. These questions will need to be addressed in order to more fully delineate which pathways involved in collagen biosynthesis, posttranslational modifications, or collagen remodeling represent attractive future therapeutic targets in the tumor stroma.

Methods for Measuring Fibrillar Collagen Stiffness

Structural alterations of the ECM during tumor initiation and progression have been shown to occur in several epithelial tumors [78, 79]. As mentioned earlier, TACS signatures predict that collagen fibers in normal tissue are curly and non-oriented, which is different from the highly linearized fibers of intra-tumoral collagen [4, 76]. The fibrotic reaction observed in the stroma of many cancers, characterized by an excess accumulation of some fibrillar collagens (especially types I, III, V, XI) as a result of desmoplasia, is considered to be a hallmark of cancer [70, 80, 81]. There are multiple collagen receptors in addition to collagen-binding integrins, such as DDRs, leukocyte-associated Ig-like receptors (LAIRs), and glycoprotein VI [82]. These receptors are (1) not necessarily expressed on tumor cells or stroma cells (LAIRs on immune cells, Glycoprotein VI (GPVI) on platelets), and (2) unlike integrins their role as mechanoreceptors with the ability to reorganize collagen has not been established.

Fibrillar collagens can be readily visualized with second harmonic generation (SHG) two-photon confocal microscopy both *in vivo* and *ex vivo* (i.e., histology sections), and its organization can be probed with SHG polarization measurement [83–85]. In SHG, an excitation wavelength of 840 nm is applied to a sample, and the resultant SHG signal is then measured, which is exactly one-half of the excitation wavelength (i.e., 420 nm). SHG polarization microscopy allows the structural details of collagen organization in the tissue to be studied, whereby for each orientation of incoming laser polarization, a set of outgoing SHG polarizations is measured revealing the second-order susceptibility component ratio in each pixel of the image. These measurements reflect the hierarchical organization of collagen in the tissue [86]. The SHG polarization measurement is influenced by several factors, including the amino acid composition and sequence of the collagen triple helix, organization of the triple helices in the collagen fibrils, arrangement of these fibrils in the fibers, and finally fiber orientation with respect to the tissue section plane [85]. In addition, the SHG analysis renders an average fiber orientation in each pixel of the image and provides information on the orientation related to the helical pitch angle of the polypeptide chain of the collagen triple helix in the tissue [87]. Hence, polarization SHG is a promising technique to detect collagen alterations in the ECM during cancer progression [88]. SHG enables pathologists to perform a live biopsy, for example, in the endoscopic setting, or provides a quick histopathology investigation possibility that does not require staining. SHG microscopy presents unique advantages compared to conventional optical techniques to investigate the 3D heterogeneous accumulation of fibrillar collagen during fibrotic pathologies [89]. Another way to analyze the fibril orientation distribution is to measure the degree of waviness or

alignment and orientation of collagen by an Image J plug-in method [90]. In this way the local collagen fiber orientation was derived from the angle of the oriented collagen structure. The shape of the distribution indicated the degree of alignment within the image, where wide and broad shapes suggested little coherence in alignment and tight peaks implied aligned structures. In another study, the collagen fiber arrangement in NSCLC tumor xenografts was measured by a novel relative linearity index [76].

Another method of studying the collagen linearity on a nanometer scale is electron microscopy, measuring how straight or “curly” an individual fiber is [91]. Accordingly, the linearity on this scale would correlate to the stiffness of individual fibers. The advantage of the SHG images is that they show collagen arrangement on a larger scale (the images are 0.5 mm × 0.5 mm), which is indicative of the stiffness or stretchiness of tissue on the micron-to-mm scale.

In a more advanced way, the self-assembly of the native collagen fibrils *in vitro* could be characterized by the use of atomic force microscopy (AFM) [92, 93]. AFM elasticity measurements are a powerful tool to directly assess mechanical stiffness on the level of individual, or groups of, fibers. In fact, AFM can be used as a microdissection tool to study the inner assembly of the collagen fibrils. The AFM technique is based on detection of forces acting between a sharp probe, known as AFM tip, and the sample’s surface [94]. To determine the elastic properties of collagen fibrils, the tip of the AFM (cantilever) was used as a nanoindenter by recording force-displacement curves [95]. It has been shown that a new variant of AFM, which is called *in situ* atomic force indentation microscopy [96], is capable of measuring stiffness changes in mammary gland tissue as it evolves from normal to malignant with exquisite spatial detail. Based on this method, in a mouse model of human breast cancer that metastasizes to the lungs, the extracellular matrix at the tumor boundary turned out to be the stiffest of all the tumor’s components. In this study, AFM was applied to measure the stiffness of the surrounding extracellular matrix as a prognostic indicator for tumor development and aggressiveness [97].

Another technique of interest for measuring ECM and tissue stiffness at the macroscopic level is shear rheology [98]. At its simplest, this approach provides high-resolution determination of the matrix and tissue elasticity by measurements of mechanical compression and nanoindentation [98]. Shear rheology is a commonly applied means of testing the mechanical properties of materials by indenting the test material with a diamond tip while measuring the force-displacement response [98]. Although the techniques described above provide accurate and useful quantitative data on the biomechanical properties of matrix and tissue, most are generally considered invasive and/or destructive methodologies [99]. Hence, there is a need to develop methods to measure elastic properties and stiffness of tissues and matrix in a noninvasive manner for clinical application. Magnetic resonance and ultrasound elastography are routinely used tools in the clinic that provide the image contrast of elastic properties of tissues [100]. Clinical *in vivo* imaging by elastography shows that malignant breast tumors tend to appear stiffer than benign breast tumors; in particular, the stiffer tissue is frequently observed at the tumor margin or the invasive edge of the tumor [100]. Newer technologies based on fluorescence resonance energy transfer (FRET) [101],

magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) [102] are being developed to image the dynamic status of ECM remodeling [103]. Advances in μ-ultrasound, optical coherence tomography (OCT), optical acoustic microscopy, and scanning acoustic microscopy (SAM) [98] are under development to facilitate imaging and quantitative measurement of stiffness at the microscopic scale [104]. In addition, increasing the resolution of many of the above techniques will be possible with improved contrast agents, such as so-called smart probes, which are MRI contrast agents that can be used to study ECM components [105–107]. More information on these techniques is available in other reviews and reference materials.

In summary, new techniques that image the dynamics of cell-ECM interactions to noninvasively quantify remodeling of the ECM at the submillimeter level will ultimately provide additional resources for basic research and in the clinic. Therefore, increased understanding of the molecular basis of mechanotransduction may lead to identification of an entirely new class of molecular targets for anticancer therapy.

Role of Fibronectin EDA in the Tumor Stroma

Fibronectin (FN) is a large modular extracellular matrix protein composed of type I, type II, and type III repeats [108]. FN RNA is alternatively spliced at three conserved regions EIIIA (EDA), EIIB (EDB), and V (CS-1). The FN gene structure and splicing have been described in detail elsewhere [109]. The EDA and EDB domains display 29% sequence identity but are each highly conserved among vertebrates [109]. Whereas a number of receptors have been described for EDA (described later), the cellular receptor(s) for the EDB domain remains largely unknown. Therefore, most of the focus has been on the EDA isoform.

The EDA and EDB isoforms are both highly expressed during embryonic development, especially in developing blood vessels [110], but are almost absent in the adult organism where vascularization and tissue reorganization are quiescent. During wound healing [111] and fibrosis and in solid tumors [112], the EDA/EDB embryonic splice variants are reexpressed [113], leading to the term "oncofetal" splice variants. Some studies suggest that these embryonic splice forms in tumors are mainly expressed in neo-vasculature [114], whereas other studies demonstrated their presence in the fibrotic stroma associated with myofibroblasts [115, 116].

The EDA domain is composed of seven antiparallel beta strands separated by loops [109]. Early studies suggested that the presence of EDA in intact FN indirectly influenced the exposure of the RGD sequence in the 10th FN type III repeat leading to higher binding affinity for integrin α5β1 to FN EDA [117]. In later studies, it was demonstrated that integrins α9β1 and α4β1 bound directly to a cryptic loop region in an EDA-containing fragment but not to the intact FN EDA [118]. Binding of these integrins to the cryptic site would thus require proteolytic cleavage of fibronectin. α4β7 integrin on lung fibroblasts has also been shown to bind directly to FN EDA

[119]. Similarly, Toll-like receptor 4 (TLR4) has been reported to be activated upon binding to the isolated EDA fragment but not upon binding to the intact fibronectin EDA [120]. Importantly, FN EDA enhances TLR4 response, which in turn has been reported to augment TGF-β signaling [121]. α9β1 on basal keratinocytes co-localizes with EDA at the dermal-epidermal junction in skin wounds, but in dermal wounds some dermal fibroblasts also express α9β1 [111]. Endothelial cells on developing and adult lymphatic vessels also express α9β1 [122]. Depending on the relative levels of different receptors, the effect of FN EDA is thus likely to vary.

Upon gross examination, mice deficient in either EDA or EDB appear normal, suggesting a redundancy for these splice forms during development [123, 124]. In contrast, mice lacking both isoforms die at E9–E10, due to cardiovascular defects and leaky blood vessels [125]. Careful analysis of fibronectin EDA$^{-/-}$ mice reveals some mild phenotypes including a mild lymph vessel impairment, due to a transient role for α9β1/fibronectin EDA during lymphangiogenesis [122]. However, other data suggests that Elastin Microfibril Interfacer 1 (EMILIN1) might play a more prominent role than FN EDA as an α9β1 ligand during lymph vessel development, especially in mature lymph vessels [126]. Whereas the expression of FN EDA clearly is a marker for certain biological processes such as wound healing, fibrosis, and a reactive tumor stroma, the exact role of EDA in these events is more complex [43].

Function of Fibronectin EDA Domain in Wound Healing

The role of EDA in wound healing has been studied in great detail. In a much-cited study, an essential role of EDA in TGF-β-stimulated myofibroblast differentiation of rat dermal fibroblasts *in vitro* was determined using neutralizing antibodies [127]. In another study, EDA induced a pro-fibrotic effect in dermal fibroblasts via binding to α4β1-mediated adhesion without affecting myofibroblast differentiation [128]. Similarly, studies of wound healing in an EDA knockout mice failed to detect any major myofibroblast differentiation defects in the granulation tissue, though reduced epithelial migration was observed at the epidermal-dermal border along with some defects in granulation tissue [124, 129]. A role for integrin α9 and EDA in keratinocyte migration was further supported by experiments where α9 was conditionally deleted on keratinocytes, resulting in epithelial thinning [129]. Independent studies using EDA blocking antibodies *in vivo* resulted in mild effects on granulation tissue. The authors of these studies suggest that the less dense granulation tissue observed in these experiments was due to defective migration of dermal fibroblasts into the wounds, rather than defective myofibroblast differentiation [130].

Function of Fibronectin EDA Domain in Fibrosis

A limited number of studies of EDA fibronectin function have been performed in fibrosis models. An *in vitro* study suggests that integrin α4β7 on lung fibroblasts stimulates myofibroblast differentiation [119]. In a mouse model, fibronectin EDA

deficiency prevented bleomycin-induced lung fibrosis [131]. Mechanistic analyses suggested an effect related to TGF-β activation in the lungs in this fibrosis model. Studies of infarcted hearts have also revealed reduced cardiac fibrosis and myofibroblast differentiation in the absence of EDA [132]. Finally, recent data suggests that FN EDA associated with TLR4 may play a role in keloids to couple a fibrotic response in the skin with an inflammatory response [121].

Function of Fibronectin EDA Domain in Tumorigenesis

In the context of tumors, *in vitro* and *in vivo* experiments have suggested different roles for fibronectin EDA. In colon carcinoma, FN EDA sustained tumor cell proliferation and induced lymphangiogenesis through VEGF-C secretion in mouse xenograft models [133, 134]. FN EDA has also been shown to induce EMT in lung and colon carcinomas, thus promoting metastasis [135, 136]. In a radiotherapeutic aspect, the presence of FN EDA reduced radiation sensitivity in head and neck carcinoma by inhibiting apoptosis of tumor cells [137]. Despite these findings, absence of either EDA or EDB did not affect tumor growth, tumor angiogenesis, α-SMA expression in the tumor stroma, or tumor metastasis in either the Rip1-Tag2 tumor model or a xenograft model [110].

In summary, FN EDA is highly expressed in granulation tissue, in fibrotic lesions, and in the tumor stroma. Critical analysis in genetic models demonstrated a moderate effect of FN EDA in wound healing and variable effects on myofibroblast differentiation in fibrosis models. It is thus notable that genetic lack of EDA was without effect in the tested tumor models. FN EDA in some cell models appears to influence myofibroblast differentiation and not in others. This might be related to the source of cells (embryonic origin, cell type, tissue, receptor repertoire expressed by cells). In the limited number of tumor studies performed in mouse models lacking EDA, the splice variant does not seem to be involved in inducing myofibroblast differentiation in the tumor stroma. Conditional deletion of both EDA and EDB forms in the tumor stroma is needed in order to exclude functional redundancy. Finally, the data from wound healing studies suggest that EDA during wound healing is more involved in stimulating recruitment of tissue fibroblasts to the area of fibroblast activation rather than affecting myofibroblast differentiation per se.

Stromal Proteoglycans

Proteoglycans (PGs), abundant at cell surfaces and in the extracellular matrix, belong to a group of glycoproteins in which the core protein is substituted with one or more polysaccharide chains (called glycosaminoglycans). PGs play important roles during different aspects of cancer progression (for review, see [138–140]). Heparan sulfate PGs (HSPGs) execute their function by binding to a variety of molecules including members of several growth factor families, chemokines,

morphogens, serine protease inhibitors, and extracellular matrix proteins [141]. Protein binding is generally mediated by their sulfated GAG chains but may in a few cases involve interaction with core proteins [142]. Examples of proteins that depend on binding to HSPGs for function include members of the FGF family and their corresponding receptors, VEGF, members of the transforming growth factor-β family, Wnt proteins, pleiotrophin, and the serine protease inhibitor antithrombin [143]. Depending on the molecule, the activity of the bound factors is mostly enhanced, although there are few examples of activities that are inhibited by the binding to HSPGs. The morphogen, Wnt, is sequestered by HSPG GAG chains at the cell surface and becomes available for receptor activation only following enzyme-catalyzed release of specific sulfate groups from the heparan sulfate chains [144].

In addition to the direct effect of PGs on growth factor signaling, the HSPG-bound factors are protected from proteolytic degradation and can be released and activated under different physiological or pathological conditions like cancer [145]. Sequestration of chemokines and cytokines plays a critical role in regulating the shape of morphogen gradients and in inducing a signal for cell migration, a first step for invasion and metastasis [146, 147]. The major PGs are subclassified into three groups depending on their localization: intracellular PGs (serglycin), cell surface-associated PGs (syndecans, glypicans), and secreted PGs (hyalectans, small leucine-rich proteoglycans, perlecan) [148]. In this chapter, we focus on the stromal PGs, the most characterized in the tumor context being shed syndecans and small leucine-rich proteins/proteoglycans (SLRPs), and summarize how their presence in tumor stroma influences cancer progression.

Syndecans

Syndecans are transmembrane heparan sulfate PGs with four members in vertebrates, syndecan-1 to syndecan-4. They are involved in diverse biological processes, such as regulating cell adhesion, cell migration, and cell differentiation as well as participating in the organization of ECM and the cytoskeleton [149]. Syndecans can serve as co-receptors on the cell surface and also provide a link between the ECM and the cytoskeleton by directly interacting with the cytoskeleton or via other molecules [150].

One interesting feature of syndecans is the shedding of the extracellular domain that enables syndecans to act as soluble factors [151], which plays an important role in tumorigenesis. The shedding occurs next to the plasma membrane and is processed by different MMPs: MMP-7 is involved in syndecan-1 and syndecan-2 shedding, and MMP-2 and MMP-9 can cleave syndecan-1, syndecan-2, and syndecan-4, whereas MMP-14 can cleave syndecan-1 and syndecan-4 [152–154]. The shedding is regulated by different growth factors and cytokines present in the tumor microenvironment, such as FGF-2 and TNF-α [155, 156]. In addition, heparanase, an enzyme that cleaves the heparan sulfate chains, regulates syndecan-1 expression and promotes syndecan-1 shedding, resulting in increased myeloma tumor growth [157].

In general, shed syndecans promote tumor progression, and it was described earlier that highly soluble syndecan-1 was associated with poor outcome in non-small cell lung cancer [158]. This correlation was also observed in myeloma and bladder carcinoma [159, 160]. In breast carcinoma, shedding of syndecan-1 from CAFs stimulates tumor cell proliferation via FGF-2, shed syndecan-1 thus serving as a paracrine mediator [161, 162]. However, another study demonstrated an inhibitory effect of shed syndecan-1 on breast adenocarcinoma cell proliferation [163]. The study interestingly suggested the duality of membrane-bound and membrane-soluble syndecan-1. In a study by Nikolova et al., transmembrane syndecan-1 promoted cell proliferation and inhibited invasion, whereas shed syndecan-1 inhibited proliferation but increased invasiveness, suggesting that both syndecan forms contributed to breast cancer progression but at different stages [163]. More recently, shed syndecan-2 has been shown to contribute to colorectal tumor growth and metastasis by upregulating MMP-7, suggesting a positive regulatory loop between these two proteins [164].

Another study suggests that shed syndecan-1 translocates to the nucleus of tumor cells, indicating that syndecan-1 may deliver growth factors (e.g., HGF) to the nucleus, and also downregulates histone acetylation, leading to increased gene transcription [165]. This mechanism is suggested to involve endocytosis of syndecan-1 growth factor complex from the cell surface and transport to the nucleus, but the exact mechanism of nuclear import has not been elucidated.

It has been reported that chemotherapeutic drugs, used in myeloma treatment, stimulate the shedding of syndecan-1 thus contributing to increased tumor growth [166]. Additionally, shed syndecan-1 contributes to chemotherapy resistance in colon cancer [167]. Targeting shed syndecans could be an effective strategy to control cancer progression; however better understanding of the molecular mechanisms of action is needed in order to avoid any potential adverse side effects.

Small Leucine-Rich Proteoglycans

Small leucine-rich proteoglycans (SLRPs) are extracellular matrix proteins rich in leucine-rich repeats, conferring a "banana" shape structure with a concave face involved in protein-protein interactions. Most SLRPs bind to fibrillar collagen and regulate collagen fibrillogenesis and matrix assembly [168]. Among the many biological processes regulated by SLRPs, tumor growth is one of the most well studied. The SLRP family encompasses 18 members, grouped into five classes (I–V) [148]. In this section, we will focus on the role of four SLRPs from classes I and II in tumor progression.

Decorin is a chondroitin/dermatan sulfate SLRP that is expressed in several tissues. Although one study associated high expression of decorin with metastasis and poor survival in breast cancer [169], decorin is often described as having antitumor properties, as listed below. Decorin expression is downregulated in bladder cancer [170], prostate cancer [171], lung cancer [172], and breast cancer

[173, 174] where a reduced expression is associated with poor survival [175]. Consistent with these observations, liver carcinogenesis was promoted in decorin-null mice [176]. Moreover, overexpression of decorin was shown to inhibit metastasis of prostate cancer [177], inhibit proliferation of bladder tumor cells [170], and inhibit colorectal carcinoma cell growth and migration [178, 179]. Systemic injection of decorin in MDA-231 triple-negative breast carcinoma xenografts induced expression of cellular adhesion molecules and promoted tumor suppressor genes, whereas inflammatory and immune response genes were downregulated [180].

From a mechanistic point of view, decorin can affect tumor progression via its interaction with tyrosine kinase receptors. It has been demonstrated that decorin can bind to the EGF receptor and mediate internalization and degradation of the receptor and induce expression of $p21^{WAF}$, an inhibitor of the cell cycle and apoptosis [181]. Decorin can also antagonize Met, a receptor for hepatocyte growth factor, via degradation of β-catenin leading to reduced cell migration and invasion [182]. The decorin/Met axis appears to be required for the induction of an oncostatic mitochondrial protein, mitostatin [183]. In addition, decorin has been shown to bind and antagonize VEGFR2, inhibiting angiogenesis through endothelial cell autophagy [184, 185], and to bind IGF-IR to inhibit tumor cell migration and invasion [186].

Based on these observations, decorin is considered as a promising therapeutic protein in cancer progression treatment [177]. However, similar to syndecan-1, decorin has also been reported to induce resistance to some chemotherapeutics [187, 188].

Biglycan, like decorin, is a chondroitin/dermatan sulfate proteoglycan, which belongs to the class I of SLRPs. Available data indicates that high expression levels of biglycan correlate with poor prognosis in pancreatic adenocarcinoma and esophageal carcinoma [189, 190]. Moreover, biglycan was shown to promote migration and invasion of gastric carcinoma through FAK signaling activation [191]. However, biglycan also displays antitumor activity, inhibiting bladder carcinoma and pancreatic carcinoma cell proliferation [192, 193].

Lumican is expressed as keratan sulfate PG in the cornea but exists as a glycoprotein substituted by non- or low-sulfated polylactosamine chains in other tissues [194]. In tumor tissues, lumican is often overexpressed by stromal cells and/or tumor cells, and the correlation of its expression to malignancy is complex [195, 196]. In advanced colorectal cancer, Seya et al. have shown that lumican expression in tumor cells is associated with poor survival [197], whereas de Wit et al. have described a correlation with good survival in stage II patients [198]. In breast cancer, lumican expression was found to decrease with the progression of disease [199]. Consistent with this observation, high expression of lumican is associated with good survival in invasive stages of breast cancer [175]. In pancreatic cancer, patient outcome is dependent on the type of cells expressing lumican. Expression in tumor cells is associated with longer survival, whereas expression in pancreatic stromal cells is associated with poor outcome [200]. However, a recent study showed that lumican expression in pancreatic stroma was only correlated with good survival

after surgery [201]. This correlation is also observed in lung adenocarcinoma patients, where patients with stromal lumican-positive tumors had longer survival than those expressing lumican in tumor cells [202]. We suggest that these differences could be related to the secretion of different glycosylated forms of lumican in different cellular contexts.

The antitumor properties of lumican have mainly been reported in melanoma, where lumican is expressed in the peritumoral stroma [203] and is suggested to serve as a biological barrier, controlling melanoma invasion. Lumican was shown to inhibit melanoma cell progression via interaction with α2β1 integrin and altering composition of focal adhesion complexes [204–206]. More recently, lumican was defined as a new inhibitor of MT1-MMP in melanoma cells, thus inhibiting tumor environment proteolysis and invasiveness [207]. Antitumorigenic activities of lumican were also found in prostate cancer [208], in colon cancer by affecting tumor cell migration through upregulation of gelsolin [209], and in pancreatic cancer, in which lumican reduced EGF receptor expression resulting in reduced Akt signaling and tumor cell growth inhibition [201].

Fibromodulin, like lumican, is a keratan sulfate SLRP that belongs to class II and is expressed in dense regular connective tissues. Although fibromodulin expression has been described in some types of cancer, its role has been poorly investigated. Oldberg et al. have shown that in experimental carcinomas, fibromodulin promotes the formation of a dense collagen matrix through the regulation of fibril diameter, leading to an increased interstitial fluid pressure (IFP), with possible adverse consequences for delivery of chemotherapeutics [210]. It is interesting to remember that other SLRPs also modulate collagen fibrillogenesis and could be thus involved in IFP regulation in different types of cancers, despite their antitumorigenic properties.

SLRPs also function to sequester TGF-β [211], a growth factor already described in this chapter, involved in EMT and fibroblast activation. A work by Maris et al. demonstrates that asporin, a member of the class I SLRPs, inhibits TGF-β activity resulting in reduced breast cancer growth and metastasis in Nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice [212]. Interestingly, asporin expression is induced by TGF-β, thus asporin and TGF-β appear to regulate each other in an intricate feedback loop.

Matricellular Proteins: Tenascins and Periostin

Matricellular proteins are secreted macromolecules that do not play a primary role in matrix structure but are able to modulate cell interactions and functions [213]. In cancer, matricellular proteins are involved in different steps of tumorigenesis due to their ability to bind different cell receptors [214]. The matricellular protein family includes thrombospondins, tenascins, SPARC, periostin, osteopontin, and CCN proteins. In this chapter, we focus on the role of tenascins and periostin in cancer progression.

Tenascins

The tenascin family is composed of four members in vertebrates, expressed in different tissues with a common role in modulation of cell adhesion and spreading [215]. Although all tenascin isoforms are expressed in different cancer forms, tenascin-C has been studied the most.

Tenascin-C is absent or lowly expressed in adult tissues, in contrast to the strong expression observed in cancer. Tenascin-C expression is induced in several solid tumors and is often associated with poor prognosis (for review, see [216]). It is clear that tenascin-C promotes tumorigenesis, acting at different steps of this process. On one hand, tenascin-C stimulates tumor growth by abolishing the cell proliferation-suppressing effect of fibronectin [217, 218]. Tenascin-C has also been demonstrated to compete with fibronectin for syndecan-4 binding, thus weakening breast carcinoma cell adhesion and spreading on fibronectin [219]; this cell adhesion inhibition leads to cell rounding that enhances tumor cell proliferation. On the other hand, tenascin-C can reduce apoptosis of pancreatic cancer cells, by activating the antiapoptotic Bcl-2 and Bcl-xL and inhibiting cleavage of caspase-3 [220].

Tenascin-C also stimulates EMT of breast cancer cells, in an αvβ1- and αvβ6-dependent manner [221, 222]. The Wnt/β-catenin signaling pathway, which is known to induce EMT [223], is enhanced in the presence of tenascin-C via the downregulation of the Wnt inhibitor Dickkopf 1, which stabilizes β-catenin [218, 224]. It is interesting to note that the tenascin-C gene was identified as a β-catenin signaling target in colorectal cancer, suggesting a feed-forward loop that could stabilize the EMT phenotype and influence invasion in this tumor type [225]. Furthermore, tenascin-C plays a role in tumor cell migration and invasion [226, 227]. In a study of invasive melanoma, tenascin-C was found to form, in addition to fibronectin and collagen I, tubular structures that were proposed to serve as channels for melanoma cell invasion [228]. Interestingly, tenascin-C can also upregulate MMP-9 and MMP-13 expression in breast cancer, thus promoting cancer cell invasion [229, 230]. Knockdown of tenascin-C in the MDA-MB-435 melanoma cell line decreased the number of lung metastasis in nude mice, demonstrating that tenascin-C may stimulate metastatic progression [231]. A more recent publication demonstrated that in lung metastatic sites, tenascin-C is overexpressed by S100A4^{+} stromal cells, most likely fibroblasts, supporting metastatic colonization [232]. In the same study, tenascin-C-null mice injected with 4T1 murine breast cancer cells displayed fewer and smaller metastatic lung nodules [232]. Another interesting study initiated by Oskarsson et al. showed that tenascin-C secretion by breast cancer cells is required to form a metastatic niche for the establishment of lung metastases [73].

Tenascin-W has been the last tenascin member to be described, and relatively little is known about this tenascin family member. Expression of tenascin-W has been shown to be regulated by TGF-β [233] and was initially observed to be strongly upregulated in the tumor stroma of breast and colon cancer patients [234, 235]. In the context of breast cancer, tenascin-W has been shown to promote

migration of breast tumor cells through interaction with α8β1 integrin [236]. In later studies, Brellier et al. determined that tenascin-W expression was also induced in melanoma and in pancreatic, kidney, and lung carcinomas; the authors suggested that tenascin-W might be a useful cancer biomarker in several solid tumors [237].

Tenascin-X is expressed in several tissues, with high expression in skin and skeletal muscle [238]. Deficiency or mutation in tenascin-X gene leads to a form of Ehlers-Danlos syndrome, characterized by skin and joint hyperextensibility [239]. In contrast with other tenascins, tenascin-X was first predicted to be antitumorigenic: its expression was strongly decreased in malignant melanoma [240], and mice deficient in tenascin-X displayed increased melanoma invasion and metastasis [241]. This was explained by an induction of MMPs, including MMP-2, in the absence of tenascin-X through JNK signaling, indicating a role of this tenascin in matrix proteolysis regulation [242]. Alcaraz et al. have suggested a different role of tenascin-X in breast cancer progression. In their study, tenascin-X was suggested to contribute to TGF-β activation via its interaction with α11β1 integrin, thus promoting EMT [243]. It will be interesting to determine if the binding of tenascin to α11β1 is direct, and if so, which part of integrin α11β11 binds to tenascin-X.

Periostin

Periostin is a matricellular protein, which is highly expressed in mesenchymal tissues during development [244]. Genetic deletion of periostin leads to tooth defects and a periodontal-like disease, which result in dwarfism [244]. Wound healing studies suggest a promoting effect of periostin in dermal myofibroblast differentiation and collagen gel contraction [245]. A pro-fibrogenic role for periostin in cardiac and skeletal muscle fibrosis has also been reported [246, 247]. Interestingly, periostin has been observed to interact with fibrillar collagen, and in the absence of periostin the collagen fibrillar diameter increases [248, 249].

In the tumor context, an early study reported reduced numbers of activated CAFs and less collagen in capsule and TME, leading to increased growth of grafted mouse tumor cell lines in postn$^{-/-}$ mice [250]. Later studies have focused on the presence of periostin in the tumor stroma of gastric cancer, melanoma, and glioblastoma and in metastatic niches [251–253]. In one study, the ability of periostin to bind Wnt was suggested to be the mechanism underlying the ability of periostin to support cancer stem cell maintenance and tumor metastasis [74, 254].

In summary, proteoglycans and matricellular proteins show different effects on tumorigenesis, sometimes with opposite effects in different tumor types. Figure 4.3 and Table 4.1 summarizes the role of stromal proteins in tumorigenesis and the experiments we have mentioned in the text, respectively.

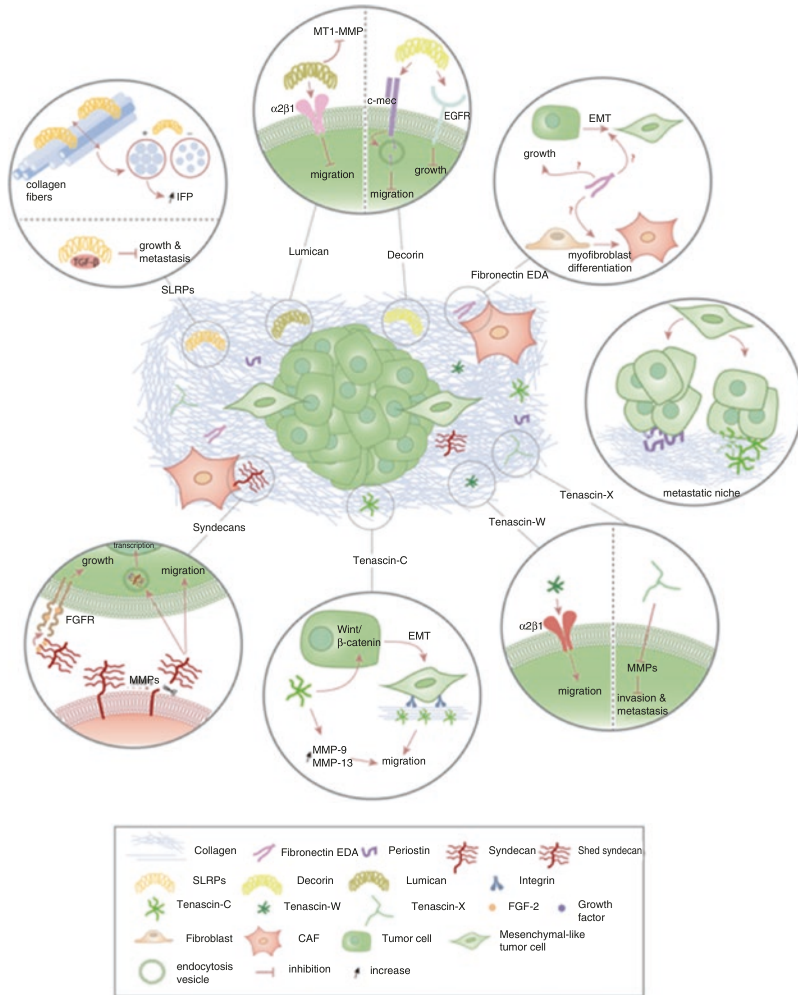

Fig. 4.3 Role of stromal ECM proteins in tumorigenesis. (**a**) SLRPs have a major function to regulate collagen fibrillogenesis. As shown for fibromodulin, SLRPs could increase thickness of collagen fibers resulting in increased interstitial fluid pressure (IFP). Some SLRPs such as asporin have the ability to bind and sequester TGF-β resulting in cancer growth and metastasis inhibition. (**b**) Lumican has been shown to inhibit cancer cell migration by interaction through α2β1 integrin and by inhibiting MT1-MMP. Decorin has been shown to interact with tyrosine kinase receptors. Binding to c-met leads to internalization of the receptor and inhibition of cancer cell migration. Binding to EGF receptor (EGFR) leads to cell cycle inhibition. (**c**) The role of EDA fibronectin is uncertain; it has been shown to mediate EMT and to stimulate cancer growth; however, absence of EDA does not seem to affect tumorigenesis. (**d**) Syndecans at the cell surface of CAFs can be shed by MMPs to induce its effect on cancer cells. Syndecans as a cofactor for FGF receptor (FGFR) stimulate tumor growth by delivering FGF-2. Syndecan-1 could be endocytosed to deliver growth factors into the nucleus leading to increased gene transcription. (**e**) Tenascin-C has been shown to contribute to EMT by stabilizing β-catenin. Tenascin-C also mediates cancer cell migration through upregulation of MMPs and by forming tubular structures with collagen and fibronectin. (**f**) Tenascin-W also mediates cancer cell migration but via interaction with integrins. In contrast, tenascin-X reduces invasion and metastasis by inhibiting MMPs. (**g**) Periostin and tenascin-C are needed in metastatic niches to support the metastatic colonization

Table 4.1 Role of some stromal ECM proteins for tumorigenesis based on experimental models

ECM protein	Knockout phenotype mice	Potential ECM receptor in tumor stroma	Localization in tumors	Effects in tumor context	Type of model
Fibrillar collagens					
Collagen I	Embryonic lethal, severe structural defects in connective tissues [255–257]	α2β1, α11β1	Stroma	Barrier Highway for metastasis	MMTV-PyMT crossed with Col1a1^{tmJae} mice [259] mammary tumors [55] Prolyly-4-hydroxylase alpha subunit-2 knockdown xenograft
Collagen III	Perinatal, lethal [258]	α2β1, α11β1	Stroma	Restrict tumor growth [51]	Mammary tumors [56] 4T1 xenografts in Col III$^{+/-}$ mice [51]
COLINBRIs:					
Fibronectin EDA	Normal Defective lymph vessels [122, 125]	α5β1 α4β1 α9β1 TLR-2/4	Biomarker for myofibroblasts in stroma [125]	No effect in Rip1-Tag2 model	Rip1-Tag2 model pancreas cancer, B16 melanoma xenograft FN EDA$^{-/-}$ mice [125]
Periostin	Tooth eruption defect [260]	αv-integrins (αvβ3, αvβ5)	Stroma	Reduced glioblastoma growth, reduced breast cancer metastasis to lungs, retain decorin, concentrate Wnt in stem cell niches [74, 253, 261, 262]	Xenograft glioblastoma stem cells with knockdown of periostin [253], PyMT breast cancer model with periostin$^{-/-}$ [254]

(continued)

Table 4.1 (continued)

ECM protein	Knockout phenotype mice	Potential ECM receptor in tumor stroma	Localization in tumors	Effects in tumor context	Type of model
Proteoglycans:					
SLRPs:	Collagen organization				
Decorin	Skin fragility [263]	Tyrosine kinase receptor [266]	Stroma	Promotes tumor suppressor genes and cellular adhesion molecules	Systemic injection in MDA-231 breast carcinoma xenograft [180]
Lumican	Skin fragility, cornea opacity [264]	α2β1 [205]	Stroma and tumor cells	Inhibited melanoma growth and invasion	Lumican-transfected B16 melanoma xenograft [271]
Syndecan-1	Normal [265]	Cooperate with integrins [267–269]	Stroma and tumor cells	-Shedding [162], increased angiogenesis [162, 270], reduced myeloma growth	Xenograft model CAG myeloma knockdown syndecan-1 [272]
Matricellular proteins:					
Tenascin-C	Viable, subtle defects hair follicles [273, 274]	Integrin ligand [276] and steric hindrance of integrin-mediated adhesion	Stroma	Important for metastatic niche function [74]	Xenografts of mammary carcinoma MDA231 and CN34 with knockdown tenascin-C [74]
Tenascin-W	ND	αvβ1, α4β1 [234]	Biomarker-activated perivascular stroma in solid tumors [237]	ND	ND
Tenascin-X	A form of Ehlers-Danlos syndrome [275]	αvβ3	Stroma	Restrict melanoma invasion and metastasis	Xenograft B16 melanoma knockdown tenascin-X [241]

Summary

The tumor stroma is complex and dynamic during tumor growth and contains an ECM with changing composition. The exact function of the tumor stroma varies with the tumor stage, and it will be important to better elucidate the function of ECM molecules at different stages of tumor growth and metastasis. To determine if the tumor stroma acts as a fertile soil, providing a supportive ECM network rich in blood vessels, or if it acts as a stiff barrier, we have to consider additional components of the stroma. In this review, we have highlighted some aspects ascribed to the insoluble ECM of the stroma, but additional consideration of the integrated roles of the immune system, paracrine signaling, and above all, inter-tumoral and intra-tumoral heterogeneity is necessary in order to fully address the central questions: Tumor stroma, friend or foe? Barrier or support?

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Chapter 5
Stromal PDGF Receptors as Prognostic and Predictive Biomarkers

Arne Östman

Abstract The PDGF family of growth factors exerts important regulatory functions on mesenchymal cells such as fibroblasts, vascular smooth muscle cells, and pericytes. Through activation of the tyrosine kinase PDGF alpha- and beta-receptors, these growth factors stimulate proliferation and migration of target cells and regulate their contractile capacity. PDGF receptors play major roles during development in settings of paracrine interactions between mesenchymal cells and epithelial or endothelial cells.

The focus of this review is a discussion of experimental and correlative studies which have explored the biological mechanisms and clinical significance of PDGF receptors in mesenchymal cells of the tumor microenvironment. Collectively these studies identify the PDGF system as critical regulators of tumor growth, metastasis, and drug efficacy.

Continued efforts in this field have the potential to advance stromal PDGF receptors to a state of clinically implemented biomarkers and therapeutic targets in selected patient populations.

Keywords PDGF • PDGF receptors • Cancer • Prognosis • Tumor microenvironment • Biomarker • Fibroblasts • Pericytes • Tumor stroma • Response prediction

Introduction

Platelet-derived growth factor (PDGF) is a family of growth factors exerting important regulatory functions on glial cells and mesenchymal cells such as fibroblasts, vascular smooth muscle cells, and pericytes.

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Early cancer-related studies on PDGF family members focused on the role of autocrine oncogenic PDGF receptor signaling, based on the discovery that one of the classical retroviral oncogenes, v-sis, encoded a variant of the PDGF B-chain [1]. These studies led to identification of rare malignancies where PDGF receptors indeed act as oncogenic drivers, some of which are now also treated with PDGF receptor-blocking tyrosine kinase inhibitors such as imatinib (reviewed in [2, 3]).

During the last 10–15 years, these studies have been paralleled by experimental and correlative studies which have analyzed the potential impact of PDGF receptor signaling in mesenchymal cells of the tumor microenvironment, such as fibroblasts and pericytes. This chapter aims at summarizing findings from these studies with separate discussions of the experimental studies (see the section "PDGF Receptor Status and Prognosis") and the studies dominated by analyses of clinical cohorts (see the section "Stromal PDGF Receptors and Response tTo Treatment" and "Future Perspectives").

These core parts of the text are preceded by brief introductions to the molecular biology (see the section "Developmental and Physiological Roles of PDGF") and the developmental and physiological roles (see the section "Tumor Phenotypes Controlled by Stromal PDGF Receptors") of the PDGF system. More detailed discussions of these subtopics of PDGF biology have been summarized in other reviews [4–6].

Molecular Cell Biology of the PDGF System

PDGFs and Their Receptors

The family of PDGFs is composed of five disulfide-linked dimeric growth factors: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD (Fig. 5.1). PDGF-A-, PDGF-B-, PDGF-C-, and PDGF-D-chains are encoded by different genes subjected to different regulatory mechanisms [5]. The PDGF A-chain gene encodes two splice variants differing in their C-terminal [7]. The mature dimeric isoforms all have a conserved receptor-binding core domain and two intra-chain disulfide bridges [8, 9].

PDGF-BB, and PDGF-AA dimers composed of the longer splice variant, includes a basic C-terminal retention signal mediating binding to cell surface and pericellular extracellular matrix and proteoglycans which restricts signaling to neighboring cells [10, 11]. PDGF-CC and PDGF-DD are both secreted as latent proteins which are activated after proteolytic cleavage of a regulatory N-terminal CUB domain [8].

The five PDGF ligands exert their biological effects through two structurally related tyrosine kinase receptors: PDGF alpha-receptors (PDGF-αR) and PDGF

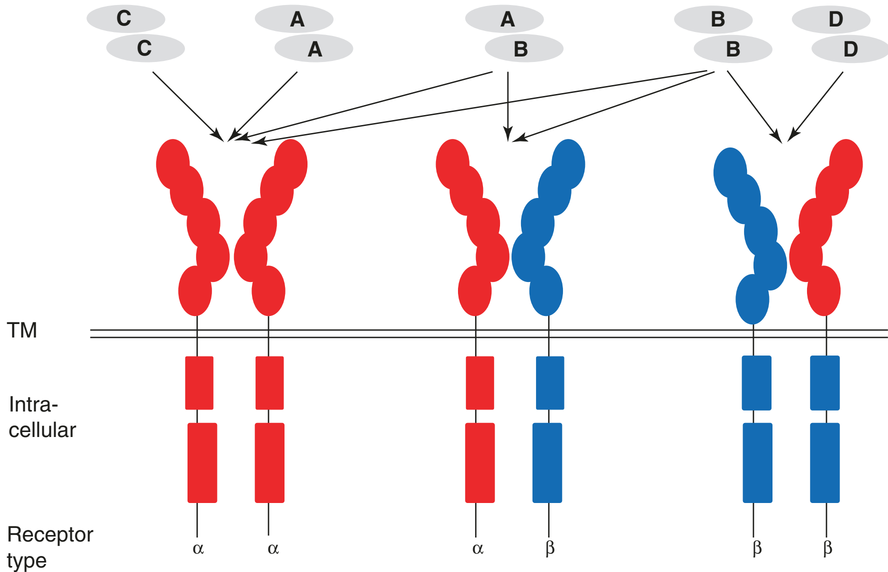

Fig. 5.1 Schematic illustration of binding specificities of the five PDGF isoforms and the structural organization of the PDGF receptors

beta-receptors (PDGF-βR) (Fig. 5.1). Both these receptors are composed of an extracellular region with five Ig-like domains, a single transmembrane segment and an intracellular part with a split tyrosine kinase domain. Whereas the intracellular parts of the two receptors show a high degree of conservation, larger differences are observed in the extracellular part involved in ligand binding [8].

The different ligands vary in their receptor-binding profiles. Based on cell culture experiments, using purified ligand isoforms, it has been concluded that PDGF-AA, PDGF-AB, PDGF-BB, and PDGF-CC bind PDGF-αR and that PDGF-BB and PDGF-DD are high-affinity ligands for PDGF-βR [4, 8] (Fig. 5.1).

Cellular Responses, Receptor Activation, and Molecular Signaling Induced by PDGFs

Typical responses to PDGF stimulation in tissue culture settings include proliferation, directed migration, and contraction of collagen gels. The molecular basis for these cellular responses has been extensively characterized [4, 5].

Ligand-induced receptor dimerization is the crucial event in receptor activation. Receptor dimerization occurs through binding of one dimeric ligand to Ig domains 1–3 of two receptor molecules. Receptor dimers are also stabilized by Ig-domain-4-mediated

receptor-receptor interactions. The ligand-induced receptor dimerization triggers autophosphorylation of tyrosine residues, which act as docking sites for SH2-domain-containing signaling molecules. These include adaptor proteins such as the p85 subunit of PI3K and the Ras-activating Grb2, as well as proteins with intrinsic enzymatic activity like c-Src and PLC-gamma. A third class of SH2-domain-containing proteins activated by PDGF receptors is the STAT transcription factor family. Mechanisms for negative regulation of PDGF receptor signaling include ligand-induced receptor internalization promoted by ubiquitination and dephosphorylation by tyrosine phosphatases.

Detailed cell biology studies indicate that different PDGFR dimers display differences with regard to their signaling activity. However, the physiological significance of these findings remains largely unknown. Genetic studies in mice have demonstrated normal phenotypes of mice expressing chimeric receptors composed of the extracellular domain of the PDGF-αR and the intracellular domain of PDGF-βR [12].

Developmental and Physiological Roles of PDGF

The roles of the PDGF system in development have been extensively studied in mouse models where the effects of knocking out different PDGF ligands and receptors have been analyzed (reviewed in [5]). Collectively these studies have identified PDGFRs as important regulators of mesenchymal cells involved in cross talk with adjacent epithelial or endothelial cells. A theme emerging from these studies is a pattern of paracrine signaling with ligands produced by the endothelial/epithelial cells that stimulate recruitment and proliferation of the PDGF receptor-expressing mesenchymal cells.

Developmental Roles of PDGF-αR

Organs with a particular PDGF-αR dependency include the lungs and the GI tract. These and other developmental roles of PDGF-αR have been extensively reviewed [5].

Knockout of PDGF-A leads to a failure of mesenchymal cell spreading into the walls of alveolar saccules of the lung and an emphysema-like phenotype [13]. Conversely, overexpression of PDGF-αR ligands results in perinatal death associated with lungs displaying a thickened mesenchyme compatible with hyperproliferation of alveolar smooth muscle cell progenitors [14]. Paracrine PDGF-A/PDGF-αR signaling also controls formation of gastrointestinal villi [15]. PDGF-A knockout mice display misshapen gastrointestinal villi. This phenotype has been concluded to reflect a critical role for PDGF-αR -signaling in the renewal and migration of mesenchymal cells that normally line the basement membrane and control villi formation. Furthermore, PDGF-αR knockout mice also display hypoplasia in the mesenchymal compartment of the skin and kidney.

Other independent studies relying on overexpression of hyperactive variants of PDGF-αR have analyzed the roles of PDGF-αR in mesenchymal precursor cells with perivascular location. Notably, PDGF-αR hyperstimulation prevented adipogenic differentiation and instead promoted development toward a pro-fibrotic ECM secretory phenotype [16].

Together these findings suggest an important role of PDGF-αR signaling for maintenance and function of mesenchymal cells involved in epithelial instructive functions during organ development.

Outside the context of mesenchymal/epithelial interactions, PDGF-αR/PDGF-A has also been shown to participate in CNS development by regulation of oligodendrocytes and astrocytes.

Developmental Roles of PDGF-βR

Analyses of PDGF-βR and PDGF-B knockout mice have identified this ligand-receptor pair as key molecules in recruitment and function of vascular mural cells [5]. Deficiency of these genes is associated with reduced pericyte coverage, endothelial hyperplasia, and abnormally variable capillary diameter [17, 18]. Defects in the formation of kidney glomeruli, including failure in recruitment of mesangial cells and an accompanying defect in capillary branching, also illustrate the importance of PDGF-βR for proper regulatory functions of perivascular cells [19]. Both in the case of angiogenesis and glomeruli formation, endothelial cells are the main source of PDGF-BB whereas the receptor is expressed on the mural cells.

The importance of the PDGF-B/PDGF-βR axis for vascular function has also been supported by human genetics data. This occurred through identifications of loss-of-function mutations of PDGF-B and PDGF-βR in familial idiopathic ganglia calcification and the concomitant demonstration of vascular defects in the etiology of the disease (reviewed in [20]).

Physiological Roles of PDGFs

Roles for PDGFs in wound healing have been implied since the original purification of PDGF from platelets and the associated demonstration of potent mitogenic and chemotactic effects on fibroblasts. These notions have been substantiated by numerous model system studies and clinically validated in phase III studies which demonstrated increased wound closure in chronic diabetic neuropathic ulcers by recombinant PDGF-BB [21]. PDGF-βR signaling in dermal fibroblasts has also been shown to counteract edema formation. The underlying mechanism involves effects of PDGF-βR-regulated fibroblasts on interstitial fluid pressure which, in turn, involves integrin-mediated interactions with extracellular matrix [22].

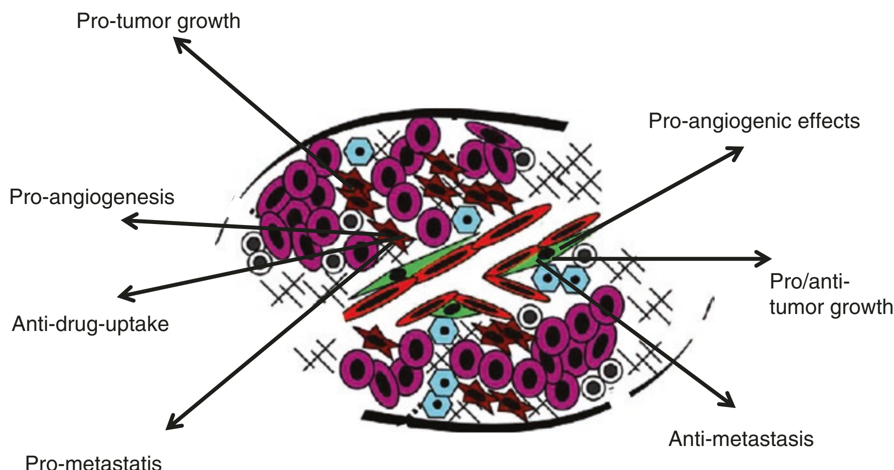

Fig. 5.2 Schematic illustration of tumor biology processes regulated by PDGFR-positive fibroblasts (*brown star-shaped cells*) and perivascular cells (*green elongated cells*) (modified from Pietras and Ostman, Exp Cell Res, 2010)

Tumor Phenotypes Controlled by Stromal PDGF Receptors

As outlined above, studies in cell and developmental biology have identified PDGF receptors as important regulators of fibroblasts and pericytes. These findings have prompted a number of experimental studies exploring how PDGF signaling in these cells impact on different aspects of tumor biology. As detailed in the following paragraphs, these experimental studies indicate that PDGF receptor signaling in fibroblasts and pericytes can stimulate primary tumor growth and metastasis and also negatively regulate uptake and efficacy of systemically delivered drugs (Fig. 5.2).

PDGF Receptors in Fibroblasts

The stimulatory effects of stromal PDGF receptors on tumor growth were first demonstrated in experiments where melanoma cells, lacking PDGF receptors, showed increased tumor formation upon overexpression of PDGF-BB [23]. Although these initial studies did not provide detailed mechanistic information, it was concluded that the growth advantage was related to increased angiogenesis and recruitment of tumor-supportive fibroblasts.

Studies using similar experimental approaches, with overexpression of PDGF ligands in receptor-negative cells, demonstrated the stimulatory effects of different PDGF ligands in models of, e.g., skin, breast, and lung cancer types, where increased PDGF-dependent recruitment of fibroblasts was implied as the underlying mechanism [24–28]. Further support for this concept has also been obtained in studies

where pharmacological inhibitors of PDGF receptor signaling displayed therapeutic effects in a genetic mouse model of skin cancer [29].

Pro-metastatic effects of PDGF-activated fibroblasts have been observed in different animal models. The PDGF inhibitor imatinib significantly reduced metastasis in an orthotopic model of colorectal cancer, with PDGF receptor expression restricted to the tumor stroma, which occurred in the absence of major effects on primary tumor growth [30]. Furthermore, tissue culture studies have demonstrated that PDGF-BB stimulation of fibroblasts enhances their ability to stimulate, in a paracrine manner, colorectal cancer cell migration and invasion [31]. PDGF-induced secretion of stanniocalcin 1 (STC1) was identified as a critical component of this paracrine pathway. Animal studies provided independent evidence for pro-metastatic effects of fibroblast-derived STC1.

Important roles for stromal PDGF receptors in determining drug efficacy have also been postulated by findings from mouse cancer model studies. These studies were prompted by initial observations in edema models, referred to above, which demonstrated an ability of PDGF-βR signaling in fibroblasts to increase interstitial fluid pressure (IFP) and thereby reduce edema (reviewed in [22]). These findings prompted studies which tested the hypothesis that increased tumor IFP, associated with reduced tumor drug uptake, could be overcome by targeting of stromal PDGF receptors. A series of studies, using different combinations of tumor models and PDGF inhibitors collectively provided strong evidence that blocking of stromal PDGF receptors indeed reduced tumor IFP, increased tumor drug uptake and enhanced the therapeutic efficacy of systemically delivered drugs [32–35]. Notably, the efficacy-enhancing effect of PDGF inhibitors was observed in studies using standard chemotherapy agents including 5-FU and Taxol, as well as macromolecules such as radiolabeled tumor-targeted antibodies.

PDGF Receptors on Perivascular Cells

The recognition of the importance of tumor angiogenesis, together with the developmental biology-derived evidence linking PDGF-βR to pericyte function, suggested that perivascular PDGF receptors could regulate tumor angiogenesis and thereby affect tumor growth and progression.

The first study exploring this concept used the PDGF-Bret/ret mice, expressing a truncated hypomorphic form of PDGF-BB, which earlier had been shown to display reduced pericyte coverage. Experiments with xenograft tumors in these mice established that attenuated PDGF-βR signaling reduced tumor angiogenesis [36]. Subsequent studies demonstrated that overexpression of PDGF-βR ligands in cancer cells enhanced growth of melanoma xenografts [37]. Analyses of the tumor stroma implied increased pericyte coverage, occurring in the absence of changes in vessel density, as the mechanism underlying the tumor-supportive effect. Later, studies in genetic mouse cancer models combined VEGF- and PDGF-βR-targeting agents and promoted a concept suggesting tumor vessel-stabilizing and pro-tumoral effects of PDGF-βR-dependent perivascular cells [38]. The general significance of

these findings have been challenged in more recent studies where perivascular PDGF-βR instead was associated with reduced tumor growth, suggesting stage- and tumor-type-specific effects [39, 40].

The impact of perivascular PDGF-βR on metastasis has been experimentally explored in studies, which have used suicide-gene-mediated depletion of PDGF-βR-positive particular cells [41, 42]. Both studies indicated that loss of PDGF-βR-positive perivascular cells induced a pro-metastatic tumor phenotype, including increased hypoxia, c-MET-dependent tumor cell stimulation, and increased angiopoietin2-dependent angiogenesis.

Additionally, links have also been made between perivascular status and tumor immune surveillance. Analyses of the "low-pericyte tumors" in the PDGF-Bret/ret mice noted an increased tumor infiltration of immune-inhibitory MDSCs [43]. This occurred together with a reduced T-cell infiltration and an immune signature implying reduced antitumoral immune activity. An interesting implication of these studies, which should be further explored, is that perivascular status might determine response to immune therapy. Relationships between perivascular PDGF-βR status and response to treatment remain unclear. Efficacy of anti-VEGF agents has been reported to be either unaffected or increased in models where perivascular PDGF-βR status has been manipulated [38, 44].

PDGF Receptor Status and Prognosis

As outlined above, experimental studies have implicated PDGF receptors as important stimulatory molecules for fibroblasts and perivascular cells. In parallel, tumor microenvironment studies have demonstrated that stromal cells contribute to tumor progression and drug response. Together, these research areas have prompted a series of studies which have analyzed potential associations between stromal PDGFR status and survival in different tumor types. Differential expression of the two PDGF receptors has indeed been identified in tumor stroma of clinical samples (Fig. 5.3).

Most of these studies have used conventional immunohistochemistry with antibodies recognizing PDGF-αR or PDGF-βR, together with manual semiquantitative scoring of stromal expression. Other studies have also been performed in which these antibodies have been used together with digital-image-analysis-based scoring [45]. These novel methods, in addition to giving quantitative data, have also allowed differential analyses of PDGF receptor expression in perivascular areas and in fibroblast-dominated tumor regions.

Some studies have employed pPDGFR antibodies to monitor expression of activated receptors [46], although concerns have been raised regarding the specificity of these reagents. A proximity ligation assay for detection of phosphorylated PDGFRs has also been described [47–49], but not yet used to report on stromal PDGFR status. A shared problem for these two approaches is the sensitivity to artifacts caused by tissue handling affecting protein phosphorylation.

PDGF-βR status in clinical samples has also, in addition to these protein-based assays, been determined by bioinformatics-based approaches. In these analyses a

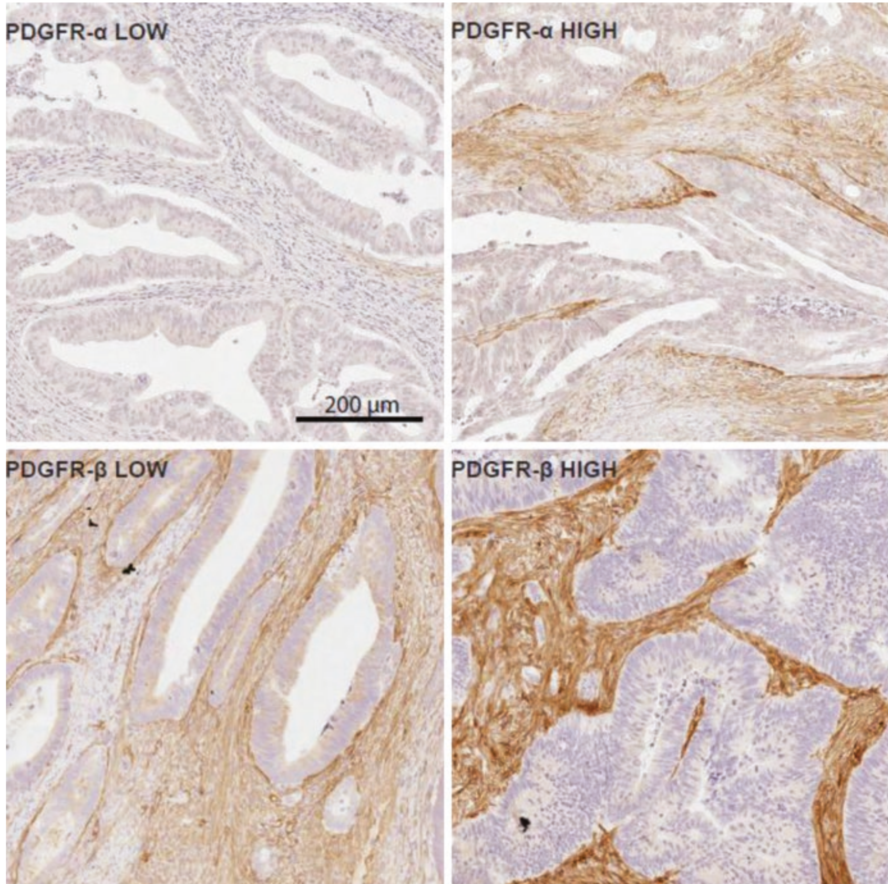

Fig. 5.3 Microphotograph illustrating variable expression of PDGF-αR and PDGF-βR in tumor stroma of human colorectal/pancreatic cancer samples (pictures courtesy of A. Mezheyeusky)

"PDGF-βR signature," derived from PDGF-BB-activated cultured fibroblasts, was used to analyze a number of different breast cancer gene expression datasets [50]. The following paragraphs discuss results from these analyses with subsections focusing on, firstly, fibroblast expression of PDGFRs and, secondly, perivascular PDGFR expression.

PDGFR Expression in Stromal Fibroblasts

The first major study on the prognostic relevance of stromal PDGF-βR expression was done in breast cancer and uncovered significant associations between high stromal expression and shorter recurrence-free and breast cancer-specific survival based on univariate analyses [51]. The study, based on approximately 290 cases, also

identified strong correlations between stromal PDGF-βR and poor prognosis markers such as high grade, high proliferation, and HER2 amplification.

Strong and robust signals linking stromal PDGF-βR signaling to poor prognosis in breast cancer were also obtained in the gene signature study, in which a tissue-culture-derived PDGF-βR signature was used [50]. In this study, gene expression data from tumors of four different breast cancer cohorts was used to dichotomize cases into "high-signature-score" and "low-signature-score" groups. The "high-signature-score" group showed consistent associations with poor prognosis in the different cohorts in analyses using recurrence-free survival or disease-specific survival/overall survival as end points. Importantly, significant prognostic association was also detected in multivariate analyses using standard clinico-pathological characteristics (HR 1.2–1.3) or other stroma-related signatures (HR 1.3–1.6). Based on subset analyses, the prognostic impact was strongest in low-grade and Luminal A tumors. As in the IHC study, strong correlations were noted between high stromal PDGF-βR and poor prognosis markers such as high grade, high proliferation, and HER2 amplification.

The impact of stromal PDGF-βR has also been analyzed in prostate cancer. This study relied on analyses of individuals subjected to "watchful waiting" and thus represents the natural course of the disease [52]. The analysis of 266 tumors demonstrated significantly shorter cancer-specific survival in the group with high PDGF-βR expression (HR 2.4 in univariate analyses). Scoring was also done on histologically normal adjacent tissue. Interestingly, similar associations with survival were detected in this dataset. Whether these findings reflect that prognosis is affected by "constitutional" inter-individually variable PDGF-βR expression or rather reflect tumor "field-effects" remains unresolved.

Other conventional IHC studies have also demonstrated associations between high PDGF-βR and poor prognosis in gastric, colorectal, and pancreatic cancer [53–55]. Analyses of rhabdomyosarcoma have provided the first evidence that stromal PDGF-βR is also relevant for prognosis in sarcomas through findings of significant associations between stromal PDGF-βR and development of distant metastasis [56].

More recently a series of studies have been performed where stromal PDGF-βR status has been determined following IHC analyses and digital-image-analyses-supported automated scoring. These studies have provided novel data from ovarian and renal cell cancer showing that, also in these tumor types, high stromal/fibroblast PDGF-βR expression is associated with poor prognosis [45, 57]. In the case of ovarian cancer, the signal from univariate analyses was maintained in multivariate analyses.

As outlined above, high PDGF-βR expression appears to be consistently associated with poor prognosis. Concerning PDGF-αR less information is available [58]. Some tissue culture studies have shown that PDGF-αR is downregulated upon activation of fibroblasts by TGF-beta [59]. It is therefore possible that PDGF-αR expression marks a resting, and possibly growth restraining, fibroblast population. Further studies are therefore warranted to explore the possibility that the two PDGF receptors mark different fibroblast subsets which might show differential associations with outcome.

Perivascular PDGF-βR Expression

Developmental biology and experimental tumor biology studies have established that perivascular cells exert important regulatory functions affecting vascular biology which in turn impact on normal physiology and the pathophysiology of tumors. Based on the established role of PDGF-βR as a key regulator of pericytes (see above), studies have been initiated exploring potential inter- and intra-case heterogeneity of perivascular PDGF-βR status and associations with other vascular features, clinico-pathological characteristics, and survival.

Analyses of colorectal tumors, using simultaneous staining with multiple markers associated with perivascular cells, have demonstrated the existence of multiple subsets of perivascular cells. These might eventually have different functions and cells of origin, as well as variable capacity for differentiation [60].

Potential independent prognostic capacity has been indicated by the fact that perivascular status is largely independent from vascular density or average size. This notion is supported by studies which have identified significant associations between poor prognosis and high perivascular PDGF-βR expression in breast, ovarian, and kidney cancer ([45, 57, 60]; Rosin et al., (pers. comm.)

The breast cancer study relied on semiquantitative scoring of sections stained with CD34 antibodies to identify tumor vessels, together with PDGF-βR antibodies. The survival association was retained in multivariate analyses with standard risk factors, and explorative sub-analyses indicated a particularly strong prognostic relationship in the low-grade and Luminal B breast cancer subgroups. Notably, in this particular study, the prognostic association was stronger for perivascular PDGF-βR than fibroblast/stromal expression of the same marker. In the kidney and ovarian cancer studies referred to above, using automated quantitative scoring, the significant associations between high perivascular PDGF-βR and shorter survival were maintained in multivariate analyses.

Stromal PDGF Receptors and Response to Treatment

Preclinical evidence, outlined above, has suggested multiple mechanisms whereby either fibroblast or perivascular PDGF-βR signaling could affect efficacy of chemotherapy and VEGF-directed anti-angiogenic therapy. A series of studies are thus expected which will use clinically well-annotated tumor collections to analyze the potential of stromal PDGFRs as response-predictive biomarkers. Notably, such studies should ideally be performed in a manner which allows a distinction between the impact of the PDGFR biomarker on natural course and on the efficacy of treatment.

Promising results from such efforts have recently been obtained in the setting of adjuvant tamoxifen treatment of early breast cancer [61]. This study relied on analyses of tumor tissue collected from women participating in two adjuvant tamoxifen studies recruiting pre- and postmenopausal women. Both studies were positive and

contributed to the implementation of this treatment [62, 63]. Following dichotomization of patients based on their stromal PDGF-βR expression, clear differences in tamoxifen efficacy were detected in the premenopausal group. Whereas the low-stromal-PDGF-βR group displayed a significant benefit of tamoxifen with regard to recurrence-free survival, no significant effects were detected in the high-stromal-PDGF-βR group. Analysis of the postmenopausal cohort yielded results with similar trends. When analyses were restricted to cases with >75% ER positivity, stromal PDGF-βR status divided the cohort into one "PDGF-βR-low" group which showed significant benefit and a second nonresponsive "PDGF-βR-high" group.

Future Perspectives

Findings from the interacting experimental and correlative studies on the roles of PDGF receptors signaling in tumor stroma set the stage for continued analyses and exploration of this growth factor system. A series of questions are suggested by recent findings concerning biological mechanisms. Improved methods for monitoring of PDGF receptor status in clinical samples should facilitate development toward use of these molecules as clinical biomarkers. Finally, therapeutic targeting of stromal PDGF receptors in selected patient populations still appears as a viable research goal.

Concerning tumor biology a series of questions are raised by the accumulating evidence for clinically relevant inter-case variability in the expression of perivascular and fibroblast expression of PDGF-αR and PDGF-βR. As of the writing of this chapter, the underlying basis for this variability remains unknown. A key task for future studies is thus to, firstly, identify the molecular regulatory systems controlling PDGF receptor expression and, secondly, to address the fundamental question about relative contribution of host and cancer genetics in the inter-patient heterogeneity. The relationships between receptor-positive fibroblasts/CAFs and perivascular cells should also be better resolved. Emerging evidence for strong intra-case correlations between PDGFR status in fibroblasts and perivascular cells suggests that these cells might share a common cell of origin, which should be further explored. Further studies addressing biological and prognostic differences between the two PDGF receptors are warranted and will possibly be guided by refined analyses of differential instructive roles of PDGF-αR and PDGF-βR during development.

Future analyses of clinical cohorts are likely to benefit from improvements in methodology for analyses of PDGF receptor status. Signals linking PDGF receptor status to prognosis or response to treatment might be enhanced in analyses which integrate further spatial information in scoring algorithms such as the relative position of positive cells to epithelial or immune cells. Advances allowing scoring of activated versus non-activated receptors also appear as an important task which can possibly be achieved by assays specifically detecting activated dimeric receptors or

multi-probe in situ profiling with PDGFR-activation-associated genes. Improved possibilities for PDGFR profiling of metastatic lesions might depend on noninvasive modalities. Eventually, this can be achieved with PET imaging combined with radiotracers such as derivatives of the preclinically validated PDGF-βR "affibody" [64].

The first generation of studies demonstrating associations between PDGF receptor status and survival should be validated in independent cohorts for ultimate clinical use. Such studies should be designed to specifically address impact on natural course and response to treatment. Concerning the latter it will be very interesting to see results from ongoing studies relating perivascular PDGF receptor status to the benefits of anti-angiogenic drugs, a class of drugs where biomarkers are strikingly absent and urgently needed. Selection for future strategies for exploitation of PDGFRs as biomarkers will also be highly dependent on yet-to-be-collected information about the potential to deduce PDGFR status in metastasis from analyses of primary tumors. Studies resolving this issue should be prioritized.

In addition to the biomarker potential, stromal PDGF receptors also remain interesting candidates for therapeutic targeting. Recent findings implying PDGF-αR and PDGF-βR in distinct biological processes provide a rational for development of isoform-specific antagonists, possibly including monoclonal antibodies. The now established inter-case variability in PDGFR status also strongly suggests that future studies using PDGFR antagonists should be performed on optimally selected patient populations. Hopefully, results from such efforts will be reported during the upcoming 5-year period.

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Chapter 6
The Function and Diagnostic Potential of Adipocyte-Derived Factors in the Tumor Microenvironment

Joan Chang and Andrew C. Dudley

Abstract Solid tumors resemble dysfunctional "organs" comprised of malignant cancer cells and heterogeneous components of the tumor microenvironment (TME). The TME includes extracellular matrix and non-cancer stromal cells (e.g., fibroblasts, immune cells, and vascular cells) that may support cancer progression. Adipocytes, despite being the most abundant cell type in certain tumor types (e.g., breast cancer), are often overlooked in the TME. It is now well established that the TME plays an important role in tumor growth and metastasis, and while multiple studies have contributed to our understanding of the TME, relatively little is known about how adipocytes, despite their role as major sources of paracrine and endocrine factors, influence tumor progression. In this chapter, we will briefly introduce the TME and its various components and then provide a comprehensive analysis of the roles of tumor-associated adipocytes and adipokines during solid tumor development. We will also highlight the potential diagnostic/prognostic value of adipose tissue and adipose-derived factors in cancer.

Keywords Tumor microenvironment • Adipocytes • Adipose tissue-derived factors • Adipokines • Obesity • Metastasis • Inflammation • Tissue injury • Adipocyte lineage tracing • Angiogenesis

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Introduction

In the United States and other developed countries, cancer death rates have continually declined in the last two decades due to research efforts into cancer prevention, detection and therapeutics, as well as better education for the general public in understanding the effects of carcinogens [1, 2]. However, cancer is still currently among the leading causes of morbidity and mortality worldwide (WHO Fact sheet No 297). This is due in part to the highly variable nature of cancer, which may exist as a "spectrum" of different tumor cells with different characteristics and functions, even within the same type of cancer (as denoted by organ site). Thus, cancer may be described as many separate diseases, since treatment options differ from cancer type to cancer type, and the treatment response among patients varies drastically. Until recently, cancer research focused almost exclusively on understanding the biology of malignant cancer cells, in particular the genetic and proteomic alterations that promote their survival and metastasis. However, it is now well established that cancer cells do not exist in a vacuum; rather, they interact closely with the extracellular matrix (ECM) around them, as well as non-cancer stromal cells that either reside in the vicinity of the cancer cells or are actively recruited from distant sites (e.g., from the bone marrow). It is thus critical to continue studies to better understand how tumor heterogeneity, among both cancer cells and stromal cells found in the TME, influences tumor progression and treatment strategies.

The Tumor Microenvironment

Cancer occurs when normal cells accumulate mutations in their genome that provide a survival advantage. However, the microenvironment or "niche" where these mutated cells are situated is also critical for supporting cancer cell survival. Indeed, all of the "hallmarks of cancer" defined by Hanahan and Weinberg are influenced directly or indirectly by the TME [3], which is a highly dynamic and interactive "ecosystem" that changes over time and may differ in primary tumors versus their metastases. Thus, solid tumors together with the TME may be considered caricatured versions of dysfunctional organs [4].

The TME consists of both noncellular and cellular components. It also harbors various growth factors and cytokines derived from different cell types (Fig. 6.1). One way tumor cells create a favorable environment for tumor growth and angiogenesis is by altering the noncellular component of the TME, namely, the ECM. The cellular constituents of the TME (i.e., non-malignant stromal cells) may also create a permissive environment for the cancer cells to thrive. Overall, research on the effects of tumor-supportive properties of non-malignant stromal cells has mostly focused on fibroblasts, which can be "activated" by cancer cells to form myofibroblasts, also known as carcinoma-associated fibroblasts (CAFs) [5, 6]. Also found within the TME are immune cells (e.g., macrophages, T cells, neutrophils [7]), bone marrow-derived progenitor cells (BMDCs) [8], and vascular cells [9]. In the case of cancers derived near fatty tissues, adipocytes may also be present [10]. Each of these

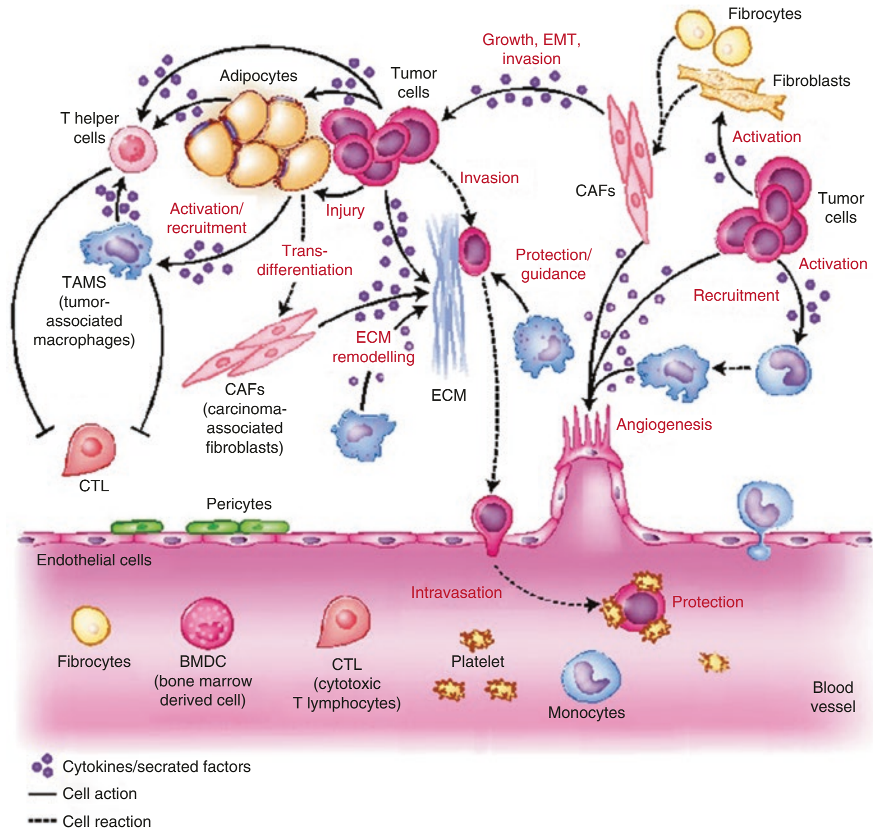

Fig. 6.1 The tumor microenvironment. Here we show the variety of host-derived stromal cells that are involved in mediating tumor progression. Tumor cells secrete factors that remodel the ECM and alter the behavior of stromal cells to often aid tumor development. These host-derived tumor-associated cells then further facilitate tumor growth/invasion by secreting additional factors that promote tumor proliferation, invasion, angiogenesis, remodeling of the ECM, and recruitment of tumor-promoting immune cells

stromal cells, together with the ECM, may promote (or inhibit depending on the context) tumor growth and metastasis via secretion of various growth factors/cytokines, which activate signaling pathways both in cancer and non-malignant stromal cells. Here, we will briefly describe each of the major components of the TME.

The ECM

The ECM is comprised of many types of proteins, which are usually large in size, complex, and highly conserved between species [11]. These include laminins, collagens, fibronectin, elastin, and tenascins. The ECM was once viewed as merely a stable supporting structure that maintains tissue morphology, but recent studies

have demonstrated the dynamic nature of the ECM, which undergoes extensive remodeling that is tightly controlled, especially during normal tissue homeostasis [12]. Aberrant ECM dynamics, such as increases in ECM deposition, altered cross-linking of ECM molecules, and changes in stiffness, leads to abnormal behaviors of the cells nearby. This results in altered integrin signaling as well as altered growth factor signaling due to changes in the presentation of growth factors bound to the ECM [11]. Indeed, abnormal ECM is often a clinical precursor of diseased states including fibrosis and cancer [13].

Carcinoma-Associated Fibroblasts

Carcinoma-associated fibroblasts (CAFs) can be derived from multiple cell types including fibroblasts in the immediate tumor environment, bone marrow-derived cells, endothelial cells (through an endothelial-to-mesenchymal transition, or EndMT), and perhaps also from the cancer cells through epithelial-to-mesenchymal transition (EMT) [6]. Compared to normal fibroblasts, CAFs typically promote tumor growth and angiogenesis and enhance the metastatic dissemination of cancer cells [5, 6], although these tumor-supportive effects may be tumor-type dependent, as recent research suggest that CAFs inhibit pancreatic cancer progression in certain contexts [14, 15]. CAFs may also travel in the circulation as fibrocytes and enhance tumor proliferation and fibrosis (i.e., chronic inflammation) once recruited to the tumor site [16]. Certain subtypes of fibrocytes may also mediate immunosuppression, while maintaining phenotypic and functional hallmarks of a traditional fibrocyte [17], and prepare secondary organ sites for metastasizing cancer cells through recruitment of monocytes [18].

Bone Marrow-Derived Cells

The bone marrow is a source for highly plastic cells that are mobilized into circulation and then migrate toward tumors. Bone marrow-derived cells (BMDCs) are recruited during the beginning stages of tumor development [19]. It is suggested that as they are incorporated into the TME, they differentiate into various different cell types including pericytes, endothelial cells, fibroblasts, and monocytes, which contribute to tumor growth and metastasis through diverse mechanisms [8]. Interestingly, BMDCs recruited by cancer cells may not necessarily “home” toward the primary tumor; instead, they may colonize specific areas in metastatic sites, in preparation for incoming metastasized cancer cells, creating what are known as “pre-metastatic niches” [20, 21].

Immune Cells

Immune cells from both the innate (e.g., macrophages and neutrophils) and adaptive immune system (e.g., T and B cells) are important defenders against bacteria, viruses, and cancer cells. As expected, immune therapies for cancer have been focused on, for example, harnessing the cytotoxic abilities of T cells (cytotoxic T lymphocytes or CTL) against tumor cells [22]. Emerging evidence has shown that the TME can co-opt and subvert these immune cells to promote tumor progression, by altering their polarization/activation status. In general, immune cells with "1" in their polarization nomenclature indicate an antitumor phenotype, whereas "2" denotes pro-tumor properties. For example, T-helper cells, which regulate the activation and proliferation of CTLs, can be Th1 or Th2. Th1-polarized cells can elicit direct cytotoxic effects against tumor cells and are responsible for activation and perseverance of the CTL population [23]. Th2-polarized cells on the other hand activate a humoral response (i.e., B-cell activation as opposed to CTL activation) and suppress Th1 responses [7, 23]. For macrophages, there are at least two types of polarization: "M1" and "M2." M1s secrete inflammatory cytokines that activate Th1-adaptive immune response, which in turn kills tumor cells [24]. Conversely, tumor-associated macrophages (TAMs) that have infiltrated tumors are typically M2 polarized [24]. M2s secrete immunosuppressive cytokines and stimulate a Th2 response, thus inhibiting the Th1 response while promoting tumor growth. M2s also secrete various proteases and angiogenic factors, which promote invasion and metastasis of the tumor cells [25]. Similar to macrophages, it has recently been demonstrated that neutrophils are also "N1" and "N2" polarized [26]. N1s have much higher cytotoxic activities against tumor cells and secrete more immune-activating cytokines/chemokines, and it was shown that TGFβ within the TME recruits the pro-tumor N2 phenotype, which in contrast to N1s contributes to immunosuppression and thus promotes tumor progression [26].

Vascular Cells

The involvement of vascular cells (endothelial cells and pericytes) in tumor progression is well-studied [27]. Tumor growth requires oxygen and nutrients, and the ability of tumors to undergo an angiogenic switch (i.e., become vascularized) is a crucial step during tumor progression. This requires both co-option of nearby vessels as well as new endothelial cells, in a process known as sprouting [28, 29]. Recently, it was shown that genetic depletion of Apelin (Apln) specifically in the vasculature diminished sprouting and reduced tumor growth [30]. Furthermore, tumor vessels are needed to remove waste products generated during tumor

metabolism as well as provide a route for metastasizing cancer cells throughout the body [9]. Interestingly, tumor endothelial cells (TECs) have very different biology compared to normal endothelial cells; for example, TECs have a unique gene expression signature, and vessels formed by TECs have excessive branching and increased leakiness [31, 32]. In addition, aberrant cross talk between TECs and immune cells such as leukocytes, T cells, and macrophages provides the tumor with both protection against immune surveillance while promoting a pro-inflammatory environment, which further fuels tumor progression [9].

Adipocytes and Cancer: An Introduction

Adipocytes have been mostly overlooked or considered innocent bystanders in the TME. This is despite the fact that adipocytes are abundant in multiple cancer types including breast, ovarian, prostate, and liver [33]. Additionally, obesity, a medical condition characterized by expansion and hypertrophy of adipocytes, is a risk factor for certain cancers [34, 35]. The prevalence of obesity has increased drastically worldwide as a result of increased affluence of the general population, as well as a shift in dietary habits. It is estimated that up to 20% of all cancer cases count obesity as a dominant causative factor [36, 37], which impacts not only cancer incidence but also treatment outcomes [38]. The relationship between obesity and cancer is complex, as an increase in body mass index (BMI) may impact cancer progression either positively or negatively depending on the type of cancer (see [33, 38]). It is known that BMI does not necessarily reflect adiposity or reflect distribution of adipose tissues accurately [39], and the latter is a risk factor as well as prognostic indicator for colorectal cancer [40], underlining the importance of adipose tissue location as well as overall adiposity in mediating cancer progression.

In kidney cancer (renal cell), obesity is a strong risk factor for cancer incidence; paradoxically, in patients already diagnosed, obesity is linked to extended survival [41]. The cause of this phenomenon is still under debate and may be attributed to the "nutritional buffer" provided by the extra adipose tissue for cancer patients undergoing chemotherapy and/or radiation therapy; this decreases treatment-related toxicities and increases treatment efficacy [38, 42]. Irrespective of confounding and complex results from epidemiologic meta-analyses associating obesity and cancer, obesity is clearly a cancer risk factor and is associated with increased mortality for kidney, colon, liver, prostate, and breast cancers [36].

The importance of white adipose tissue (WAT, see the section "Types and Functions of Adipocytes" for different types of adipocytes that comprise adipose tissue) was questioned when studies using an A-Zip/F-1 "fatless" mouse demonstrated that these mice have accelerated tumor formation compared to wild-type mice [43, 44]. The authors concluded from these observations that adipokines, which are soluble factors secreted mainly by white adipocytes, are not important in

mediating tumor progression. Instead, the authors argued, it is the inflammatory status that contributes to obesity-related cancer progression [44, 45]. However, the A-Zip/F-1 mice are severely diabetic, they have elevated systemic glucose/insulin/free fatty acids/triglycerides, and they have severe chronic inflammation as indicated by the highly elevated levels of inflammatory cytokines present in the serum. Surprisingly, despite being "fatless," the A-Zip/F-1 mice have a much higher body weight as well as body length compared to their age-matched littermates [44]. These observations indicate that perhaps the results using the A-Zip/F-1 mice are not sufficient to conclude what role white adipose tissue plays in cancer progression, as any effects of adipocyte-related influence on tumor development may have been masked by the "cytokine storm" that characterizes this model. In addition, the adipokine adiponectin was significantly lower in the A-Zip/F-1 mice when compared to the wild-type mice. While this is not surprising as WAT is the predominant source of adiponectin, obese individuals were reported to have significantly lower circulating adiponectin levels [46], likely due to negative regulation by multiple adipose tissue-derived factors that are elevated during obesity (e.g., TNF-α, IL-6). Additionally, adiponectin levels in general are inversely correlated with cancer, suggesting that adiponectin may have tumor-inhibitory effects (discussed in details in the section "Adipokines and Cancer" of this chapter).

Studies from a few decades ago demonstrated that both spontaneous and induced mammary carcinomas are increased in A^{vy} mice [47–50], which have ubiquitous expression of the appetite-stimulating agouti protein and thus have a much higher body weight [51]. Mice made obese by injection of gold thioglucose (GTG) also have a higher incidence rate for spontaneous mammary tumor development [52], although when the ovaries were removed from the obese mice, the cancer incidence was significantly lower than the control mice. This result suggested a confounding effect of sex hormones on obesity-related tumorigenesis [53]. Additionally, obese mice, induced by a high-fat diet, also had the highest rate of tumor growth when compared to lean mice [54]. Similar tumor-promoting effects of obesity were observed in various other cancers such as colorectal [55–57], liver [58, 59], prostate [60–63], and skin (both melanoma and non-melanoma, [64, 65]). These studies all suggest an important role for white adipose tissue and/or obesity in mediating cancer progression.

The Structure of Adipose Tissues

There are three main types of adipose tissues: (1) subcutaneous, which is below the skin and includes deep hypodermic depots; (2) mammary, which as the name suggests, is present in the breast; and (3) visceral, which surrounds the inner organs and is divided into omental, mesenteric, retroperitoneal, gonadal, perivascular, and pericardial depots [66]. In addition to these adipose depots, the bone

marrow also contains a rich source of adipocytes, ranging from 15 to 60% of bone marrow volume in humans depending on age [67]. Adipose tissue was long thought of as mostly inert—providing insulation for the body against the cold, giving support and protection for vital organs, passively maintaining energy homeostasis by acting as a reservoir to store excess nutrients in the form of fat, and releasing the fat when required in the form of fatty acids. However, adipose tissue is rather a dynamic "organ" that actively secretes both paracrine and endocrine factors, which exerts an influence at both local and systemic levels; as such, dysfunction of the adipose tissue leads to metabolic syndromes such as hyperglycemia (increased blood glucose), dyslipidemia (increased circulating lipids), and insulin resistance [68]. Damaged or dysfunctional adipose tissues (e.g., adipocyte hypertrophy due to obesity) also release pro-inflammatory cytokines, which contribute to an inflammatory environment. The effects of WAT in cancer may not only be due to the systemic response of the body toward the obese condition (i.e., metabolic syndromes leading to the imbalance of hormones) but also due to a local response (e.g., increase in adipose-derived factors surrounding the tumor which alter the TME).

Adipose tissue is comprised of a heterogeneous population of cells, the predominant cell type being adipocytes. The other component, the stromal vascular fraction (SVF), contains endothelial cells, lymphocytes, resident monocytes, macrophages, fibroblasts, and other precursor/progenitor cells [69]. Adipose SVF, in particular adipose progenitor cells, has been shown to promote tumor progression. For example, it was demonstrated that SVF cells from WAT is actively recruited by cancer cells to the primary tumor site, they are incorporated into specific niches created by various stromal cells (e.g., the vasculature), and they promote tumor growth [70]. In diet-induced obese mice engrafted with tumors, the number of circulating adipose-derived SVF cells increased dramatically, which were then incorporated into the developing primary tumor. This recruitment contributed to the stabilization of tumor blood vessels and promotion of tumor growth [71]. Thus, the SVF from adipose tissue is a rich source of tumor-promoting cell types, which the cancer cells can co-opt to further their progression.

Adipocytes

Not all adipocytes are identical—while the image of "white adipose tissue" comes to mind when the word "fat" is mentioned, research has uncovered at least three different types of adipocytes, each with distinct biology and functions, namely, "white," "brown," and the reversible "beige." In addition, the anatomical location of the adipose tissue also determines the proportion of various "colored" adipocytes and their overall systemic function [72].

Types and Functions of Adipocytes

Classic white adipocytes, their color being reflective of their high lipid content, represent the majority of the cells in visceral and subcutaneous adipose tissue depots, which are areas that expand with obesity [68]. They are the predominant cell type involved in energy storage and release of hormones and other factors such as cytokines (see the section "Adipose-Derived Factors and Cancer" of this chapter), and they have a unilocular lipid droplet structure [72, 73]. Brown adipocytes, on the other hand, have high mitochondria content and thus an abundance of cytochromes, which gives the tissue a "brownish" tint. They are characterized by multilocular lipid droplets and are involved in non-shivering thermogenesis, where fatty acids within the cells undergo beta-oxidation, followed by the uncoupling of electron transport from ATP production, thereby creating heat. This uncoupling is carried out by uncoupling protein 1 (UCP1) located in the mitochondrial membrane [73]. As such, in highly generalized terms, white adipocytes are characterized as being "UCP1 low" and involved in metabolic responses, whereas brown adipocytes are "UCP1 high" and involved in temperature regulation.

Until recently, brown adipocytes were thought to be limited to, at least in humans, neonates that have not acquired the ability to shiver and generate body heat. However, adipose tissue with both metabolic and thermogenic activities in human adults was recently reported [74]; subsequent research suggests that white adipocytes can undergo what is known as a "browning" process, forming "beige cells" [75]. These beige cells, also known as "brite" (brown in white) cells, are similar to brown adipocytes in the sense that they have multilocular lipid droplets and express a handful of other brown fat-specific genes [76]. However, they resemble white adipocytes as they have low basal levels of UCP1, which can be stimulated (e.g., through cold adaptation) to the levels observed in bona fide brown adipocytes [77]. In contrast, beige adipocytes can also undergo a "whitening" process, whereby warm adaptation causes them to change their morphology and upregulate white adipocyte-specific genes [78]. Recent research suggests that brown and white adipocytes arise from distinctive precursor cells—brown adipocytes are derived from Myf-5+ myogenic lineage, whereas white adipocytes are derived from adipogenic precursors [79, 80]. Beige cells, on the other hand, may arise from adipogenic precursors (as a result of transdifferentiation from white adipocytes [81, 82]). However, this may not be the primary source, as demonstrated by lineage tracing methodology [83]. Skeletal muscle-residing precursor cells [84], or bipotential precursor cells expressing platelet-derived growth factor receptor alpha (PDGFRα), can differentiate directly into white or beige adipocytes depending on the stimulus [85].

In the following section, we will focus on the role of white adipocytes in cancer, as these are the major cell types in the usual adipose depot surrounding an expanding

Table 6.1 List of adipose tissue-derived factors

Metabolism
Apolipoprotein E (ApoE)
Autotaxin
Desnutrin
Free fatty acids
Glycerol
Lipoprotein lipase
Lysophosphatidic acid
Zinc-a2-glycoprotein (ZAG)
Steroid/hormones
Estradiol
Estrone
Resistin
Testosterone
Growth factors/cytokines
Fibroblast growth factors (FGF)
IL-1
IL-6
IL-8
IL-10
IL-17
IL-18
Insulin-like growth factor I (IGF1)
Lipocalin 2
Macrophage migration inhibitory factor (MIF)
Nerve growth factor (NGF)
Tumor necrosis factor (TNFα)
Vascular endothelial growth factor (VEGF)
Tissue factor (TF)
Transforming growth factor beta (TGFβ)
Chemokines
CC-chemokine ligand 2 (CCL2/monocyte chemotactic protein 1, MCP1)
CC-chemokine ligand 7 (CCL7)
C-reactive protein (CRP)
CXC-chemokine ligand 5 (CXCL5/RANTES)
Vasoactive factors
Adipocyte-derived relaxing factor
Angiotensinogen
Angiopoietin-1
Angiopoietin-2
Angiopoietin-related protein 4 (Angiopoietin-like 4/Fasting-induced adipose factor)
Angiotensin II
Atrial natriuretic factor
Monobutyrin
Eicosanoids
Prostacyclin (Prostaglandin I2)
Prostaglandin E2
Prostaglandin F2α
Complement system
Complement factor B
Complement factor C
Complement factor C1q
Complement factor C3
Acylation-stimulating protein (ASP/C3adesArg)
Complement factor D (Adipsin)
Binding proteins
A1-acid glycoprotein
Apelin
Ceruloplasmin
Cholesterol ester transfer protein (CETP)
Haptoglobin
Insulin-like growth factor mRNA-binding proteins
Intercellular adhesion molecule 1 (ICAM1)
Metallothionein
Osteonectin
Pentraxin family member 3 (PTX3)
Retinol-binding protein
Serum amyloid A (SAA)
Tumor necrosis factor receptors
Vascular cell adhesion molecule 1 (VCAM1)
Extracellular matrix
Collagen IV
Fibronectin
Matrix metalloproteinases (MMP)
MMP1
MMP3
MMP7
MMP9
MMP10
MMP11
MMP14
MMP15
Adipokines
Adiponectin
Leptin
Other Adipose-tissue derived factors
Adipose-specific fatty acid-binding protein (a-FABP)
Adipophilin (perilipin 2)
Agouti protein
Chemerin
High mobility group box 1 (HMGB1)
Intelectin-1 (omentin)
Irisin
Plaminogen activator inhibitor 1 (PAI1)
Resistin-like molecules (RELM)
Secreted frizzeled-related protein 5 (SFRP5)
Visfatin (PBEF/NAMPT)
Vaspin

tumor. Additionally, white adipocytes are prominent secretory cells, secreting a diverse range of molecules including, but not limited to, adipokines, growth factors, hormones, ECM proteins, signaling molecules, and free fatty acids (Table 6.1, adapted from [73] and Table 5.3 of [86]); this highlights the potential for white adipocytes as an important and pervasive cell type within the TME.

Adipocytes and Cancer

As adipocytes mature from precursor cells, there is an increase in the secretion of basement membrane ECM proteins such as laminins and type IV, V, and VI collagens [87–89]; as such, normal mature adipocytes are generally separated from epithelial cells by basement membranes. During normal body homeostasis, such as mammary gland involution, the separation between cells is disrupted for the extensive restructuring of the gland. This breaking down of basement membranes and co-mingling of different cell type mimics the process of invasive breast tumors [90]. It is likely that during tumor expansion and invasion, cancer cells will first come into contact with adipocytes. As adipocytes are fragile cells with thin membranes, these physical interactions may induce injury to the adipocytes (i.e., "squeezed" by the expanding tumor cells), causing rupture or damage to the adipocyte membranes, thus releasing their cellular contents, which in turn induces inflammatory responses in the tumor site [91, 92]. Indeed, recent research demonstrated that tumor cells actively alter the adjacent adipose tissue, whereby the size of adipocytes is reduced, indicating lipolysis and modification of both intracellular lipid droplets and basement membrane components surrounding the adipocytes [91, 93, 94]. Extensive fibrosis in the tumor-associated adipose tissue was also evident using Picrosirius Red stain, and the release of lipids/triglycerides into the TME as a result of adipocyte injury was suggested to trigger macrophage activation and inflammation [91, 95]. This release of lipids and triglycerides may have a twofold effect—in addition to recruitment of immune cells, these molecules may be taken in by cancer cells as an energy source, thereby accelerating tumor growth [96].

Injury to the adipocytes may also in turn lead to necrotic cell death, causing release of damage-associated molecular patterns (DAMPs), such as the nonhistone chromatin-associated protein high mobility group box 1 (HMGB1), as well as pro-inflammatory cytokines such as IL-6 into the TME [91, 92]. Peritumoral adipose tissue shows an extensive inflammatory response and an increase in macrophages, which may be derived from proliferating resident macrophages within the SVF or mobilized from other sources such as the circulation [92]. Thus, through recruitment of pro-tumor immune cells, tumor-associated adipocytes exacerbate tumor-associated inflammation in the TME and promote tumor progression. This is perhaps best demonstrated by studies showing that when tumors are implanted in sites distant from adipose tissues, they show a growth delay compared to those implanted in adipose-rich areas [92, 97].

In addition to physical injury, there may also be pro-tumor responses due to heterotypic cross talk between tumor/stromal cells and adipocytes. It is well-established that tumor cells release enzymes that modify the ECM [12, 13]; as these factors chew through the basement membrane, the once-sequestered adipocytes can now interact with other cell types (including tumor cells) within the adipose depot and the TME. While it is challenging to differentiate between the effects of adipocytes on other cells and vice versa in vivo, one can postulate that tumor-educated adipocytes elicit very different responses from naive adipocytes one example would be the increased angiogenesis in tumor-associated adipose tissues compared to control adipose tissues—when placed in ex vivo culture [92]. Thus, heterotypic cross talk between cancer cells and adipocytes appears to favor tumor progression as co-injection of murine adipocytes and human breast cancer cells demonstrated that adipocytes promote tumorigenesis [98]. Mature adipocytes, but not pre-adipocytes, also promote breast cancer proliferation in matrix [99], whereas in colon cancer both pre-adipocytes and mature adipocytes promote proliferation [100]. Adipocytes were also shown to increase prostate cancer cell proliferation [101] and migration [62], as well as promote pancreatic cancer in mouse models [102]. Taken together, these results clearly demonstrate a pro-tumorigenic role for adipocytes in many different contexts.

Using an experimental metastasis model, cancer cells previously exposed to adipocytes during culture prior to tail vein injection yielded more lung metastases than those cultured without adipocytes [103]. In the same study, the authors also demonstrated that cancer cells co-cultured with adipocytes, or in adipocyte-conditioned media (from both normal and cancer-associated adipocytes), have higher in vitro invasive capabilities [103]. Similarly, migration/invasion as well as wound-healing abilities of the cancer cells are increased after co-culture with adipocytes [89], and adipocyte-conditioned media promotes breast cancer cell proliferation [98]. As the cancer cells were not in direct contact with adipocytes in the latter experiments, it is suggested that this increase in invasion is mediated through secreted factors from adipocytes and does not require direct cell-cell contact. Co-culture experiments also demonstrated that adipocytes elicit a protective effect on breast cancer cells against irradiation, through the activation of Chk1 and prevention of cell death. The authors also described an increase in IL-6 expression in tumor cells after co-culture with adipocytes, which may be the mediator underlying this radioresistant phenotype [104]. Thus, it is evident that adipocyte-derived factors are important in mediating multiple aspects of tumor progression, including invasion and responses to therapy.

Adipocytes are among the first cell types that interact with emerging tumor cells; thus it is not surprising that adipocytes may be diminished even at an early stage of tumor development. Research has demonstrated a dynamic desmoplastic response elicited by adipocytes at the edge of the tumor, where adipocytes are shown to be high in abundance. As the focus is shifted toward the tumor center, adipocytes are no longer detected, and instead CAFs are in abundance [93, 103]. Furthermore, adipocytes co-cultured with cancer cells in vitro undergo extensive phenotypic changes, showing an elongated fibroblast-like cell morphology [89, 103], as well as

a decrease in adipocyte marker expression and an increase of proteases and inflammatory cytokines [103]. Peritumoral adipose tissue also displays atrophied adipocytes with a decrease in expression of adipocyte-specific markers [91]. These results raised the possibility that tumor-educated adipocytes may dedifferentiate to form other pro-cancer stromal cell types that promote cancer progression.

Adipocytes are derived from mesenchymal precursors and are believed to be post-mitotic. Though it was suggested that adipocytes juxtaposed to tumors could dedifferentiate and reenter the cell cycle, it has been challenging to rule out interference from other stromal cell types such as pre-adipocytes or fibroblasts [70, 71, 103]. Previous research has suggested "transdifferentiation" between white and beige adipocytes, so it is plausible that mature adipocytes, when under the right stimulus, could dedifferentiate into other cell types. This is supported by studies that showed adipocytes co-cultured with cancer cells acquire fibroblast-like features [103]. Notably, Bochet et al. demonstrated that in athymic nude mice with engrafted GFP-expressing adipose tissues containing breast cancer cells, there is an emergence of GFP-expressing stromal cells expressing FSP-1 (a marker for fibroblasts) within the tumors. Interestingly, these green stromal cells do not express the classical CAF marker α-SMA [105]. They also showed that in human breast cancer specimens, there is an increase of FSP-1 expressing cells as the focus shifts from the adipose tissue surrounding the tumor toward the tumor core [105]. With an in vivo lineage tracing model whereby all mature adipocytes are indelibly marked in an immunocompetent mouse (the *adipoq-Cre:ZSgreen* mice, where the ZSgreen fluorescent protein is produced in adiponectin-driven Cre-expressing cells, i.e., adipocytes [106]), and implanting syngeneic cancer cells, one could precisely follow the progression of adipocytes as tumors develop and explore how cancer cells impact the differentiation of adipocytes in vivo. Recently, we have injected syngeneic murine mammary carcinoma cells into the mammary fat pad of immunocompetent *adipoq-Cre:ZSgreen* mice and showed the presence of green fluorescent cells with a spindle-shaped morphology within the tumor core (Fig. 6.2). These preliminary findings indicate that as tumors progress, the adipocytes (the honeycomb-like structures surrounding the tumor) incorporated into tumors change their morphology. Future studies will be needed to elaborate how the function of these spindle-shaped, adipocyte-derived cells might differ from their mature adipocyte counterparts in tumors and what role they play during tumor growth.

In addition to the role of adipocytes in promoting tumor growth and invasion/metastasis at the primary tumor site, adipocytes residing in secondary organ sites may also promote cancer progression by providing a favorable environment for cancer cells that have disseminated from the primary site. One good example would be the bone marrow (BM), which is a prime metastatic target site for many cancers such as multiple myeloma, breast, and prostate cancers. Brown et al. first showed that prostate cancer cells are attracted to adipocytes residing within the metabolically active red bone marrow [107, 108]. More recently BM adipocyte-derived factors CXCL1 and CXCL2 were shown to promote osteolysis in prostate cancer, in turn facilitating metastatic colonization of the cancer cells [109]. BM adipocytes also

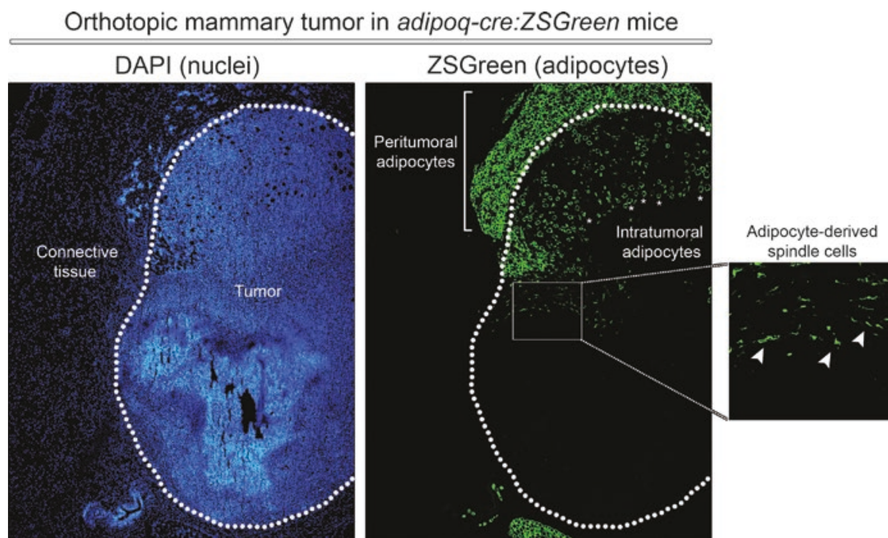

Fig. 6.2 Co-option and de-differentiation of adipocytes in mammary tumors. E0771 murine mammary carcinoma cells (1 × 10^6) were injected orthotopically into the 4th mammary fat pad of 8-week old *adipoq-Cre:ZSgreen* female mice. Tumors grew for a week before mice were sacrificed and tumors (together with the surrounding mammary fat pad) were collected, fixed, frozen, and sectioned. 15 μm sections were cut and nuclei were counterstained with DAPI. Tile-scans of the whole section were carried out using confocal imaging at 10× magnification. *Left:* tile-scan of DAPI staining of a tumor with surrounding connective tissues. The tumor is outlined with dotted white lines. *Right:* the same section with green fluorescence. Green cells are adiponectin-expressing adipocytes. As seen from the image, peritumoral adipocytes (green cells) have a typical honeycombed appearance. Within the tumor, individual green cells are observed that were likely co-opted as the tumor expanded (*marked with asterisks*). Towards the center, green cells with a spindle morphology can be clearly observed. *Inset:* higher magnification of the tumor core (40×) showing the adipocyte-derived spindle cells

promote metastatic tumor growth in breast and prostate cancer through upregulation of FABP4, IL-1β, and HMOX1, which in addition to their effects on proliferation also promote the invasiveness of the disseminated cancer cells [110].

Adipose-Derived Factors and Cancer

Adipokines and Cancer

As summarized in Table 6.1, adipose tissue secretes a wide range of molecules; for some of the mentioned molecules—collectively known as “adipokines”—the adipocytes are the predominant source. Here, we will focus on the two major adipokines and discuss their role in cancer progression.

Leptin

Leptin is a 16-kDa protein encoded by the *Ob* gene and is predominantly produced by white adipose tissue. There are also other sites of production, albeit at vastly lower quantities, including the placenta, ovaries, bone marrow, intestine, stomach, pituitary gland, liver, brain, mammary epithelial cells, and skeletal muscle [111]. As leptin is produced by white adipocytes, its expression is positively correlated with body mass. However, leptin production is also controlled by levels of various factors, such as insulin, tumor necrosis factor alpha (TNFα), glucocorticoids, sex hormones, prostaglandins, as well as hypoxia (low oxygen levels, commonly observed in solid tumors) through HIF-1 regulation [112, 113]. Leptin controls satiety and regulates energy homeostasis by acting on the arcuate nuclease of the hypothalamus [114]. The leptin receptor has six isoforms (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, Ob-Rf), which are from the extended class I cytokine receptor family and dimerize with one another without intrinsic kinase activity [115]. The receptor isoform expression varies according to tissue/cell type and is autoregulated by ligand stimulation [116, 117]. After binding to the receptors, leptin activates various signaling pathways such as the JAK/STAT, MAPK, PI3K (also known as AKT), AMPK, and IRS pathways [118–120].

Previous research has shown that leptin promotes proliferation, migration, and invasion in tumor cells, identifying it as a cancer-promoting factor [112, 121]. Leptin promotes proliferation of prostate cancer through the activation of MAPK, PI3K, and JNK/MAP kinase pathways [122]. It also promotes proliferation of breast cancer cells and colorectal cancer cells through the activation of JAK/STAT3, MAPK-ERK1/2, and PI3K pathways [123–125]. Leptin trans-activates ErbB2 and EGFR and interacts with IGF-1, thereby promoting invasion and migration. It also increases estrogen levels by stimulating aromatase expression and can directly activate the estrogen receptor (ER), thus further promoting the growth of ER-positive breast cancer cells [126–128]. Apoptosis is also inhibited by leptin in colorectal cancer [123–125], and invasion is increased by leptin-promoted proteolytic cleavage of ECM [129]. In addition to its effects on cancer cells directly, leptin stimulates inflammatory cytokine (e.g., IL-6, TNFα) production in macrophages, i.e., favoring the tumor-promoting M2 subtype [130]. Additionally, leptin has been shown to regulate endothelial cell proliferation and increase endothelial COX-2 expression [131, 132]; it also promotes angiogenesis in prostate and colorectal cancers by inducing the expression of VEGF, FGF2, MMP2, and MMP9 [133, 134] and increases the expression of VEGF-receptor 2 (VEGF-R2), further eliciting an angiogenesis-promoting effect in breast cancer [135].

Adiponectin

Adiponectin is a 30-kDa protein that belongs to the complement-1q family [136]. It is primarily secreted in the monomeric form but can further oligomerize to form trimeric, hexameric, or multimeric forms [111]. It can also be cleaved by leukocyte elastase, generating a globular oligomeric complex [137]. Adiponectin is expressed

by adipocytes in high concentrations and is generally thought of as adipocyte specific, although studies have reported expression in the skeletal muscle, liver, colon, salivary glands, bone marrow, fetal tissue, placenta, and cerebrospinal fluid [138], albeit at much lower levels. Paradoxically, even though adiponectin is predominantly expressed by adipocytes, the circulation levels are negatively correlated with BMI and body fat percentage, which is likely due to negative regulation by various other adipose-secreted proteins, such as TNFα, IL-6, and IL18 [139, 140]. There are two main receptors for adiponectin: adiponectin receptor 1 (adipoR1) and adiponectin receptor 2 (adipoR2). AdipoR1 preferentially binds to globular adiponectin, whereas adipoR2 binds to other higher-molecular weight forms more readily [141]. These receptors then either homo- or heterodimerize and activate downstream signaling events through phosphorylation, such as AMPK [142, 143], acetyl-CoA carboxylase [144], and MAPK [141]. Adiponectin is an insulin sensitizer [145] and has anti-atherogenic [146] and anti-inflammatory [147] effects. It also maintains the vasculature within adult adipose tissue and is likely an angiogenesis inhibitor [148].

In general, adiponectin and leptin have opposing effects on cancer cells and can antagonize one another, e.g., adiponectin suppresses leptin-induced IL-6 signaling, by inhibiting autocrine IL-6 production, decreasing soluble IL-6 receptors, and increasing sgp130 (inhibitor of the IL-6/IL-6receptor complex) [149, 150]. Where leptin administration increases proliferation, migration, and invasion of cancer cells, adiponectin treatment in mouse models was shown to inhibit tumor growth by decreasing proliferation and increasing apoptosis of cancer cells; it also inhibits invasion of cancer cells and leads to decreased vessel density in tumors [151–155]. As such, adiponectin is widely viewed as an antitumor factor. Adiponectin was shown to inhibit proliferation of prostate cancer cells through androgen receptor-dependent (inhibition of STAT3 signaling [156]) or androgen receptor-independent mechanisms (activation of AMPK and thus inhibition of mTOR/PI3K/AKT pathways [157]). It also inhibits TNFα in breast cancer cells, thereby decreasing estrogen synthesis and inhibiting NFκB signaling, leading to a decrease in proliferation [158]. This inhibition of TNFα also suppresses VEGF production, in turn causing a decrease in angiogenesis and subsequent increase in apoptosis/necrosis within the tumor mass [148, 157]. The effects of adiponectin in endometrial cancer is, unsurprisingly, similar to that observed in breast cancer, as both tissues respond to estrogens and express high concentrations of VEGF [111, 159]. In addition, adiponectin can induce cell cycle arrest and apoptosis through PTEN-dependent (PI3K/Akt) or PTEN-independent (Erk1/2 and cyclin E2) mechanisms in endometrial cancer [160]. In terms of colorectal cancer, adiponectin also has antiproliferative and pro-apoptotic effects through the activation of AMPK and inhibition of the mTOR pathway [161].

Other Adipose Tissue-Derived Factors and Cancer

In this section, we will discuss a few other adipose tissue-derived factors and their roles in cancer. These molecules are frequently grouped together with adiponectin and leptin as "adipokines"; however, as these molecules may not be predominantly secreted by adipocytes, but rather derived from the adipose tissue (which includes

the SVF), we refer to them here as "adipose tissue-derived factors" instead. Many of these factors have intricate relationships with the aforementioned adipokines. Additionally, as briefly discussed in the previous section, injured adipocytes likely passively release factors (as opposed to "secrete," which suggests an active and controlled process) that fuel tumor progression.

Visfatin/PBEF/NAMPT

Visfatin, a 52-kDa protein, is also known as pre-B-cell colony-enhancing factor (PBEF) or nicotinamide mononucleotide denylytransferase (NAMPT). It was first identified as PBEF due to its effects on the development and maturation of B-lymphocytes [162]. It was then identified as an adipokine that is highly expressed in visceral fat, with circulating levels correlating with the size of the visceral fat depot (but not subcutaneous fat) and thus was termed "vis(ceral)fat" in [33, 163]. Fukuhara et al. also described it as a hormone that binds and activates the insulin receptor, thereby mimicking insulin signaling. However, this study was retracted in 2007, throwing the physiological relevance of visfatin into question. Nonetheless, in a later study, it was described as NAMPT, a key enzyme involved in NAD biosynthesis from nicotinamide [164], thus considerably expanding its biological implications beyond adipose tissue. Additionally, visfatin was shown to be released predominantly from macrophages rather than adipocytes residing in visceral adipose tissues [165] (although visfatin circulation levels are usually increased in obesity), as such visfatin is involved in inflammation. The pro-angiogenic effects of visfatin are also well documented [166], which occurs through Erk1/2 activation [167], VEGF/MMP2/MMP9 production [168], FGF-2 upregulation [169], and MCP1/CCR2 induction [170].

In terms of visfatin and cancer, it was shown that visfatin inhibition confers greater sensitivity toward chemically-induced apoptosis in fibrosarcoma cells [171], and exogenous expression of visfatin in prostate cancer increases proliferation through Erk1/2 and p38, as well as the expression of MMP2/9, suggesting another link with increased invasive capabilities [172]. Similarly, the inhibition of visfatin suppresses in vitro proliferation and in vivo tumor growth of prostate cancer cells, with a further sensitization of the cancer cells toward chemotherapeutic treatment [173]. Visfatin was also demonstrated to promote proliferation through Notch1 signaling in breast cancer cells [174]. Of note, one known small molecule inhibitor of visfatin, FK866/APO866, is being evaluated as a cancer therapeutic [166] and has completed Phase II clinical trials. Another, CHS828/GMX1777, despite promising preclinical results [166], could not complete clinical trials due to financial constraints.

PAI-1

Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor that is secreted by endothelial cells, stromal cells, and visceral WAT [175]. It affects adipocyte differentiation and insulin signaling, and circulating levels are positively associated with obesity as a result of increased PAI-1 production in obese

adipocytes [176]. PAI-1 inhibits urokinase-type and tissue-type plasminogen activators (uPA and tPA), thereby blocking fibrinolysis and remodeling of the ECM. PAI-1 both promotes and inhibits angiogenesis depending on the concentration [177]; therefore, its expression plays a critical role in tumor growth, invasion, and metastasis [178]. uPA expression is positively associated with tumor growth, invasion, and metastasis [179], whereas PAI-1, an inhibitor of uPA, is associated with both anti- and pro-cancer activity.

PAI-1 was reported to inhibit prostate tumor growth, angiogenesis, invasion, and metastasis [180] and appeared to negatively impact mammary adenocarcinoma progression [181]. However, PAI-1 was also shown to promote tumor invasion and angiogenesis, where host-derived PAI-1 is more important than tumor-secreted PAI-1 [182]. Additionally, PAI-1 treatment conferred protection against chemotherapeutics in prostate cancer and leukemia cell lines, through inhibition of apoptosis [183]. Loss of PAI-1 also reduces tumor growth, invasion, and metastasis in fibrosarcoma and non-melanoma skin cancer [184, 185]. The contrasting results suggest that the effects are contextual based on the levels of PAI-1—where low levels (nearer physiological levels) increase angiogenesis and elevated levels does the opposite [179]. However, the effects of PAI-1 on cancer progression are not restricted to angiogenesis—PAI-1 interacts with vitronectin [186] and integrins, thus regulating adhesion [187] and migration properties of cancer cells [188]. It is also implemented in neutrophil recruitment and the inflammation response [189]. Thus, PAI-1 both positively and negatively mediates tumor progression depending on the context and tumor type.

TNFα

Tumor necrosis factor alpha (TNFα) is a 25-kDa cytokine that plays an important role in the adaptive immune system. It is a transmembrane protein with signaling potential both as a membrane-integrated protein and as a soluble cytokine released after proteolytic cleavage (see [190] for a detailed review). TNFα is a key growth factor that is secreted by macrophages, although adipocytes are also known to secrete it [191], and TNFα levels are elevated in obese individuals, again likely reflecting a chronic inflammatory state [59, 192]. It has two receptors: TNFR1, which is found in most cell types and activated by soluble TNFα, and TNFR2, predominantly found on hematopoietic cells that preferentially bind the transmembrane TNFα. Antitumor effects of TNFα were reported in gastric cancer, where it activates caspase-3 and thus apoptosis [193], and TNFα was thought to be a strong candidate as a cancer therapeutic as its name suggested [190]. However, as research demonstrated that TNFα is not only produced by cancer cells but also present in high amounts in the TME, it is increasingly clear that TNFα can promote carcinogenesis, likely through the activation of NFκB, which in turn promotes the expression of factors that stimulate inflammation, proliferation, survival, invasion, and metastasis [194, 195]. TNFα further fuels cancer progression by inducing the production of other pro-cancer cytokines, angiogenic factors, and MMPs, thereby

promoting growth and invasion of tumor cells [196]. Furthermore, using dietary/genetically induced obese mouse models, it was shown that liver inflammation and tumorigenesis are enhanced through increased TNFα and IL-6 expression [59], further highlighting the link between "disease state adipocytes," adipose-derived factors, and tumor-promoting activities. As such, TNFα is an attractive therapeutic target as it promotes tumor progression on several levels. TNFα has tumor-cytotoxic properties in certain contexts; however, it requires careful administration to ensure direct contact with the cancer cells, in order to obtain maximal benefit [190]. On the contrary, TNFα inhibitors showed some success in clinical trials for various cancers and may be considered good candidates as combination therapies either by increasing sensitivity to chemotherapy [197, 198] or by re-education of the TME [199].

IL-6

Similar to TNFα, interleukin-6 (IL-6) is a well-known inflammatory cytokine that is secreted by macrophages as well as adipocytes (albeit in lower amounts) and is elevated in obese patients [59, 192]. Additionally, IL-6 expression levels are elevated in cancer-associated adipocytes [91, 103]. The role of IL-6 in cancer has been debated, as IL-6 was shown to be both pro- and anti-apoptotic in breast cancer cells [200–203]; nonetheless, IL-6 is generally thought of as pro-cancer, where IL-6 treatment induces malignant features in mammospheres from ductal breast carcinoma as well as normal mammary gland [204]. IL-6 induces the production of many proangiogenic molecules including VEGF, which contributes to tumor growth and metastasis through the angiogenic switch [205]. It also activates/increases aromatase expression involved in estrogen synthesis and therefore is likely to have a major role in cancer progression in postmenopausal female patients [202]. IL-6 knockout mice demonstrated a resistance toward carcinogen-induced tumorigenesis in liver cancer [204], and IL-6 has been shown to increase the transcription factor Jagged-1, in turn enhancing self-renewal capabilities of breast cancer stem-like cells [111]. As mentioned above, IL-6 is upregulated in obese mice with liver cancer, again potentially linking adipocytes with tumor-promoting functions [59].

Diagnostic Value of Adipocytes in Cancer

Based on in vitro and in vivo evidence, adipocytes appear to be important in mediating cancer progression in the TME. But how do adipocytes in the TME impact clinical outcomes? Studies have been conducted to evaluate the prognostic values of local adipose tissue invasion by cancer cells at the tumor edge and reported a correlation between high adipose tissue invasion with poor patient outcomes for breast, prostate, pancreas, kidney, and colon cancers [206–208]. Histological studies on steatosis (adipocyte infiltration of the liver) and adipocyte infiltration of the pancreas have both been linked to an increased risk of hepatocellular and pancreatic

cancers, and in terms of established cancers, adipocyte infiltration in pancreatic cancer and breast cancers are both associated with increased aggressiveness and faster disease progression [208, 209].

Interestingly, IL-6 expression in tumor-surrounding adipocytes had been shown to be elevated in human breast cancer patients with higher tumor grade and/or lymph nodes involvement; similarly, this increase of IL-6 in tumor-surrounding adipocytes was also observed in prostate cancer [210]. These results suggest that in addition to detection of adipocyte infiltration into tumor tissues, assessing the levels of adipose-derived factors in tumor-associated adipose tissue may also be prognostic. All of these studies using human patient samples highlight the feasibility of using adipocytes to assess cancer incidence, as well as predict disease outcome and progression.

Diagnostic Value of Adipose-Derived Factors in Cancer

Circulating Adipokines/Adipose Tissue-Derived Factors

Many of the adipocyte-derived factors are influenced by a multitude of other soluble factors, which may be highly variable between patients (e.g., estrogen levels); in addition, these factors also influence the activity and/or expression of one another (e.g., leptin, adiponectin, TNFa, IL-6). Thus, the effectiveness of studying the serum levels of adipokines in cancer patients is often complicated, and contradictory reports in correlating serum levels of adipokines with cancer progression is not unusual.

High serum leptin levels have been associated with increased risk for colorectal cancer [211], but mixed reports exist [212–217], where higher leptin levels may be indicative of better prognosis [218]. In terms of breast cancer, after unifying contradictory results in case-control studies, three out of ten studies showed positive correlation between leptin levels and breast cancer, while the others showed no association [219]. In this instance, it is challenging to draw a conclusion on the relationship between leptin and breast cancer stage, as the various studies did not have a unified study design (e.g., sample collection from fasting/non-fasting patients, stratification of women into premenopausal or postmenopausal statuses, history of leptin-related diseases such as diabetes, etc.). Interestingly, where the three studies showing positive correlation between serum leptin and breast cancer regardless of menopause stage, one study showed that in premenopausal patients, an inverse association of leptin and breast cancer was reported [220]. This result was also found in a later study where postmenopausal breast cancer patients showed a correlation between high leptin levels and tumor grade/stage [221], thus indicating that postmenopausal women may be particularly affected by leptin, likely due to the increased importance of the adipose tissue as a source of estrogens [221, 222]. A small study in Turkish patients indicated that higher circulating leptin levels are observed in patients with papillary thyroid carcinoma [223], but more studies need

to be conducted to confirm this finding. Surprisingly, in a small case-control study on patients with renal cell carcinoma, leptin was inversely associated with cancer risk [224]; however, there was no adjustment for sex or tumor stage. In a later study with a much larger patient cohort, a significant association between high leptin concentration and increased renal cell carcinoma was observed, although the authors also conceded this correlation may be dependent on the racial background [225]. Furthermore, higher leptin serum levels were also shown to be a predictor for shorter progression-free survival [226].

Adiponectin levels are inversely related with risk, incidence, and tumor grades of prostate cancer [227], but its relationship with female breast cancer is dependent on the menopausal state, where a correlation between low adiponectin levels and breast cancer is only observed in postmenopausal women [228]. In contrast, in endometrial cancer, a link between low circulating adiponectin levels and cancer risk is strongly represented in premenopausal women, independent of BMI status [159, 229]. Contradicting results have been reported between adiponectin and colorectal cancer [214, 230], although low circulating adiponectin is mostly associated with an increase in colorectal cancer incidence [231–233]. Conversely, in pancreatic cancer, case-controlled studies suggest that increased circulating adiponectin levels are associated with cancer incidence [234, 235], although in male smokers, an opposite trend was reported [236]. Low circulating adiponectin is also strongly associated with renal cancer incidence, with a further inverse correlation between adiponectin levels and occurrence of metastases; this is despite a lack of consistency between adiponectin levels and tumor grade [237–239]. In addition, in patients with end-stage renal disease, who have a higher risk of cancer incidence, low circulating adiponectin levels are an independent predictor of malignancy [240]. This suggests that in other chronic diseases that have a link with cancer incidence (e.g., Crohn's disease), circulating adiponectin levels may also be useful as a malignancy predictor.

Serum levels of visfatin were shown to be significantly higher in endometrial cancer patients and are correlated with visfatin expression within the tumor [241]. Additionally, in colorectal cancer patients, high circulating visfatin levels were identified as a significant risk factor for both early and advanced diseases, and levels were positively correlated with tumor stage progression [214, 242]; a similar trend was reported in gastric cancer patients [243], further confirming a strong clinical relevance for visfatin as a biomarker of cancer incidence and progression.

For the diagnostic value of TNFα, high circulating levels of TNFα and its soluble receptor (in particular sTNFR2) were shown to be correlated to a higher risk of endometrial cancer in a case-control study [244], and TNFα levels in blood are also positively correlated with disease stage and severity in prostate cancer [245, 246].

Increased serum IL-6 levels are linked to a significant increase in endometrial cancer risk [247], and interestingly, one potential reason why IL-6 may contribute to endometrial carcinogenesis in postmenopausal women is through adipocytes, where an increase in adipocyte-derived estrogen as a result of IL-6 stimulation drives hyperplasia and ER-positive tumor growth [248]. Circulating IL-6 levels are also positively correlated with tumor grade and worse overall outcome in prostate

cancer patients [245, 249], multiple myeloma [250], metastatic renal cell carcinoma [251] and breast cancer [205, 252, 253] and are identified as a risk factor for colorectal cancer in postmenopausal women [211]. Interestingly, it was reported that reduced IL-6 or sIL-6R (soluble IL-6 receptor) levels in the serum of patients indicate a response to therapy [254], suggesting the potential of using adipose-derived factor levels as an indicator for treatment response.

Studies in breast cancer demonstrated that a combination of both leptin and adiponectin circulating levels (i.e., ratio between the two adipokines) may be a better prognostic predictor for patient outcome [255–258]. This highlights the complex relationships between various adipokines and adipose tissue-derived factors and suggests a multifactorial analysis where all of these factors must be taken into account in order to utilize them as prognostic indicators.

Expression of Adipokines/Adipose Tissue-Derived Factors in Tumor Tissues

Despite the shortcomings of using circulating levels as a biomarker for cancer progression, adipokines/adipose tissue-derived factors may still be used as prognostic markers through detection of their expression(s) in biopsy samples. For example, leptin and its receptor are overexpressed in human primary and metastatic breast cancer, with the highest levels detected in poorly differentiated tumors, which are usually associated with worse patient outcome [259]. Additionally, differentiating between the subtypes of leptin receptor in these breast cancer patients may also be a prognostic factor, where patients with high Ob-Ra-only have longer disease-free survival compared to patients with a high Ob-Rb/Ob-Ra expression ratio [260]. Similarly, leptin is overexpressed in colorectal cancers and is significantly correlated with tumor grade [261]. Leptin and leptin receptor expression levels in papillary thyroid cancer are positively associated with aggressiveness of the disease [262, 263], and high leptin receptor levels in renal cell carcinoma were associated with venous invasion/tumor grade/presence of lymph node metastasis [226].

In terms of adiponectin, the expression of AdipoR1 and AdipoR2 are increased in colorectal cancer cells [264]. However, in renal cancers, a decrease of receptor expression was reported suggesting that the use of AdipoR1/R2 as a diagnostic marker may be dependent on the cancer type. Visfatin expression, on the other hand, is positively correlated with stage and myometrial invasion in patients with endometrial cancer [241]. High expression of visfatin in breast cancer tissues is associated with ER and PR status, as well as poor disease-free and overall survival in patients [265]. Interestingly, visfatin expression could predict poor response toward doxorubicin chemotherapy [266], and patients with high visfatin expression have decreased recurrence rates after hormone therapy (but not radio- or chemotherapy [265]), suggesting the potential of visfatin in stratifying treatment options for breast cancer patients. Similarly, PAI-1 expression can also predict treatment response to tamoxifen in recurrent breast cancer patients [267] and act as a predictor for shorter overall as well as disease-free survival in women with breast cancer [268].

Polymorphisms of Adipokines/Adipose Tissue-Derived Factors

In addition to using expression levels in tumors or surrounding tissues as an indicator for disease progression, mutations in the genes encoding adipocyte-derived factors may also be used for cancer risk assessment, similar to the *BRCA1/BRCA2* mutations in breast cancer patients. Polymorphisms of leptin (*LEP*) and leptin receptor (*Ob-R*) were associated with higher risk of breast cancer in women, and the association was strongest in obese postmenopausal women [269–272]. PAI-1 promoter polymorphism (known as *4G/5G* polymorphism, where the *4G* allele translates into higher PAI-1 expression) has also been linked to cancer incidence/prognosis. In breast cancer patients, *PAI-1 4G* is increased in patients with aggressive tumor characteristics [273], and another reported that *5G/5G* homozygosity is a positive prognostic marker for aggressive diseases [274], although a third study found no link between PAI-1 polymorphism and breast cancer incidence/outcome. *PAI-1 4G* was found to be a major contributor to early stages of oral cancer [275], and PAI-1 polymorphisms may also be good prognostic markers in high-grade gliomas [276]. TNFα-308, a polymorphic form of TNFα, is a risk factor for breast [277], gastric [278], and liver cancer [279], and a single-nucleotide polymorphism in the IL-6 promoter (in particular the 174 G/C polymorphism) has been associated with more aggressive breast cancer types [280, 281].

Conclusion

As discussed in this chapter, there is great potential for adipocytes as a histological prognostic tool in cancer patients; however, the use of circulating adipose-derived factors for prognosis requires further investigation, which may involve improved patient stratification and multifactorial analyses. Nonetheless, apart from being prognostic markers, adipokines/adipose tissue-derived factors may also be useful in measuring treatment response. It should be noted that some of the molecules discussed here are considered good therapeutic targets and some small molecule inhibitors are already in clinical trials for cancer. This highlights the importance of further exploring the role of the adipose tissue and adipocytes in mediating cancer progression and metastasis.

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Chapter 7
Inflammation and Cancer: The Role of Lipid Signaling in the Continuum Between Two Ends of the Tumor Spectrum

Megan L. Sulciner, Molly M. Gilligan, Bruce R. Zetter, and Dipak Panigrahy

Abstract Inflammation and cancer have a long and contentious history. Currently, there are two lenses through which the role of inflammation in cancer can be viewed. Substantial evidence suggests that inflammation can not only propagate, but even initiate cancer pathogenesis. However, emerging studies indicate that inflammation may alternatively enhance host containment and destruction of tumorigenic cells. Herein, we explore how our understanding of inflammation in cancer has evolved, from the first identification of excessive inflammation in tumors two millennia ago to the complex association between inflammation and cancer pathogenesis with the recent emergence of immune-harnessing cancer therapies. We discuss the dynamic roles of various immune cells, cytokines, and specific lipid autacoid signaling in cancer, focusing on fatty acid-derived lipid mediators such as prostaglandin E_2. We contrast the pro-tumorigenic and anti-tumorigenic functions of immune cells and lipid mediators, while highlighting how their functions can be dramatically altered by the tumor microenvironment.

Keywords Inflammation • Cancer • Tumor • Lipids • Cytokines • Immune cells • Macrophages • Natural killer cells • T cells (T lymphocytes) • B cells (B lymphocytes) • Dendritic cells • Inflammatory score • Prostaglandin E_2 • Annexin A1 • C-reactive protein (CRP)

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History

The association between inflammation and cancer dates back as early as two millennia ago, when Claudius Galen expanded on Hippocrates' theory of cancer as an excess of black bile (melancholia) by suggesting that cancer was a swelling resulting from excessive inflammation [1, 2]. In 1861, Dr. Rudolph Virchow, a German pathologist, delivered a twenty-part lecture series at the Pathological Institute of Berlin in which he was the first to describe immune cell infiltrates in tumors. Specifically, he noted white blood cells in tumor tissue and suggested that immune cells release factors that stimulate the proliferation of tumor cells [3]. Thirty years later, Dr. William B. Coley alternatively linked immune stimulation to cancer regression. At the New York's Academy of Medicine, he reported three cases of tumor regression resulting from inoculating sarcoma patients with a strain of *Streptococcus* bacterium [4]. Later coined "Coley's toxin," inoculation with this bacterium is now known to have elicited tumor regression through tumor necrosis factor-α (TNFα)-mediated activation of cytotoxic immune cells.

Within the last 50 years, seminal progress has been made towards understanding inflammation and cancer. In 1986, Harvard Medical School pathologist Dr. Harold Dvorak elegantly characterized tumors as "wounds that do not heal," noting that while tumors elicited an immune response, they exhibited persistent inflammation rather than the gradual cessation of acute inflammation associated with wound healing [5]. Drs. Lisa Coussens, Douglas Hanahan, and Zena Werb later demonstrated that premalignant tissue became malignant with the assistance, or "co-conspiracy," of inflammatory cells, such as mast cells, macrophages, and T lymphocytes [6, 7]. It is now recognized that an inflammatory tumor microenvironment plays a key role in every stage of tumor development. Several immune cell types, including macrophages, neutrophils, mast cells, and regulatory T cells, secrete tumor growth-promoting chemokines, pro-angiogenic factors, and metastasis-promoting extracellular proteases [8, 9]. By 2011, Drs. Douglas Hanahan and Robert Weinberg included inflammation as a defining hallmark of cancer [10].

Despite inflammation commonly being viewed as "the other half of the tumor" [11], a growing body of evidence suggests immune cells and their mediators may play more ambiguous roles, in the prevention of prevent cancer progression. In 1977, Dr. Alberto Mantovani demonstrated that antibody-dependent cellular cytotoxicity (ADCC) inhibited tumor cell growth via both cytostatic (inhibiting cell growth and division) and cytolytic (lysis-inducing) mechanisms [12]. Mantovani's work became the foundation for future studies characterizing the antitumor potential of immune surveilliance. As Coley's century-old observations suggested, [c]ancer cells express antigens that can be identified as "nonself" by surveillance immune cells, such as natural killer cells and cytotoxic T cells, and can elicit an antitumor immune response. Mantovani's later studies characterizing the ability of "tumor-associated macrophages" to both stimulate and inhibit tumor growth further highlighted the contrasting pro- and anti-tumorigenic functions of the immune system [13]. In 1987, Dr. Frances Balkwill and her colleagues demonstrated that intraperitoneal treatment

with the pro-inflammatory cytokines TNFα and interferon γ (INFγ) prolonged survival of ovarian tumor-bearing mice [14]. Building on Coley's and Mantovani's pioneering studies, her findings suggested provoking an inflammatory immune response may alternatively elicit endogenous anti-tumorigenic activity. Balkwill later characterized novel mechanisms through which TNFα can be simultaneously pro- and anti-tumorigenic depending on the specific tumor microenvironment [15].

Self-limited and localized, or acute, inflammation serves as the body's endogenous defense against the outside world. However, unresolved acute inflammation can become chronic. Chronic inflammation and inflammatory diseases are in fact risk factors for over ten different cancer types: chronic *Helicobacter pylori* predisposes to stomach cancer, inflammatory bowel diseases predispose to colon cancer, etc. [16]. However, chronic inflammation may not always progress to a related organ system cancer. For example, psoriasis (chronic inflammation of the skin) is not always linked to skin cancer incidence [11]. Moreover, 19 cancers have been associated with prior bacterial, viral, or parasitic infection [16]. Chronic inflammation can even be necessary for cancer pathogenesis, as mice engineered to develop hepatitis (a chronic inflammatory disease of the liver) failed to develop hepatocellular carcinoma when TNFα was neutralized [17]. Importantly, NFκβ, a pro-inflammatory transcription factor critical in innate and adaptive immune activation, was also suppressed in these mice [17]. The Karin laboratory demonstrated that activation of NFκβ perpetuates inflammation and contributes to colon tumor growth, while inhibition of NFκβ downregulates inflammation, resulting in tumor regression [18]. The inflammatory component of malignancies is further evidenced by the clinical effectiveness of anti-inflammatories, including nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, as anticancer therapies [19]. As we strive to untangle the complex, multifaceted relationship between inflammation and cancer, our understanding of the disease has shifted radically. Cancer is increasingly illuminated as a war of self, the outcome hinging on whether our immune systems keep our mutated cells in check or assist in their progression and eventual malignant escape.

Tumor Microenvironment

The tumor microenvironment has become a focus in cancer research as its constituents are the major contributors of tumor-promoting inflammation. Here, we focus specifically on the diverse roles of immune cells, cytokines, lipid mediators, and inflammatory biomarkers that actively mediate inflammation in the tumor microenvironment. While inflammation has traditionally been associated with cancer progression, current studies continue to identify both pro- and anti-tumorigenic mechanisms enacted by our immune system. We explore the pro-tumorigenic mechanisms of infiltrating immune cells, pro-inflammatory cytokines lipid mediators, and pro-inflammatory biomarkers, while examining how these immune cells and their mediators may paradoxically play an anti-tumorigenic role in the context of cancer.

Immune Cell Biomarkers

Both the innate and adaptive immune systems contribute to cancer-associated inflammation. Immune cells such as macrophages, dendritic cells, natural killer cells, T lymphocytes, and B lymphocytes play critical pro-tumorigenic and anti-tumorigenic roles in inflammation-associated cancer pathogenesis.

Macrophages

Macrophages are traditionally viewed as scavengers of the immune system. As monocytes, their immature predecessors, they circulate in the blood until they receive a signal to migrate into tissues, where they mature into macrophages. Macrophages are dual-functional: while they participate in the innate immune response by surveying tissues and destroying antigens via phagolysosomes, they also function as antigen-presenting cells within the adaptive immune system to mount specific T and B cell responses against foreign particles, microbes, or even cancer cells.

While previously thought to be predominantly anti-tumorigenic, our understanding of macrophages in cancer has evolved since the 1980s to encompass their more recently characterized pro-tumorigenic actions. In the 1980s and 1990s, resident liver macrophages known as Kupffer cells were reported to possess anti-tumorigenic activity [20]. Specifically, Kupffer cells were shown to phagocytose tumor cells, and their systemic depletion led to increased metastases [21]. Tumor-bearing rats lacking macrophages, a result of exogenous depletion, were shown to exhibit increased tumor differentiation and decreased survival due to aggressive tumor growth [22]. In contrast, the depletion of monocytes and macrophages in animal tumor models demonstrated reduced tumor incidence, as well as inhibition of tumor growth and angiogenesis [23]. The anti-tumorigenic nature of macrophages is further exemplified by the evolution of tumor cells to evade macrophage detection. Leukemia cell expression of CD47, an anti-apoptotic and autophagic marker, was demonstrated to inhibit macrophage phagocytosis, enabling tumor immune evasion and cancer progression [24].

Macrophages have been characterized in various tumorigenic and metastatic disease settings. For instance, glioblastoma has been associated with increased numbers of circulating monocytes [25]. Further, phagocytosis of circulating breast cancer exosomes by distant macrophages has been shown to increase macrophage secretion of pro-inflammatory cytokines via NFκβ activation [26]. Importantly, this implicates macrophages as potential mediators of metastasis, as breast cancer commonly metastasizes to the lung and brain. Indeed, studies have characterized the pro-metastatic role of macrophages in genetically engineered murine breast cancer models [27].

It is now appreciated that functionally distinct subsets of macrophages exist, and their polarization is influenced by their surroundings. Two of these polarizations are

referred to as "M1" and "M2" phenotypes. However, the traditional M1/M2 categorization of macrophages appears to be an oversimplification [28, 29], as recent studies have expanded the role of M2-phenotypic macrophages to include mediating the resolution of inflammation. During the resolution of inflammation, an active process, lipid autacoids stimulate M2 macrophages to efferocytose apoptotic cellular debris [30–33]. Glioblastoma-associated myeloid cells are characterized as having an "M0," non-polarized phenotype. However, these non-polarized macrophages have been found to express some characteristic M2 markers, namely TGF-β and IL-10 [25]. M2-polarized macrophages in a non-cancer setting are characterized as anti-inflammatory, participating in wound healing and tissue repair; however, in a cancer setting, they are characteristically viewed as "pro-tumorigenic." Specifically, M2 macrophages have been shown to promote disease progression in numerous cancers, including lung, breast, and ovarian [34–36]. In genetically engineered murine models of lung adenocarcinoma, depletion of M2-polarized alveolar macrophages inhibited tumor growth [34]. Additionally, the polarization of macrophages to their M2 phenotype has been shown to accelerate breast cancer growth [35]. Similarly, M2-phenotype macrophages have been shown to be present in ascites fluid taken from ovarian cancer patients [36]. Interestingly, when these macrophages were cultured with lipopolysaccharide (LPS), an inflammatory stimulus, they adopted an M1 phenotype, exhibiting toll-like receptor activation and upregulating the cytotoxic activity of natural killer (NK) cells [36]. Thus, the reprogramming of M2 macrophages to an M1 phenotype can promote their antitumor activity.

In contrast to M2-polarized macrophages, M1 macrophages have been characterized as anti-tumorigenic. In non-cancer settings, these macrophages mount an inflammatory response via their phagocytic and antigen-presenting activity [30, 31]. Current cancer immune-based therapies include polarizing macrophages to their M1 phenotype. Blocking TGFβ signaling in combination with stimulation of toll-like receptor 7 (characteristic of innate immune activation) results in the polarization of macrophages toward an M1 phenotype with specific antitumor activity [37].

In addition to M1 macrophages, studies have identified CD169+ macrophages, non-phagocytic mediators of immune tolerance, as important anti-tumorigenic cells. While CD169+ macrophages are unable to phagocytose cells and debris, they are of particular importance in immune tolerance [28]. Interestingly in an inflammatory setting, these macrophages have been shown to activate cytotoxic CD8+ T cells with a greater range of targets than dendritic cells [38]. As cancers have a wide variety of constantly mutating antigenic targets, this is a potential mechanism correlating CD169+ macrophages with documented antitumor activity in a variety of cancers, including endometrial carcinoma and melanoma [39–41]. CD169+ macrophages inhibit melanoma growth in orthotopic murine models via their ability to bind tumor-derived extracellular vesicles in draining lymph nodes and subsequently present them to B cells [41]. These various host macrophage-mediated mechanisms induce an adaptive immune response against the tumor, thus highlighting enhancement of the immune system as a potential cancer therapy.

Dendritic Cells

Similar to macrophages, dendritic cells exhibit both pro- and anti-tumorigenic activity in cancer. In inflammatory reactions, dendritic cells are the primary antigen-presenting cell. After binding an antigen at the site of inflammation, dendritic cells migrate to lymph nodes and activate antigen-specific immune responses from B and T lymphocytes. Dendritic cells are present in a variety of tumor types, including breast cancer [42, 43]. In a genetically engineered murine model of breast cancer (MMTV-PyMT), dendritic cells were demonstrated to be the most prevalent immune cell in the tumor tissue. Subsequent depletion of dendritic cells ultimately inhibited tumor growth and lung metastasis [44]. While dendritic cells are pro-tumorigenic, they can also possess antitumor activity. The difference in roles may be related to tumor progression. During the initial states of tumor progression, dendritic cells demonstrate antitumor activity. However, with tumor progression, dendritic cells lose their antitumor activity, unable to perform antigen presentation to T cells, and can even become immunosuppressive against T cells [45]. This highlights the adaptability of dendritic cell function within the tumor microenvironment and provides a possible approach for future immunotherapy. In fact, dendritic cells are currently being utilized in cancer vaccination clinical trials [46–48]. Tumor-associated dendritic cells exposed to tumor antigens ex vivo are then reintroduced to glioblastoma patients. These results have demonstrated harnessing dendritic cells antigen-presenting activity increases overall and progression-free survival in glioblastoma patients [49].

Natural Killer Cells

Natural killer (NK) cells also play a key role in inflammation and cancer. NK cells mediate antibody-dependent cellular cytotoxicity within the innate immune system. Recent studies have also implicated NK cells in adaptive and memory immunity [50]. NK cells not only promote nonalcoholic steatohepatitis (inflammation and fat accumulation of the liver), but also play a pivotal role in its progression to hepatocellular carcinoma (HCC) [51]. Specifically, NK cells interact with CD8+ T cells to release pro-inflammatory cytokines, contributing to tumor progression [51]. Further, specific subsets of NK cells have been implicated in halting natural host immune responses in breast cancer patients via expression of TGFβ and IL-10 [52].

Conversely, studies have identified various antitumor activities of NK cells. Prostate cancer patients with increased peripheral natural killer cells achieved improved overall survival. [53] Specifically, NK cells in these patients express NKp46, DNAm-1, and NKG2D, which contributed to the lysis of prostate tumor cells [53]. Additionally activation of NK cells in the presence of IL-12 and IL-15

allows for the mounting of cytotoxic response against breast cancer stem cells [54]. Importantly, IL-12 and IL-15 activate NK cells and enhance their cytotoxic activity.

T Cells

The adaptive immune system, comprised of T and B cells, also plays a critical role in inflammation and cancer. T cells, including CD4+ T helper cells and CD8+ T cytotoxic cells, are antigen-specific cells that have a range of known phenotypes [55]. CD4+ T cells assist in B cell antibody class switching, and activation of CD8+ T cells and can have both pro- and anti-tumorigenic activities. CD4+ T cell infiltration is increased in skin dysplasia, as well as in squamous cell carcinoma. Their role in tumor pathogenesis is evident as demonstrated in a CD4+ T cell knock-out murine model, in which tumor growth is suppressed [56]. However, it appears the tumorigenic role of CD4+ T cells varies across malignant tissues. Specifically, the loss of CD4+ T cells in the liver has been shown to accelerate tumor growth both in murine models and human patient samples of hepatocarcinogenesis. This mechanism may be due to an increase in hepatocytes' lipid concentration, resulting in CD4+ T cell death and subsequent disease progression from nonalcoholic fatty liver disease to cancer [57]. T cells with cytotoxic phenotypes (CD8+) are anti-tumorigenic, specifically in genetically engineered murine breast cancer models. Their cytotoxicity against tumor cells is dependent on IL-15, and knocking out IL-15 facilitates a marked increase in tumor growth [58]. Similarly, in genetically engineered murine non-small cell lung cancer models, knocking out CD8+ T cells resulted in stimulation of tumor growth and reduced survival [59]. In recent clinical studies, CD8+ T cells appear to play a key role in antitumor immunity in both pancreatic and colorectal cancers. An increase in both CD8+ T cells and CD4+ T cells resulted in increased overall and disease-free survival in patients with pancreatic ductal carcinoma [60]. Reduced metastasis has been correlated with increased numbers of cytotoxic cells in colorectal cancer patient samples [61]. Similarly, depletion of CD8+ T cells in murine colorectal cancer models resulted in accelerated tumor growth. Thus, T cells represent the flexibility of the adaptive immune system by hindering and promoting tumor growth.

B Cells

B lymphocytic cells, another essential arm of the adaptive immune system, are best known for their role in humoral immunity, providing immune responses from a distance. B cells secrete antigen-specific antibodies, which have a broad range of activities including marking cells for destruction (opsonization), providing a physical barrier on an antigen, or facilitating the creation of immune

memory. Consistent with immune cells we have explored previously, B cells also appear to have variable roles in tumor pathogenesis. Most notably, B cells have been implicated in pancreatic cancer progression. The depletion of B cells in mice inhibited orthotopic pancreatic adenocarcinoma tumor progression. The underlying mechanism is believed to be associated with B cells' role in macrophage polarization, specifically polarizing macrophages to an M2 phenotype [62]. Similarly, pancreatic tumors injected into mice lacking functional B cells exhibited decreased tumor growth as compared to wild-type mice. Alternatively, transplanting B cell-deficient mice with wild-type B cells promote tumor growth, further highlighting the pro-tumorigenic role of B cells [63]. The presence of mature B cells has also been correlated with higher epithelial ovarian tumor grade. Additionally, increased levels of plasma cells, which are terminally differentiated B cells, have been correlated with decreased overall and ovarian cancer-specific survival [64]. While largely pro-tumorigenic, B cells have also been shown to have anti-tumorigenic functions. Gene expression studies have revealed that the presence of B cells in the microenvironment of basal-like breast tumors correlates with increased progression-free survival [65]. While the specific mechanisms through which B cells exert anti-tumorigenic action is unclear, it is evident that B cells can be both anti- and pro-tumorigenic depending on the specific tumor environment.

Cytokine and Chemokine Biomarkers

We will next explore the role of cytokines and chemokines in cancer-associated inflammation. Cytokines and chemokines are small protein signaling molecules that enable crosstalk between immune cells and direct immune cell trafficking, respectively. While cytokines and chemokines in non-cancer settings may be considered pro- or anti-inflammatory, similar to immune cells, their role in tumor progression varies. While there is an enormous number of cytokines and chemokines that have been implicated in cancer, we focus on TNFα, TGFβ, IL-1, IL-6, IL-10, and CCL2.

Tumor Necrosis Factor α

Tumor necrosis factor α (TNFα) is perhaps the most iconic cytokine in cancer-association inflammation. However, as noted by Balkwill, "tumor necrosis factor" is likely a misnomer [15]. While the name "tumor necrosis factor" would lead one to believe it has antitumor activity, recent research has supported a dual role in tumorigenesis for TNFα. TNFα is traditionally implicated in septic shock and is characterized as a pro-inflammatory cytokine. TNFα has also been demonstrated to have both pro- and anti-tumorigenic roles. Its contribution to cancer pathogenesis

is believed to be via activation of immune cells, including B cells, which recent studies have shown are likely responsible for skin carcinogenesis. Selectively knocking out TNFα in B cells resulted in a reduction of papillomas in tumor-bearing mice [66]. Similar observations have been made in orthotopic glioblastoma tumor models, in which knocking out TNFα significantly increases survival rates [67]. TNFα has also been shown to be increased in non-small cell lung and pancreatic cancers, and its expression in ovarian carcinoma patient tissue correlates with high-grade serous carcinomas and endometrioid carcinomas [68, 69, 70]. While it would appear TNFα could be an appealing target for cancer therapy, anti-TNFα therapies have failed in the clinic to date [71]. Insight into its therapeutic failure may be due to TNFα's role as an anti-tumorigenic cytokine. If TNFα is expressed directly by tumor cells, it could then exert an autocrine antitumor activity. Mice injected with tumor cells genetically modified to secrete high levels of TNFα have little to no tumor growth as compared to control. This model has been recapitulated in breast, melanoma, and lung carcinoma models [72]. This in turn could possibly be utilized for future gene therapy, to stimulate ones' own tumor to exert autologous antitumor activity.

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is widely studied for its activation of regulatory T cells and Th17 cells. TGF-β activation of regulatory T cells has been shown to dampen inflammation and activate self-tolerant immune mechanisms. Elevated levels of TGF-β secreted from natural killer cells, along with IL-10, have been reported in breast cancer patients [52]. TGF-β has also been implicated in the progression of cervical squamous cell carcinoma. TGF-β activation has been demonstrated to be the result of thrombospondin-1, an acute phase inflammatory protein, secretion due to the interaction between cancer cells and cancer-associated fibroblasts [73]. TGF-β receptor 1 and 2 knockout mice exhibit markedly decreased pancreatic tumor growth. However, selective knockout of the TGFbeta receptor in epithelial cells significantly promotes pancreatic tumor growth [74]. This highlights the role of the tumor microenvironment in mediating pro- and anti-tumorigenic inflammation signals. Anti-TGF-β therapy in a recent clinical trial proved to have preliminary tumor reduction on advanced melanoma and renal cell carcinoma, with no apparent toxicity [75]. This establishes TGF-β as a putative viable cytokine target for future cancer therapy.

It has been demonstrated in murine pancreatic cancer models that TGF-β may also have antitumor activity. In orthotopic and genetically engineered murine models, pharmacological inhibition of TGF-β signaling in the pancreas using a TGF-β receptor antagonist contributed to pancreatic ductal adenocarcinoma progression [76]. Like other inflammatory mediators in the tumor microenvironment, TGF-β exhibits both pro- and anti-tumorigenic activity and its actions may require further studies.

Interleukin-1

Other cytokines and chemokines have ambiguous roles in inflammation associated with tumor pathogenesis. Within the IL-1 family, IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra) tend to be a focus of many studies. IL-1 is known to enhance CD4+ T cell proliferation and differentiation of B cells in standard inflammatory settings. It is most often associated with induction of fever in the early phase of the acute inflammatory reaction. However, it has been shown that inhibition of IL-1α and IL-1β in murine myeloma models decreases survival rates. In this model, IL-1α and IL-1β increase Th1 cell secretion of pro-inflammatory cytokines, which in turn activate cytotoxic macrophage responses toward tumor cells [77]. IL-1α is the dominant family member in acute inflammation. However, as acute inflammation progresses to become chronic, IL-1β becomes the predominant mediator [78]. As recent studies have demonstrated, IL-1β appears to play an integral role in inflammation and cancer. Decreased IL-1β secretion by tumor cells or stroma correlates with a decrease in progression-free survival in prostate cancer patients, thus highlighting the potential antitumor mechanisms associated with IL-1β in the tumor microenvironment [79]. Whereby, other studies have suggested IL-1β exhibits pro-tumor effects. Specifically, infiltrating neutrophils in a colitis model will secrete IL-1β that in turn contributes to colitis-associated tumorigenesis via upregulating the secretion of IL-6 [80]. IL-1Ra is a competitive antagonist to IL-1α and IL-1β and has been shown to inhibit their pro-inflammatory mechanisms [81]. While IL-1Ra may appear to be an alluring target for cancer therapy, recent data suggests its role in cancer progression is more complex than previously viewed. An increase in IL-1Ra has been correlated with decreased event-free and overall survival in T cell lymphoma patients [82]. Similarly, IL-Ra levels are increased in women with newly diagnosed breast cancers, as compared to breast cancer negative controls [83]. Taken together, the IL-1 cytokine family activity appears to be rather situational in its pro- and anti-tumorigenic activity.

Interleukin-6

Similar to IL-1, IL-6 is another cytokine implicated in acute inflammation and more specifically fever. IL-6 is synthesized and secreted predominantly by macrophages and enhances macrophages' ability to present to T cells via upregulation of B7 expression following recognition of pathogen-associated molecular patterns. Unlike the IL-1 family mediators, IL-6 has been shown to be predominantly pro-tumorigenic. In 2014, Karin and Taniguchi described IL-6 as one of the "critical lynchpins" associating inflammation and cancer [84]. They implicated IL-6 downstream signaling as contributing to tumor cell survival and proliferation, as well as to inflammation in the tumor microenvironment. Similarly, they highlighted that IL-6 is not only associated with acute inflammation, but also participates in T cell

activation throughout chronic inflammation. IL-6 has also been associated with at least 12 cancer types in humans, including but not limited to stomach, pancreatic, liver, intestinal, uterine, breast, lung, esophageal, prostate, bladder, and kidney cancers [84]. In a murine model of pancreatitis (a chronic inflammatory condition), the deletion of IL-6 has led to the recovery of normal pancreatic tissue as compared to wild-type mice, which ultimately develop pancreatic tumors [85]. IL-6 secretion from fibroblasts has even been implicated as a mechanism for angiogenesis, again highlighting the pro-tumorigenic role of IL-6 [86]. IL-6 has been implicated in the progression of both triple-negative breast cancer and pancreatic cancer [70, 85, 87]. Blocking the IL-6 receptor on breast cancer cells has been shown to render the tumor cells unable to adhere to endothelium, which is a key step in metastasis [87]. Thus, IL-6 not only possesses the potential to promote primary tumor growth but also tumor angiogenesis and metastasis.

Interleukin-10

In noncancer settings, IL-10 is traditionally characterized as an anti-inflammatory cytokine, for its inhibition of NFκβ, a transcription factor implicated in both cancer and inflammation. Recent studies, however, have shown that IL-10 may contribute to tumor growth and even cancer therapy resistance. IL-10 is increased in breast tumor tissue, with a corresponding increase in macrophage infiltration [88]. Further, increased secretion of IL-10 by macrophages in the tumor stroma has been associated with drug resistance in breast cancer [88]. This again presents an interesting paradigm, in which a characteristically anti-inflammatory cytokine contributes to tumor growth.

Chemokine Ligand 2

Chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP1), is involved in macrophage chemotaxis, signaling macrophages to traffic to a specific tissue site. CCL2 plays a critical role in inflammation and cancer, particularly for its role in breast cancer metastasis. CCL2 secreted by tumor cells and macrophages increases metastatic seeding of breast cancer cells via stimulating the secretion of CCL3 [27]. Interestingly, CCL3, also known as macrophage inflammatory protein 1-α (MIP1-α), is known to play a role in acute inflammation, again demonstrating the intertwined role of inflammation and cancer. Further, a recent clinical trial implicated antagonizing CCL2 activity in pancreatic tumor inhibition. Pharmacologically inhibiting CCR2, the receptor for CCL2, in combination with chemotherapy significantly inhibited tumor growth [89]. In this study, inhibition of CCR2 also decreased tumor-associated macrophages and regulatory T cells while increasing CD8+ and CD4+ T cells.

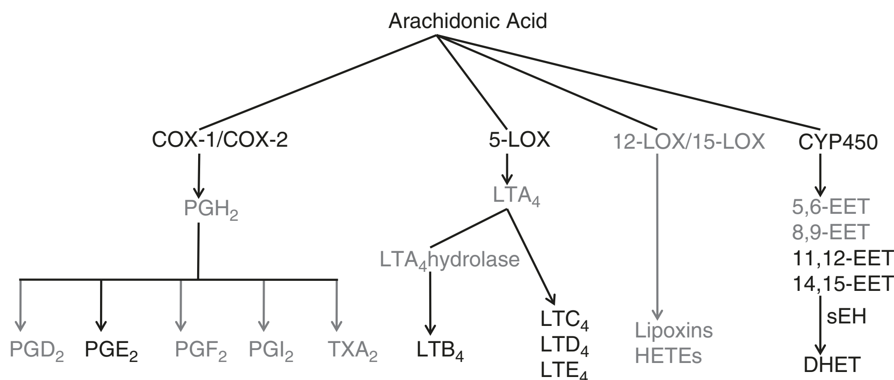

Fig. 7.1 Arachidonic acid metabolism via cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) enzymes. Lipids highlighted include PGE_2, LTB_4, LTC_4, LTD_4, LTE_4, 11,12-EET, and 14,15-EET for their diverse roles in inflammation-associated tumor pathogenesis

Lipid and Protein Biomarkers

Another lens to view inflammation and cancer is through inflammatory lipids and proteins. Lipids biosynthesized from arachidonic acid are termed eicosanoids, potent locally acting mediators of inflammation (Fig. 7.1). We discuss specifically prostaglandin E_2 (PGE_2), epoxyeicosatrienoic acids (EETs), omega-3 fatty acids, and leukotrienes, as well as emerging protein biomarkers annexin A1 and C-reactive protein (CRP).

Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a characteristically pro-inflammatory bioactive lipid synthesized from arachidonic acid initially by cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) and then by PGE synthase. Both PGE_2 and COX-2 expression have been implicated in inflammation and cancer. PGE_2 has been most notably characterized in colon cancer, where neutralizing PGE synthase and thus inhibiting PGE_2 synthesis decreases colon tumor formation in genetically engineered murine models of colon cancer [90]. Further, levels of PGE_2 positively correlate with cancer stem cell markers in colorectal cancer patient tumor samples. PGE_2 has also been shown to be pro-metastatic, as administration of PGE_2 results in increased tumor and liver metastasis in a genetically engineered model of colorectal cancer [91]. Mechanistically, PGE_2 has been demonstrated to promote tumor growth through stimulation of angiogenesis and immune suppression in several cancers, including colon cancer [92]. PGE_2 generated by tumor cells additionally stimulates myeloid-derived suppressor

cells to inhibit natural killer cells, contributing to immune suppression [93]. In addition to the well characterized activity of PGE_2 in colon cancer, the deletion of COX or PGE synthase in melanoma cells resulted in tumor rejection in immunocompetent mice [94]. However, in Rag1 knockout (KO) mice, melanoma tumor cells lacking PGE synthase form growing tumors [94]. As Rag1 KO mice are unable to generate mature T and B lymphocytes, this result highlights the relationship between PGE_2, immune cells, and tumor progression. We analyze the characterization of PGE_2 within this paradigm in regard to its relationship with omega-3 fatty acids and aspirin in the sections entitled "Omega-3 Fatty Acids and Derivatives" and "Inflammation and Cancer: Clinical Applications," respectively.

Epoxyeicosatrienoic Acids

Epoxyeicosatrienoic acids (EETs) are locally acting lipid signaling molecules with a short half-life and exhibit both autocrine and paracrine activities. EETs are the products of arachidonic acid metabolism by cytochrome P450 enzymes. EETs are then further metabolized by soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acid (DHET). EETs have been vastly studied in various inflammatory diseases. While EETs are known to mediate proliferation, migration, and inflammation in human tissues, their molecular mechanisms in doing so remain poorly characterized [95]. EETs are known to play a role in coronary arteriole dilation [96], stimulate tissue and organ regeneration [97], promote wound healing [98], delay seizure onset [99], and participate in many other disease processes with an inflammatory component [100]. Interestingly, their role in specific inflammatory diseases appears to be somewhat complex. EETs have been shown to be cardioprotective, inhibit pathogenesis of diabetes, and exert renal and neuronal protection. EETs also promote tumor cell proliferation and regulate host antitumor immunity [101, 102]. Thus, their role in cancer continues to be an active area of study. It has been demonstrated that both exogenous systemic and endogenous endothelium-derived EETs promote not only tumor growth and angiogenesis in murine models but also metastasis and tumor dormancy escape [101]. The EETs pro-tumor activity in these models was in part due to increasing VEGF secretion from endothelium in the tumor microenvironment [101]. In fact, the pro-tumorigenic role of EETs has also been demonstrated in human breast tumor tissue. Increased levels of 14,15-EET correlate with greater malignancy potential in breast cancer patients [103]. While the role of EETs in cancer requires further elucidation, modulation of EET levels via sEH has been purposed as a possible new direction in cancer therapy [95, 104]. Specifically, the promotion of EET metabolism by the endogenous over-expression of sEH in transgenic mice has been shown to reduce tumor burden [101]. In summary, the role of EETs in cancer remains an interesting vantage point whereby to view inflammation and cancer, while also providing insight into possible new therapeutic targets.

Omega-3 Fatty Acids and Derivatives

Omega-3 fatty acids are essential dietary polyunsaturated fatty acids that are metabolized in the human body to other essential lipid metabolites, including prostaglandins, thromboxane, and leukotrienes. Omega-3 fatty acids and their derivatives have recently gained widespread public attention for their potential wide-ranging health benefits. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are omega-3 fatty acids that are frequently marketed as dietary supplements to promote heart health. The potential role of these fatty acids in cancer pathogenesis is also of interest, as diets rich in omega-3 fatty acids correlate with a reduction in cancer-related deaths [105]. The anti-inflammatory mechanism to activity of omega-3 fatty acids has been primarily attributed to its metabolites, EPA and DHA. These molecules saturate enzymes that classically metabolize arachidonic acid into pro-inflammatory molecules and instead generate lipid autacoids with more anti-inflammatory characteristics [106]. EPA and DHA can additionally activate peroxisome proliferator-activated receptors (PPARs), transcription factors with known anti-inflammatory effects [106], and, interestingly, antitumor activity [107, 108]. Dietary supplementation of omega-3 fatty acids has been associated with decreased risk of colorectal cancer mortality [109]. Of note, chronic inflammation, such as ulcerative colitis, is a risk factor for colorectal cancer pathogenesis [110].

Omega-3 fatty acid supplementation has also been demonstrated to decrease prostate cancer progression in murine models via a macrophage-mediated mechanism [111]. Dietary DHA in these models decreased tumor-associated macrophage viability, as well as pro-inflammatory cytokines and NFκβ-mediated gene expression. Induction of tumor cell apoptosis may mediate, at least in part, the antitumor activity of omega-3 fatty acids [112]. Studies have further indicated that omega-3 fatty acids are capable of inducing apoptosis in a range of solid cancers in vitro, ranging from gastrointestinal origin to neural tissue, and even hematological cancers [112]. While the underlying mechanism remains to be fully elucidated, it appears that omega-3 fatty acids play a role in inducing both the intrinsic and extrinsic apoptosis pathways [112]. In addition to inducing apoptotic programs, omega-3 fatty acids play an anti-tumorigenic role through increasing tumor cell susceptibility to cytotoxic therapies and are being investigated as adjuvant cancer therapies [113]. Omega-3 fatty acid supplementation was also found to enrich tumor cell membranes in unsaturated fatty acids, in turn making the cells more susceptible to destruction by free radicals [113].

An important process in tumor malignancy is epithelial-mesenchymal transition (EMT). This transformation is critical for tumor cell invasion into neighboring tissues and sets the foundation for tumor metastasis. DHA has proven to be efficacious in inhibiting epithelial-mesenchymal transition in colorectal cancer models [114]. The mechanism of DHA's direct action in halting this process remains relatively unknown. As previously described, PGE_2 and COX-2 are characteristically pro-tumorigenic. Recent studies in endometrial cancer suggest that omega-3 fatty acid supplementation exerts its antitumor activity through downregulating COX-2 expression and thus subsequently decreasing endogenous PGE_2 levels [115]. This

mechanism has also been demonstrated in a colon cancer model, in which EPA and DHA supplementation increased lipoxin A_4 (LXA_4), an anti-inflammatory endogenous lipid mediator [116]. While the intricate workings of omega-3 fatty acids still required further research, their potential in cancer therapeutics appears promising.

Leukotrienes

Similar to EETs, leukotrienes are another class of eicosanoid metabolites of arachidonic acid generated by members of the lipoxygenase enzyme family. Leukotrienes are traditionally viewed as pro-inflammatory molecules and have been highly studied in inflammatory lung diseases. Similar to the other lipid mediators, their role in inflammation is being actively elucidated. Leukotrienes are mainly synthesized by leukocytes, contributing to both innate and adaptive immunity responses [117]. In acute inflammatory settings, leukotriene B_4 (LTB_4) increases leukocyte trafficking, as well as pro-inflammatory cytokines such as IL-6 and TNFα. In fact, it has been demonstrated that cellular secretion of LTB_4 is a crucial first step in potentiating inflammation-induced tumorigenesis in a lung cancer model [118]. The mechanism of LTB_4 tumorigenic contribution was demonstrated to be mediated by signaling through binding of its receptor BLT1, characteristically expressed on peripheral blood leukocytes [118]. LC-MS-MS-based profiling demonstrated an increase in leukotrienes LTC_4 and LTE_4 correlates with tumor progression in aggressive murine lung cancer models [119]. Interestingly, in these models, resident alveolar macrophages demonstrated high expression of 5-lipoxygenase (5-LOX) and subsequent increases in LTB_4, LTC_4, and LTD_4 secretion, as compared to infiltrating macrophages which did not produce leukotrienes [119]. This study provides insight into leukotrienes' locally acting inflammatory mechanisms in a cancer setting. Interestingly, the deletion of 5-LOX in a murine lung cancer model stimulates primary tumor growth and liver metastasis through the regulation of T cells [120].

Recent evidence implicating 5-LOX as an inhibitor of tumor growth suggests that leukotrienes produced by this enzyme potentially enact anti-tumorigenic programs in addition to their well-characterized pro-tumorigenic activities. Inhibition of 5-LOX in murine models has been demonstrated to reduce polyp burden in intestinal mucosa, a known mechanistic step in the APC-driven adenoma-carcinoma sequence of colon cancer [121]. In this model, the inhibition of 5-LOX was further accompanied by a decrease in inflammatory infiltrate, including cytokines and immune cells [121]. Further, the inhibition of 5-LOX has been shown to selectively induce apoptosis in prostate cancer cells via decreased expression of c-Myc mRNA [122]. C-Myc is a commonly mutated gene in various cancers, allowing for unregulated cell proliferation. In pancreatic cancer models, 5-LOX knockout mice were shown to have decreased pancreatic lesions, precursors to pancreatic ductal adenocarcinoma [123]. Thus, while further studies on the role of leukotrienes in cancer are needed, it is apparent that their modulation could provide important insight into cancer pathogenesis and potentially open new therapeutic avenues.

Annexin A1

A protein of growing interest in inflammation and cancer is annexin A1, an anti-inflammatory protein that inhibits acute inflammation by blocking phospholipase A_2. This neutralization results in decreased eicosanoid production and inhibits leukocyte adhesion and infiltration. In breast cancer patients, high levels of annexin A1 have been associated with decreased overall survival and poor breast cancer-specific survival [124, 125]. Similarly, lung cancer patients have been shown to have a significant increase in annexin A1 levels in plasma [126]. Further, annexin A1 mRNA and protein levels are increased in lung tumor tissue as compared to adjacent, noncancerous tissue [126]. Thus, annexin A1, an anti-inflammatory protein, may prove to be an important biomarker for tumor progression.

C-Reactive Protein

C-reactive protein (CRP) is released by macrophages and enhances complement recognition and subsequent phagocytosis by macrophages. CRP is clinically used as a biomarker for various inflammatory diseases, including inflammatory bowel disease, pelvic inflammatory disease, arthritis, autoimmunity, and even heart disease. While C-reactive protein's role in cancer has remained elusive, it has been shown to be a biomarker for various cancers. Elevated CRP levels are validated biomarkers in both Crohn's disease and ulcerative colitis, both chronic inflammatory diseases of the colon. Elevated levels of CRP have also been associated with an increased risk of colorectal cancer development [127]. Moreover, increased levels of CRP correlate with decreased overall survival and more aggressive tumor recurrence and metastasis in oral squamous cell carcinomas [128]. CRP levels have also been associated with an increased risk of epithelial ovarian carcinoma and breast cancer [129–131]. Increased levels of CRP are correlated with decreased cancer-specific survival in pancreatic cancer patients [132]. While the relationship between CRP and cancer risk and progression remains unclear, research suggests that CRP is largely pro-tumorigenic and could serve as a clinical biomarker for several types of cancer.

Inflammation and Cancer: Clinical Applications

Chronic inflammation is a known risk factor for various cancers. For example, pancreatitis is a known risk factor for pancreatic cancer, ulcerative colitis and Crohn's disease are known risk factors for colon cancer, and *Helicobacter pylori* infections are known to increase the risk of stomach cancer. Other associations include cystitis

and bladder cancer, Barrett's esophagus and esophageal carcinoma, and bronchitis and lung cancer [16, 133]. However, the extent to which underlying inflammation contributes to tumor growth remains elusive. Inflammatory reactions appear to have a threshold for which they can either play a pro-tumorigenic role or anti-tumorigenic role. Thus, characterizing the point at which inflammation transitions from facilitating tumor inhibition to promoting disease progression has been the aim of many recent clinical studies. These studies aim to evaluate the "overall inflammatory score" in relation to specific cancers. Further, these clinical studies seek to highlight a potential threshold between acute and chronic inflammation that, when surpassed, could contribute to cancer pathogenesis.

In a recent study, in which the inflammatory score reflected the neutrophil to lymphocyte ratio, an increased neutrophil to lymphocyte ratio is an independent predictive marker or clinical outcomes in head and neck squamous cell carcinoma patients. This increased ratio was also associated with a decrease in overall survival and recurrence-free survival [134]. Another study found men who demonstrated either acute or chronic inflammation in prostate tissue with a negative prostate biopsy have a decreased risk in prostate cancer pathogenesis 2 years post-biopsy. However, 4 years post-biopsy, decreased risk in prostate cancer was found to be positively associated with acute inflammation [135]. In a similar study on prostate cancer, chronic inflammation, defined by the presence of lymphocytes, plasma cells, and macrophages, was associated with lower prostate tumor volume [136]. In a large-scale study evaluating the overall risk of cancer, a combination of CRP levels and leukocyte count was used as an inflammatory score. An increase in the inflammatory score correlated with an increase in prostate, lung, and colorectal cancer risk [137]. A correlation between decreased inflammation and increased cancer survival has also been shown in cervical cancer, in which a decreased platelet to lymphocyte ratio correlates with an increase in overall and disease-free survival in patients [138]. Recent studies further highlight the importance of lymphocyte to monocyte ratio in several cancers. Interestingly, decrease in lymphocyte to monocyte ratio was found to be consistent with decrease in overall survival in colorectal cancer, lung cancer, pancreatic cancer, and Hodgkin's lymphoma [139]. An additional large-scale, retrospective study demonstrated that a greater ratio of lymphocytes to monocytes resulted in increased overall and disease-free survival in non-small cell lung cancer patients [140].

Thus, while the mechanisms underlying inflammation's contribution to cancer progression remain an area of ongoing research, it is apparent that inflammation has an intimate connection with tumor pathogenesis. Therefore, it is no surprise that in efforts to prevent cancer progression, widely used anti-inflammatory medications have come into focus as prophylaxis. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have been the subject of small- and large-scale cancer patient studies. Beginning in the late 1980s, there has been a growing body of evidence implicating aspirin's ability to decrease colorectal cancer risk [141, 142]. Although yet to be elucidated, aspirin's antitumor mechanism appears to be its multifaceted inhibition of inflammation through COX acetylation. Aspirin acetylation

of cyclooxygenase inhibits prostaglandin synthesis, thus decreasing the pro-inflammatory and pro-tumorigenic activities of PGE_2 as well as decreasing platelet activation. It has been hypothesized that aspirin also inhibits immune cell activation [142]. Aspirin regiments have been associated with decreased risks of epithelial ovarian cancer and gastric cancer [143–145]. Aspirin has also been associated with decreased risk of breast cancer-related death following breast cancer diagnosis [146]. However, retrospective studies assessing aspirin's anticancer benefits remain controversial. One review concluded that there was no statistically significant correlation between low-dose aspirin use and reduction in overall cancer risk and that aspirin's chemopreventive activity was limited to colorectal cancer risk [147]. Similarly, other nonsteroidal anti-inflammatory drugs have proved to decrease risk of colorectal and prostate cancer [148–150]. The mechanism of NSAIDs within the tumor microenvironment has also been characterized as their ability to inhibit tumorigenic potential and tumor immune tolerance, as well as to enhance immunosurveillance [110]. Interestingly, this activity was found to be mediated in part through the reduction of PGE_2 [110]. However, anti-inflammatories such as aspirin and COX-2 inhibitors have faced therapeutic challenges in the clinic, as they cause severe side effects such as kidney toxicity, stomach bleeding, and heart complications. Thus, more efforts to characterize the anti-tumorigenic mechanisms of aspirin and other NSAIDS are needed to develop novel viable clinical therapeutics that harness their broad anticancer activity.

Summary

Extensive evidence has established a link between inflammation and cancer, from the first observation of inflammation in tumors hundreds of years ago to current studies that have characterized the malleable and complex roles inflammatory cells and their mediators play in cancer progression. Experimental and clinical studies provide mechanisms that can be harnessed for future cancer therapy, with the aim to halt pro-tumorigenic inflammatory processes or harness the anti-tumorigenic pathways embedded in the human immune system. Many of the lipid mediators discussed above have potential as cancer biomarkers and exhibit a dual role in tumorigenesis, highlighting their diverse biological activity in various tumor microenvironments (Fig. 7.2). The frame through which to view inflammation and cancer is not one of strictly "pro-tumor" or "antitumor"; rather there exists a multitude of environmental influences that ultimately direct the role inflammation plays within the context of cancer. Future studies will be required to elucidate both the pro- and anti-tumorigenic roles inflammation plays, as well as to provide novel therapies that harness the immune system to inhibit or prevent cancer.

Biomarker	Level in Tumor Microenvironment	Tumorigenesis Associations
TNFα	Increased	Promote ovarian and endometrial carcinoma[68], non-small cell lung cancer[69], pancreatic cancer[72] Inhibit breast, melanoma and lung cancer[72]
	Decreased	Inhibit papillomas[66] and glioblastoma[67]
TGFβ	Increased	Promote breast carcinoma[52] and cervical squamous cell carcinoma[73]
	Decreased	Promote pancreatic ductal adenocarcinoma[59] Inhibit pancreatic tumors[74], melanoma and renal cell carcinoma[75]
IL-1	Increased	Promote colon tumors[80]
	Decreased	Promote myeloma[77], prostate carcinoma[79]
IL-6	Increased	Promote stomach cancer, pancreatic cancer, liver cancer, intestinal cancer, uterine cancer, breast cancer, lung cancer, esophageal cancer, prostate cancer, bladder cancer and kidney cancer[84], triple negative and pancreatic cancer[87,70,85]
	Decreased	Inhibit pancreatic tumors[85]
IL-10	Increased	Promote breast carcinoma[88]
	Decreased	–
CCL2	Increased	Promote breast cancer metastasis[9]
	Decreased	Inhibit pancreatic ductal adenocarcinoma[89]
PGE_2	Increased	Promote colon cancer [91], and angiogenesis[92]
	Decreased	Inhibit colon cancer[90] and melanoma[94]
EETs	Increased	Promote melanoma, fibrosarcoma, lung, prostate and liposarcoma tumor growth, angiogenesis and metastasis[101], breast tumor[103]
	Decreased	–
LTB_4	Increased	Promote lung tumors[118,119]
	Decreased	–
Annexin A1	Increased	Promote breast cancer progression[124,125]; lung cancer progression[126]
	Decreased	–
C-reactive protein	Increased	Promote colorectal cancer[127], oral squamous cell carcinoma[128], epithelial ovarian and breast carcinoma[129, 130, 131], pancreatic carcinoma[132]
	Decreased	–

Fig. 7.2 Biomarkers in the tumor microenvironment can simultaneously promote (*red*) and inhibit (*blue*) tumor pathogenesis, highlighting the fluidity of the role of inflammation in cancer

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Chapter 8
Cancer Immunity and Immune Evasion Mechanisms

Stalin Chellappa, Einar M. Aandahl, and Kjetil Taskén

Abstract Understanding the role of the immune system in cancer development and progression is a challenging process. The collective efforts unequivocally show that the immune system is playing a dual role in promoting and inhibiting tumor development. The tumor microenvironment is highly infiltrated by immune cells, which includes innate (macrophages, mast cells, neutrophils, dendritic cells, and myeloid-derived suppressor cells) and adaptive (T and B) cells. This diverse set of cells contributes to the secretion of different pro-inflammatory immune mediators creating a microenvironment that influences cancer growth in a pleiotropic manner. It is the composition of inflammatory mediators and the activation status of different immune cells that interact with the tumor to dictate either tumor regression or tumor progression. CD4+ and CD8+ T cells play a pivotal role in anticancer immunity. The CD4+ T cells are instrumental in eliminating cancer cells by secreting various cytokines and activating and recruiting other cell types such as macrophages and granulocytes. However, CD4+ T cell-mediated activation of CD8+ T cells and subsequent cytotoxic activity of the CD8+ T cells represent the major effector mechanism of antitumor immunity. Here, we review and discuss the current knowledge with respect to the functional role and prognostic significance of individual T cell subsets in various malignancies.

Keywords CD4+ T cells • CD4+ Th cell subsets • CD8+ CTLs • Tregs • GATA3 RORγt • T-bet • FOXP3 • Cytokines • Immunosuppression • Plasticity • Tumor-infiltrating T cells • Prognosis

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Cancer Immunoediting and Tumor Immune Evasion Mechanisms

While the role of the immune system in controlling microbial pathogens is well appreciated, the notion that the immune system can also control tumor development and progression has been a controversy for over a century. In 1909, Paul Ehrlich was the first to suggest that the immune system could protect the host from malignancies [1]. Nearly 50 years later, Thomas and Burnet predicted that adaptive immunity is responsible for preventing tumor formation and progression in an immunocompetent host and proposed the concept of cancer immunosurveillance [2, 3]. However, due to the absence of experimental support, the cancer immunosurveillance concept was abandoned. This was largely due to the lack of mouse models with pure genetic backgrounds available at that time. By the 1990s, with improved genetically modified mouse models available, several seminal works have validated the role of cancer immunosurveillance in both chemically induced and spontaneous tumor models [4]. Multiple components of the immune system have been identified as having central roles in cancer immunosurveillance, such as T cells, B cells, natural killer (NK) cells and INFγ, and perforin [4, 5]. Similarly, several experimental and clinical studies have confirmed the existence of cancer immunosurveillance (T cell-mediated cancer immunosurveillance is described in detail in the following sections) [5]. Collectively, these findings suggest that cancer immunosurveillance can function as a microenvironmental tumor suppressor. However, despite the presence of an active cancer immunosurveillance process, many immunocompetent individuals still develop cancer. This paradox has been explained via seminal mice studies showing that the immune system not only eliminates but also reduces the immunogenicity of the tumor but also has the capability to promote tumor growth [4]. This led to a significant revision of the original cancer immunosurveillance theory wherein Robert Schreiber and colleagues proposed a new concept termed "cancer immunoediting," which emphasized the cancer-promoting and cancer-suppressing role of the immune system during tumor growth [4, 6]. Cancer immunoediting consists of three phases, elimination, equilibrium, and escape, and termed as "three Es of cancer immunoediting" [6]. The elimination phase represents the original concept of cancer immunosurveillance, in which the cooperative actions of innate and adaptive immunity eliminate the tumor before it is clinically manifest. Several studies suggest that the immune component required for the elimination of tumors depends on specific-tumor characteristics such as origin (spontaneous vs. carcinogen-induced), anatomical location, histology, and growth rate. During the elimination phase, rare tumor cell variants may survive and enter into an equilibrium state. In this period, tumor cells undergo antigenicity sculpting by immune cells applying a selective pressure leading to the survival of the fastest growing cells that escape elimination by the immune system. This process induces reduced immunogenicity and acquired resistance to immune effector cells. The equilibrium state is the longest phase, and it extends throughout the life of the host. The end stage of the equilibrium phase results in generation of several tumor clones with the most immunoevasive

Table 8.1 Tumor immune evasion mechanisms

Evasion strategy	Mechanism
Impaired tumor antigen presentation	• Downregulation of tumor antigens or antigen-processing machinery (e.g., lack of LMP and TAP proteins) [8] • Downregulation of MHC genes [9]
Impaired trafficking of immune cells into tumor microenvironment	• Epigenetic silencing of chemokine expression [10] • Lack of endothelial adhesion molecules [11–13] • Physical barrier by stroma [14] • Lack of tumor antigens in lymphoidal organs [15]
Immune cell dysfunction or subversion	• Immune suppression mediated by CD4+ FOXP3+ regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [16–20] • Secretion of suppressive cytokines (TGF-β, IL-10, etc.) [21–23] and other soluble immunosuppressive factors (prostaglandins, VEGF, RCAS1, extracellular adenosine, reactive oxygen, nitrogen species, etc.) [24–28] • Expression of IDO in tumor cells leading to secretion of immunosuppressive tryptophan metabolites [29] • Induction of T cell tolerance by expressing cognate ligands for T cell checkpoint inhibitory receptors such as CTLA-4, PD-1, LAG-3, and Tim-3 [30, 31] • Apoptosis of immune cells induced by tumor cell expression of CD95L (FasL) (tumor counterattack) [32] triggering CD95 (Fas)-mediated T cell apoptosis • Immune cell deviation and plasticity [33–36]
Tumor cell resistance to apoptosis	• Abnormal expression of anti-apoptotic molecules (Bcl-2 and IAPs family protein) [37] • Mutations or loss of pro-apoptotic molecules (TRAIL and CD95 receptors) [37] • Interference with granzyme/perforin pathway [38, 39]

mutations and epigenetic instability. These cells ultimately enter the escape phase and develop into visible tumors and successfully avoid immune destruction, which is now considered as an emerging hallmark of cancers as described by Hanahan and Weinberg [7]. Tumor cells evade the protective immunity by several mechanisms as presented in Table 8.1. Currently, targeting one or more of these mechanisms clinically holds the most promising approach to improve antitumor immunity [24].

T Lymphocytes and Cancer Immunity

T cells are generally classified into two lineages: CD4+ T cells and CD8+ T cells. CD4+ T cells are further classified into CD4+ T helper cells (Th) that mediate tumor immunity and CD4 + FOXP3+ regulatory T cells (Tregs) that suppress antitumor immunity (described later). Naïve T cells that express a unique T cell receptor (TCR) on the surface develop through stringent positive and negative selection pathways in the thymus. T cells migrate through tissues and scan for cognate antigen

peptide-MHC complex that activates their TCR, resulting in functional differentiation into a variety of subsets [40]. Here we focus on conventional TCRα/β T cell subsets and their role in tumor immunity.

CD4+ T Cells in Anticancer Immunity

CD4+ Th cells are crucial in orchestrating humoral and cell-mediated immune responses [41]. However, their role in anticancer immunity is complex and reflects the diverse role of various CD4+ Th cell subsets [33]. The CD4+ Th cell TCR recognizes antigenic epitopes in the form of 12–20-residue long peptides, presented by major histocompatibility complex II (MHC-II) expressed on professional antigen-presenting cells (APCs) which include dendritic cells (DCs), macrophages, and B cells [42]. Upon recognition of antigen on the APC surface by the TCR along with appropriate interaction of co-stimulatory receptors such as CD28 on T cells with ligands such as CD80/86 on APCs leads to naïve CD4+ Th cell activation [43], which results in clonal expansion, triggered effector functions, and subsequent memory formation. During this period, the fate and functional specialization of activated CD4+ Th cells are largely dependent on the concentration and source of antigen, the type of APC engaged by CD4+ Th cells, the co-stimulatory receptors expressed by APCs, and, most importantly, the polarizing cytokine milieu of the microenvironment at the time of activation that drives the naïve CD4+ Th cells toward a particular Th cell subtype [40]. Together, these polarizing factors contribute to the specific expression of key subset-defining transcriptional factors and the subsequent secretion of effector cytokines that defines the functional subsets of CD4+ Th cells [40]. The cytokines secreted by CD4+ Th cells then activate and recruit a variety of other immune effector cells that together define the type of immune response [41]. Table 8.2 summarizes the CD4+ Th cell subsets in the human and murine systems, the polarizing cytokines that drive their development, their master transcription factors, and the effector cytokines they secrete.

Table 8.2 CD4 + Th cell subsets: polarizing cytokines, master transcription factors, and effector cytokines

Th subset	Polarizing cytokine	Transcription factor	Effector cytokine
Th1	IL-12, IL-18, INFγ, IL-27	T-bet, STAT4	IL-2, IL-10, INFγ, TNF-α, TNF-β (LT-α), CCL2, CCL3
Th2	IL-4, IL-25, IL-33, TSLP	GATA3, IRF4, STAT6	IL-4, IL-5, IL-9, IL-10, IL-13, IL-21, IL-31, TNF-α
Th17	TGF-β, IL-1β, IL-6, IL-21, IL-23	RORγt, RORα, IRF4 Batf, STAT3	IL-17A, IL-17F, IL-21, IL-22, IL-26 (human), TNF-α, CCL20
Th22	IL-6, IL-13, TNF-α	AHR, Batf, STAT3	IL-10, IL-13, IL-22, IL-21, TNF-α, IL-26 (human)
Th9	TGF-β, IL-4	PU.1, IRF4	IL-9, IL-10
Tfh	IL-6, IL-21	Bcl6, BATF, c-MAF	IL-4, IL-10, IL-12, IL-21, INFγ

Conventional Role of CD4+ T Cells in CD8+ Cytotoxic T Cell (CTL) Responses

CD4+ Th cells play an essential role in priming, activation, and expansion of CTL responses, a concept known as CD4+ T cell help [44–46]. CD4+ T cell help is complex and involves multiple mechanisms broadly classified into direct and indirect help. During the primary immune response to the tumor, the major indirect help from activated CD4+ Th cell comes through CD40/CD40L interaction with APCs that leads to maturation of the APCs [47–49]. This process provides all three necessary signals for CD8+ T cell activation, including antigen-mediated TCR triggering, co-stimulation, and stimulatory cytokines, most notably IL-12, which are all critically important for naïve antigen-specific CD8+ T cells to differentiate into CTLs. Alternatively, CD4+ Th cells can directly activate CTLs through CD40/CD40L [50]. Furthermore, activated CD4+ Th cells also directly help CTLs through secretion of IL-2, which supports growth and expansion [51, 52]. Furthermore, secretion of INFγ by CD4+ Th1 cells upregulates the expression of MHC molecules on the surface of tumor cells leading to a feed-forward loop of enhanced CTL responses as well as CD4+ Th responses [53]. In addition to priming the primary CTL response, CD4+ Th cells also help during the post-priming stage that takes place at the tumor site [54, 55]. Moreover, tumor-specific CD4+ Th cells have been shown to enhance the expansion of both low-avidity [56] and cognate [57] CTLs at the tumor site and enhance tumor rejection. In addition to their support to optimize CTL responses, CD4+ Th cells also play an essential role in generation and maintenance of memory CD8+ T cells during active CTL responses and homeostatic proliferation [58, 59]. Hosts lacking CD4+ Th cells have been shown to have reduced number of CD8+ memory T cells and impaired secondary CD8+ T cell responses [60].

Unconventional Role of CD4+ T Cells in Tumor Immunity

CD4+ Th cell-mediated antitumor immunity is primarily thought to involve activation and maintenance of CTL responses. However, more recent studies have shown that CD4+ Th cells also play independent roles in antitumor immunity. Here we discuss the specific roles of different CD4+ Th cell subsets in antitumor immunity.

CD4+ Th1 Cells in Tumor Immunity

In 1986, Mossman and Coffman demonstrated that antigen-specific mouse CD4+ Th cells can be categorized into two types, Th1 and Th2, based on their pattern of cytokine production [61]. In 1991, Romagnani and colleagues discovered that human CD4+ Th clones specific for intracellular *Mycobacterium tuberculosis* were mostly Th1 cells, whereas the CD4+ Th clones specific for the extracellular helminth *Toxocara canis* were mainly Th2 cells [62]. This firmly established the Th1/Th2 paradigm in both human and mice. The Th1 lineage is controlled by the key

transcription factor T-bet and the key polarizing cytokine IL-12 [40, 63–65]. Th1 cells secrete a set of cytokines that includes IL-2, INFγ, and TNF-α and the chemokines CCL2 and CCL3 that attract macrophages (Table 8.2), and they are best characterized for their role in clearance of intracellular pathogens such as viruses and their role in the pathogenesis of autoimmune conditions [66]. Th1 cells are considered to have potent antitumor activity due to their secretion of INFγ, IL-2, and CD40/CD40L co-stimulation to help initiate CD8+ T cell responses as described earlier [58]. Several human Th1 cells can also mediate antitumor immunity independently of helping CTL responses. INFγ plays a crucial role in antitumor responses and acts directly on tumor cells as well as promoting immune cell responses against tumor cells [67, 68]. Interestingly, an earlier study in mice demonstrated that Th1 cell-mediated INFγ secretion in the tumor microenvironment is essential for inhibiting angiogenesis and regression of tumors that do not express MHC-II [69]. Similarly, a study of mouse B cell cancer suggests that Th1 cell-mediated INFγ secretion in the tumor microenvironment is essential for eliminating MHC-II-negative tumor cells through activation of type 1 macrophages and angiogenic inhibitors like IP-10 [70]. However, their mechanistic relevance in human cancer is yet to be determined. Furthermore, a key function of Th1-derived INFγ in tumor-bearing hosts is to substantially increase the IL-12 secretion by DCs, which serves to further polarize the naïve CD4+ T cells into a Th1 phenotype, thereby contributing to their own development and maintenance [71]. In addition, secretion of cytokines and chemokines by Th1 cells also leads to recruitment and activation of pro-inflammatory type 1 macrophages (M1) and natural killer (NK) cells at the cancer site [68, 72, 73]. The cytotoxic mediators secreted from type 1 macrophages and NK cells have multiple antitumor properties [74, 75]. In line with this, patient studies show that the presence of Th1 cells and increased levels of their associated cytokines correlate with superior antitumor immunity and good clinical outcome in a majority of cancers [76]. Despite their potent antitumor role, Th1 cell functions are efficiently hindered by tumor cells by varying suppressive factors (Table 8.1 and described later), and imbalance or alterations in Th1/Th2 ratio in many human cancers lead to poor clinical outcome [77]. Owing to their importance, Th1 cells are also being utilized in clinical studies. Adoptive transfer of tumor antigen-specific Th1 cells in patients with metastatic melanoma [78] and metastatic cholangiocarcinoma [79] was recently shown to induce regression of the tumor for prolonged periods. In contrast, responses in melanoma patients that received only autologous, in vitro-expanded, tumor-infiltrating CD8+ T lymphocytes (TILs) [80] were found to be suboptimal and suggest the importance of inducing tumor antigen-specific Th1 cells for successful antitumor immunity.

CD4+ Th2 Cells in Tumor Immunity

CD4+ Th2 cells are recognized for their role in the host defense against extracellular parasites and their involvement in allergy and asthma. In both mice and humans, Th2 lineage commitment is controlled by the transcription factor GATA3 and

exposure to the polarizing cytokine IL-4 in the microenvironment of APC-naïve CD4+ Th cells [40, 81, 82]. Th2 cells then produce their signature cytokines such as IL-4, IL-5, IL-13, and IL-10 (Table 8.2). These cytokines mutually antagonize the development of Th1 cells [40, 64]. Th2 cells have been extensively studied for their role in antitumor immunity and in the context of disease progression and disease outcome. Initial studies from murine models and in vitro studies showed that IL-4 secreted from Th2 cells has a direct anti-angiogenic and tumoricidal activity [83–85]. IL-4 and IL-13 are critical for the recruitment of eosinophils and macrophages and in some cases neutrophils and CD8+ T cells to the tumor site and result in regression of tumor [86–90]. Conversely, Th2 cytokines also interfere with antitumor activity, which is largely attributed to Th2 cytokines that antagonize the development of INFγ-secreting Th1 and CTLs at the cancer site. IL-4 and IL-13 have an anti-apoptotic role [91–94], and IL-13 also has a pro-fibrotic role [95, 96] that may affect antitumor activity. Numerous studies indicate that the Th1/Th2 ratio is altered in a variety of cancers [76, 77]. Initial murine studies suggested that both Th1 and Th2 cells contribute to antitumor immunity [73, 97, 98]. However, the increased presence of Th2 cells was found to be pro-carcinogenic in many human cancers [33, 76, 99, 100]. These pro-tumorigenic roles of Th2 cells were proposed to be cancer specific rather than a global effect, as the Th1 response in these patients was not impaired [101, 102]. Multiple tumor-derived factors may favor the development of Th2 cells. Tumor cell-derived IL-10 induces skewing toward Th2 cells and inhibits the maturation of dendritic cells (DCs), which effectively reduces the secretion of INFγ and IL-12 from T cells resulting in impaired antitumor activity [103, 104]. Early reports demonstrated that human renal cell carcinoma and non-small cell lung cancer actively produced Th2-polarizing cytokines [105, 106]. Pancreatic cancer, an aggressive malignancy, is typically infiltrated by Th2 cells [107]. A clinical study from pancreatic cancer patients showed that the skewing toward Th2 was primarily due to the secretion of thymic stromal lymphopoietin from cancer-associated fibroblasts that activate DCs to produce Th2-associated cytokines and polarize T cells toward Th2 cells [108]. A similar mechanism was observed in mouse models of breast cancer [109], and chronic gastritis [110], which is the causative factor for gastric cancer. Similarly, studies in mice have shown that the expression of the human tumor antigen EpCAM strongly promotes Th2 skewing despite of the presence of strong Th1-polarizing conditions [111]. Thus, the involvement of Th2 cells in antitumor immunity is still controversial and that their effect may be context dependent.

CD4+ Th17 Cells in Tumor Immunity

In 2005, a third subset of CD4+ Th cells was identified in mice and named as Th17 cells based on the production of the cytokine IL-17 [112, 113]. Two years later, the existence of Th17 cells was confirmed in the human immune system [114, 115]. The development of Th17 cells are controlled by the master transcription factor RORγt and multiple polarizing cytokines [116–118] (Table 8.2). Owing to their

inflammatory properties, Th17 cells have been studied in a number of diseases both in mice and in humans and found to be important in the host defense against extracellular bacteria and fungi, but pathogenic in many inflammatory and autoimmune diseases [34, 116, 119, 120]. Th17 cells are shown to infiltrate several cancer types in both mice and humans [34]. However, their exact role in antitumor immunity is controversial and still elusive. Contradictory findings with respect to their role in antitumor immunity versus a pro-carcinogenic role may be due to the existence of multiple flavors of Th17 cells that are fostered by different cancerous cell types and mediators in the cancer microenvironment. Furthermore, the use of a variety of mouse tumor models adds complexity to this issue. Evidence for the role of Th17 cells in antitumor immunity came from studies with established models of B16 melanoma [122], and B16/F10 lung metastatic melanoma [123] in mice, in which adoptive transfer of in vitro-expanded, tumor antigen-specific Th17 cells induced regression of the cancer to a larger extent than Th1 cells transferred in a parallel experiment. The transfused Th17 cells were found to promote the infiltration of DCs and enhanced cross-antigen presentation to naïve CD8+ T cells as well as to induce the secretion of CCL20 from cancer-residing lung cells to further recruit CD8+ CTLs into the tumor site [123]. Therefore, the Th17 cells were proposed to have a synergistic function with CD8+ CTLs. In contrast, other tumor models in mice, which included leukemia [124], cervical cancer [125], non-small cell lung cancer [126], lung cancer [127], and colon cancer [128], suggested that Th17 cell-secreted inflammatory cytokines in the tumor microenvironment promoted neutrophil recruitment and secretion of elastase, a pro-tumorigenic factor [129]. They also promoted the secretion of pro-angiogenic factors and pro-inflammatory cytokines from tumor cells, which promote angiogenesis and cancer progression [129]. Recent studies with genetically modified mice with colon cancer [130] and pancreatic cancer [131] showed that the preinvasive epithelial layer expressed large amounts of IL-17R that facilitated the infiltration of Th17 cells further substantiating the above findings. Subsequently, the IL-17A derived from Th17 cells triggered the oncogenic signal through the IL-17R-STAT3 pathway and accelerated the transformation of epithelial cells into invasive neoplasia. Recently, β-catenin signaling was also implicated in the development of Th17 cells in colon cancer [132]. Similar dichotomous findings were observed in human cancer patients where infiltration of Th17 cells was positively associated with CD8+ T cell count and better survival in ovarian cancer [133] and esophageal cancer [134], whereas increasing evidence suggests the opposite in many solid tumors [34, 76].

Th17 cells are also found to be a major fraction of TILs in human cancers, attracted by tumor-derived RANTES and MCP-1 [135, 136]. Human Th17 cells also undergo plasticity (secreting cytokines of other lineages) [117, 120]. Interestingly, in vitro-expanded, tumor antigen-specific Th17 clones from melanoma and breast and colon cancer produced large amounts of polyfunctional cytokines including IL-8 and TNF-α, but not IL-2, IL-4, IL-12, or IL-23 [135]. Furthermore, the same authors also suggested that Th17 cells can be converted into FOXP3-expressing, Treg, cells that produce IL-10 and TGF-β1, indicating a possible regulatory function [137]. In contrast, other studies suggest that in vitro-expanded, tumor antigen-specific Th17 clones from colon cancer and ulcerative colitis mainly produced IL-2, TNF-α, INFγ, and GM-CSF and exhibited plasticity

to convert into FOXP3- and INFγ-expressing cells with suppressive properties [129, 133, 138]. These findings were contrasted by the proposed cytokine signature of freshly isolated Th17 cells from healthy patients [139] and argue that these differences may arise from in vitro induced changes or may reflect their actual function in the cancer microenvironment. The conversion of Th17 cells into Th1 cells is well documented in autoimmune diseases and cancer [117, 120]. However, recent findings have shown that ex vivo-isolated Th17 cells from peripheral blood mononuclear cells (PBMCs) of human pancreatic cancer patients can also produce Th2 and Th17 cytokines [140]. Notably, these findings demonstrate that Th17 cells from human cancers not only correlate with IL-17 secretion but can also acquire Th1- or Th2-associated features. To summarize, Th17 cell-mediated antitumor immunity is due to enhancement of DC and CD8+ CTL function. However, Th17 cells also contribute to cancer-promoting inflammation and angiogenesis. Further, their plasticity-associated complexity in the tumor microenvironment may determine their pro-tumorigenic, suppressive, or anti-tumorigenic role that may influence cancer prognosis.

CD8+ Cytotoxic T Lymphocytes (CTLs) in Cancer Immunity

CTLs recognize their cognate antigen through binding of their TCR to antigen-MHC-I complex expressed on the surface of tumor cells. Th cells also provide help to CTL responses (see the section "Unconventional Role of CD4+ T Cells in Tumor Immunity"). CTLs potentially eliminate the tumor cells and have been shown to correlate with good prognosis in almost every type of human malignancy (Table 8.3). CD8+ T cells use multiple mechanisms to kill tumor cells mediated by granzyme B, perforin, and the triggering of the Fas signaling pathway through Fas ligand (FasL). FasL expressed on CTLs binds to its cognate receptor on the tumor cell surface and induces apoptosis. Similarly, perforin secreted by activated CTLs forms pores on the surface of tumor cells that aid in directed delivery of granzyme B into the tumor cell that subsequently induces apoptosis. In addition, naïve CD8+ T cells also differentiate into different subsets such as Tc1 (Tbet+ Eomes+ INFγ+), Tc2 (GATA3+ IL-4+), and Tc17 (RORγt+ Tbet+ IL-17+) cells, which are driven by master transcription factors and polarizing cytokines similar to those described for Th1, Th2, and Th17 cells (Table 8.2) and also produce key cytokines similar to that of Th subsets (Fig. 8.1). Since type 1-, 2-, and 17-related cytokines are mainly produced by Th subsets rather than Tc subsets in the cancer microenvironment, their functional relevance is not yet clearly known. However, recent studies in mice suggest that T cells secrete INFγ, but not IL-4 and TNF-α, in a directional way (at the immunological synapse) onto the target cell [205]. It is possible that INFγ secreted by tumor-infiltrating Tc1 cells can have direct antitumor activity by enhancing MHC expression on cancer cells, inducing angiostatic effects, and also recruiting macrophages [68]. The role of IL-4-secreting Tc2 cells in the cancer microenvironment is largely unknown, although a study from breast cancer [206] showed their association with cancer progression. In contrast to Tc1 cells, IL-17-secreting Tc17 cells were found

Table 8.3 The association of tumor-infiltrating T cell subsets and prognosis

Cancer type	CD8+ T cells	CD4+ Th1 cells	CD4+ Th2 cells	CD4+ Th17 cells	CD4+ Treg cells
Head and neck cancers	Good [141, 142]				Good [142]
Esophageal cancer	Good [143, 144]	Good [145]		Good [134]	
Lung cancer	Good [146]	Good [146]		Poor [147]	Poor [148]
Pancreatic cancer	Good [149, 150]		Poor [108]	Poor [151]	Poor [151, 152]
Distal bile duct cancer	Good [153a] Poor [153b]				Good [153a]
Breast cancer	Good [154]	Good [155]	Good [156]	Poor [157]	Poor [158, 159] Good [160]
Gastric cancer	Poor [161, 162]	Good [163]	Poor [163]	Good [164] Poor [165]	Good [165] Poor [166]
Hepatocellular carcinoma	Good [167, 168] Poor [168]	Good [169]		Poor [170]	Poor [168, 171]
Colon cancer	Good [172–178]	Good [172–174]	None [173]	Poor [173, 179, 180]	Good [173, 180–192] Poor [183] None [176]
Ovarian cancer	Good [184]	Good [185, 186]	Poor [186]	Good [133]	Good [187, 188] Poor [189]
Renal cell carcinoma	Good [190]	Good [191]			Poor [191]
Prostate cancer	Good [192]				
Urothelial carcinoma	Good [193]				
Endometrial cancer	Good [194]				
Cervical cancer	Good [195] Poor [196]				
Melanoma	Good [197, 198]				None [199] Poor [200, 201]
Follicular and Hodgkin's lymphoma			Good [202]		Good [203, 204] Poor [202]

to be impaired in cytotoxic activity [207, 208]. However, adoptive transfer studies in mouse tumor models have shown that Tc17 cells inhibited tumor growth, which was primarily associated with their plasticity to convert into Tc17/1 cells that produced INFγ along with IL-17A [209]. However, Tc17 cells identified in gastric cancer [161], hepatocellular cancer [210], distal bile duct cancer [153b], cervical cancer [196], breast cancer [206], and endometrial carcinoma [211] were primarily found to be less cytotoxic and promoted cancer. Especially in gastric [161] and cervical cancer [196], Tc17 cells were shown to promote angiogenesis and to recruit suppressor cells, including myeloid-derived suppressor cells (MSDCs) and Tregs. Therefore,

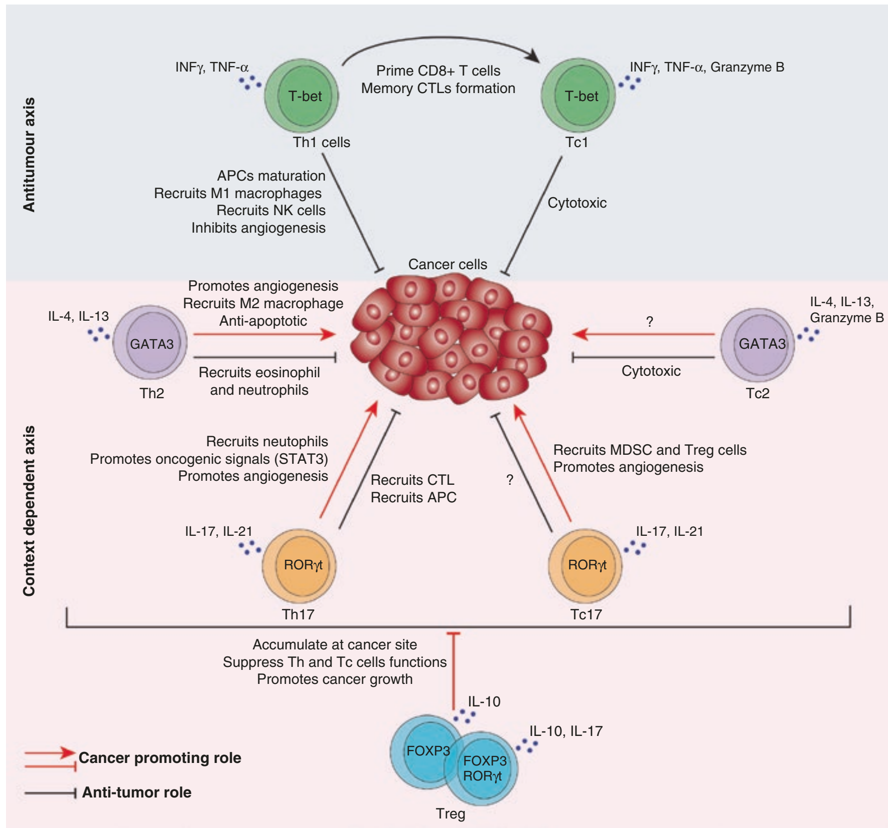

Fig. 8.1 Role of T cell subsets in antitumor immunity. Th1 cells express T-bet, INFγ, and IL-12. Th1 cell is superior in antitumor activity primarily due to activating APC, M1 macrophages, NK cells, and prime CTL (Tc1 cells) response. Both Th1 cells and Tc1 cells associate with good prognosis in many cancers and form a prominent antitumor axis in humans. Th2 cells express GATA3, IL-4, and IL-13 and contribute to cancer regression via recruiting eosinophils and neutrophils. Furthermore, cytokines produced by Th2 cells also contribute to angiogenesis, recruit M2 macrophages, and have an anti-apoptotic role. Their counterpart the Tc2 cells contribute to cancer regression through their cytotoxic activity, but their possible cancer-promoting features are not clearly known. Th17 cells contribute to cancer regression via activating APC and CTL. However, they may also contribute to cancer progression by various mechanisms. Similarly, their counterpart Tc17 also primarily contributes to cancer progression by recruiting suppressor cells into cancer stroma, mainly Tregs and MDSCs. Both Th17 and Tc17 cells contribute to angiogenesis. Tregs contribute to cancer progression by suppressing the effector functions of Th and Tc cell subsets. Tregs also largely accumulate at the cancer site, and their phenotypic heterogeneity and plasticity also contribute to pro-carcinogenic inflammation and cancer progression. Therefore, Th2, Th17, Tc17, and Treg subsets form a context-dependent axis in antitumor immunity in human malignancy

emerging results suggest that the cytotoxic activity of CD8+ T cells is context dependent, and under specific polarizing conditions, they may potentially lose their cytotoxic activity.

Tumor-Infiltrating T Cell Subsets and Their Prognostic Value

Despite the associations described above of various types of Th and Tc subsets with different cancers, the use of phenotyping of tumor-infiltrating T cell subsets as a prognostic marker is a complicated endeavor. In addition to the complex interactions in the tumor microenvironment, CD4+ Th cells in the tumor can be found in different maturation states such as activated, exhausted, or regulatory. Moreover, they may share phenotypic markers with other immune cells adding more complexity to analyses and interpretations of individual patient TIL profiles. Conflicting conclusions with respect to TIL phenotype could also potentially be due to differences in methodologies used, such as immunohistochemistry (IHC), multicolor flow cytometry, and polymerase chain reaction (PCR). Nonetheless, similar conclusions drawn for a particular cancer type by several groups substantiate the need for studying the link between Th cell subsets and prognosis and/or response to therapy. Here we summarize the prognostic value of analyzing the abundance of Th1, Th2, Th17, and CD8+ T cell subsets in several human malignancies (see Table 8.3). Th1 cells and CD8+ T cells are strongly associated with good prognosis in many human cancers including esophageal cancer [143–145], colon cancer [172–178], head and neck cancer [141, 142], lung cancer [146], pancreatic cancer [149, 150], distal bile duct cancer [153a], breast cancer [154, 155], gastric cancer [163], prostate cancer [192], urothelial cancer [193], ovarian cancer [184–186], endometrial cancer [194], cervical cancer [195], hepatocellular carcinoma [167–169], melanoma [197, 198], and renal cell carcinoma [190, 191]. Despite this, the presence of CD8+ T cells has also been reported to associate with poor outcome, particularly in hepatocellular carcinoma, gastric cancer, distal bile duct cancer, and cervical cancer (Table 8.3), which is thought primarily to be due to conversion of CD8+ T cells into Tc17 cells [153b, 161, 162, 196]. In contrast to Th1 cells and CD8+ T cells, Th2 and Th17 cells correlate with either good or poor prognosis (Table 8.3). Th17 cells have been associated with good prognosis in esophageal cancer [134], ovarian cancer [133], and gastric cancer [164] but correlated with poor prognosis in colon cancer [173, 179], lung cancer [147], pancreatic cancer [151], breast cancer [157], gastric cancer [165], and hepatocellular carcinoma [170] (Table 8.3). Whereas the presence of Th2 cells is associated with good prognosis in breast cancer [156] and follicular and Hodgkin's lymphoma [202], their presence associates with poor prognosis in pancreatic cancer [108], gastric cancer [163], and ovarian cancer [186], but does not appear to have an impact on colon cancer prognosis [173] (Table 8.3). Interestingly, in gastric cancer accumulation of Th17 cells have been shown to associate with either good prognosis irrespective of the cancer stage [164] or poor prognosis at early stage of the cancer [165]. These disparities could originate from differences in experimental setup and markers used to define Th17 and Th2 cells. Some of the abovementioned studies used only IL-17 as a predictor, investigating the CD4+ IL-17+ T cells. This may affect the results as other immune cell types including γδ T cells, myeloid cells, and innate lymphoid cells (ILCs) can also produce IL-17 [41, 121]. In addition, as we described earlier (see the section "Unconventional Role of CD4+ T Cells in Tumor Immunity"), Th17 cells also undergo plasticity, and therefore the conflicting observation of Th17 cells and Th2 cells may also reflect the fundamental differences in the inflammatory tumor

microenvironment and stress the importance of well-delineated Th lineage analysis in these patients. In addition, Galon and colleagues earlier proposed a concept termed "immune contexture" in which the location and density of CD8+ T cells and CD4+ Th cells in both the invasive margin and intra-tumoral region predicted a favorable outcome in colorectal cancer patients [172, 212]. Recently, this particular immune contexture has also been demonstrated in other cancer types [153a, 213, 214]. These findings provide a framework to further standardize the studies that involve T cell subset association with prognosis in human cancer.

CD4+ T Cells Suppressing Antitumor Immunity

CD4 + FOXP3+ Regulatory T Cells (Tregs)

Tregs are crucial in the maintenance of peripheral tolerance and prevention of autoimmunity [215]. The transcription factor FOXP3 is essential for the development and function of Tregs [215]. Various CD4+ Treg subsets have been identified in humans that can be broadly divided into thymus-derived (tTregs) and peripherally induced Tregs (pTregs). The essential function of Tregs is to suppress the activation, clonal expansion, and effector functions of various immune cells including CD4+ T cells, CD8+ T cells, natural killer T (NKTs) cells, and antigen-presenting cells (APCs) through a myriad of mechanisms [216, 217]. Accumulating evidence suggests that Tregs are specifically attracted by chemokines secreted by cancer cells [16]. Similarly, the cancer microenvironment provides a niche to strongly expand Tregs [218] where the Tregs contribute to the suppression of antitumor immunity initiated by Th cells, CTLs, and other innate immune cell tumors [17]. The conversion of Th cells into pTreg cells has been suggested to account for the high number of Tregs in tumor tissue. However, recent findings using epigenetic analysis of Tregs from tumor sites from mice and human found that a significant proportion of intra-tumoral Tregs were of nTreg origin and suggested that Th to Treg conversion is only partly contributing to the expansion of the Treg population [219]. Apart from their suppressive function, IL-10 secreted by Tregs can also skew Th subset tumor into a Th2 phenotype, which is associated with poor prognosis in many tumor types (Table 8.3). In addition, recent evidence from many solid tumors especially colon cancer [183, 220, 221], pancreatic cancer [140], and breast cancer [222] suggests that IL-17+ FOXP3+ Tregs retain their suppressive function but also contribute to Th17-associated inflammation, which is associated with poor prognosis in these tumor types (Table 8.3).

Tumor-Infiltrating Tregs and Their Prognostic Value

Tumor-infiltrating Tregs have been extensively studied and the prognostic value of their presence varies in different tumors. Tregs have been reported to correlate with poor outcome in colon cancer [183], lung cancer [148, 223], pancreatic cancer [151, 152], breast cancer [158, 159], gastric cancer [166], ovarian cancer [189], renal cell

carcinoma [191], and hepatocellular carcinoma [168, 171] as well as melanoma and follicular and Hodgkin's lymphoma [202]. In contrast, the presence of Tregs was found to be associated with good prognosis in colon cancer [173, 181, 182], head and neck cancer [142], distal bile duct cancer [153a], gastric cancer [165], ovarian cancer, and breast cancer [160], as well as follicular and Hodgkin's lymphoma [203, 204] (Table 8.3). Interestingly, associations with both good and poor prognosis were observed within the same cancer type for colon, breast, gastric, and ovarian cancer and Hodgkin's lymphoma (Table 8.3). Moreover, some studies have reported that the presence of Tregs has no impact in colon cancer and melanoma (Table 8.3). These discrepancies in prognostic value may arise from the use of different markers to define Tregs. Other factors that may contribute to these discrepancies are the presence of tumor subtypes where the impact of antitumor immunity varies, tumor stage, and the location of the characterized Tregs (within the tumor tissue, at the margin of the tumor or in the inflamed tissue outside the tumor). Finally, the role of Tregs in cancer progression may also be dependent on whether the cancers were preceded, or stimulated, by inflammation. In addition, many of these studies have not reported Treg-suppressive function or their phenotypic plasticity. The positive impact of Tregs in some tumor types may reflect their anti-inflammatory role in suppressing tumor-promoting inflammation. Moreover, discrepancies within the same tumor type such as colon, breast, and gastric cancer may indicate that Tregs may predominantly share other Th lineage phenotypes, such as IL-17+ FOXP3+ Treg, which have been found to be the major Treg pool in colon, breast, and pancreatic cancer patients [140, 183, 222]. Nonetheless, these data suggest that the original view on Tregs in suppressing antitumor immunity is oversimplified and that Tregs may have multiple roles in influencing inflammation and shaping the tumor microenvironment as well as in suppressing antitumor immunity.

Conclusion

Experimental and clinical studies now indicate that T cells play a pivotal, albeit sometimes paradoxical role in shaping antitumor immunity (Fig. 8.1). Nonetheless, the presence of Th1 and CTL cells is strongly associated with favorable outcomes in many tumor types and indicates that active cancer immunosurveillance is an integral part of many human malignancies. However, the potency of CTL function in several malignant tumors is generally compromised. The main factors contributing to tumor immune evasion include reduced MHC-I and MCH-II expression by tumor cells to eliminate the direct detection by CTLs, along with reduced help from CD4+ Th tumor cells. In addition, the differentiation of CD8+ T cells into less cytotoxic and pro-inflammatory subsets under polarizing conditions in the tumor microenvironment together with Treg-mediated immunosuppression at the cancer site contributes to the functional defect in tumor-specific Th1 cells and CTLs that ultimately lead to tumor progression. In addition, Th2, Th17, and Tregs are largely associated with poor outcome in many tumor types. The bifurcation of the pro- and

anti-tumorigenic nature of T cell subsets is too complex to predict, as it largely depends on cytokines secreted in the cancer microenvironment. To add to this complexity, recent reports suggest that T cells share different lineage-specific transcription factors and exhibit heterogeneity and plasticity. This may explain the paradoxical role of Th2, Th17, and Treg subsets observed, as many earlier studies assessed the prognostic value of individual subsets, but did not consider the potential of phenotypic plasticity. It is also inevitable that the location of T cells and the niche they share with other immune cells, cancer cells, and stromal cells along with their complex interactions dictate their functional status. An integrated picture of all these factors will shed more light on the role of T cells in cancer and enable us to better tailor T cell therapies in the future.

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Chapter 9
Drivers of EMT and Immune Evasion

Rolf A. Brekken and Katarzyna Wnuk-Lipinska

Abstract The heterogeneity of tumor cells and the complexity of the surrounding microenvironment make the process of predicting patient outcome and selection of the most suitable treatment regimen very difficult. Over the past decades, many biomarkers have been evaluated for prognostic value. The advent of immune therapy as a frontline treatment for some cancers has moved immune phenotyping into the forefront of biomarker and predictive marker research. Here, we review some of the regulatory mechanisms of the host immune response and epithelial plasticity and highlight their potential as biomarkers of the hallmark of immune evasion.

Keywords TAM receptors • Phosphatidylserine • EMT • Immunosuppression • Inflammation

Introduction

Tumors are a complex network of transformed cells, immune cells, stromal cells, vascular and lymphatic vessels, and extracellular matrix (ECM). Tumor progression is dependent on the interaction between tumor cells and the cells and ECM of the surrounding microenvironment. The immune component of the tumor microenvironment is of particular interest as it controls or promotes tumor progression and is highlighted by the fact that tumor-associated inflammation and immunoevasion have been recognized as hallmarks of cancer [1]. Indeed, every type of immune cell can be found within a tumor, including dendritic cells (DCs), macrophages, myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells, lymphocytes, mast cells and B cells.

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It is clear that location, density and the level of interaction between different immune cell populations impact clinical outcome [2]. For instance, high infiltration of tumors by CD3$^+$CD8$^+$ cytotoxic T cells (CTLs) and T helper 1 cells (T_H1) is generally associated with good clinical outcome [2]. In contrast, elevated levels of CD4$^+$ regulatory T cells (T_{reg}) in tumors are typically linked with poor prognosis [3, 4]. It is not just the presence of immune cells but also the activity of those cells that affects tumor progression. For example, in many tumors, NK cells are present at a high level but are anergic, as evidenced by downregulation of such markers as NKp30, NKp80, DNAX, or CD16 in lung, cervical, and ovarian carcinomas [5, 6]. This anergic state is maintained by tumor-derived suppressive factors, such as TGFβ, IDO or PGE2, which downregulate NK cell effector functions and allow tumors to escape from NK-mediated recognition [2]. In addition, B cells have been observed to enhance the progression of spontaneous murine tumors due to release of anti-inflammatory IL-10, which stimulates polarization of macrophages into a pro-tumorigenic (M2) phenotype [7, 8] and enhances immune suppression. However, B cells are not always associated with poor prognosis, as in some subtypes of breast and ovarian carcinomas, the presence of B cells was found to be beneficial, because they can function as antigen-presenting cells and participate in the induction of memory T cells [9].

The cytokine/chemokine pathways that control immune cell infiltration and activity in tumors are complex, yet there is a strong impetus to determine if quantification of the factors that drive these pathways has predictive value for the outcome of therapy. Here we discuss some of those mechanisms. In particular, we focus on molecules that can drive the immunosuppressive state in the tumor microenvironment and can potentially serve as biomarkers for poor prognosis. These markers include the TAM (Tyro3, Axl, Mer) family of receptors, externalized phosphatidylserine (PtdSer), and vascular endothelial growth factor (VEGF). We will also highlight the link between epithelial plasticity (e.g., EMT programs) and immune escape in tumors.

TAM Receptors in Inflammation

TAM receptor tyrosine kinases (RTKs), Tyro3, Axl, and Mer, are involved in the clearance of apoptotic cells in healthy adult tissues [10], the regulation of the innate immune response [11, 12], viral infection [13], as well as cancer progression and metastatic dissemination [14–16]. TAM receptors are particularly important for the processes driven by sentinel cells (DCs and macrophages), such as innate inflammatory responses and the clearance of apoptotic cells by means of phagocytosis [12]. TAM receptor signaling is pivotal in the inflammatory cycle and constitutes an “off switch” for dynamic innate immunity. This regulation prevents prolonged inflammation from occurring and restores homeostasis to adult tissues [12].

An innate immune response in sentinel cells often initiates with the activation of Toll-like receptors (TLRs) by danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) resulting in the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), IL-12 and type I interferons (IFNs) [17], which form a positive feed-forward loop with their receptors, resulting in amplification of the pro-inflammatory cascade [18] (Fig. 9.1a). Constitutive secretion of TNF-α, and other pro-inflammatory molecules, can lead to chronic inflammation and subsequent endotoxic shock [19]. Hence, the levels of these cytokines are tightly controlled. Simultaneous with TLR activation, as proposed by Rothlin et al. [12], IFN α/β receptor (IFNAR) and STAT1 form a complex with ligand-activated TAM receptors. This results in the activation of STAT1, which translocates to the nucleus and triggers the expression of the cytoplasmic suppressors of cytokine signaling 1 and 3 (SOCS1 and SOCS3). SOCS1 mediates polyubiquitination and subsequent degradation of the TLR connecting molecule myelin and lymphocyte protein MAL, preventing MAL-dependent p65 phosphorylation and transactivation of NF-κB leading to inhibition of inflammatory responses [20] (Fig. 9.1b). SOCS3 prevents ubiquitination of TLR downstream effector TNF receptor-associated factor 6 (TRAF6), thereby inhibiting the TLR signal transduction pathway [21] (Fig. 9.1c). As described above, TAM-regulated signal transduction pathway is pivotal for the suppression of inflammatory responses [11, 12, 22].

Furthermore, SOCS1 and SOCS3 can inhibit JAK/STAT1 signaling and subsequently impair synthesis of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, CCL9, CCL10, and TNF-α [23]. Additionally, the activation of Axl and/or Mer stimulates Twist activation, which transcriptionally suppresses TNF-α expression [24, 25]. Thus, as a consequence of the interaction with SOCS proteins, TAM receptors govern the repression of the pro-inflammatory cascade by blocking TLR signaling and hindering cytokine-receptor feed-forward signaling loops.

TAM signaling is also essential for efferocytosis, the phagocytic clearance of apoptotic cells [26]. Efferocytosis initiates an immunosuppressive signal in phagocytic cells, which is a critical defense against autoimmunity and is required for the maintenance of tissue homeostasis [27]. However, tumors are able to co-opt this biologic process to evade immune surveillance [28]. TAM receptors regulate phagocytosis of apoptotic cells and inhibit inflammation and immune activation; however, the exact mechanism remains unclear.

As described above, TAM signaling profoundly influences the function of sentinel cells. Specifically, TAM activation has an inhibitory effect on the innate immune response, and the downregulation of TAM signaling promotes autoimmunity. TAM receptors, despite their similarity, are likely to perform distinct functions. Axl and Mer were identified as central in the control of the innate immune system, yet they exhibit divergent expression and activity depending on the condition of surrounding tissue. While both are immunosuppressive phagocytic receptors, Mer appears to be a principal RTK in the tolerogenic environment, whereas Axl operates during

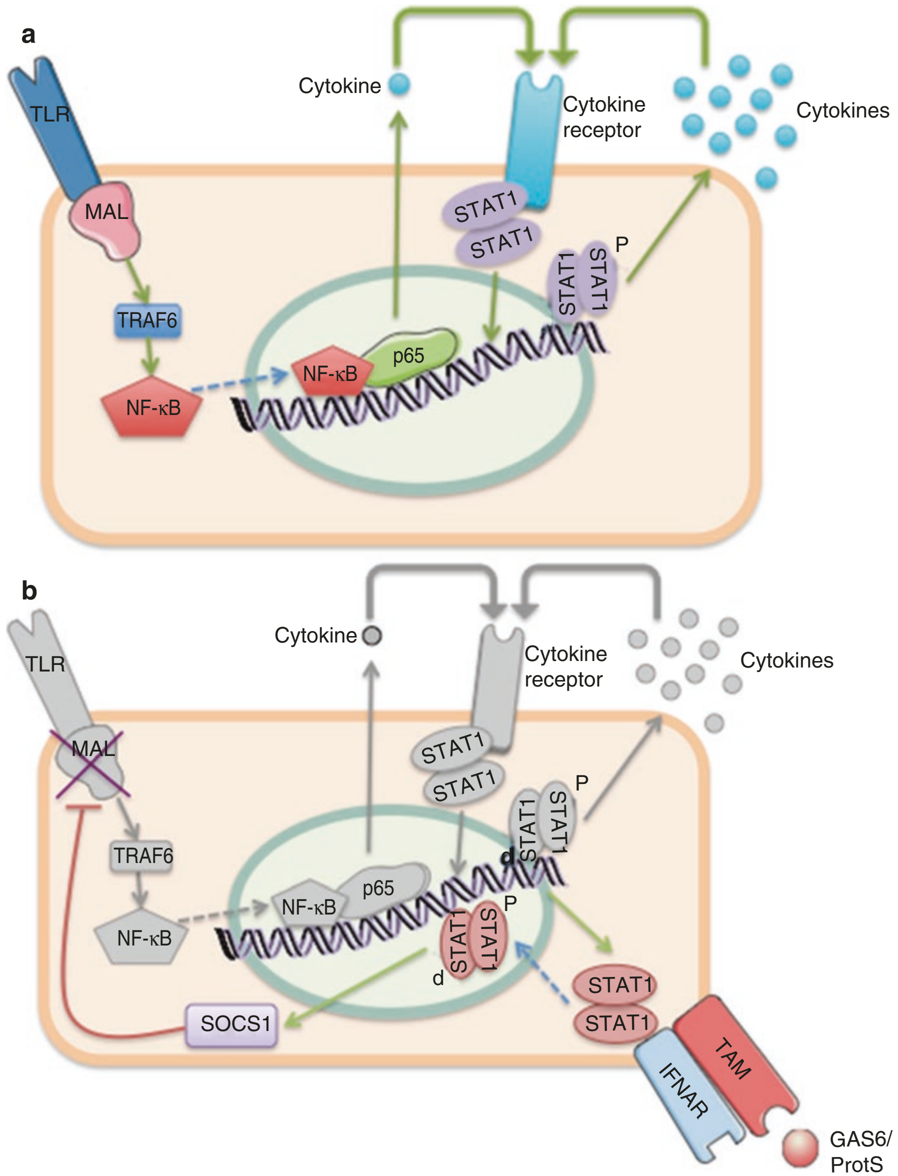

Fig. 9.1 TAM-regulated suppression of inflammatory response in sentinel cells of the innate immune system. Activation of TLR results in the secretion of pro-inflammatory cytokines, which are in positive feed-forward loop with their receptors (**a**). TAM-mediated activation of SOCS1 (**b**) and SOCS3 (**c**) inhibits signaling of MAL and TRAF6, subsequently resulting in immunosuppressive environment

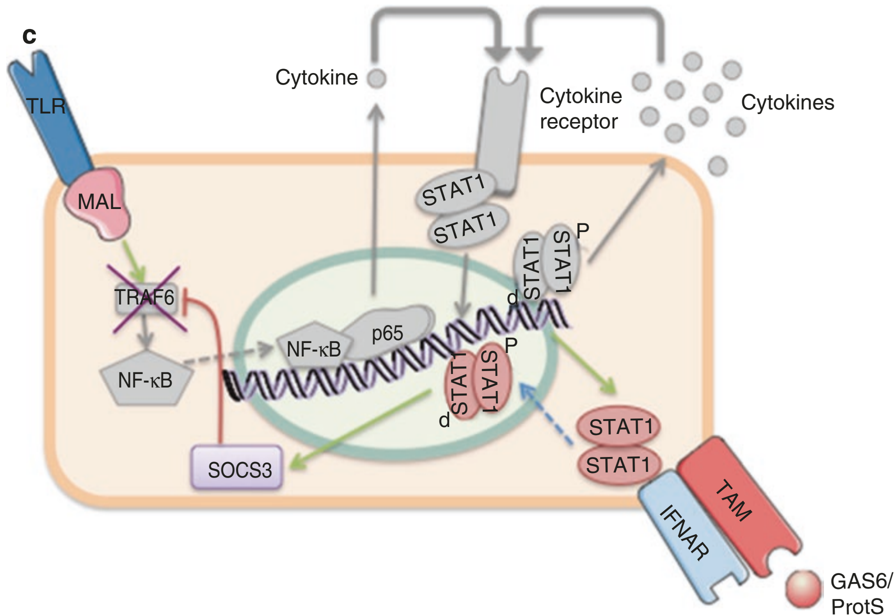

Fig. 9.1 (continued)

inflammation [29]. Furthermore, Axl and Mer are differentially dependent on their ligands. Axl is activated by dimerization through binding to Gas6 in a 2:2 stoichiometry [30]. Gas6 is a ligand for each TAM receptor, whereas Tyro3 and Mer are also activated by protein S [31]. Gas6 is required for the activation of Axl, whereas Axl maintains Gas6 expression in vivo [29]. This codependence and continuous presence of Gas6-Axl complex suggest that there may be another trigger of full Axl activation. Indeed, it was shown that basal Gas6-Axl activity was greatly enhanced upon exposure to apoptotic cells with membranes rich in externalized PtdSer, and therefore PtdSer appears to be a critical stimulus driving Axl activation [29]. How PtdSer contributes to Gas6-induced Axl activation is still not clear. The immunoglobulin-like (Ig) domains of Axl function as a docking site for the laminin G-like (LG) domains of Gas6, which drives Axl dimerization [30]. PtdSer appears to stabilize Axl dimerization and allows optimal Axl signaling [32]. Furthermore, Axl can be activated through ligand-independent dimerization with other TAM receptors or members of other RTK families, such as ErbB [33, 34]. In contrast Mer has only one docking site for Gas6 and exhibits a much lower affinity for its ligands.

TAM signaling is also important for the maturation and differentiation of NK cells [35]. NK cells are an essential component of the innate immune system that recognizes infected or malignant cells in the absence of “education” or priming [36]. In the presence of Gas6 or protein S, TAM receptors expressed by immature NK cells are activated. TAM signaling stimulates the acquisition of inhibitory, e.g., members of Ly49 and CD94 families, or activating receptors (CD69) required for target-cell recognition [37, 38]. Thus, TAM-deficient NK cells lack inhibitory and activating

receptors and fail to secrete certain cytokines, such as macrophage-inducing IFNγ even though they produce normal levels of perforins and granzymes [35]. This results in NK cells that have a tenfold lower activity against target cells [35].

The signaling of principal TAM receptors, Axl and Mer, is particularly important in sentinel cells of the immune system [12]. Signaling of either receptor drives an intrinsic negative feedback for the immune activation. Importantly, activation of Axl and Mer is specific for different environments, where Axl is specialized to function in inflammatory and Mer in tolerogenic settings [29]. Accordingly, the functional divergence of these receptors can have adverse repercussions for the development of new therapies. TAM activation can be beneficial for patients suffering from autoimmune diseases [39]. Long-term inhibition of Mer could possibly disturb homeostasis of healthy tissues and may result in many adverse side effects. On the other hand, prolonged Axl inhibition may be less harmful and potentially advantageous for cancer patients. Hence, antibody-based [40] or highly selective targeted treatments [41] are promising approaches to treat human disease through modulation of TAM signaling.

Phosphatidylserine in Inflammation

PtdSer is an anionic phospholipid that is actively segregated to the inner leaflet of the plasma membrane. In apoptotic cells PtdSer is flipped to the outer leaflet of the membrane where it functions as a signal for macrophages to engulf the cell [42]. PtdSer is externalized on the vascular endothelial cells in tumors [43] and is constitutively present in the outer leaflet of some tumor cells [44]. PtdSer externalization on tumor cells is significantly increased when they undergo apoptosis upon chemo- or radiotherapy [45, 46]. PtdSer is an evolutionarily conserved driver of immunosuppression in the tumor microenvironment [47], a topic that has recently been reviewed [48]. As mentioned above, PtdSer is required for full activation of TAM receptors [29, 32], whose signaling has an inhibitory effect on the innate immune response [12]. Specifically, low basal activity of Axl-Gas6 complex is significantly reinforced upon bridging with PtdSer [29]. Furthermore, formation of the Axl-Gas6-PtdSer complex is necessary for phagocytosis of apoptotic cells by macrophages [10].

PtdSer-dependent efferocytosis triggers secretion of anti-inflammatory mediators TGFβ and IL-10 by macrophages [49]. Furthermore, tumor microenvironments rich in PtdSer are characterized by diminished adaptive immune response. For instance, intratumoral dendritic cells, upon engulfment of PtdSer-opsonized cells, maintain an immature phenotype, resulting in failed antigen presentation [50]. Also, PtdSer has been linked to suppression of cytotoxic T-cell responses [51]. PtdSer-rich microvesicles, released by lymphocytes upon TCR activation, also transmit cell-death signals to activated T cells [52, 53]. Lymphocyte-derived microvesicles may induce apoptosis and nonreversible inhibition of T-cell function by suppression of CD3-ζ chain T-cell receptor, which plays an important role in antigen recognition

[53] or by expression of apoptosis-inducing ligands, such as Fas ligand (FasL), programmed death ligand (PDL), and TNF-related apoptosis-inducing ligand (TRAIL) [54]. Further, PtdSer present on the lymphocyte-derived microvesicles has been postulated to modulate T-cell activation in a reversible fashion [51]. As such, PtdSer seems to enhance the activity of diacylglycerol kinase-α (DGK-α) [51], which are found in excessive amounts in anergic T cells [55]. Although primarily reversible, suppression of T-cell function through contact with PtdSer may become nonreversible, as a consequence of chronic exposure to PtdSer-rich membranes and result in T-cell exhaustion [51, 56].

PtdSer also activates a second class of receptors, T-cell immunoglobulin and mucin receptors (TIMs) [57]. There are three subtypes of TIM receptors (TIM-1, TIM-3, and TIM-4). All TIM family members have been shown to recognize PtdSer through a conserved N-terminal IgV extracellular domain [58–60]; however, their expression varies between different immune cells [57]. TIM-1 is highly expressed on T helper 2 (T_H2) cells and functions as a co-stimulatory receptor important for T-cell activation [57]. TIM-3 is preferentially expressed on T_C1 (a subset of CD8+ lymphocytes producing IFNγ) and T_H1 cells and mediates apoptosis of those cells [57]. TIM-3 expressed by dendritic cells and macrophages enhances phagocytosis of apoptotic cells and antigen presentation [57]. TIM-4 is important for the maintenance of a tolerogenic state and phagocytosis of apoptotic cells and is solely expressed by antigen-presenting cells [57, 60].

TIM-3 is known to be immunosuppressive when activated and is thought to be a biomarker for T-cell exhaustion [61]. Hence, its function in cancer has been studied extensively. Recent studies on TIM-3 signaling revealed that TIM-3 abrogates Lck function and prevents TCR signal transduction [62]. Lck-mediated phosphorylation can be however rescued by HLA-B-associated transcript 3 (Bat3), which was postulated to positively regulate catalytic activity of Lck and, as a result, prevent the induction of T-cell exhaustion [63]. Consequently, the shortage of Bat3 expression in T cells causes accumulation of inactive Lck [63], which reduces the efficacy of the T-cell receptor (TCR) complex.

In summary, PtdSer, by impinging upon multiple innate and adaptive pathways, functions as a negative regulator of immune responses in the tumor microenvironment. It has shown that PtdSer can serve as a versatile biomarker of tumor cells [64] and tumor vasculature [43], as well as a novel target for therapeutic approaches [47].

Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells adopt a mesenchymal phenotype [65]. EMT endows epithelial cells that normally are polarized and interact with a basement membrane, with motile and invasive properties, and increased production of extracellular matrix components (ECM) [65].

EMT is critical for morphogenesis in the developing embryo [66]. In the adult tissue, EMT is restricted and occurs primarily during wound healing or organ regeneration and fibrosis [67, 68]. More recently, EMT has been linked to cancer progression [69] and is thought to be a critical mechanism in the acquisition of a metastatic phenotype in epithelial tumors [70, 71].

Physiologically, regulatory networks exist that function to support the homeostasis of every cell; however, when these networks are disturbed, dedifferentiation may be triggered [72]. It was proposed that during primary tumor formation, tumor cells become receptive to EMT inducers whose source is often the tumor-associated stroma [65, 71]. Thus, changing microenvironmental conditions regulate epithelial-mesenchymal plasticity of tumor cells. There are several major players involved in EMT induction, such as HGF, EGF, PDGF, and TGF-β [73–76]. These in turn act on a range of transcription factors that directly or indirectly orchestrate EMT machinery [70]. EMT and its reverse process MET are regulated by at least four different regulatory networks, transcriptional control, noncoding RNA regulation, differential splicing and posttranslational control, where EMT-inducing transcription factors play a central role in this network, as they are interconnected with remaining regulatory layers [72, 77–79].

To undergo EMT, an epithelial cell must be in a permissive state, allowing it to circumvent normalizing cues from the microenvironment [72, 78]. In the case of tumors, inflammation and hypoxia are commonly known environmental factors that disturb the equilibrium between these regulatory networks [69] and promote cellular transition [78]. Response to oxygen levels is mainly regulated by hypoxia-inducible factor 1α (HIF1α), expression of which correlates with metastasis in many cancers [80–82]. HIF1α upregulates expression of zinc transcript protein 1 (SNAI1), which in turn functions as a negative regulator of transcription of the cell-cell adhesion molecule E-cadherin [83]. In consequence of disturbed formation of adherens junctions within the epithelium, cells become motile and able to avoid anoikis [84–86].

There are more transcription factors, such as zinc finger protein 2 (SNAI2), zinc finger E-box-binding homeobox 1 (ZEB1), zinc finger E-box-binding homeobox 2 (ZEB2) and Twist family BHLH transcription factor 1 (TWIST1) [87–89], that repress the expression of several junctional proteins such as E-cadherin, claudins or desmosomes and promote EMT [84–86]. EMT transcription factors also have exceptionally high potency in causing genome-wide changes in gene expression by their interaction with epigenetic modulators [90]. Among the epigenetic mechanisms, DNA methylation, histone modifications, and microRNA expression changes have been observed in cells undergoing EMT [91]. As an example SNAI1 expression is associated with methylation of regulatory regions of *CDH1* [92], where methylation represses *CDH1* expression and promotes EMT [93, 94]. SNAI1 was also found to stimulate histone deacetylase activity, which in turn silences the *CDH1* promoter [95]. High SNAI1 expression also correlates with immunosuppression in melanoma, which is demonstrated by impaired DC recruitment and poor infiltration of cytotoxic T cells [96].

Among the microRNAs (miRNAs) that are implicated in EMT are members of the miR-200 and miR-34 families. They are in a reciprocal feedback loop with ZEB1/ZEB2 [97, 98] and SNAI1 [99], respectively, and are known to be drivers of an epithelial phenotype [100]. Therefore miR-200 and miR-34 family members are considered to be tumor suppressors [101]. A recent work by Chen et al. [102] suggests that the miR-200/ZEB1 axis regulates EMT and PD-L1 expression on tumor cells, and it is therefore an indirect cause of immunosuppression. Hence, miR-200 and ZEB1 are in a negative feedback loop, and, while tied to EMT regulation, they mediate CD8+ T-cell impairment via increased PD-L1 expression, which promotes tumor growth and metastasis.

Overexpression of EMT-related transcription factors, such as SNAI1, SNAI2, ZEB1 and ZEB2, and TWIST1, also leads to increased expression of Axl [14]. Moreover, high levels of Axl expression support the maintenance of a mesenchymal phenotype by stimulating SNAI1, SNAI2 and TWIST expression [14, 41]. Bearing in mind that Axl is involved in sustaining mesenchymal traits, such as motility and invasiveness, the essential role of Axl in tumor initiation and metastasis takes on additional clarity [41, 103]. The elevated Axl expression is linked with unfavorable overall prognosis in breast cancer [14], non-small cell lung carcinoma [104], pancreatic cancer [105], glioblastoma multiforme [106], esophageal adenocarcinoma [107], acute myeloid leukemia [108] and ovarian cancer [109]. Significantly, abundant expression of Axl is predominantly related to metastatic dissemination and survival, rather than primary tumor growth [14, 41, 103, 110].

Many recent studies have revealed elevated expression of Axl in tumors resistant to chemotherapies [34, 73, 111–114]. More importantly, these studies suggest that chemoresistance is a repercussion of EMT [115], while inducing MET restores sensitivity. Axl expression is associated with an aggressive mesenchymal phenotype and with immunosuppression. Thus inhibition of Axl signaling has the potential to reduce tumor invasion and immunosuppression. However, while high Axl levels have been correlated with poor clinical outcome, the predictive value of Axl expression for immune phenotype has not been yet evaluated.

Normal and neoplastic epithelial cells that undergo EMT acquire stem cell properties [116, 117]. Interestingly, EMT seems to serve as a universal mechanism for the acquisition of stem-like traits, and therefore epithelial cells within tumors appear to undergo the same program as normal antecedent cells [116, 118]. The mesenchymal phenotype is associated with increased motility, invasiveness, and resistance to apoptosis, all traits that predispose to metastatic dissemination [119, 120]. Furthermore, cancer stem cells (CSCs) are thought to have the ability to initiate tumors at distant sites upon MET [121] and form macrometastases through self-renewal [118]. The invasive phenotype of these cancer stem cells is a reflection of their plasticity and ability to reversibly change phenotype across the epithelial-mesenchymal axis [122]. Importantly CSCs are associated with drug resistance and tumor recurrence [121, 123–128]. As a result, CSCs are thought to be a major cause of treatment failure, and development of targeted therapeutics directed against them is an attractive concept [121]. However, while

CSCs can give rise to a new tumor that includes non stem-like tumor cells, the reverse is also true, implying that CSCs may be regenerated from non-cancer stem cells within a treated carcinoma [129]. Therefore, propitious therapy should include agents targeting CSC and normal cancer cells, eliminating both CSCs and their descendants [130].

Immunoregulation

EMT, genotypic and phenotypic heterogeneity, and dynamic epigenetic interactions have been linked with tumor relapse and resistance to systemic therapy [131]. Also, reciprocal interactions between tumor cells and components of the surrounding microenvironment, such as soluble inflammatory mediators, can confer resistance to therapy [132]. Additionally, therapy-induced injury, which occurs in response to systemic treatment disrupting established tumor structure, causes secretion of pro-inflammatory cytokines, which can mediate tumor cell plasticity. For instance, chemotherapy, in parallel to killing cancer cells, stimulates macrophages to produce TNF-α and IL-6. TNF-α in turn promotes chemoresistance by activating its downstream effector nuclear factor-κB (NF-κB) and then CXCL1 and CXCL2 [133]. Interestingly, TNF-α also has a protective effect toward BRAF-mutant melanoma cells, in which case TNF-α and NF-κB signaling enable cancer cells to bypass apoptosis induced by BRAF inhibitors [134]. Furthermore, TNF-α and IL-6 were found to elicit transition of tumor cells to a mesenchymal phenotype by modulating the expression of EMT-related transcription factors, such as Twist, Snail, or Slug [135–139]. Accordingly, plasma TNF-α was proposed to be suitable as a biomarker, when combined with TNM classification of malignant tumors, for predicting survival of head and neck squamous cell carcinoma and may be useful for designing treatment strategies [140].

Also, T cells are part of the reciprocal interactions between immune and tumor cells. Melanoma-specific cytotoxic T leukocytes (CTLs) secrete IFNγ, which in turn induces expression of PD-L1 by tumor cells. PD-L1 inhibits the function of the effector T cells by binding to its receptor PD1 [141, 142]. It is, however, possible to block the formation of the PD1/PD-L1 axis by treatment with immune checkpoint inhibitors and thereby restore a pro-inflammatory environment with tumor-specific CTLs [141, 143]. The possibility of reactivation of CTLs in the tumor microenvironment is one of the indications of the functional plasticity of the tumor immune component.

Additionally, alterations in metabolic pathways contribute to the interplay between tumor and immune cells. For instance, hypoxia was proposed to stimulate the expression of CCL28, followed by recruitment of regulatory T cells (T_{reg}) stimulating tumor tolerance [144]. Hypoxia-induced release of VEGF by tumor cells also contributes to the recruitment of T_{reg} cells [145], indicating that immune tolerance and angiogenesis are closely related mechanisms mediating immune evasion [146].

VEGF and Inflammation

To maintain the dynamic interactions between tumor and immune cells, there is an extensive vascular network. The complex organization of blood vessels within the tumor may allow mesenchymal tumor cells to escape and encourage metastatic spread. On the other hand, dense vasculature favors the recruitment of the immune cells to the tumor environment. VEGF is a key inducer of the tumor neovascularization and is abundantly produced by tumor cells [147]. VEGF release is regulated by VEGF receptor 2 (VEGFR2)-dependent activation of mTOR. This autocrine feed-forward loop intensifies the primary angiogenic signal and leads to the formation of new blood vessels in cancer [148]. This step is essential for tumor growth, further expansion and metastatic dissemination [1, 149]. Moreover, it was suggested that CXCL1/CXCR2 stimulates VEGF release through the activation of its downstream JAK/STAT3 signaling cascade [150]. The CXCL1/CXCR2 axis amplifies VEGF signaling and possibly rescues the proangiogenic phenotype upon VEGF inhibition. It was also revealed that activation of Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP-2) by Axl inhibits vascular endothelial growth factor receptor 2 (VEGFR2) during morphogenesis of endothelial cells [151]. Interestingly VEGFR2 is expressed not only on the surface of endothelial cells but also on macrophages, neutrophils, myeloid-derived suppressor cells (MDSCs), DCs and T cells [152–156]. Hence, it is not surprising that antiangiogenic therapy affects the immune function in the tumor microenvironment. Further, anti-VEGF therapy can reduce macrophage and MDSCs infiltration and increase mature DCs in the tumor suggesting that anti-VEGF therapy can function to restore a pro-inflammatory tumor microenvironment [157–159].

Concluding Remarks

Tumors are a complex network of dependencies between tumor cells, immune cells and the microenvironment. The immune contexture varies between tumors and may be associated with different overall prognosis, depending on the histological and molecular type of the tumor, stage and organ-specific microenvironment. There are different approaches to modulate immune response. Certain approaches, for instance immune checkpoint blockade or adaptive cell transfer, can result in long-term durable efficacy. However, multiple evolutionarily conserved programs active in the tumor microenvironment promote immune suppression. For example, the processes of efferocytosis and EMT function to promote local immune suppression, and these programs are co-opted by the tumor to evade immune surveillance. These pathways exploit signaling modules including those driven by the TAM receptors, PtdSer and EMT-inducing growth factors, and as a result these molecules or pathways are attractive as targets for prognostic exploration.

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Chapter 10
Inflammatory Biomarkers for Cancer

Alexandre Corthay and Guttorm Haraldsen

Abstract Cancer is associated with various degrees of inflammation both locally and systemically, resulting from an immunological response towards malignant lesions. Here, we critically evaluate several inflammatory parameters as biomarkers and prognostic tools for cancer. Colorectal cancer (CRC) represents a paradigm of the causative relationship between chronic inflammatory disease and cancer development. However, close examination reveals that for CRC, risk in patients with inflammatory bowel disease (IBD) has been largely overestimated. In fact, IBD patients only have a slightly increased risk of developing CRC (standardized incidence ratio ~ 1.7), which only weakly supports the link between chronic inflammation and cancer. However, long-term immunosuppressive treatment of IBD patients is associated with an increased risk for overall cancer, particularly haematologic and skin cancers. In contrast, there is a strong association between infection with the bacterium *Helicobacter pylori*, gastritis, and gastric cancer. Therefore, *H. pylori* seropositivity or the associated gastritis may be used as predictive biomarkers for cancer, although the rate of false positives is high. We have also reviewed several cytokines of the interleukin-1 family and cytokines that converge on STAT3 signalling because they are very well suited to illustrate the multitude of cytokine actions that makes interpretation of one single cytokine as a biomarker of cancer very complex. In addition, we describe in more detail the biology of IL-33, the most recently identified member of the IL-1 family, because it has not yet been subject to review in the context of cancer immunology.

Keywords Inflammatory biomarkers • Inflammation • Inflammatory bowel disease • Colorectal cancer • Gastritis • Gastric cancer • *Helicobacter pylori* • Interleukin TGF

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Introduction

Cancer elicits an inflammatory immune response within and around tumours as well as systemically. The role of the immune system in cancer is complex as both tumour-promoting and tumour-suppressive effects have been observed [1]. Some chronic inflammatory diseases are associated with increased cancer risk, suggesting a causative relationship between chronic inflammation and cancer development [2]. Therefore, one may consider using a diagnosis of inflammatory disease as a predictive biomarker for cancer. In the first two sections of this chapter, we present current knowledge about two inflammatory conditions that are reported to predispose to cancer, namely, inflammatory bowel disease and gastritis. The usefulness of these diseases as risk biomarkers of cancer is critically discussed. In the following sections, we critically review several cytokines as biomarkers of inflammation and cancer prognosis.

Inflammatory Bowel Disease and Colorectal Cancer Risk

Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of inflammatory bowel diseases (IBD). People with CD have 50% greater mortality than the general population, whereas those who suffer from UC have 10% increased mortality [3]. An increased risk of developing colorectal cancer (CRC) has been reported for patients with IBD. In fact, colon cancer represents a paradigm of the causative relationship between chronic inflammatory disease and cancer development [2]. In the official guidelines from the American Gastroenterological Association concerning CRC risk, surveillance colonoscopy is recommended every 1–3 years for patients with IBD [4]. A fundament for these guidelines is a landmark meta-analysis published in 2001 by Eaden et al. that reported the cumulative probabilities of patients with UC to develop CRC of 2% by 10 years, 8% by 20 years, and as high as 18% by 30 years [5]. These numbers have been much referred to and represent a pillar for the paradigm of chronic inflammation causing cancer. However, the validity of these high estimates has been questioned since their publication. In Northern California, Herrinton and colleagues (see Table 10.1) reported the incidence of CRC from 1998 to 2010 among 16,500 individuals with IBD which was only 60% higher than in the general population and was stable over time [6]. The standardized CRC mortality ratios were 2.3 and 2.0 for individuals with CD and UC, respectively [6].

In a recently published prospective study of >19,000 patients with IBD in France, the standardized incidence ratio (SIR) of colorectal cancer (CRC) was 2.2 for all patients [7]. Patients with IBD and long-standing extensive colitis were found to have an increased risk for CRC with a SIR of 7.0 [7]. In contrast, CRC risk was lower among IBD patients receiving thiopurine therapy [7]. Thus, these two studies are consistent with a 1.6–2.2 times increased risk of developing CRC in patients with IBD. These numbers are in accordance with a meta-analysis of population-based

Table 10.1 Reported risk of colorectal cancer in patients with ulcerative colitis (UC)

First author	Publication year	Country	No. of UC patients	SIR[a]	Reference
Stewenius	1995	Sweden	471	2.1	PMID:7636371
Wandall	2000	Denmark	801	1.7	PMID:10766327
Palli	2000	Italy	689	1.8	PMID:10982757
Jess	2006	USA	378	1.1	PMID:16618397
Jess	2007	Denmark	1575	1.1	PMID:17206705
Söderlund	2009	Sweden	4125	2.7	PMID:19422077
Herrinton	2012	USA	10,895	1.6	PMID:22609382
Jess	2012	Denmark	32,911	1.1[b]	PMID:22522090
van den Heuvel	2016	The Netherlands	1644	0.7	PMID:27170593
Cheddani	2016	France	474	0.9	PMID:27481308

[a]Standardized incidence ratio
[b]This study reported relative risk (RR) instead of SIR

Table 10.2 Reported risk of colorectal cancer in patients with Crohn's disease (CD)

First author	Publication year	Country	No. of CD patients	SIR[a]	Reference
Palli	2000	Italy	231	1.4	PMID:10982757
Jess	2004	Denmark	374	1.6	PMID:14984375
Jess	2006	USA	314	1.9	PMID:16618397
Jess	2007	Denmark	641	1.4	PMID:17206705
Söderlund	2009	Sweden	3482	2.1	PMID:19422077
Herrinton	2012	USA	5603	1.6	PMID:22609382
Jess	2012	Denmark	14,463	0.9[b]	PMID:22522090
Beaugerie	2013	France	11,759	2.4	PMID:23541909
van den Heuvel	2016	The Netherlands	1157	2.0	PMID:27170593
Cheddani	2016	France	370	2.5	PMID:27481308

[a]Standardized incidence ratio
[b]This study reported relative risk (RR) instead of SIR

cohort studies that was published in 2013 and found a SIR for CRC of 1.7 for patients with IBD (PMID: 23448792) [8]. Close examination of population-based cohort studies from several countries reveals a consistent but modest increased risk for CRC in the range 0.7–2.7 both for patients with UC (Table 10.1) and CD (Table 10.2).

There is strong evidence that the risk of CRC for IBD patients may have decreased considerably over the past 30 years [9, 10]. A large cohort study in Denmark with >47,000 patients with IBD over a 30-year period (1979–2008) showed that the overall risk of CRC among patients with IBD was comparable with that of the general population [10]. However, increased CRC risk was observed for subgroups of patients with UC such as patients diagnosed in childhood or adolescence, those with

long duration of disease, and those with concomitant primary sclerosing cholangitis [10]. For patients with UC, the overall relative risk for CRC decreased from 1.34 in 1979–1988 to 0.57 in 1999–2008. For CD patients, the relative CRC risk was 0.85 and did not change over time. Therefore, the authors of this large Danish cohort study concluded that a diagnosis of UC or CD no longer appears to increase patients' risk of CRC, presumably due to improved therapies for patients with IBD [10]. Similarly, a nationwide study in the Netherlands including 78 general hospitals concluded that the risk of developing CRC in IBD patients was very low [11]. Interestingly, reduced CRC incidence was observed in IBD patients treated with immunosuppressive therapy or by tumour necrosis factor α (TNF-α) blockade. [11]. Thus, one can conclude that the CRC risk in IBD has been overestimated in the past, presumably due to bias in patient inclusion and flaws in statistical analysis. A major pitfall appears to be the use of referral centre-based rather than population-based cohorts which tend to overestimate the risks by including patients with more severe disease (PMID:23448792). An approximately twofold increased risk of developing CRC in patients with IBD is a more reliable estimate, which only weakly supports the proposed link between chronic inflammation and cancer. In contrast, long-term immunosuppressive treatment of IBD patients is associated with an increased risk for overall cancer including haematologic and skin cancers (PMID:27170593), consistent with a key role of the immune system in preventing cancer. Thus, a diagnosis of IBD may be considered a risk biomarker for immunosuppression-associated cancers rather than for CRC only.

Helicobacter pylori Infection and the Risk of Gastritis

Helicobacter pylori is a Gram-negative bacterium which colonizes the stomachs of about half of the world population. The prevalence of *H. pylori* infection varies widely according to geographic area [12]. Sequencing data from a worldwide collection of *H. pylori* strains suggest that anatomically modern humans were already infected with the bacterium before their migrations out of Africa [13]. A complete *H. pylori* genome was recently recovered from the stomach of a 5300-year-old mummy of an early European farmer (*the Iceman*), formally demonstrating that *H. pylori* has been a human pathogen for >5000 years [14]. Although the vast majority (>90%) of *H. pylori* infected people remain asymptomatic, *H. pylori* is considered the main causative agent behind gastritis and peptic ulcer. *H. pylori* (originally named *Campylobacter*) was first isolated from stomach biopsies by the Australian scientists J. Robin Warren and Barry Marshall [15]. *H. pylori* was reported to grow in close contact with the epithelium of the stomach, presumably near the neutral end of the pH gradient and protected by the overlying mucus [15]. Importantly, *H. pylori* was almost always detected in patients with active chronic gastritis, suggesting a causal relationship between *H. pylori* infection and inflammation. This hypothesis was strengthened by the analysis of biopsy specimens from 100 consecutive patients, revealing that the bacterium was present in almost all patients with active chronic

gastritis, duodenal ulcers, or gastric ulcers [16]. Several studies have confirmed this association. Among 274 Italian patients with gastritis, *H. pylori* was found in 84%, while none of the 36 gastritis-free stomachs harboured the bacterium [17]. A case-control study in Finland with 33 patients with gastric ulcer and as many controls showed that *H. pylori* infection was significantly more frequent in the ulcer patients (57%) than in the non-ulcer cases (33%, $p < 0.05$) [18]. Notably, in the non-ulcer group, *H. pylori* infection was strongly associated with gastritis: 11 of 11 (100%) of the *H. pylori* infected patients had atrophic gastritis, as compared with 7 of 22 (32%) of the noninfected patients ($p < 0.01$) [18].

Helicobacter pylori Infection and Gastric Cancer Risk

Several studies have revealed that infection with *H. pylori* is associated with an increased risk of developing gastric cancer. For example, in a cohort of American men of Japanese ancestry living in Hawaii, 94% of patients with gastric carcinoma, but only 76% of the matched controls, had a positive test for *H. pylori* antibodies, implying an odds ratio of 6.0 [19]. As the level of antibody to *H. pylori* increased, there was a corresponding increase in the risk of gastric carcinoma [19]. Another American study in California with 109 patients with gastric adenocarcinoma reported 84% infection rate among patients and 61% for the matched controls [20]. Similar findings were made with patient cohorts in Japan and Norway [21] [22] [23] . Thus, although most *H. pylori* infected individuals remain healthy, there is a strong association between *H. pylori* infection and gastric cancer. Essentially, all patients with gastric cancer are colonized by *H. pylori*. Infection by *H. pylori* is now established as a risk factor for developing gastric cancer. In fact, *H. pylori* was classified as a carcinogen by the World Health Organization (WHO) in 1994 [24]. Thus, gastric carcinoma is considered a paradigm of infection-associated cancer.

Can *H. pylori* Infection or Gastritis Be Used as Biomarkers for Gastric Cancer Risk?

Although it is well known that most people infected with *H. pylori* never develop gastric cancer, several investigators have attempted to use *H. pylori* infection and/or gastritis as predictors of gastric cancer development. In a prospective study, 1526 Japanese patients with various gastrointestinal diseases (duodenal ulcers, gastric ulcers, gastric hyperplasia, or non-ulcer dyspepsia) at the time of enrolment were followed. Gastric cancer developed in 36 of 1246 *H. pylori* infected patients (2.9%) but in none of the 280 uninfected patients ($p < 0.001$) [25]. Interestingly, gastric cancer did not develop in any of the 253 patients with *H. pylori* infection who received eradication therapy [25]. In a second prospective study in Japan, *H. pylori* antibodies and serum pepsinogen I and II (used a markers of gastric atrophy) were

measured for a total of 9293 participants representing the healthy Japanese population [26]. A total of 43 persons developed gastric cancer during the follow-up period. A significantly higher incidence of gastric cancer was observed for individuals with an 'atrophic' pepsinogen status. In this study several patients who developed gastric cancer were negative for *H. pylori* antibodies [26]. The authors speculated that in the most advanced cases of gastric atrophy, *H. pylori* levels may dramatically decrease and *H. pylori* antibodies may actually disappear [26]. This hypothesis is supported by a Finnish study in which 47 men with advanced *H. pylori*-positive atrophic gastritis were followed for 10 years [27]. *H. pylori* antibodies disappeared spontaneously in almost one-fourth of patients with advanced gastritis ($n = 11$), and this was accompanied by no or only a mild improvement of the gastric mucosa [27]. If *H. pylori* antibodies and the bacterium itself may spontaneously disappear in some patients with advanced gastritis and if gastritis after the disappearance of *H. pylori* still predisposes to cancer, this weakens the validity of *H. pylori* detection as a biomarker for gastric cancer. In conclusion, *H. pylori* seropositivity or the associated gastritis may be used as biomarkers to predict development of gastric cancer. This may be particularly useful in populations with a high incidence of gastric cancer such as in Japan. However, there is a very high rate of false positives.

Cytokines as Biomarkers of Inflammation and Cancer Prognosis

Central to the consideration of inflammation-associated biomarkers is the important role of soluble cytokines and other signalling factors that serve to initiate and maintain inflammation. Some of these are released from the liver, whereas others originate either from leukocytes or tissue-resident cells of inflammatory lesions. In the following section, we will focus on some selected cytokines. We give a detailed account of their sources, targets, and the signalling pathways they activate, as this information is critical to understanding inflammatory biomarkers in cancer.

MyD88 and Interleukin-1 (IL-1) Family Members

IL-1 is elevated in various types of cancers, and it is known that patients with IL-1-producing tumours have poor prognosis [28]. This knowledge has called for strategies to target IL-1 that will be detailed below. However, before we explain the possible functions of IL-1 and put them into the context of other IL-1-like cytokine effects (IL-18 and IL-33), we shall first describe the mechanisms by which these cytokines affect the immune system and may act to affect tumour development. Signalling induced by most members of the IL-1 family of cytokines and also the Toll-like receptor (TLR) family converges on MyD88 (myeloid differentiation primary response gene 88), a central adapter of the IL-1 receptor/TLR superfamily

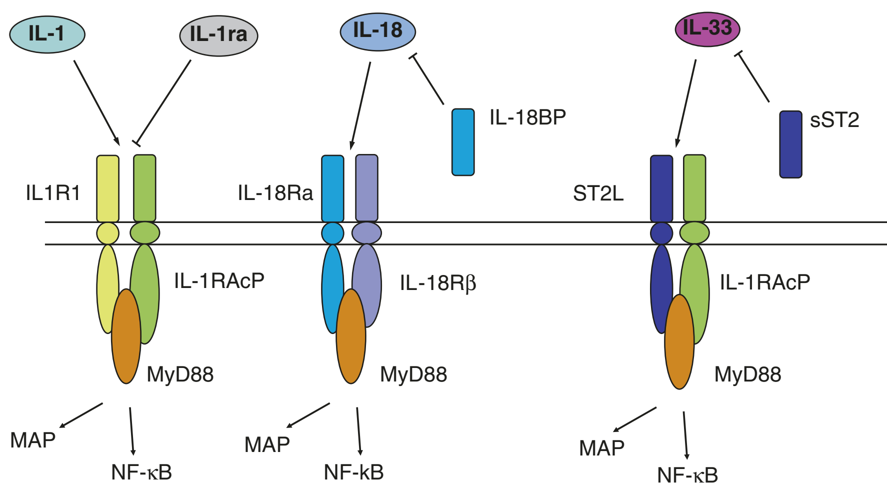

Fig. 10.1 Schematic representation of receptors for IL-1, IL-18, and IL-33. IL-1 receptor (IL1R1) forms a heterodimer with the IL-1 receptor accessory protein (IL-RAcP, aka IL1R3) upon binding of IL-1α or IL-1β, leading to intracellular recruitment of MyD88 and downstream signalling to NF-kB and p38MAP. Interleukin-1 receptor antagonist (IL1Ra, aka IL1F3) competes with IL-1 for binding to IL1R1. IL-18 receptor (IL-18Rα, aka IL1R5) forms heterodimer with IL-18Rβ (aka IL1R7) to mediate signalling after binding IL18. IL18-binding protein (IL18BP) acts as decoy to intercept IL-18. IL-33 receptor (IL-33Ra, aka ST2 or IL1R4) forms heterodimer with IL-RAcP upon binding of IL-33. Soluble IL33R/ST2 acts as decoy to intercept IL-33

[29]. This central position of MyD88 (Fig. 10.1) in inflammation has boosted a strong interest in assessing tumour development when MyD88 is inactivated, and starting this account of the IL-1 family can be better explained by first examining the signalling events downstream from MyD88 activation.

MyD88

The majority of studies imply that MyD88 signalling promotes carcinogenesis in many cancer models. In chemical carcinogenesis models of skin and liver, tumour induction was inhibited in response to genetic lack of *MyD88* [30, 31]. Reduced intestinal tumour growth was also observed in *Myd88*$^{-/-}$ mice subjected to multiple injections of the carcinogen azoxymethane (AOM) in comparison with WT controls [32]. Furthermore, MyD88 signalling was shown to be required for AOM-enhanced colon carcinogenesis in *Il10*$^{-/-}$ mice [33] and in the *Apc*$^{Min/+}$ mouse model of spontaneous intestinal tumorigenesis; MyD88 signalling contributed to adenoma growth and progression [32].

On the other hand, when combining AOM with the chemical irritant DSS, MyD88 was found to protect against the development of colitis-induced cancer [34], contrasting the findings obtained in response to AOM alone [32]. Moreover,

MyD88 activation protects against the development of myeloproliferative neoplasia (MPN) [35]. A protective role of MyD88 against cancer may be explained by the fact that the immune system naturally protects against cancer and that certain types of inflammation prevent malignancies, while other types promote cancer [1, 36, 37].

These observations have nevertheless raised the following question: what are the signals that act upstream of MyD88? Central to the activation of IL-1 and IL-18 is their cleavage by caspase-1, generated by the inflammasome, a composite protein complex strongly involved in the regulation of inflammation and autoimmunity [38]. Indeed, inflammasome defects have been shown to increase tumour growth in several colitis-derived murine cancer models [39–41]. However, the biological effects of IL-1 and IL-18 differ in many respects. Despite the fact that both cytokines signal via MyD88, IL-1 is a strong driver of NF-kB signalling and the MAPK p38 pathway, whereas IL-18 signal transduction mainly involves the latter [42].

Interleukin-1

In carcinogenesis-driven experimental skin cancer, lack of IL-1β led to slower tumour growth, and, conversely, when IL-1 receptor antagonist was lacking, tumour growth was accelerated [43]. In the same vein, IL-1 contributes to the development of preneoplastic gastric lesions in the *Helicobacter*-driven model of intestinal neoplasia [44]. On the other hand, genetic lack of IL-1R does not alter outcome of the AOM/DSS-driven colitis model [34]. Several studies in mice have also documented protective functions of IL-1α and IL-1β against cancer [1, 45].

The effect of targeting IL-1 signalling in cancer treatment has been assessed in some clinical trials. A phase 2 clinical trial with patients with smouldering or indolent multiple myeloma indicated that blocking of IL-1 activity by recombinant IL-1 receptor antagonist (Anakinra) may result in prolongation of progression-free disease [46]. Moreover, a human antibody to IL-1α was tested in a phase I trial of patients that were refractory to antitumour therapies and losing weight and found to induce a significant increase in lean body mass [47].

Interleukin-18

IL-18 is a member of the IL-1 family of cytokines best known for its role in promoting IFN-γ production and Th1 polarization of T cells [48] and a cytokine that has also attracted interest in the field of immuno-oncology. In the *Helicobacter*-driven model of intestinal neoplasia, IL-18, in contrast to IL-1, appears to prevent the onset of gastric cancer [44], perhaps by mediating conversion of T cells to a regulatory phenotype [49]. Likewise, mice lacking either IL-18 or IL-18R were highly susceptible to tumour formation in a colitis-driven model of colorectal cancer [34]. Indeed,

several studies have implicated IL-18 production as the main mediator that confers protection against colorectal tumour formation downstream of the Nlrp3 inflammasome [39]. In fact, in a model lacking caspase-1, substitution with bioactive IL-18 could reverse epithelial dysplasia [41]. IL-18 administration also mediated regression of melanoma and sarcoma [50], apparently mediated by IFN-γ [51] and perhaps involving the antiangiogenic effect of IFN-γ-responsive chemokines CXCL9 and CXCL10 [52]. It deserves mention that IL18-primed human NK cells develop a distinct helper phenotype that shows reduced cytotoxic function and instead, via production of IFN-γ, promote tumour-specific Th1 and CTL responses [53].

The experimental evidence of an antineoplastic role of IL-18 is in apparent contradiction to the elevated levels of IL-18 seen, for example, in human ovarian cancer [54]. However, it appears that while tumour cells have the capacity to synthesize high levels of IL-18, it is the full-length pro-IL-18 that has not been processed by capase-1, and accordingly, it has no biological activity [54]. Likewise, elevated levels of soluble IL-18 binding protein have the capacity to neutralize the effect of IL-18 [55, 56]. The preclinical efficacy of IL-18 has initiated clinical trials of IL-18 alone or in combination, showing that IL-18 has low toxicity in man but a limited therapeutic effect as a single agent [48].

Interleukin-33

Interleukin-33 (IL-33, also known as IL1F11) is the most recently identified member of the IL-1 family of cytokines [57]. Its involvement in cancer development has not yet been reviewed, and it will therefore be dealt with in more detail. This is underscored by the fact that, like IL-1α, IL-33 has a complex biology based on the observation that it acts as a nuclear factor in many cell types, yet when released by damaged cells, it binds to a more conventional surface membrane receptor (IL-33R, also known as ST2 or IL-1R4) that resembles other members of the IL-1/Toll-like receptor (TIR) superfamily (Fig. 10.1). It is important to understand this dual function when interpreting results in cancer biology studies that modulate the function of IL-33 and its receptor.

The identification of IL-33 was initiated by the discovery that serum-stimulated fibroblasts expressed a molecule that partly resembled the IL-1 receptor [58]. This putative IL-1 family receptor member was designated ST2 and widely characterized as an orphan receptor until scientists working at Genentech identified IL-33 as its ligand [57]. In a genome-wide search and modelling for novel members of the IL-1 family, Schmitz et al. [57] revealed a sequence that was first characterized as a transcript induced in vessels in a model of vasospasm after subarachnoid haemorrhage [59]. This protein was later found to be abundantly expressed in the nuclei of endothelial cells in most healthy human tissues, as well as in some epithelia and in the fibroblastic reticular cells of lymphoid tissues [60, 61]. In lesions of inflammation, it is also expressed by activated fibroblasts, and the repertoire of epithelial cells that express IL-33 is expanded [62]. There are also important species differences [62],

but as a general principle, IL-33 is expressed in intact cells as a nuclear protein thought to affect transcriptional behaviour.

The other aspect of IL-33 biology occurs when IL-33 is released by necrotic or otherwise damaged cells and acts as an active cytokine that binds to IL-33 receptor (IL-33, previously designed ST2) and initiates a signalling cascade that also involves signalling via MyD88, p38MAPK, and NF-kB (Fig. 10.1) [57]. Very interestingly, the signalling pathways activated by IL-33 show very strong similarities to those well characterized in response to IL-1. Indeed, despite numerous papers that analysed IL-33 signalling, only one publication reported a side-by-side comparison of the transcriptional response, concluding a virtually identical profile and instead describing mechanisms of regulation related to expression of IL-33R in relation to cell cycle [63]. Further complicating the biology of ST2 is the fact that alternative splicing of mRNA results in transcripts that also encode a shorter, soluble form of ST2 (sST2) that lacks the transmembrane region and is thought to act as a decoy receptor (Fig. 10.1).

Serum levels of IL-33 are reportedly elevated in breast, lung, and gastric cancer and are associated with tumour progression and metastasis [64–66]. On the other hand, multiple myeloma patients had an inverse association between IL-33 levels [67], whereas a study reporting on serum values from patients with hepatocellular carcinoma found no difference when compared to controls [68]. Nevertheless, elevated serum levels of soluble ST2 were identified as a negative prognostic factor in hepatocellular carcinoma [68]. Soluble ST2 is also a negative prognostic factor in oestrogen receptor-positive breast cancer [69]. At the level of tissue expression, increased numbers of IL-33-positive tumour cells were reported to correlate with poor prognosis in carcinoma of the tongue [70]. By contrast, IL-33 has been associated with prolonged patient survival in hepatocellular carcinoma, based on the degree of IL-33-expressing cells in relation to survival [71].

There are also studies that have explored the mechanistics of IL-33 activity. In fibroblasts isolated from head and neck squamous cell carcinomas, IL-33 is upregulated when compared to fibroblasts from healthy gingival tissues [72]. Moreover, recombinant IL-33 promotes the invasive behaviour of such cancer-associated fibroblasts (CAFs), stimulating an epithelial-mesenchymal transformation [72]. Gastric cancer-derived cell lines respond to recombinant IL-33 with increased migration, invasion, and production of the matrix metalloprotease MMP3 as well as IL-6 [66]. The strongest functional evidence for a role of IL-33 in cancer development comes from a recent study that revealed a nonredundant role of IL-33 in myeloproliferative neoplasia (MPN) [35]. Based on the known association of inflammation and MPN, Mager et al. used a model of MPN-like disease in which *styx*, a mutant of the inositol phosphatase SHIP, leads to increased numbers of granulocyte-macrophage progenitors and myeloid cell proliferation in multiple organs. First, demonstrating that deletion of MyD88 abrogated fatal MPN-like disease [35], a systematic search for involved upstream receptors identified IL-33/ST2 signalling as a nonredundant requirement in this process because deletion of ST2 prevented the aberrant haematopoiesis in *styx* mice. Moreover, IL-33 was shown to originate from tissue-resident,

non-haematopoietic cells, as lethally irradiated IL-33$^{-/-}$ mice reconstituted with *styx* bone marrow failed to develop MPN-like disease.

While IL-33/ST2 signalling affected the development of MPN-like disease, it is unclear what initiates the release of IL-33. While IL-33 is released from damaged cells, secretion from live cells remains an unsettled issue. Nevertheless, upon disease progression in the *styx* mutants, infiltrating immune cells cause progressive tissue damage to the lung and intestine, and it is conceivable that necrotic damage provides the IL-33 that supports development of MPN-like disease.

Cytokines that Converge on STAT3 Signalling

STAT3 (signal transducer and activator of transcription 3) is a transcription factor that has received much attention in cancer biology because of its function as an oncogene [73]. While much focus has been given to IL-6 and its signalling from the IL-6 receptor towards activation of STAT3, several other cytokine pathways converge on this central transcription factor, and we shall give examples of how this should be considered in different settings of cancer development.

Interleukin-6 Family

The interleukin-6 family of cytokines is defined by the shared use of the gp130 receptor beta-subunit. IL-6 family members, with the exception of IL-31, can activate the STAT3 pathway, as well as the Erk and PI3K pathways. Included in this family is IL-6, recognized for its role as a systemic acute phase mediator, and IL-11, a cytokine that stimulates platelet production. IL-6 is one of the best characterized tumorigenic cytokines [74]. Elevated IL-6 levels in patients with CRC are associated with advanced stage cancer and an independent prognostic marker of reduced survival [75]. A common feature of IL-6 family signalling is the downstream activation of STAT3, and it is clear that manipulation of STAT3 signalling indeed affects tumorigenesis and that its activation can be driven by other upstream mediators that signal via STAT3.

First, evidence suggests that IL-11 is more strongly correlated to elevated STAT3 activation than IL-6 in gastrointestinal cancers [76]. This became evident from the observation that TGF-β (see below) can drive the stromal production of IL-11 in this context [77]. Additionally, STAT3 signalling can induce microsatellite instability in colorectal cancer by activation of the human mismatch repair mediator MSH3 (mutS homologue 3) [78]. Moreover, excessive STAT3 activation is a prominent feature of the majority of solid cancers, and, indeed, some carcinomas also show activating somatic mutations in *STAT3* and *gp130*, as well as epigenetic silencing of SOCS3 that encodes a critical negative regulator of STAT3 signalling [79, 80].

IL-22

Several cytokines not related to the IL-6 family of cytokines also signal via STAT3. IL-22 is a member of the IL-10 family produced by T helper 17 (Th17) cells, Th22 cells, and innate lymphoid cells. IL-22 is involved in the resolution of tissue damage [81], and, in fact, IL-22-deficiency delays wound healing [82]. A possible involvement of IL-22 in cancer development and progress comes from the following studies: Elevated levels of IL-22 are associated with non-small cell lung cancer [83], and in human CRC, elevated IL-22 is associated with resistance to chemotherapy [84, 85]. At a functional level, exogenous IL-22 promoted the growth of non-melanoma ectopic skin cancers in nude mice [86] and conversely, in a model of colorectal cancer. In fact, neutralization of IL-22 blocked several target genes of the IL-22/STAT3 axis and led to a reduction in dysplasia and invasiveness [87]. Moreover, transgenic IL-22 expression driven by the albumin promoter increased susceptibility to carcinogen-induced liver cancer [88], and, in addition, IL-22 stimulates synthesis of VEGFa – itself a STAT3 activator.

TGF-β

TGF-β is a pleiotropic cytokine that has complex roles in cancer, wound healing, and tissue homeostasis. In breast and prostate cancer, TGF-β appears to induce a variety of prometastatic programmes [89], whereas in the development of colorectal cancer, TGF-β is generally thought of as having a tumour-suppressive function [67]. Whereas normal cells may produce small amounts of TGF-β, cancer cells often secrete large quantities [90]. TGF-β also affects the functions of the non-transformed cells that are present in the tumour mass, in particular by inhibiting immune cells, presumably as a mechanism to dampen the antitumour immune response. For various cancer types, an association has been reported between elevated serum levels of TGF-β and poor prognosis [90]. Amazingly, TGF-β has the ability to function both as a tumour suppressor and a tumour promoter, this duality being known as the TGF-β paradox [91].

In early stages of tumorigenicity, TGF-β potently induces growth arrest of cancer cells. Downstream mediators of TGF-β signalling are SMAD2/3, which interact with SMAD4 to regulate gene expression. In a model of colorectal cancer induced by *Helicobacter bilis*, Smad3-deficient mice showed more rapid disease development, and intriguingly, Smad4-deficiency is associated with poor prognosis in human CRC and leads to elevated levels of CCL15, thought to recruit CCR1+ myeloid cells to liver metastases [83, 92].

In contrast, in later stages of cancer development, TGF-β signalling pathways are severely dysregulated, and TGF-β promotes tumour growth instead [91]. Perhaps accordingly, high levels of TGF-β are associated with poor prognosis for patients with established CRC. This paradox may rest on the recent demonstration that

TGF-β drives synthesis of IL-11 in cancer-associated fibroblasts that feeds back on tumour cells to promote STAT3 signalling and metastasis, perhaps by suppressing apoptotic stimuli encountered during colonization [67].

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Chapter 11
Immunology of Solid Tumors Beyond Tumor-Infiltrating Lymphocytes: The Role of Tertiary Lymphoid Structures

Gert G.G.M. Van den Eynden, Roberto Salgado, and Karen Willard-Gallo

Abstract Immune cells and other constituents of the immune system make up an important part of the tumor microenvironment. Due to increased knowledge on the biology of the immune system in solid tumors and the successes with the treatment of patients with drugs that target its function, interest in immuno-oncology has increased enormously since the first successful trials. The first part of this chapter gives an overview of our current understanding of the role of the immune system in solid tumors, with a focus on the role of tumor-infiltrating lymphocytes (TILs) and their organization in structures called tertiary lymphoid structures (TLS). The increased interest in immuno-oncology has also triggered the search for predictive and prognostic biomarkers. One of the best characterized tissue-based biomarkers of the immune response in solid tumor is the presence of TILs. The second part of the chapter, which focuses on breast cancer, describes currently available data on TILs as a prognostic biomarker, challenges on the assessment of TILs, and TLS and the efforts of the International Immuno-Oncology Biomarker Working Group on standardization of its assessment.

Keywords Immunology • Solid tumors • Tumor-infiltrating lymphocytes • Tertiary lymphoid structure • International guidelines • Breast cancer • Organized immune responses • Methodology • Assessment

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Introduction

The immune system is an important constituent of the tumor microenvironment. Due to better understanding of its vast complexity and also its power and flexibility, as well as the development of drugs that target some of its key molecules or processes, interest from the cancer community has emerged to exploit the possibilities for novel treatments of patients with solid tumors.

An important challenge is the identification, characterization, and assessment of tissue-based prognostic and predictive biomarkers related to the immune system. The value of tumor-infiltrating lymphocytes (TILs) as a prognostic biomarker has been extensively studied, and standardization efforts are ongoing in breast cancer and other tumors. The transition of using this parameter in research settings to routine clinical practice is expected to yield significant benefits to patients and physicians. However, it is more challenging to find biomarkers for the organization of the immune response. Not only the intensity of the immune reaction, but also its organization, plays a major role in patient outcome. Our knowledge about this organization and the factors involved in the temporal and spatial aspects is rapidly increasing, although it remains a challenge to translate this knowledge from the lab to the bedside and use it in the treatment of patients with cancer.

The purpose of this chapter is to give an overview of our current understanding of the role of the immune system in solid tumors, followed by a discussion of practical issues of the assessment of tissue-based biomarkers of the immune response in solid tumors, such as TILs and tertiary lymphoid structures (TLS).

Organized Immune Responses in Solid Tumors

Adaptive Immune Responses Originate in Organized Lymphoid Structures

The physiological functions of the immune system are to defend against infectious microbes, recognize and help remove aberrant or dead cells, and maintain constant surveillance of self/not-self. Immune responses are commonly generated in encapsulated secondary lymphoid organs (SLOs), with the spleen and lymph nodes as major active sites in humans. Their organ architecture is highly organized with segregated T and B cell zones designed to favor specific cellular interactions that generate adaptive immune responses to foreign antigens. SLO structural support is provided by a dedicated reticular network that is intercalated with specialized high endothelial venules and lymphatic vessels used for leukocyte trafficking. Dendritic cell foci along with follicular dendritic cell networks are other characteristic features of SLOs.

SLO formation is part of a genetic program initiated during gestation that progresses in the absence of antigens. Embryonic lymphoid organ development begins with aggregation of lymphotoxin-producing lymphoid tissue inducer cells (reviewed in [1, 2]). Subsequently, lymphoid tissue inducer cells interact with lymphotoxin

β-receptor-expressing mesenchymal stromal organizer cells to initiate a sequence of events that includes vasculature development and recruitment of specialized hematopoietic cells eventually ending with an encapsulated, functional SLO. Interestingly, in addition to the thymus and spleen, lymph nodes are also thought to arise at precisely determined sites along blood vessels in response to specific developmental signals [3].

Nonencapsulated lymphoid tissues also develop prenatally as a salient feature of the mucosal immune system [4]. These tissues are responsible for protecting barrier surfaces and include Peyer's patches, tonsils, and location-termed mucosa-associated lymphoid tissue (MALT), bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT), or nasal-associated lymphoid tissues (NALT). After birth, inducible lymphoid tissues (iBALT, iGALT, etc.) can also form at sites of infection or injury. These de novo lymphoid tissues are referred to as ectopic or tertiary lymphoid structures or organs (hereafter referred to as TLS). They have an architecture similar to SLOs with distinct T cell zones and B cell follicles containing reactive germinal centers (GC) that incorporate follicular dendritic cells, follicular helper T cells (Tfh), and maturing B cells (reviewed in [5, 6]). TLS are credited with resolving acute inflammation in a variety of situations, while under chronic inflammatory conditions, their presence has been shown to be either a contributing disease factor or provide protective immunity (reviewed in [5, 7]). Figure 11.1 gives an overview of human diseases associated with the presence of TLS.

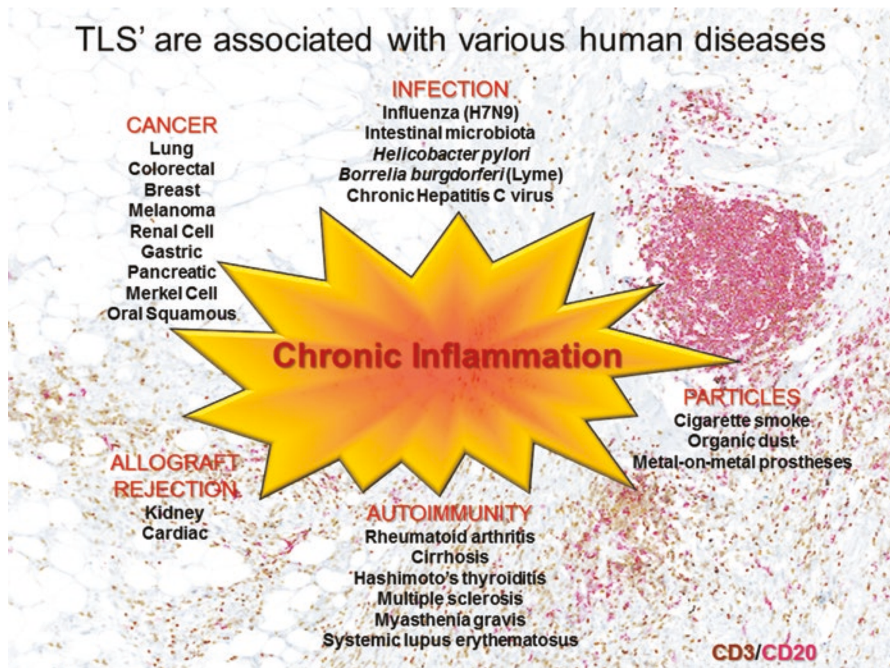

Fig. 11.1 Schematic overview of human diseases associated with the presence of TLS

Ectopic Lymphoid Tissue in Autoimmunity and Infectious Diseases

In chronic inflammatory diseases, TLS arise at sites of inflammation or infection due to persistent antigen and a continuing imbalance between recruitment and clearance of immune cells in the inflamed tissue. TLS are thought to be a key contributing factor to the chronic inflammation associated with autoimmune diseases, including rheumatoid arthritis [8], Sjögren's syndrome [9], and systemic lupus erythematosus (SLE) [10]. Rheumatoid arthritis patients develop TLS in inflamed synovial tissues where their formation has recently been associated with a specific subset of endothelial cells and precursor follicular dendritic cells [11]. Earlier studies associated high frequencies of circulating activated B cells and Tfh cells with disease activity [12] and CXCL13-producing $CD4^+$ T cells with the neogenesis of lymphoid structures in the synovium [13]. These data suggest that interactions between specific immune subpopulations in the inflamed tissue microenvironment foster lymphocyte recruitment and TLS development in rheumatoid arthritis.

SLE is an autoimmune disease characterized by autoreactive antibodies to nuclear antigens. SLE patients frequently develop renal inflammation (lupus nephritis) characterized by autoantibody complexes whose deposition in the kidney can lead to can lead to organ failure [10]. Initially, it was thought that these immune complexes arrived in the kidney via the circulation, but evidence now suggests they are produced in situ in association with TLS neogenesis [14]. Lymphocytic infiltration also characterizes the salivary glands of patients with Sjögrens syndrome where up to 40% develop TLS in parallel with increased autoantibody production and more severe disease [9]. Studies have further shown that active Epstein-Barr virus (EBV) infection can be associated with TLS and disease-specific autoreactive B cells resident in patient's salivary glands [15]. Lymphoid neogenesis and TLS in nervous tissues of patients with multiple sclerosis have also been associated with disease pathogenesis and virus infection [16, 17].

Viral, fungal, and parasitic and bacterial pathogens often enter the host via mucosal surfaces, such as those in the respiratory and gastrointestinal tracts. The mucosal immune system, responsible for protecting these surfaces, must both maintain a commensal microbiota and protect the host from pathogenic microorganisms [4]. Mucosal lymphoid tissues charged with these tasks include Peyer's patches and tonsils, fat-associated lymphoid tissues, and ectopic TLS (the latter induced in response to infection or inflammation) as well as the draining lymph nodes. Viruses are a good example of a mucosal response to infection because many have envelope proteins containing repetitive B cell epitopes, such as the hemagglutinin protein of influenza [18]. Murine models have shown that TLS develop in the mucosal tissues of animals acutely infected with influenza [19, 20] and that this can still occur in the absence of SLOs [21]. Patients chronically infected with hepatitis C virus [22], *Helicobacter pylori* [23], or *Mycobacterium tuberculosis* [24] characteristically develop TLS at infected sites. EBV has also been associated with other autoimmune

diseases (rheumatoid arthritis, Myasthenia gravis) where virus-infected autoreactive B cells accumulate in TLS or the target lymphoid organ and produce pathogenic autoantibodies [25].

Lymphoid neogenesis and TLS are not limited to autoimmune diseases and microbial infection. Pulmonary TLS have been associated with cigarette smoking [26] and in the joints of patients receiving metal hip replacements [27], both thought to be in response to the particulate matter. Acute and chronic allograft rejection has been associated with TLS formation at the organ site in kidney [28, 29], cardiac [30], and lung [31] transplant patients. Studies of cardiac allograft recipients found a strong association between the presence of TLS and earlier times of rejection post-transplantation [30]. Latent cytomegalovirus infection in virus-naïve cardiac transplant patients was also associated with TLS and accelerated allograft rejection [32]. Recent studies have provided intriguing evidence, principally in murine models, that TLS can promote graft tolerance or rejection depending upon the surrounding inflammatory microenvironment (reviewed in [33]). A common denominator among all of these pathological conditions is the presence of a persistent inflammatory response to antigens or particulate matter and a microenvironment conducive to lymphoid neogenesis.

Cancer-Associated TLS in the Tumor Microenvironment

The immune response is responsible for the continuous elimination of aberrant cells in an attempt to defend the host against malignant cell growth. Ultimately, its failure to recognize and remove defective cells at an early stage permits their persistence in an indolent state and can promote the accumulation of synergistic defects favoring transformation. Immune responses are also thought to play critical and decisive roles in tumor progression, invasion, and metastasis through active engagement with other cells in the tumor microenvironment. Thus, while the dynamic activities of immune cells may initially restrain abnormal cell growth as malignancy progresses, the developing tumor frequently acquires attributes that redirect the immune response, at least in part, toward a pro-tumor role.

An increasing number of studies have associated the presence of tumor-infiltrating lymphocytes (TIL) with good clinical outcomes in solid tumors, including melanoma [34], colorectal carcinoma [35], non-small cell lung cancer [36], and breast cancer [37]. Figure 11.2 illustrates a breast carcinoma with a mild and a breast carcinoma with a dense lymphoid infiltrate. Breast cancer gene expression studies were the first to establish a correlation between an immune signal, signifying the presence of infiltrating leukocytes, and clinical outcomes [38–42]. This association varies between gene expression-based breast cancer molecular subtypes [43–45], being strongest in the high-risk triple-negative (TN) and HER2+ BC subtypes. High expression of two immune response gene signa-

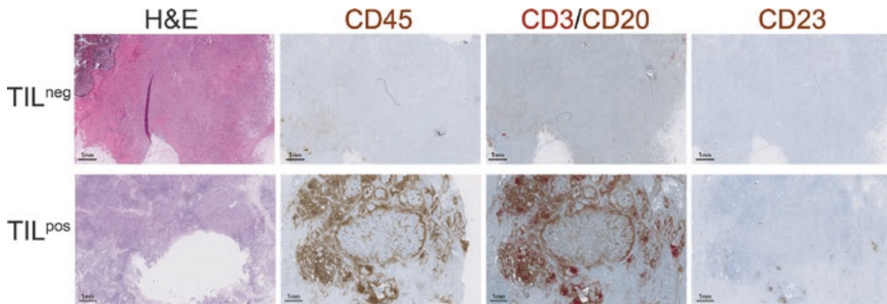

Fig. 11.2 A breast carcinoma with a mild (*upper row*, TILneg) and a breast carcinoma with a dense (*lower row*, TILpos) lymphoid infiltrate. H&E overview (first column); immunohistochemical stainings with a leucocytic marker, such as CD45 (second column); and T and B cell markers, such a CD3 and CD20, respectively (third column), emphasize the difference in inflammatory infiltrate between both tumors. The immunohistochemical double staining with CD3/CD20 (third column) and an immunohistochemical staining with CD23, a marker of follicular dendritic cells, demonstrate the presence of several TLS in the carcinoma with a dense TIL infiltrate. See also Fig. 11.3 (*TIL* tumor-infiltrating lymphocyte, *CD* cluster of differentiation)

tures were consistently and significantly associated with increased pathologic complete response rates after preoperative chemotherapy [46]. The level of immune cell infiltration was also associated with a benefit to preoperative chemotherapy [47–49] with the most robust correlation observed in TN and HER2+ breast cancer (reviewed in [37]).

Subsequent reports demonstrated a significant association in BC between the presence of specific immune cell subsets and clinical responses. For example, tumors with extensive T cell infiltration ($CD8^+$, $CD4^+$ Th, and Tfh T cell subsets) were strongly associated with prolonged survival [50, 51]. A regulatory $CD4^+$ T cell presence was initially shown to signal worse clinical outcomes [52, 53] although subsequent data suggest that their numbers may parallel the extent of the immune infiltrate [54]. Further assessment of large clinical trials suggests that despite the functional heterogeneity of TIL, the degree of global infiltration assessed on hematoxylin and eosin (H&E)-stained tumor sections has predictive and prognostic value for TN and HER2+ breast cancer [47, 55–58].

Initial investigations into the disposition of TIL at tumor sites revealed the presence of lymphocyte aggregates in more extensively infiltrated colorectal tumors [59]. A retrospective study of non-small cell lung cancer identified these aggregates as lymphoid structures (initially termed Ti-BALT for tumor induced but now called TLS) and positively correlated their presence with clinical outcome [60]. In colorectal cancer, active TLS were also observed and shown to predict clinical outcome [61, 62]. Gastrointestinal or respiratory tract tumors arise in tissues that are at a major interface with the external environment and thus are normally protected by the mucosal immune response.

In breast cancer, as in other internally resident solid tumors, lymphoid aggregates have been observed for years but not studied in detail [53, 63–65]. Gu-Trantien et al. described lymphoid structures in breast cancer, an internally resident tumor, showing that they are organized like lymph nodes, including characteristic T cell zones and B cell follicles containing follicular dendritic cells, $CD4^+$ follicular helper T cells (Tfh), and maturing B cells within active GC [51, 66]. These studies were the first to show that $CD4^+$ Tfh cells are a specific component of tumor-associated TLS and link their presence with positive clinical outcome in breast cancer. Known for their critical role in helping to generate B cell-mediated immune responses, the infiltrating Tfh cells are predominantly located in TLS in the peritumoral stroma. Tfh cells play an important role in secondary lymphoid organs by initiating GC reactions that lead to B cell differentiation and plasma and memory cell generation.

Current knowledge of the role(s) that Tfh and B cells play in human tumor immunity is rather limited and controversial, with some studies correlating their presence with a better prognosis while others suggest worse outcomes for various tumor types [67]. Data from TLS-positive tumors indicate that the position and prevalence of T and B cells within the tumor dictate their responsiveness, with extensively infiltrated tumors successfully sequestering the majority of leukocytes in organized TLS. In contrast to autoimmune diseases where TLS promote and sustain disease in solid tumors, TLS may functionally organize immune cells in a failed effort to eliminate the tumor. This effort may, however, produce a sufficiently strong antigen-specific response to generate immunological memory capable of controlling residual disease in some patients. Long-term survival studies suggest that TLS are an important biomarker for patients with melanoma [68, 69], colorectal cancer [70, 71], non-small cell lung cancer [60, 72, 73], breast cancer [51, 74, 75], ovarian cancer [76], renal cell cancer [77], oral squamous cell carcinoma [78], and pancreatic cancer [79].

Significantly, successful efforts to vaccinate against oncogenic viruses have been associated with specific immune responses and lymphoid neogenesis at the lesion site. Vaccination of mice administered with a single dose of recombinant vaccinia vector [80] led to iBALT formation. More pertinently, in patients intramuscularly vaccinated with a therapeutic vaccinia vector expressing the human papillomavirus (HPV) 16 proteins E6 and E7, lymphoid neogenesis was observed in the distant cervical lesion [81]. The TLS induced by vaccination had characteristic T cell zones and B cell follicles with active GC. Recent clinical trials using this therapeutic HPV vaccine demonstrated higher rates of lesion regression in patients with HPV-16 or HPV-18-positive cervical inter-epithelial neoplasia [82, 83]. In patients with regression of the lesions, increased $CD8^+$ T cell infiltration was detected at a higher frequency in HPV-vaccinated, compared to placebo-treated, individuals [82]. Intraepithelial $CD8^+$ T cells were used as a surrogate marker since they were previously shown to be associated with TLS formation at the lesion site [81]. These

studies suggest that when it is possible to identify the appropriate target, such as in virus-associated cancers, then appropriate education of the immune response may be sufficient for long-term control.

The advent of immunotherapeutic agents, capable of manipulating the immune system by targeting immune checkpoint molecules, has shown their power to achieve durable clinical benefit in patients with melanoma and kidney cancer, tumors well known as being immunogenic [84, 85]. Surprisingly, responses have also been observed in traditionally chemoresistant neoplasms such as non-small cell lung cancer [86] and tumors considered to be nonimmunogenic (bladder and prostate cancer) [87]. A common denominator that is emerging from studies of patients treated with immunotherapy is the necessity of a preexisting immune response to the tumor. Because the use of these new agents is costly and associated with significant side effects [88], there is an urgent need for biomarkers like TLS to identify patients with specific antitumor immune response and who most likely will have a benefit. Overall, these studies suggest that a variety of immune-based approaches, tailored to different tumor types, may generate sufficient immunological memory to effectively control residual disease in cancer patients.

Assessment of the Immune Reaction in Solid Tumors: The Breast Cancer Example

TILs and Survival

The best characterized tissue-based marker of the immune reaction in solid tumors are TILs; samples of over 10,000 patients have now been analyzed for TILs in order to assess their prognostic or predictive importance in breast cancer patients. Most data have been gathered in prospective–retrospective phase III clinical trials, and TIL levels assessed on H&E-stained slides can be considered to have level 1 evidence for prognosis in triple-negative breast cancer (triple-negative breast cancer) according to Simon et al. [89]. Additionally, Loi et al. confirmed the prognostic significance in a pooled analysis of triple-negative breast cancer trials [90]. The results summarizing the prognostic and predictive evidence of the main TILs in breast cancer in the adjuvant setting are summarized in Table 11.1.

Methodological Challenges in the Assessment of TILs

Scoring of TILs by pathologists remains a challenge. TILs may be located diffusely across a tumor and may have a very heterogeneous pattern; TILs are either concentrated heterogeneously and spatially within a single tumor bed or may be associated with a punctate pattern across the tumor bed. There can also be a gradient of TILs

Table 11.1 Adjuvant studies on the prognostic role of tumor-infiltrating lymphocytes in breast cancer

Reference	Study	Regimen	Tumor tissue assay	Sample size	Correlation with outcome
[58]	BIG 02–98	A → CMF or AC → CMF	Full section H&E	2009 total	None
				256 TNBC	Stromal TILs (sTIL) (continuous, per 10% increase) Univariate: HR 0.84 ($P = 0.02$, DFS) HR 0.82 ($P = 0.02$, OS) Multivariate: HR 0.85 ($P = 0.02$, DFS) HR 0.83 ($P = 0.02$, OS)
				297 HER2+	None
				1078 HR+	None
[57]	E2197 E1199	AC versus AC AC → docetaxel or paclitaxel	Full section H&E	481 TNBC	sTIL (continuous, per 10% increase) Univariate: HR 0.86 ($P = 0.02$, DFS) HR 0.81 ($P = 0.01$, OS) Multivariate: HR 0.84 ($P = 0.005$, DFS) HR 0.79 ($P = 0.003$, OS)

(continued)

Table 11.1 (continued)

[56]	FinHER	Docetaxel or vinorelbine → FEC With trastuzumab if HER2+	Full section H&E	934 total	None
				134 TNBC	sTIL (continuous, per 10% increase) Univariate: HR 0.79 ($P = 0.03$, DDFS) HR 0.80 ($P = 0.08$, OS) Multivariate: HR 0.77 ($P = 0.02$, DDFS) HR 0.81 ($P = 0.14$, OS)
				209 HER2+	sTIL (continuous, per 10% increase) correlate with DDFS (HR 0.82, $P = 0.025$ univariate) only with trastuzumab, not OS
				591 HR+	None
[37]	Four studies including NEAT clinical trial		TMA CD8, FOXP3 immunohistochemistry	12 439	CD8+ T cells in tumor and stroma was associated with 28% and 21% reduced risk of BCSS. Greater benefit in ER-negative disease and ER = /HER2

[50]	Consecutive	CMF	TMA CD8-immunohistochemistry	1334	Binary high versus low: total CD8 correlates with BCSS (HR 0.55, $P = 0.001$ multivariate training set; HR 0.58, $P < 0.002$ multivariate validation set)
[91]	Consecutive	MF, AC, FAC, or no chemotherapy	TMA CD8-immunohistochemistry	1985 HR+	None
				216 HER2+	None
				496 TNBC	Binary any versus none: CD8 correlates with BCSS, multivariate iTIL (intratumoral TILs) HR 0.48, $P < 0.001$
[92]	Institutional	Varied-chemotherapy not specified	PD-L1 mRNA TILs	636	Higher PD-L1 mRNA associated with better recurrence-free survival PD-L1 mRNA correlated with TILs
[93]	Consecutive	CMF, AC, CEF, or CAF	TMA CD3-immunohistochemistry	255	Binary high versus low: total CD3 correlates with DFS in anthracycline group (HR 0.25, $P = 0.0056$)

(continued)

Table 11.1 (continued)

[94]	N9831	ACT (arm A) or ACT + trastuzumab followed by trastuzumab alone (arm C)	Full section H&E	489 patients (arm A) and 456 patients (arm C)	sTILs, cutoff 60% Univariate: arm A: HR 0.23 ($P = 0.01$; RFS) arm C: HR 1.26 ($P = 0.63$; RFS) Multivariate: arm A: NS arm C: HR 1.01 ($P = 0.04$)
[95]	Consecutive TNBC	Institutional	Full section H&E	897	sTILs, continuous variable Univariate: TILs significant predictor of better DFS, DDFS and OS ($P < 0.0001$) Multivariate: Each 10% increase in TILs independent predictor of DFS, DDFS and OS Stratified analysis: results similar in all subgroups

[96]	Two multicentric randomized trials	Adjuvant anthracyclines versus no chemotherapy	Full section H&E	816 patients	sTILs, itTILS, continuous Multivariate: sTILS: HR 0.89 ($P = 0.005$, OS) itTILS: HR 0.85 ($P = 0.003$, OS) Effect limited to TNBC and HER2+ BC TILs did not predict for efficacy of anthracyclines

Adapted from Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26 [2]:259–71 with permission

within a single tumor, ranging from high TILs to zones of low TILs. TILs located within the stroma that is associated with invasive cancer may be continuous with sometimes extensive localization of TILs within normal lobules and also with TIL infiltration around foci of ductal carcinoma in situ (DCIS). In addition, some specific tumor growth patterns may be associated with different TIL patterns. A tumor with a diffuse and solid growth pattern, constituted by solid tumor nests with only limited stroma between the tumor nests, is rapidly defined as having a high level of TILs since the area of the stromal compartment is low compared to a more infiltrative and dissociative tumor growth pattern. In addition, the TIL infiltration may be located almost solely at the periphery of the tumor bed, sometimes located at >1 or 2 high-power fields from the invasive edge, thus obscuring where the borders of the invasive tumor really end. In addition, different subtypes may add additional hurdles to pathologists when scoring TILs. In the classical subtype of invasive lobular adenocarcinoma, distinguishing infiltrating tumor cells from TILs may not always be that straightforward. In addition, in some tumors a remarkable perivascular location of TILs is encountered, with minimal stromal infiltration by TILs, rendering the evaluation more difficult. TILs can not only be found within the stromal compartment, but also within the tumor cell nests, although in a lower frequency than usually encountered within the stromal compartment. Clearly distinguishing intratumoral TILs from tumor cells may be problematic without the use of immunohistochemistry. All the abovementioned variables may potentially affect the inter- and intra-observer variability of pathologists.

The First International Recommendations on the Evaluation of Tumor-Infiltrating Lymphocytes [37]

Considering the abovementioned variables, there was a need to develop international guidelines that can be used in standard histopathological practice, in a research setting, as well as in clinical trials. Therefore, a group of experts convened and published a guidance document demonstrating step by step how TILs should be assessed, whether it be on core biopsies or on full sections (Table 11.2, for a detailed description, we refer to [37]), clearly distinguishing the recommendations for actual practice from those areas that are still to be considered investigational, such as the use of immunohistochemistry and machine learning algorithms for assessing TILs. The approach for developing this methodological guidance was based on a method originally developed by Denkert and colleagues that has proved to be clinically valid in several retrospective–prospective phase III clinical trials and that was subsequently refined as more experience accumulated. The evaluation of TILs on samples after neoadjuvant treatment still needs more methodological experience and evidence of clinical utility of the chosen method before formal recommendations by

Table 11.2 Recommendations for assessing tumor-infiltrating lymphocytes (TILs) in breast cancer

1. TILs should be reported for the stromal compartment (=% stromal TILs). The denominator used to determine the % stromal TILs is the area of stromal tissue (i.e., area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (i.e., fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei)
2. TILs should be evaluated within the borders of the invasive tumor
3. Exclude TILs outside of the tumor border and around DCIS and normal lobules
4. Exclude TILs in tumor zones with crush artifacts, necrosis, and regressive hyalinization as well as in the previous core biopsy site
5. All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes are excluded
6. One section (4–5 μm, magnification ×200–400) per patient is currently considered to be sufficient
7. Full sections are preferred over biopsies whenever possible. Cores can be used in the pretherapeutic neoadjuvant setting; currently no validated methodology has been developed to score TILs after neoadjuvant treatment
8. A full assessment of average TILs in the tumor area by the pathologist should be used. Do not focus on hotspots
9. The working group's consensus is that TILs may provide more biological relevant information when scored as a continuous variable, since this will allow more accurate statistical analyses, which can later be categorized around different thresholds. However, in daily practice, most pathologists will rarely report for example 13.5% and will round up to the nearest 5–10%, in this example thus 15%. Pathologist should report their scores in as much detail as the pathologist feels comfortable with
10. TILs should be assessed as a continuous parameter. The percentage of stromal TILs is a semiquantitative parameter for this assessment, for example, 80% stromal TILs means that 80% of the stromal area shows a dense mononuclear infiltrate. For assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be taken into account. Lymphocytes typically do not form solid cellular aggregates; therefore, the designation "100% stromal TILs" would still allow some empty tissue space between the individual lymphocytes
11. No formal recommendation for a clinically relevant TIL threshold(s) can be given at this stage. The consensus was that a valid methodology is currently more important than issues of thresholds for clinical use, which will be determined once a solid methodology is in place. Lymphocyte-predominant breast cancer can be used as a descriptive term for tumors that contain "more lymphocytes than tumor cells." However, the thresholds vary between 50% and 60% stromal lymphocytes

From Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26 [2]:259–71 with permission

the International Working Group can be drafted. In a similar vein, the method by which TILs in lesions containing merely in situ lesions should be characterized is so far unexplored, and more analytical evidence and corresponding clinical validity need to be gathered before formal recommendations can be presented.

It is emphasized in this guidance document that pathologists should try to score TILs as accurately as possible and document these in clinical and research studies as continuous variables, enabling categorization in subsequent analyses if this is required. An important concept that was introduced is the total allowable error margin (TEM) between pathologists, defined as the allowable margin that does not affect clinical practice. Currently, the scope of TIL assessment is based on the evaluation of the full H&E slide, without focusing on TIL hot spots and evaluating the often heterogeneous patterns. This element of "eye-balling" and the corresponding subjectivity may explain the interobserver discordance found between pathologists. However, the TEM may be different when assessing TILs related to prediction, where quite probably the allowable error margin between pathologists may be less wide than if a prognostic association is sought after, where a larger error margin might be allowed. The TEM is likely to affect the implementation of machine learning algorithms that aim for a more accurate determination of the amount of TILs, irrespective of the potential use of it.

Methodological Issues in the Assessment of TLS

In the guideline document, the assessment of TLS in the peritumoral region is not recommended, except for research purposes or in studies to confirm their potential clinical relevance [37]. This is mainly due to the fact that the assessment of TLS on H&E slides is difficult and subject to interobserver variability. It is difficult to distinguish denser lymphocytic infiltrates/aggregates without a germinal center from TLS, which by definition have a germinal center containing B lymphocytes and follicular dendritic cells. The use of immunohistochemistry can improve the detection of TLS in breast cancer specimens. One option is the use of CD20 as a marker for B cells or CD23 as a marker for follicular dendritic cells. In our studies we prefer to use an IHC double staining with antibodies against CD3 and CD20. TLS are then characterized by structures with a dense center of CD20-reactive B cells surrounded by a ring of T cells (Gert Van den Eynden, personal communication). Figure 11.3 illustrates the difference between a lymphoid aggregate and a true TLS and the importance of immunohistochemistry to distinguish both.

Another issue is the localization of the TLS; although these structures can be found within the tumor mass, they are often found at the border of the tumor mass or even at a distance outside the tumor. Furthermore, they are frequently found in and around DCIS lesions or in and around normal lobules. This makes the assessment again sometimes challenging and leads to less reproducible results. Nevertheless, standardization of the methodology and inter-pathologist tuning again improves the assessment of TLS (Gert Van den Eynden, personal communication).

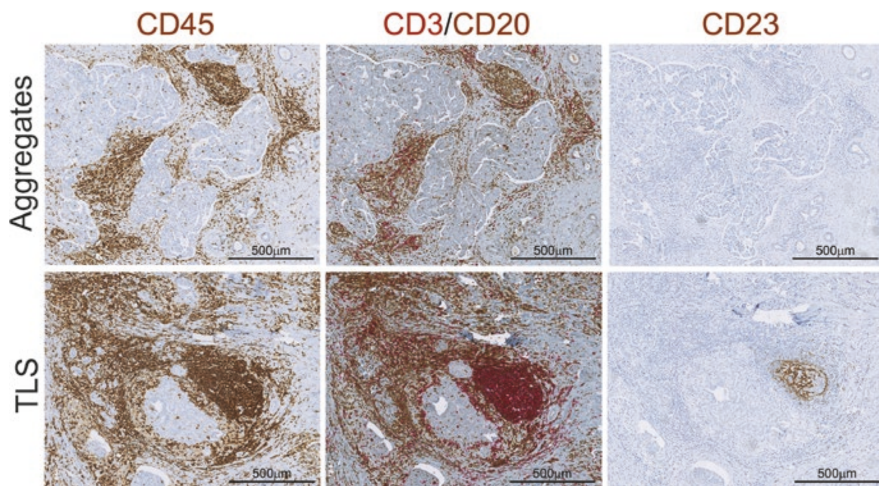

Fig. 11.3 The difference between a lymphoid aggregate (*upper row*) and a true TLS (*lower row*) is difficult on (immunohistochemical) stainings that do not differentiate between B and T lymphocytes, such as CD45 (first column). An immunohistochemical double staining with CD3/CD20 (second column) that differentiates between T and B lymphocytes, respectively, visualizes a dense (follicle) center of B lymphocytes in the TLS, in contrast to a lymphoid aggregate. Furthermore, a CD23 immunohistochemical staining (third column) shows the presence of a network of follicular dendritic cells in the follicle center of the TLS, which is absent in a "simple" lymphoid aggregate (*TLS* tertiary lymphoid structure, *CD* cluster of differentiation)

Conclusion

Immune therapy, in all its forms, is expected to become an important part of the arsenal to treat solid tumors. An important challenge will be the identification, characterization, and assessment of tissue-based prognostic and predictive biomarkers of the immune system. The role of TILs in the immune response to solid tumors has become more and more characterized, and its value as a tissue-based prognostic biomarker in triple-negative breast cancer has been extensively documented. The pathology community has performed intensive efforts to standardize the assessment of TILs in breast cancer and other solid tumors. Nevertheless, some methodological challenges remain. Furthermore, TILs as a biomarker do not provide information on the spatial and temporal organization of the immune response. As in autoimmune and infectious diseases, this organization is crucial for the immune response against solid tumors. The presence and number of TLS are parameters that include such organizational information. However, the methodology of the assessment of TLS remains to be further optimized and standardized.

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Chapter 12
The Role of Platelets in the Tumor Microenvironment

Kelly E. Johnson, Sandra S. McAllister, Joseph E. Italiano Jr., and Elisabeth M. Battinelli

Abstract Platelets are small, anuclear cells found in the circulation that have an important and well-defined role in hemostasis and wound healing. Known as the "band-aids of the blood," these cells rapidly activate, aggregate, and release a potent milieu of growth factors, cytokines, and other biological mediators at the site of vascular damage, forming a clot. Compelling evidence has revealed that tumors can co-opt the normal functions of platelets in order to advance tumor progression and metastasis. Indeed, we now know that platelets are a key component of the tumor microenvironment and that they promote cancer progression in a myriad of ways; platelets drive tumor cell invasion and epithelial to mesenchymal transition, they promote angiogenesis, they facilitate intravasation and extravasation of tumor cells, they protect disseminated tumor cells from shear forces and immune surveillance within the circulation, and they function as long-distance cargo carriers that transmit signals between primary tumors, metastases, and the bone marrow. In this chapter, we will examine the current body of evidence on the role of platelets in cancer along with the underlying mechanisms and explore platelet-targeted therapies as a novel and promising approach to cancer treatment.

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Platelets are best known for their role in thrombosis and hemostasis. These tiny, anuclear circulating cells form clots at sites of vascular damage to initiate the wound healing process. However, we now also know that platelets are a critical component of the tumor microenvironment (TME) and can profoundly affect tumor progression and metastasis. For example, platelets aid disseminating tumor cells by protecting them from high shear forces and immune surveillance within the circulation, by forming tumor cell-platelet aggregates that facilitate embolization, by promoting adhesion of tumor cells to the vascular endothelium, and by releasing a variety of soluble factors that promote tumor growth and metastasis. Platelets are, by design, carriers of a myriad of cytokines and growth factors, many of which are known to affect disease progression. Cytokines released from activated platelets not only impact the function of tumor cells but also other cells in the TME such as endothelial cells, fibroblasts, and immune cells [1–3]. In this chapter, we will discuss what is known about the complex cross talk between platelets, tumor cells, and other host cells that occurs in malignancy and highlight features of this communication that may be vulnerable to therapeutic intervention.

Platelet Function

Before exploring the role of platelets in cancer, it is beneficial to review normal physiological platelet function. Platelets are small (1–3 μm), discoid-shaped cell fragments that are released from progenitor cells called megakaryocytes in the bone marrow. Their concentration in the blood is between 1.5 and 3.5×10^8/μL in healthy individuals, and they have a half-life in circulation of only 7–10 days [4]. Structurally, platelets are anuclear and contain three distinct types of granules; alpha-granules, dense granules, and lysosomes. Each platelet contains ~50–80 alpha-granules, thus making them the most abundant type of platelet granule [5]. Over 300 biologically active factors, including cytokines, adhesion molecules, and coagulation mediators (e.g., VEGF, PF4, P-selectin, and fibrinogen), are contained within alpha-granules, which can be selectively released upon platelet activation [6]. Dense granules contain a variety of signaling intermediates such as serotonin, ATP, and ADP that can enhance platelet activation [7, 8]. Lysosomal granules are packaged with proteases and glycosideases [9]. Although they do not have nuclei, platelets contain some mRNA and translational machinery that are present in the cytosol; hence, protein synthesis can occur to a limited extent [10]. The platelet surface is coated with glycoproteins, adhesion molecules, and signaling receptors, thus enabling them to interact with other cells and to become activated upon contact with agonists such as thrombin, collagen, ADP, thromboxane, and epinephrine [7].

Platelets are often thought of as the "band-aids of the blood"; they prevent blood loss during injury by forming a clot at the site of vascular damage. By design, they must be able to remain inactive, or resting, under normal conditions but be able to quickly activate and form a clot upon contact with damaged endothelium within the high shear, high-pressure conditions of the circulation. Damage to the vascular wall causes exposure of subendothelial collagen and von Willebrand factor, which serve to attract circulating platelets by engaging glycoproteins on the platelet cell surface, such as GPIbα, thereby leading to adhesion at the site of damage [11]. Local sources of collagen and thrombin at the wound site initiate platelet activation via GPVI and PAR receptors, respectively, causing platelets to undergo a drastic shape change and to release their granule contents [11]. GPIIbIIIa on the platelet surface is activated by fibrinogen allowing for platelet aggregation and the formation of fibrinogen bridges that stabilize the clot [11]. Activated platelets release pro-coagulation factors and serve as a surface for clotting factors to assemble, further strengthening the platelet plug [7] (Fig. 12.1a).

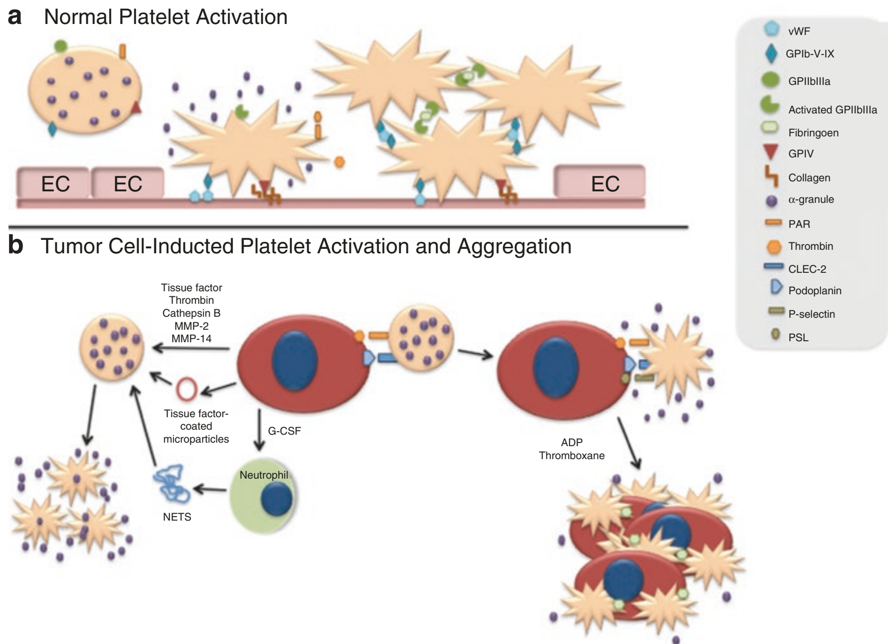

Fig. 12.1 Mechanisms of platelet activation. Under normal, physiological conditions, platelets rapidly form a clot upon contact with a site of vascular damage (**a**). Damage to the endothelium leads to exposure of underlying extracellular matrix proteins which engage glycoproteins on the cell surface of circulating platelets. Platelets activate, undergo a shape change and release their granular contents. Fibrinogen bridges form between platelets to strengthen aggregates. Granule contents increase platelet activation, trigger coagulation, and participate in the wound healing process (**a**). Tumors trigger platelet activation and aggregation through a variety of direct and indirect mechanism (**b**). Direct contact with tumor cells can cause platelet activation through a variety of ligand/receptors pairs. Tumor cell-secreted factors or microparticles also stimulate platelet activation while tumor cell-activated neutrophils can release platelet-activating NETS (**b**)

It is easy to imagine how activation of platelets at inappropriate times or locations could lead to adverse situations. Improper activation and aggregation can lead to the formation of blood clots, while release of growth factors and inflammatory cytokines from alpha-granules can promote atherosclerosis and tumor progression [12]. Indeed, pathological platelet function has been shown to occur in a variety of cancer types and platelets are accepted as key players in a number of the processes underlying disease progression and metastasis.

Identifying a Role for Platelets in Cancer

A link between cancer and abnormal coagulation was first noted in the 1800s when Jean-Baptiste Bouillaud reported a case of deep vein thrombosis associated with cancer [13]. French physician Armand Trousseau is widely credited as the first to definitively propose a link between cancer and hypercoagulabilty of the blood when he noted that patients with cancer were more likely to develop a blood clot than the general population and that blood clots could be predictive of an undiagnosed malignancy [14]. Platelets were specifically implicated in cancer in 1872 when a link between elevated platelet count and malignancy was reported [15]. Levin and Conley published a detailed examination of thrombocytosis (elevated platelet count) and cancer in the 1960s, finding that thrombocytosis was present in 38% of patients with inoperable tumors [16]. Since then, thrombocytosis has been correlated with poor outcomes in a variety of solid tumor types including cancers of the breast, lung, ovary, colon, kidney, and brain [17–22]. Thrombocytosis is also associated with increased risk of venous thromboembolism (VTE) in many cancer patients [23]. Cancer patients have a four to sevenfold greater risk of developing a pulmonary embolism or a deep vein thrombosis compared to healthy individuals [24].

Is elevated platelet count merely coincidental, or do platelets play a direct, active role in cancer progression? To answer this, Gasic et al. depleted platelets from mice prior to injecting tumor cells in an experimental murine model of metastasis [25]. Depletion with neurominidase or antiplatelet serum decreased metastasis, while infusion of platelet-rich plasma reversed this effect, suggesting that platelets play an active role in cancer progression. Subsequent mouse studies show that disruption of platelet function also reduces metastasis formation; a greater than 50% reduction in metastasis was seen in both GPVI and P-selectin knockout mice [26–29]. Interestingly, metastasis was reduced by 80% in a mouse model of gray platelet syndrome, a disorder in which platelets lack alpha-granules [30]. These animal studies verified that platelet activation and alpha-granule release were involved in metastasis.

Taken together, observations in cancer patients along with experimental mouse models clearly demonstrate that platelets play a necessary role in metastatic spread. Nevertheless, questions remain about how, mechanistically, platelets influence the metastatic process. Numerous research efforts have focused on answering this question, and in this chapter we will examine research demonstrating the role of platelets

at every stage of cancer progression, from the primary tumor site to the tumor cell's journey through the circulation and finally during extravasation and metastatic growth.

Tumor Cell-Induced Platelet Activation and Aggregation

Normally, platelets are only activated at sites of vascular injury and remain inert (resting state) while in contact with healthy cells and tissues. However, tumors and their microenvironment are far from normal and have been described as "wounds that never heal" due to persistent inflammation and tissue remodeling [31]. The TME has developed a variety of ways to induce inappropriate platelet activation and co-opt platelet function for the tumor's benefit. In vivo, activated platelets have been observed within primary tumor tissue [32]. Angiogenic vessels associated with tumors are often abnormal and leaky, with gaps between endothelial cells and areas of exposed collagen, allowing platelets entrance and access to tumors [33]. Tumor cells can activate platelets by producing the potent activator, thrombin, and elevated thrombin levels have been observed within the TME of several types of cancer [34–36]. Tumor-derived cathepsin B, matrix metalloproteinase (MMP)-2, and MMP-14 have all been shown to activate platelets, and tissue factor (TF) can also be aberrantly released from tumor cells, indirectly activating platelets through initiation of the coagulation cascade [37, 38]. Direct contact between platelets and tumor cells can also lead to activation; for instance, tumor cell podoplanin or mucins can interact with and activate platelet CLEC-2 and P-selectin, respectively [39–42].

Interactions between platelets and tumor cells either at the primary tumor site or within the circulation often lead to a phenomenon called tumor cell-induced platelet aggregation (TCIPA). TCIPA occurs when tumor cells activate platelets, leading to activation and release of platelet-derived ADP and generation of thromboxane to further trigger aggregation [43–45]. In this process, fibrin is generated, thereby cross-linking tumor cells and platelets, while glycoproteins such as GPIIbIIIa strengthen the platelet-tumor cell aggregates through fibrinogen bridges [46]. Aggregates composed of platelets and tumor cells have been observed within the circulation since the 1970s [47, 48], and tumor cell lines of breast, colon, prostate, lung, and pancreatic origin, to list a few, have been shown to aggregate platelets in vitro [49–52]. These aggregates can be observed in the blood of patients and are implicated in tumor cell immune evasion and embolization.

In addition to activation by direct platelet-tumor cell interaction, tumor cells can induce long-range activation of distant platelets. For instance, tumor cells release TF-coated microparticles that can travel through the circulation and may be involved in cancer-associated VTE [53, 54]. Another mechanism of indirect platelet activation can occur when tumor cells secrete G-CSF, causing circulating neutrophils to release platelet-activating neutrophil extracellular DNA traps (NETS) [55, 56].

The cross talk between platelets and tumor cells that mediates activation and aggregation is thought to be crucial for platelets to support tumor progression. Overall,

tumor cells have a diverse arsenal of mechanisms to induce platelet activation, and the specific methods utilized by a particular tumor may depend on the cancer type, stage, or location. For instance, some glioblastoma and pancreatic cell lines release thrombin to induce TCIPA, while MCF-7 breast tumor cells can release MMP-2 or ADP to achieve TCIPA [43, 44, 57, 58]. But regardless of the specific mechanism, activation of platelets seems to be a nearly universal phenomenon in cancer progression (Fig. 12.1b). In the next sections, we will discuss in detail how activated platelets and platelet-tumor cells aggregates are thought to influence cancer progression.

Platelets in Tumor Growth and Invasion

Platelets are packed with a myriad of biologically active growth factors and cytokines that are critically important during wound healing but can be detrimental when co-opted by tumors. Activated platelets release cargo into the peritumoral space, thus impacting tumor growth, migration, and invasion. In vitro studies have shown that PDGF, thromboxane, and platelet-activating factor (PAF) directly drive tumor cell proliferation [59–61]. However, the evidence that platelets have a role in influencing primary tumor proliferation and growth in vivo is limited [59–61]. A vast body of evidence both in vitro and in vivo suggests that, instead, platelets in the primary TME predominantly influence tumor progression by driving invasion.

Platelets promote invasion through a variety of mechanisms. Epithelial to mesenchymal transition (EMT) is a critical process that tumor cells undergo in order to become invasive. During EMT, tumor cells of epithelial origin lose their cell-to-cell adhesions and polarity, becoming more mobile and developing the characteristics and markers of mesenchymal cells. Platelets induce expression of key EMT regulators such as twist, snail, slug, vimentin, and fibronectin, while downregulating E-cadherin [62]. Findings from these studies also demonstrated that platelet-derived TGF-β1 drives EMT though activation of the TGF-β1 receptor and NF-κB signaling pathways in the tumor cells, with which they are in direct contact [62, 63]. Furthermore, conditional ablation of platelet TGF-β1 reduced metastasis in mice [62]. While TGF-β1 released from platelets has been identified as the main factor responsible for platelet-induced EMT, hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) may contribute to EMT as well [64]. Platelet-derived autotaxin has also recently been shown to directly induce breast tumor cell migration and invasion [65–67].

Another mechanism by which platelets promote tumor cell invasion is to alter the TME to favor migration and invasion. Simply adding live platelets or releasate from activated platelets significantly increases migration and invasion of tumor cells in culture [68]. By releasing MMPs directly into the peritumoral space, platelets break down the extracellular matrix to enable tumor cell migration. Furthermore, platelets induce MMP expression in other components of the microenvironment including tumor cells and endothelial cells [69–71]. Stromal cells in the TMEs are also influenced by platelet-derived factors as indicated by studies showing that tumor-

promoting cancer-associated fibroblasts (CAFs) proliferate and differentiate in response to signals from activated platelets [72, 73]. Finally, platelets are thought to facilitate tumor cell migration across the vascular endothelium by releasing factors that weaken junctions between vascular endothelial cells, thus allowing for tumor cells to escape into the circulation [74]. For example, results from studies using transendothelial migration assays demonstrated that platelet-derived ADP, TGF-β, and VEGF can all facilitate tumor cell migration across an endothelial barrier [65, 74, 75].

Platelets Promote Angiogenesis

Angiogenesis, the growth and expansion of the vasculature, is critical for most solid tumors to survive and grow beyond a diameter of 1–2 mm. A role for platelets in tumor angiogenesis was first proposed by Judah Folkman in 1998, and, indeed, platelets are now known to be intimately involved in the angiogenesis process [76]. Platelets are packed with various different pro- and anti-angiogenic regulators, but the net effect of releasates from tumor cell-activated platelets tends to strongly promote angiogenesis [77]. Over 80% of circulating VEGF, a potent pro-angiogenic mediator, is carried within the platelets of both healthy individuals and cancer patients, and VEGF levels within platelets correlate with disease progression [78–80]. In vitro, platelets and platelet releasates increase endothelial cell proliferation, migration, and capillary tube formation [77, 81]. Use of in vivo angiogenesis assays has also confirmed that platelets are required for vessel development. To that end, platelet depletion was demonstrated to decrease retinal neovascularization, corneal angiogenesis, and tumor angiogenesis [32, 82, 83].

Platelets package different angiogenic mediators into distinct alpha-granules that can be released differentially depending on the specific agonist bioavailability or receptor activation. ADP activation leads to VEGF release and a pro-angiogenic releasate, while activation with thromboxane causes retention of VEGF and release of the anti-angiogenic protein endostatin, leading to a platelet releasate with net anti-angiogenic effects. Platelet activation via the thrombin receptor PAR1 mediates VEGF release, while stimulation of the PAR4 receptor leads to endostatin release and retention of VEGF [3, 84]. These studies show that platelets are able to make “choices” about which contents to release based on the stimulus they receive. Tumor cells are able to harness this differential release for their own benefit and mediate the preferential release of VEGF over endostatin, and releasates from tumor cell-activated platelets have a strong net pro-angiogenic activity [77]. Platelet inhibition with aspirin prevents MCF-7 breast tumor cell-induced VEGF release and, in fact, leads to a net anti-angiogenic effect of platelets [77]. Taken together, these studies provide strong evidence that platelets, upon activation by tumor cells, release potent pro-angiogenic mediators to provide the growing tumor with a blood supply.

Angiogenic vessels within the tumor tend to be immature and leaky, and platelets seem to preferentially adhere to angiogenic vessels over normal, mature vessels [85]. This differential adherence may be one way in which platelets are attracted to

and enter the TME. Once inside the TME, platelets not only drive angiogenesis but can also function to stabilize angiogenic vessels [86]. Specifically, platelets seem to support pericyte coverage in angiogenic vessels and angiopoietin-1, and serotonin released from platelets may promote vessel maturation [83, 87]. In this way, platelets normalize tumor vasculature and prevent intratumoral hemorrhage. While angiogenesis is primarily a pro-tumorigenic process, vessel stability is more complex and may have antitumor effects. Therefore, more research is needed to parse the specific signals, conditions, events, and intermediates that favor platelet-induced angiogenesis or vessel stabilization.

Platelets are also capable of taking up molecules from their environment. Angiogenic factors including VEGF and bFGF are taken up by platelets from the site of the tumor, stored, trafficked, and delivered to other locations such as distant metastatic sites [88–90]. A recent study, using a murine model of luminal breast cancer, demonstrated that platelets sequester angiogenic regulators from the site of an aggressively growing primary tumor and deliver them, via the circulation, to indolent tumors located at distant anatomical sites where these platelets contribute to the growth and angiogenesis of the otherwise indolent tumor [32]. Platelet inhibition with aspirin prevented this effect, suggesting that platelets were, in fact, responsible for delivering angiogenic signals from one tumor to the other [32]. These studies highlight the potential for platelets to serve as long-haul cargo carriers, shuttling signals between distant sites as orchestrated by the tumor. However, little is currently known about the mechanism by which platelets endocytose proteins from their environment, leaving a critical gap in our knowledge. A deeper understanding of these processes should provide a source of potential therapeutic targets.

Overall, platelets contribute significantly to tumor angiogenesis via a number of mechanisms; they release potent pro-angiogenic factors upon stimulation by tumor cells, they mature and normalize unstable tumor-associated vessels, and they collect angiogenic mediators and deliver them to distant sites, propagating the angiogenic signal from the tumor. Angiogenic neovasculature not only nourishes the tumor but also provides a route for tumor cells to escape into the circulation.

Platelet-Tumor Cell Interactions in the Circulation

The circulation is a very hazardous environment for newly disseminated tumor cells, one in which tumor cells face harsh shear stresses and constant immune surveillance. The vast majority of tumor cells are destroyed within hours of introduction into the circulation, well before they can ever successfully form metastases [91, 92]. As previously discussed, contact between platelets and tumor cells causes aggregates of the two cell types to form. These aggregates can be readily identified in the circulation of cancer patients and form within minutes of tumor cell introduction into the blood stream of mice, suggesting that tumor cells fair better in circulation when coated in platelets [63]. The mechanical forces exerted on tumor cells in the blood are far greater than what was experienced in the TME and is often enough to cause their destruction [93]. Platelets are naturally suited to thrive within the

vasculature and provide protection by coating tumor cells, shielding them from shear stress [94].

Circulating tumor cells are under constant assault from immune surveillance particularly from NK cells, and platelets play a crucial role in protecting them. Activated platelets express glucocorticoid-induced tumor necrosis factor receptor ligand (GITRL) on their surface, which binds to the GITR on NKs, leading to inhibition of NK cell activity [95]. Platelets can also inhibit NK cells by modulating expression of the NKG2D receptor on the NK cell surface [96]. NK cells use this receptor to identify and lyse tumor cells. TGF-β released from activated platelets can cause downregulation of NKG2D on the NK cell surface, making NKs less able to identify and destroy tumor cells [96]. Platelet-derived TGF-β also inhibits NK cell IFNγ production and stimulates the development of immune-dampening T-regulatory cells [97, 98]. Furthermore, platelets can protect tumor cells from NK destruction by transferring MHC class I molecules to the tumor cell surface [99]. Tumor cells may also avoid lysis in the blood by aberrantly expressing integrins normally found on platelets in a phenomenon known as platelet-mimicry [100, 101]. Although a great deal has been discovered regarding how platelets disrupt immune surveillance in the circulation, relatively little is known about how platelets interact with immune cells within the TME and if these mechanisms may also apply at these sites.

Extravasation

Tumors cells must find ways to successfully exit the circulation in order to seed a new metastatic site. Immobile platelet-tumor cell aggregates have been observed in the microvasculature [102, 103], and it was historically assumed that this was a passive process with aggregates simply getting stuck within narrow vessels. We now know that arrest and extravasation are active processes and that platelets are key players in both of them. Platelet surface selectins mediate rolling along the endothelium slowing their velocity in circulation and allowing for further association with endothelial cells. P-selectin on activated platelets interacts with the endothelium while simultaneously mediating binding to tumor cells, thus tethering tumor cells to the endothelium [28, 41]. The importance of P-selectin in this process has been demonstrated in mice through pharmacological blockade as well as genetic ablation of P-selectin [42]. Next, platelet integrins mediate arrest on the endothelium, while platelet-derived factors such as MMP-1, TGF-β, and ADAM12 break down junctions between endothelial cells, allowing tumor cells to cross the now leaky endothelial barrier and enter the surrounding tissue parenchyma [103, 104].

Once disseminated tumor cells have arrived at new metastatic sites, activated platelets promote colonization, angiogenesis, and ship signals to and from distant sites. To quote Yan and Jurasz, "… perhaps a small revision is required to Paget's 'seed and soil' hypothesis of metastasis to include 'seed, soil, and fertilizer', in which platelets take on the unenviable but critical role of 'fertilizer'" [105]. However, it remains unclear if platelets support tumor cells at secondary sites through the same mechanisms employed at the primary tumor and this question warrants further investigation.

Platelets Coordinate the Systemic Effects of Tumors

As discussed previously, tumors can activate, alter, and use platelets to carry molecular signals to distant locations throughout the body, making platelets an integral part of the systemic communication and coordination that occur in cancer. Platelets can propagate messages that serve to mobilize bone marrow progenitors, alter bone function, and even prepare sites to accept future metastases. Tumors recruit bone marrow-derived cells (BMDCs) and endothelial progenitor cells to the TME. SDF-1, VEGF, and ANGPT-1 released from activated platelets have been implicated in mobilizing BMDCs and progenitor cells from the bone marrow [86, 106, 107]. Platelets also appear to promote metastasis within the lung by recruiting pro-metastatic granulocytes to platelet-tumor cell aggregates during extravasation through the release of CXCL5 and CXCL7 [63]. Kuznetsov et al. also demonstrated that platelets, acting as a long-range communication system between primary tumors, distant tumors, and the bone marrow, cooperate with BMDCs to promote vascularization of the distant tumors [32].

Bone remodeling often occurs in the setting of metastatic disease and platelets may mediate this process as well. The presence of a primary melanoma or prostate tumor increased bone formation in mice, while platelet depletion reversed this effect [108]. In these two models, platelets traffic tumor-derived MMP-1 and TFG-β to the bone where they promote bone formation. Conversely, platelets are also capable of increasing bone resorption to facilitate breast cancer metastasis to the bone. Boucharaba and colleagues demonstrated that platelets promote osteolytic bone loss by a complex mechanism in which lysophosphatidic acid (LPA) released from activated platelets drives IL-6 and IL-8 secretion from tumor cells [109]. These cytokines then stimulate bone-destroying osteoclasts. Furthermore, platelets release autotaxin from their alpha-granules, a molecule that catalyzes the production of LPA and guides tumor cells to the bone by interacting with tumor cell αvβ3 integrins [108].

Platelets clearly help orchestrate the complex coordination of events that allow tumors to metastasize. More studies are required to parse the precise role of platelets in the spread of specific tumor types and in the homing of tumor cells to particular sites of metastasis. Additionally, it is necessary to confirm that these mechanisms occur in human patients and, if so, determine potential therapeutic interventions (Fig. 12.2).

Platelet Microparticles and the Tumor Microenvironment

Recently, interest in platelet-derived microparticles (PMPs) and their potential role in cancer has been growing. PMPs are shed from platelets following activation or shear stress and consist of membrane-bound proteins and cytoplasmic components. In vitro, PMPs have similar pro-angiogenic and pro-metastatic potential as live platelets and increase EC migration and tube formation as well as promote tumor cell MMP production and invasion through matrigel [110–112]. PMPs may also transfer membrane receptors and adhesion molecules to the surface of tumor cells, conferring a more invasive phenotype. In vivo, Lewis lung carcinoma cells were

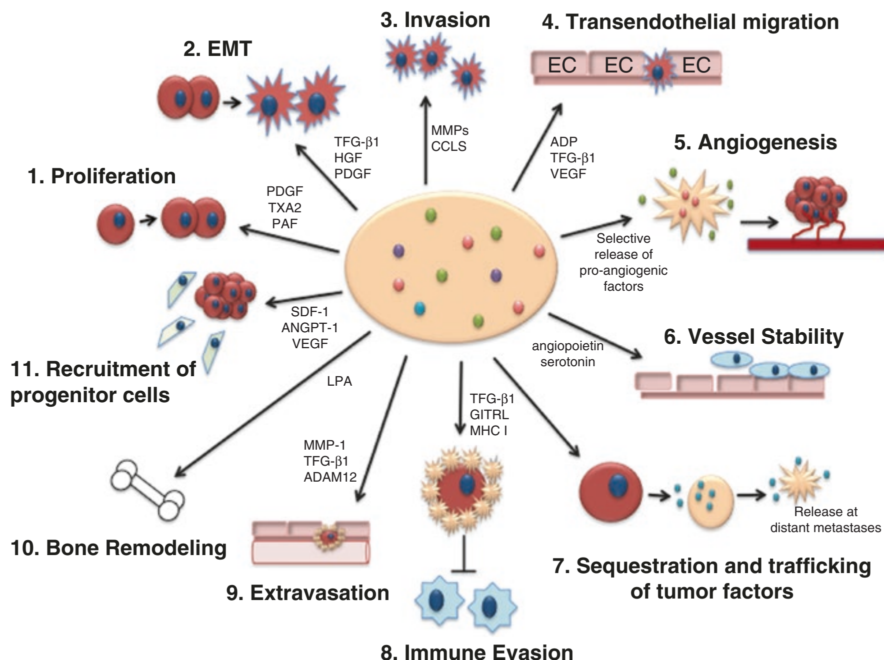

Fig. 12.2 Mechanisms by which platelets promote cancer. To date, platelets have been found to employ a wide variety of strategies to promote tumor progression and metastasis. These include direct effects on tumor cells including driving proliferation, invasion, and EMT [1–3]. Platelet-tumor cell cross talk also affects the vasculature in ways that promote angiogenesis, impact vessel stability, and facilitate intravasation and extravasation of tumor cells [4–6, 9]. Tumor cells instruct platelets to aid in immune evasion, remodeling of bone marrow, and recruitment of tumor-promoting progenitor cells [8, 10–11]. Additionally, platelets carry and deliver signals between distant sites including primary tumors, secondary tumors, and the bone marrow [7]. In this schematic, these mechanisms and any factors thought to be involved are highlighted

more metastatic when coated with PMP prior to injection, and, in an ischemia model, introduction of PMPs increased angiogenesis [113, 114].

Overall, PMPs recapitulate many of the same metastatic and angiogenic effects that are observed with whole platelets. They may potentially provide a mechanism for tumor mimicry, with tumor cells incorporating platelet markers to their cell surface after fusion with PMPs. They may also serve as a way for activated, spent platelets to continue to play a role in the TME and should be considered when conceptualizing the complex cross talk that occurs in cancer.

The Role of Platelets in Hematological Malignancies

So far most of our knowledge about the function of platelets in cancer comes from studies of solid tumors, particularly carcinomas. Relatively little is known about the role of platelets in hematological malignancies, and little can be extrapolated from

work in solid tumors due to vast differences in the tumorigenesis processes and the TMEs. However, there are some studies that can offer insights.

Unlike cell lines derived from solid tumors, many leukemia cells do not activate platelets and have been shown to inhibit activation and aggregation of platelets [115–117]. Patients with leukemia often present with thrombocytopenia (low platelet count), and their platelets display lower numbers of dense granules [118, 119]. Conversely, a few AML and CML cell lines are able to activate and aggregate platelets, and the resulting releasate increases tumor cell proliferation and survival [120–122].

The role of platelets in multiple myeloma seems even more complicated. These patients tend to have thrombocytopenia but also have elevated soluble P-selectin and TPO levels and are at increased risk of developing VTEs [123–125]. Platelets contain and release factors known to support multiple myeloma progression such as IL-6, SDF-1, and IGF-1, suggesting that platelets are poised to play a role in multiple myeloma. However, studies have not yet been done to determine if these platelet-derived factors do, in fact, mediate the progression of this disease.

Overall, the role of platelets in hematological malignancies is not well explored. Because thrombocytopenia is a feature of many hematological malignancies, it stands to reason that the role of platelets may not be as important in that context as has been observed in solid tumors. However, since platelets are shown to be altered in hematological cancers, it would be wise to examine the interactions of these tumor cells with megakaryocytes, particularly in cancers such as multiple myeloma where the bone marrow serves as a the TME. It may be that platelets serve different functions in the different types of hematological cancers and detailed investigations into each type could be beneficial.

Platelets Are Altered in Cancer Patients

Interestingly, platelets isolated from cancer patients are fundamentally different from those of healthy individuals. Platelets from breast, prostate, lung, and colon cancer patients display higher baseline activation, suggesting that they may be more reactive and have a lower threshold for activation than platelets from healthy donors [126–129]. Elevated surface levels of the activation marker P-selectin as well as increased platelet markers in the plasma such as CD40 ligand, β-thromboglobulin, and soluble P-selectin have been observed in cancer patients, and these markers tend to correlate with disease progression and poor prognosis [130, 131]. Patients presenting with elevated soluble P-selectin are more than twice as likely to develop a VTE compared to cancer patients with low levels [131].

Platelet contents are also altered in patients; total numbers of alpha-granules are higher, and pro-tumorigenic factors such as VEGF are enriched in platelets from cancer patients compared to those from healthy donors [89, 132]. It was reported that platelets from cancer patients also contain altered mRNA transcripts [133]. These transcripts appear to come from two sources: platelets take them up from tumor cells, or tumor cells induce the production of alternative splice variants within platelets.

Evidence suggests that mRNAs may be produced and packaged at higher levels at the megakaryocyte level in addition to being taken up from the tumor environment [134].

Thrombocytosis is associated with nearly every type of solid tumor suggesting that the presence of a tumor likely influences platelet production. Thrombopoietin (TPO) is the dominant driver of megakaryocyte differentiation and maturation. Preclinical studies of ovarian cancer reveal that tumor-derived IL-6 drives TPO production in the liver, leading to a boost in platelet production by megakaryocytes in the bone marrow [135]. IL-6 levels in patients correlate with platelet count, and anti-IL-6 therapy reverses this trend [136, 137]. However, more studies are needed across all tumor types to determine if this mechanism is broadly responsible for tumor-associated thrombocytosis. Another hypothesis posits that tumor cells themselves provide a source of TPO, but this has only been observed in vitro [138]. Other mechanisms that have been proposed are based on reactive thrombocytosis observed in conditions of systemic inflammation. Pro-inflammatory cytokines such as G-CSF, GM-CSF, and IL-1α are often elevated during inflammation as well as cancer progression and have also been implicated in megakaryocyte maturation and platelet production [139–142].

Although an abundance of evidence proves that platelet function, contents, and numbers are altered in cancer patients, we have only begun to understand how this occurs. Studies are needed to elucidate the effect of tumors on megakaryocyte biology and the platelets that result. Understanding the mechanism or mechanisms underlying cancer-associated thrombocytosis remains crucial, as therapies directed at this process could prevent tumors from producing an ever-increasing army of platelets that can add fuel to the fire of tumor progression.

Antiplatelet Therapy and Cancer

Based on their multifaceted role in cancer, platelets are a very attractive therapeutic target. Disrupting the communication between platelets and tumor cells by targeting platelets could theoretically block mechanisms of invasion, EMT, angiogenesis, metastasis, and activation of other host cells in the microenvironment and bone marrow. Platelet mimicry, along with many shared surface markers between platelets and tumor cells, suggests that platelet-targeted drugs could also impact the tumor as well.

Preclinical data is quite promising and reveals that targeting a number of different platelet receptors may be an effective approach for limiting cancer progression. Antiplatelet drugs that are currently available for the treatment of cardiovascular disease are now being explored as antitumor agents. For example, GPIIbIIIa blockers have been shown to inhibit lung metastasis in a murine model but have not yet been studied in patients with cancer [143]. Clopidogrel, a P2Y12 antagonist used to treat cardiovascular disease, shows antitumor properties in vitro and can prevent bone destruction and metastasis in mice [144]. Anticoagulants including fondaparinux and low molecular weight heparins (LMWH) inhibit tumor cell-induced platelet activation and attenuate the angiogenic potential of platelets in vitro [145]. These drugs make attractive candidates since they are often already given to cancer patients

due to their tendency to develop clots. However, clinical data from the use of LMWH in cancer patients shows mixed results [146–149]. Large-scale clinical trials are needed to access the efficacy of currently available antiplatelet drugs.

Aspirin is perhaps the most intriguing antiplatelet agent that has been studied to date. A massive, long-term epidemiological study by Rothwell et al. revealed that individuals who take aspirin daily are less likely to be diagnosed with cancer and show improved survival if they do develop cancer [150]. The mechanism of action for aspirin's efficacy in cancer was originally thought to be due to decreased inflammation via COX inhibition. However, the doses taken were not high enough to prevent inflammation but do cause platelet inhibition. Subsequent studies also point to a platelet-based mechanism; platelet inhibition with aspirin diminishes platelet activation, protein release, and ability to induce angiogenesis [77]. Mouse models also confirm that platelet inhibition with aspirin decreases metastasis and improves outcomes [25, 32, 151]. Aspirin seems most effective in chemoprevention but may also be beneficial if taken as part of treatment [152–154]. The exact mechanism by which aspirin inhibits platelet function is not well understood, and answering this question could lead to the development of drugs that are more specific and efficacious than aspirin but work on the same principle (Fig. 12.3).

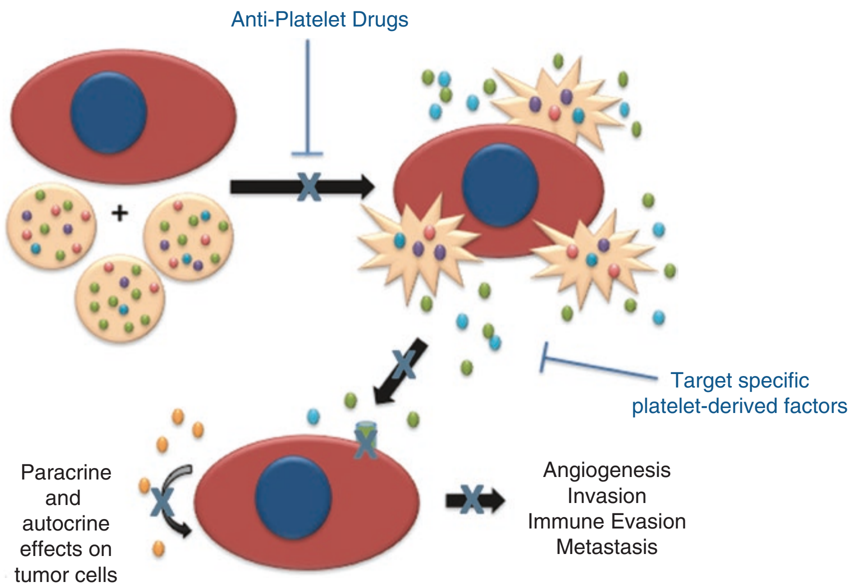

Fig. 12.3 Antiplatelet agents as novel cancer therapeutics. Because platelets play an active role in the progression and spread of many solid tumor types, they present an attractive target for therapy. Platelet inhibitors including aspirin, anticoagulants, and antiplatelet agents such as GPIIbIIIa blockers and P2Y12 antagonists may limit cancer progression by preventing tumor cell-induced platelet activation. Targeting specific platelet-derived factors and/or cognate receptors also present a currently underexplored approach to therapy that could prevent the tumor-promoting effects of platelets while sparing their important hemostatic and thrombotic functions

Conclusions

Platelets are now known to be key players in cancer progression and metastasis. These little cells supply the tumor with growth factors and mediators of invasion, provide potent pro-angiogenic regulators, and help maintain tumor vessel integrity, protect circulating tumor cells from shear stress and immune attack, and help set up new metastatic niches. Platelets also serve as long-haul cargo carries, delivering messages to and from the tumor in ways that allow the cancer to progress. These systemic changes also lead to alterations in platelet function, content, and number. Overall, research into the role of platelets in cancer has rewarded us with an abundance of novel factors, receptors, and signaling pathways that could serve as powerful new biomarkers or as potential therapeutic targets in the fight against cancer.

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Chapter 13
Regulation of Tumor Progression and Metastasis by Bone Marrow-Derived Microenvironments

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Abstract Activating mutations in driver oncogenes and loss-of-function mutations in tumor suppressor genes contribute to tumor progression and metastasis. Accordingly, therapies targeting key tumor cell-intrinsic signaling pathways are being used in clinical trials, and some have met FDA approval. However, these treatments benefit only a small proportion of patients harboring key driver mutations, and acquired resistance to these therapies presents a major impediment to effective treatment. More recently, the contribution of the tumor microenvironment (TME) has been an area of active investigation and has begun to provide critical insights into carcinogenesis. The host stromal cells in the TME coevolve with tumors and contribute to carcinogenesis in several ways. Among the host cells, bone marrow (BM)-derived cells constitute a significant fraction and directly contribute to proliferation, invasion, intravasation, extravasation, and outgrowth at the metastatic site. While the tumor-reprogrammed BM cells constitute attractive targets for anticancer therapy, recent studies have also begun to unravel their role as prognostic and predictive molecular markers of the disease.

Keywords Metastasis • Tumor microenvironment • Bone marrow • Anti-cancer therapy • Pre-metastatic niche • Tumor progression • Bone marrow-derived cells

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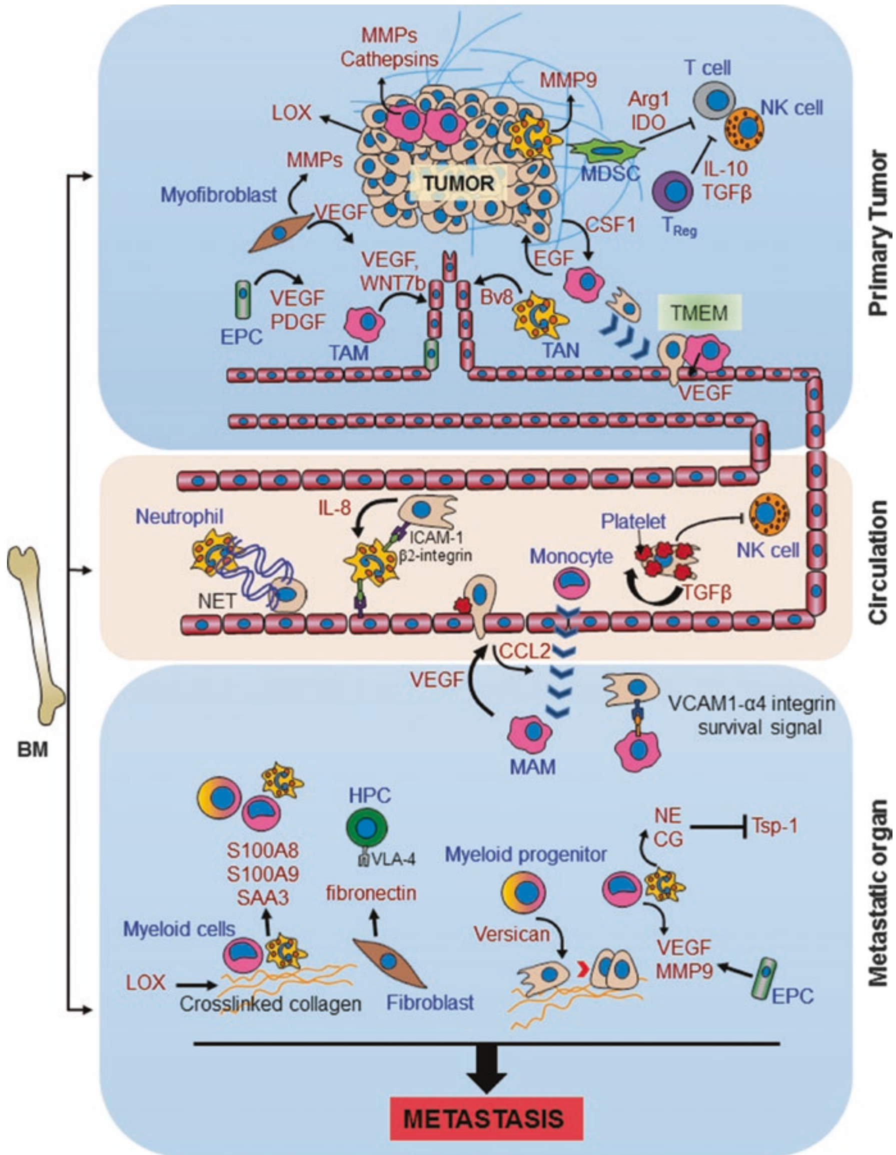
Fig. 13.1 The bone marrow (BM) contributes to primary tumor growth and the metastatic cascade. *In the primary tumor*, TAMs secrete VEGF and WNT7b, to promote angiogenesis, and MMPs and cathepsins, to mediate ECM degradation and tumor cell invasion. TANs secrete Bv8 and MMP9, promoting angiogenesis and ECM degradation. Myofibroblasts can also secrete MMPs and VEGF, contributing to angiogenesis. EPCs secrete angiogenic factors like VEGF and PDGF, generating a paracrine angiogenic signal, in addition to incorporating into nascent vessels. Furthermore, MDSCs suppress the activity of T cells and NK cells by secreting Arg1 and IDO, while T_{Reg} accomplish immunosuppression by secreting IL-10 and TGFβ. A paracrine loop exists, whereby tumor cells secrete CSF1 to recruit macrophages, which in turn secrete EGF to promote tumor cell migration. Tumor cells, TAMs, and ECs establish the TMEM, where perivascular TAMs secrete VEGF, increasing local permeability and allowing tumor cell intravasation. *In the circulation*, tumor cells activate platelet aggregation. Platelets protect tumor cells from shear stress and NK cell attack. Furthermore, platelets promote epithelial-to-mesenchymal transition (EMT) by secreting TGFβ. Platelets promote tumor cell adhesion to blood vessels at the secondary site via P-selectin. Moreover, tumors in circulation recruit neutrophils via IL-8. Neutrophils bridge tumor cells to blood vessels via neutrophil-expressed β2 integrin and tumor cell- and EC-expressed ICAM-1. Neutrophils also trap tumor cells in NETs. *In the metastatic organ*, tumor cell-derived CCL2 recruits inflammatory monocytes, which differentiate into MAMs and in turn secrete VEGF to allow tumor cell extravasation. Macrophages also promote tumor cell survival via α4 integrin signaling to VCAM-1 on tumors cells. Tumor-derived factors generate a premetastatic niche, characterized by the recruitment and activation of myeloid cells in response to S100A8 and S100A9 chemokines and SAA3 inflammatory mediator. Tumor-derived LOX crosslinks collagen in the premetastatic niche, trapping recruited myeloid cells. Furthermore, tumor-derived factors induce the secretion of fibronectin in fibroblasts, promoting the recruitment of HPCs via VLA-4. Recruited myeloid progenitor cells induce mesenchymal-to epithelial transition (MET) via their secretion of versican, promoting metastatic outgrowth. Moreover, macrophages and neutrophils secrete angiogenic factors, and neutrophil serine proteases NE and CG degrade the antiangiogenic factor Tsp-1, enhancing metastatic outgrowth. Finally, recruited EPCs incorporate into the nascent tumor vasculature, and secrete angiogenic factors, inducing the angiogenic switch and contributing to macrometastasis formation. *Arg1* arginase 1, *BM* bone marrow, *Bv8 Bombina variegata* peptide 8, *CCL2* chemokine (C-C motif) ligand 2, *CG* cathepsin G, *CSF1* colony-stimulating factor 1, *ECM* extracellular matrix, *EGF* epidermal growth factor, *EPC* endothelial progenitor cell, *HPC* hematopoietic progenitor cell, *ICAM-1* intercellular adhesion molecule-1, *IDO* indoleamine 2,3- dioxygenase, *IL-8* interleukin-8, *IL-10* interleukin-10, *LOX* lysyl oxidase, *MAM* metastasis-associated macrophage, *MDSC* myeloid-derived suppressor cell, *MMP* matrix metalloprotease, *NE* neutrophil elastase, *NET* neutrophil extracellular trap, *NK* natural killer, *PDGF* platelet-derived growth factor, *S100A8* S100 calcium binding protein A8, *S100A9* S100 calcium binding protein A9, *SAA3* serum amyloid A3, *TAM* tumor-associated macrophage, *TAN* tumor-associated neutrophil, *TGFβ* transforming growth factor beta, *TMEM* tumor microenvironment of metastasis, T_{Reg} regulatory T cell, *Tsp-1* thrombospondin-1, *VCAM-1* vascular cell adhesion molecule-1, *VEGF* vascular endothelial growth factor, *VLA-4* very late antigen-4, *WNT7b* Wingless-Type MMTV Integration Site Family Member 7b



In this chapter, we will focus on recent advances and emerging concepts of the contribution of BM-derived cells in various steps of primary tumor progression and the metastatic cascade (Fig. 13.1) and discuss future directions in the context of novel diagnostic and therapeutic opportunities. General descriptions of the contribution of the BM-derived TME to tumor growth and metastasis are covered in several excellent reviews [1–4].

Primary Tumor Growth

The BM contributes significantly to the TME and supports tumor progression and metastasis by regulating angiogenesis, inflammation, and immune suppression. Most solid tumors harbor an immune infiltrate consisting of myeloid and lymphoid cells, whose phenotype and activation status has been shown to change with the stage of malignancy [4].

Hematopoietic stem cells (HSCs) are maintained in the BM compartment and anchored to the endosteal surface by calcium-sensing receptors present on their surface [5]. Major adhesion mechanisms that mediate HSC anchorage in the BM niche include receptor tyrosine kinase TIE2-angiopoietin-1 (ANG1) interactions [6] and chemokine (C-X-C motif) receptor 4 (CXCR4)–stromal-derived factor-1 (SDF-1) interactions [7]. Furthermore, HSCs also adhere to osteopontin in the bone via β1 integrin [8].

Secreted tumor-specific factors systemically stimulate the quiescent BM compartment, resulting in the expansion, mobilization, and recruitment of BM progenitor cells. For instance, matrix metalloprotease 9 (MMP9) secreted by primary tumors systemically degrades osteopontin [9, 10] and mediates cleavage of SDF-1 [11], thereby releasing BM cells from the bone niche [12]. Similarly, tumor-secreted granulocyte colony-stimulating factor (G-CSF) mobilizes HSCs from the niche by promoting neutrophil elastase-mediated degradation of SDF-1 [13].

The mobilized BM-derived cells are recruited into tumor beds in response to chemoattractants. For instance, SDF-1 secreted by primary tumors recruits $CXCR4^+$ BM-derived cells to the TME [12]. Other tumor-secreted factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) bind to $VEGFR1^+$ BM cells, while monocyte colony-stimulating factor (M-CSF) recruits monocytes and macrophages [12]. Furthermore, chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemotactic protein (MCP-1), was identified as a tumor-derived chemokine that recruits circulating monocytes into the TME, where they undergo differentiation into tumor-associated macrophages (TAMs) [14]. Stromal-derived CCL2/MCP-1 and colony-stimulating factor (CSF1) are also involved in the recruitment of TAMs [15–17]. Additionally, hypoxia-inducible factor 1 alpha (HIF1α) in hypoxic tumors promotes the recruitment of BM-derived myeloid and endothelial progenitor cells (EPCs), which increase the bioavailability of VEGF via their secretion of MMP9, enhancing tumor angiogenesis [18]. Infiltrating BM cells also provide paracrine mitogenic signals to induce proliferation of tumor cells via their secretion of growth factors such as epidermal growth factor (EGF) and cytokines including interleukin-6 (IL-6) and tumor necrosis factor (TNFα) [12].

Macrophages are the most abundant myeloid cells present among the recruited BM-derived cells [19]. Notably, increased macrophage infiltration has been correlated with poor prognosis, as shown in Hodgkin's lymphoma, breast cancer, and lung cancer [20–22]. Classically activated macrophages, defined by an M1 phenotype, generate host responses against the growing tumors, whereas "alternatively" activated M2 macrophages, representing "educated" TAMs, are major perpetrators

of tumor progression and metastasis. M1 macrophages are characterized by an elevated expression of inducible nitric oxide synthase (iNOS), producing nitric oxide (NO) for effective pathogen killing [23], and the proinflammatory cytokine IL-12 [24]. Tumor-derived mediators, such as IL-4, IL-10, IL-13, transforming growth factor beta (TGFβ), and prostaglandin E2 (PGE2), mediate polarization of TAMs toward the tumor-promoting, M2 phenotype [25]. M2 TAMs are characterized by elevated expression of arginase (Arg1) and decreased expression of iNOS–$ArgI^{high}$ $iNOS^{low}$ [26]. M2 TAMs promote immune suppression by expressing high levels of IL-10 and downregulating IL-12 [27].

M2 TAMs also induce extracellular matrix (ECM) degradation and angiogenesis by producing MMPs and VEGF, respectively [25]. Furthermore, IL-4 secreted by pancreatic tumor cells induces the expression and secretion of members of the cysteine protease family cathepsins [28, 29] in TAMs. Specifically, cathepsins B and S have been shown to promote tumor growth, invasion, and angiogenesis [30]. By stimulating angiogenesis and tumor invasiveness, TAMs ultimately promote metastasis. In the MMTV-PyMT model of breast cancer, genetic ablation of macrophages via CSF1 deletion impairs angiogenesis and suppresses metastasis to the lung, mainly due to the VEGFA-mediated angiogenic action of TAMs [31–33]. Furthermore, deleting an effector of CSF1 signaling, Ets2, in macrophages, induced the expression of antiangiogenic factors thrombospondin 1 (Tsp-1) and Tsp-2 by macrophages, resulting in decreased angiogenesis in PyMT primary tumors and suppression of lung metastasis [34]. Moreover, macrophages promote angiogenesis and metastasis in PyMT primary tumors via their expression of WNT7b [35]. TAMs also associate with newly formed blood vessels induced by EC-derived angiopoietin 2 (ANG2), enhancing angiogenesis and tumor cell dissemination [36].

M2 TAMs promote an immunosuppressive TME by producing IL-10, which promotes Th2 cell polarization [14]. In turn, Th2 cells produce IL-4, which in a feedback loop activates M2 TAM polarization [37]. M2 TAMs also secrete CCL22, which recruits regulatory T cells (T_{Reg}) [37]. TAMs also produce TGFβ and process latent TGFβ, releasing its active form [38], hence reducing T-cell cytolytic and antitumor activity [39]. TAMs express programmed death ligand 1 (PD-L1) on their cell surface, which binds the immune inhibitory receptor, programmed death 1 (PD-1), on the surface of T cells, resulting in T-cell inactivation and apoptosis [40]. TAMs inhibit T-cell growth by depleting arginine, on which T-cell growth and survival are dependent, in the local microenvironment through expression of Arg1 [41].

TAMs contribute to chemoresistance in the MMTV-PyMT breast cancer model [21], and inhibiting macrophage recruitment using a CSF1R signaling antagonist enhanced the antitumor effect of cytotoxic chemotherapeutics via a $CD8^+$ T-cell-dependent mechanism [21]. Interestingly, TAMs that underwent CSF1R signaling blockade failed to elicit $CD8^+$ T-cell responses and exhibited reduced immunosuppressive activity [42].

Myeloid-derived suppressor cells (MDSCs) have been observed in cancer patients and contribute to tumor progression. MDSCs are a heterogeneous population of immature myeloid cells [43, 44], which are activated in response to S100 calcium-binding protein A8 (S100A8) and S100A9 proinflammatory mediators

[45]. MDSCs contribute to tumor immune evasion by suppressing the CD4$^+$ and CD8$^+$ immune response partly via arginase production, by expanding T_{Reg} cells, and by inhibiting the cytolytic activity of natural killer (NK) cells [3, 46]. MDSCs also express the interferon gamma (IFNγ)-inducible enzyme indoleamine 2,3-dioxygenase (IDO), a well-known suppressor of T-cell activation [47]. IDO catalyzes tryptophan catabolism, depleting tryptophan from the local microenvironment and blocking T-cell activation [48]. IDO expression by MDSCs was found to be STAT3-dependent in breast cancer [49]. Interestingly, in a model of melanoma, tumor-derived IDO was described to recruit and expand MDSCs via a T_{Reg}-dependent mechanism, leading to an immunosuppressive microenvironment [50].

Tumor-associated neutrophils (TANs) exert a pro-tumorigenic effect at the primary site, promoting angiogenesis and suppressing immune responses [51–53]. In mice, CD11b$^+$ Gr1$^+$ neutrophils, recruited by primary tumor-derived G-CSF, contribute to refractoriness to anti-VEGF therapy and promote angiogenesis via the expression of *Bombina variegata* peptide 8 (Bv8) [54, 55]. CD11b$^+$ Gr1$^+$ immature myeloid cells recruited to colon and lung tumors promote angiogenesis and vessel maturation via their MMP9 production, increasing VEGF bioavailability, as well as by incorporating into tumor blood vessels [56]. Interestingly, the pro-tumorigenic effects of neutrophils are TGFβ-dependent, whereupon TGFβ blockade, neutrophils switch from the "N2" pro-tumorigenic phenotype to the "N1" anti-tumorigenic phenotype [57].

Dendritic cells (DCs) are a class of antigen-presenting cells that uptake, process, and present antigens, including tumor-derived antigens, to antigen-specific T cells, resulting in T-cell activation and expansion. In melanoma, tumor-associated DCs do not present tumor antigens and fail to activate T cells [58]. Interestingly, tumor-associated DCs in ovarian cancer were found to be immunosuppressive, promoting tumor progression. In this context, lipid peroxidation by-products induce endoplasmic reticulum (ER) stress, activating an ER stress response factor, Xbp1, which reduces the ability of tumor DCs to present antigens and activate T cells [59].

In addition to the perivascular contribution of BM-derived hematopoietic cells, BM-derived VEGFR2$^+$ EPCs, recruited to early avascular tumors in response to tumor-derived VEGF, provide an alternative source of endothelial cells, which contributes to the neovascularization of certain tumors in mice and humans [60–63]. The contribution of EPCs to tumor vessel formation has been reported to be variable [64]. However, EPC ablation was associated with angiogenesis inhibition both in primary tumors and metastatic outgrowth [62, 65, 66]. EPCs also contributed to vascular rebound following administration of vascular disrupting agents [67], and chemotherapeutics rapidly induced circulating endothelial progenitor (CEP) mobilization and subsequent tumor homing [68]. Despite these studies, confusion has prevailed due to the extensive variability in EPC contribution to vessel formation in different tumor model systems [64], and some studies have even claimed lack of EPC contribution [69]. However, in addition to vessel incorporation, EPCs have been shown to secrete proangiogenic factors, including VEGF and platelet-derived growth factor (PDGF) [65, 70], suggesting that along with providing stability to nascent vessels, EPCs contribute to vessel recruitment through paracrine mecha-

nisms at a critical early stage of tumor growth. These observations are consistent with other studies demonstrating that paracrine signaling by specific populations of perivascular cells may have significant biological effects. For example, depletion of myeloid cell-derived VEGF caused vessel normalization even when abundant sources of VEGF were present in the TME [71]. Similarly, endothelial cell-autonomous VEGF and not the abundant extracellular VEGF was shown to be critically required for the homeostasis of blood vessels [72].

Furthermore, the BM contributes to myofibroblast population in the tumor stroma, as shown in a mouse model of pancreatic insulinoma that was transplanted with donor GFP+ BM [73, 74]. BM-derived myofibroblasts support angiogenesis in the primary tumor by secreting a host of proangiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), TGFβ, PDGF, and hepatocyte growth factor (HGF), and they remodel the ECM via several MMPs and ADAMs [75]. The BM is also a source of pericytes, cells that support vessel maturation, where BM-derived pericyte progenitor cells are mobilized to remodel the vasculature in tumors [76–79].

Primary Tumor Invasion and Intravasation

In order to metastasize, BM-derived cells in the TME promote tumor cell invasion into the basement membrane followed by transendothelial migration and intravasation into the circulation. BM-derived CSF1R+ TAMs secrete EGF which stimulates cancer cells to form elongated protrusions that enable invasion into the adjacent matrices and stimulate production of CSF-1, which, in turn, stimulates TAMs via CSF1R to increase production of EGF. Pharmacological or genetic abrogation of either EGF or CSF1 significantly impedes the migratory behavior of both cell types, which further confirms this positive feedback loop [80, 81]. Intratumoral CD4^{+} T cells in PyMT tumors also induce the expression of EGF in macrophages, via IL-4 [37]. Furthermore, cancer-derived CSF1 signaling through TAM CSF1R activates Wiskott-Aldrich syndrome protein (WASP), promoting macrophage migration and EGF expression [82]. Interestingly, although breast tumor cells that express ErbB3 or the CXCR4 receptor invade in response to the ligands heregulin beta1 (HRGβ1) and SDF-1, respectively, their invasion is still dependent on the EGF-CSF1 paracrine loop, such that blocking this signaling loop results in suppression of invasion in response to other ligands [83].

Moreover, Mena, the mammalian ortholog of Drosophila Enabled (Ena), contributes to cell motility by regulating actin dynamics [84]. Breast cancer cells expressing Mena^{INV}, an invasion-specific isoform, exhibit multicellular streaming and increased intravasation dependent on the EGF-CSF1 paracrine signaling loop between tumor cells and macrophages [85]. In this context, transendothelial migration of breast tumor cells occurs in microanatomical structures known as "tumor microenvironment of metastasis" (TMEM) [86]. TMEMs are composed of one TIE2^{high}/VEGF^{high} perivascular macrophage in physical contact with a Mena^{INV}-

expressing cancer cell and an underlying endothelial cell [85–87]. Macrophages induce RhoA GTPase activity in tumor cells, triggering actin-rich invadopodia that allow tumor cell transendothelial migration [88]. Furthermore, transient vascular permeability was observed at the TMEM, where TIE2^{hi} TMEM macrophages secrete VEGFA, causing local loss of vascular junctions, transient permeability, and tumor cell intravasation [86].

In pancreatic cancer, tumor-derived IL-4 induces the expression of the cysteine proteases cathepsin B and cathepsin S in macrophages, which enhance tumor cell invasion and intravasation by altering the extracellular matrix (ECM) constituent proteins such as E-cadherin collagen, laminin, and fibronectin [30]. Moreover, an in vitro study suggested that pancreatic cancer cells activate toll-like receptor 4 (TLR4) signaling in macrophages, inducing IL-10 expression and M2 polarization. Significantly, TAM TLR4/IL-10 signaling promotes epithelial-to-mesenchymal transition (EMT) in tumor cells, characterized by downregulation of E-cadherin, and upregulation of vimentin and snail, as well as induction of MMP2 and MM9 proteolytic activity, suggesting a mechanism for TAM-driven tumor cell migration [89].

Neutrophils recruited by malignant fibrosarcomas and prostate cancer cells enhance angiogenesis and intravasation in primary tumors by secreting MMP9 [90]. In intrahepatic cholangiocarcinoma, neutrophils recruited by tumor-derived CXCL5, a ligand of CXCR2, enhance metastasis [91]. CXCR2 knockout hosts exhibited smaller tumors and reduced metastasis of breast cancer cells, as well as a decrease in tumor cell proliferation and angiogenesis, coinciding with significantly suppressed recruitment of CD11b^{+} Gr1^{+} myeloid cells and F4/80^{+} macrophages [92]. These observations suggest a role of the infiltrating pro-inflammatory immune cells in tumor progression and metastasis. Additionally, myeloid cells recruited to mammary tumors harboring a Tgfbr2 deletion secrete MMPs and TGFβ1 that mediate tumor cell invasion and metastasis [93].

In a mouse model of colorectal cancer, collective invasion of cancer cells occurs via a paracrine loop between CD34+ immature myeloid cells (iMCs) and tumor cells. Tumor cells secrete CCL9, which recruits CCR1^{+} iMCs to the invasive front, where they express MMP2 and MMP9 and promote tumor collective migration [94]. As another mechanism, BM-derived mesenchymal stem cells (MSCs) which secrete CCL5 to enhance the migration and dissemination of CCR5+ breast cancer cells [95].

Tumor Cell Survival in Circulation and Extravasation into Metastatic Organs

As part of the metastatic process, cancer cells from the primary tumor intravasate into the peripheral circulation as circulating tumor cells (CTCs) [96]. Following intravasation, CTCs induce platelet coagulation by secreting thrombin, enabling platelets to shield tumor cells from shear stress encountered in circulation [97]. Platelets also protect tumor cells from the immune activity of NK cells [98, 99].

Platelet depletion or disruption of clot-forming components inhibited metastasis in mouse models [100]. Platelets have also been shown to be a major source of TGFβ1 in the circulation, and platelet-induced EMT enhanced metastasis in vivo [101].

Clot formation also recruits macrophages, which in turn protect circulating tumor cells. Tumor-initiated clot formation induces the expression of vascular cell adhesion molecule 1 (VCAM-1) and vascular adhesion protein 1 (VAP1) on endothelial cells, which recruit macrophages [102]. Macrophages expressing integrin α4 (CD49b) bind to VCAM-1-expressing tumor cells and propagate AKT-dependent survival signals to them [103].

The next challenge for CTCs is to exit the circulation and colonize the surrounding tissue of the metastatic organ. The first step of extravasation requires that a CTC properly adhere with the endothelial wall. Tumor-derived IL-8 recruits neutrophils and increases their expression of β2 integrin, promoting the interaction between tumor cell intercellular adhesion molecule-1 (ICAM-1) and neutrophil β2 integrin [104]. ICAM-1 is also expressed on endothelial cells [97], enabling neutrophils to anchor tumor cells to the endothelium, enhancing extravasation and metastatic foci formation in lungs [104] and liver [105]. Neutrophils also form structures called neutrophil extracellular traps (NETs), composed of extruded DNA and antimicrobial proteases. Neutrophils trap circulating tumor cells in NETs that form in liver and lung capillaries, in a model of postoperative infection, increasing metastasis [106].

Platelets contribute to tumor cell extravasation by promoting the adhesion of tumor cells to ECs at the distant site. In addition to EC P-selectin, platelet-expressed P-selectin promoted lung metastasis of breast cancer and melanoma [107]. CTCs themselves express the selectin ligands sialyl Lewis-a (sLe^a) and sialyl Lewis-x (sLe^x) [108]. These ligands allow tumor cells to adhere to endothelial cell E-selectin and confer increased metastatic potential [109, 110].

Moreover, inflammatory monocytes, recruited to the premetastatic lungs via the CCL2–CCR2 axis, increased tumor cell extravasation from the vasculature into the lung parenchyma by increasing VEGF-induced vessel permeability, resulting in transendothelial migration [111] during breast cancer metastasis to the lungs [112] and colorectal cancer metastasis to the liver [113]. Studies for ovarian cancer and melanoma have shown that specific recruitment of regulatory T cells protect disseminated cancer cells from immune attack [114, 115].

Tumor Cell Colonization and Initiation of Metastasis in Distant Organs

Metastatic tumors establish a BM-derived microenvironment in the distant site of metastasis, known as the premetastatic niche. This niche functions as a permissive hub for supporting colonization and outgrowth of disseminated tumor cells following extravasation. BM-derived cells at the metastatic site also influence tumor cell tropism and promote metastatic outgrowth.

Contribution of the Premetastatic Niche in Colonization and Initiation of Metastasis

In 1889, Stephen Paget proposed the "seed and soil" hypothesis, in which he suggested that cancer cells, being the "seed," had an affinity for and only colonized organs that were conducive to their growth, or had the proper "soil" [116]. Indeed studies have shown that conducive microenvironments are required for disseminated tumor cells to engraft at distant sites, in agreement with the "seed and soil" hypothesis. Strikingly, metastatic primary tumors systemically generate BM-derived "premetastatic niches" in distant organs that serve as hubs for supporting future metastases [117].

The first account of the premetastatic niche described the recruitment of VEGFR1$^+$ hematopoietic progenitor cells to the lungs as preceding and necessary for tumor metastasis. In this model, Lewis lung carcinoma and melanoma primary tumors systemically induced the expression of fibronectin in lung fibroblasts, leading to the recruitment of BM-derived VEGFR1$^+$ and CD11b$^+$ myeloid cells to the lungs via their fibronectin receptor, VLA-4 [118]. CD11b$^+$ myeloid cells are also recruited to premetastatic lungs by the chemoattractants S100A8 and S100A9, expressed in response to primary tumor-derived VEGFA, TGFβ, and tumor necrosis factor α (TNF-α) [119]. The recruited CD11b$^+$ cells in turn express TNF-α and TGFβ, which enhance tumor cell metastasis [119]. In addition, serum amyloid A3 (SAA3), induced by S100A8 and S100A9 in lungs, activates NFκB signaling via TLR4 on myeloid cells, potentiating the inflammatory response and accelerating lung metastasis [120]. Furthermore, primary tumor-derived CCL2 signaling through CCR2 on lung endothelial cells induces the secretion of S100A8 and SAA3, which increases vascular permeability, resulting in hyperpermeable foci that attract leukocytes and tumor cells [121].

In a similar intercellular feedback system, primary tumor-derived VEGF induces the expression of MMP9 in CD11b$^+$ myeloid cells and endothelial cells in the premetastatic niche [122]. MMP9 in the premetastatic niche releases VEGF from the ECM, promoting angiogenesis [123], and soluble KIT ligand, which further recruits KIT receptor-expressing BM cells [124]. Furthermore, hypoxia from primary tumors induces the accumulation of MDSCs in premetastatic lungs, suppressing the cytotoxic function of NK cells [125].

Lysyl oxidase (LOX), a hypoxia-inducible secreted amine oxidase, is also critical in the generation of premetastatic niches in solid tumor metastasis [126]. Secreted LOX from hypoxic primary breast cancer cells co-localizes with fibronectin in both pulmonary and hepatic premetastatic niches and cross-links collagens in the local microenvironment. This modification of the ECM promotes the recruitment of CD11b$^+$ BM cells, creating a niche permissive for the colonization of metastasizing tumor cells at these secondary sites [126]. The inhibition of LOX at primary tumors abrogates the establishment of premetastatic niches and decreases metastatic burden in secondary organs. Similarly, the targeting of CD11b$^+$ cells restricts the establishment of tumor-supportive premetastatic niches, reducing metastatic burden [126]. In addition, LOX expression and activity during the onset and

development of both chemical- and radiation-induced lung and liver fibrosis has been shown to be responsible for fibrosis-enhanced metastasis to these organs. The action of LOX generates tumor cell supportive niches high in fibrillar collagen, which increase seeding, tumor cell persistence, and survival [127]. While the involvement of BM-derived cells was not directly investigated, the changes occurring during fibrosis dramatically recapitulate those observed in premetastatic niche remodeling, suggesting common overlapping mechanisms [128]. More recently, LOX has also been shown to induce the formation of premetastatic osteolytic lesions in the bone. In this case, elevated levels of LOX secreted by hypoxic primary tumors alter the homeostatic balance between osteoclasts and osteoblasts. LOX modulates the BM stroma to drive de novo osteoclastogenesis while decreasing osteoblast proliferation, both in vitro and in vivo. The net result is unbalanced coupling, osteolysis, and premetastatic niche generation within the bone. These LOX-driven premetastatic niches, in turn, support circulating tumor cell colonization and the development of overt bone metastases [129].

In a bladder cancer model, the tumor cell-derived proteoglycan, versican, enhances metastasis to the lungs via a mechanism involving increased lung CCL2 chemokine expression and increased macrophage infiltration [130]. Consistent with these data, the recruitment of CCR2-expressing monocytes and macrophages to the lungs in response to tumor cell-derived and host-derived CCL2 enhances breast tumor metastasis to lungs [112].

Immature myeloid cells expressing the stem and progenitor cell marker CD117 are involved in premetastatic niche formation [118, 126]. Similarly, mature myeloid cells, such as $CD11b^+$ $Ly6C^+$ monocytes, are recruited to premetastatic lungs by CCL2, and $CD11b^+$ $CD68^+$ $F4/80^+$ macrophages are recruited by fibrin clots in the premetastatic lungs, where they enhance metastasis of B16 melanoma and breast cancer cells, respectively [131, 132]. Macrophages in the premetastatic niche are derived from circulating BM-derived monocytes, which are recruited via CCL2 [112], and this implies that primary tumor-secreted factors can systemically recruit myeloid progenitors at different stages of differentiation to premetastatic sites, where they differentiate into metastasis-promoting macrophages. Interestingly, primary prostate and breast tumors with a low metastatic potential systemically induce the expression of the antiangiogenic factor Tsp-1 in myeloid cells recruited to the premetastatic lungs [133], indicating that even nonmetastatic tumors can modify the microenvironment in distant organs.

Primary tumor-secreted G-CSF recruits $Ly6G^+$ neutrophils to premetastatic lungs, where they contribute to the formation of lung metastasis via their expression of Bv8, which promotes tumor cell migration [134]. Interestingly, the CCL2–CCR2 axis was also shown to recruit $CCR2^+$ neutrophils to the premetastatic lung [135]. However, instead of promoting metastasis, these neutrophils inhibited the survival of disseminated cells through CCL2-dependent activation of H_2O_2-mediated killing [135]. These results suggest that CCL2 both promotes and blocks metastasis initiation; hence insights into these processes will be critical for developing anti-metastatic therapies. Notably, the maintenance of the premetastatic niche was reported to depend on the activation of sphingosine-1-phosphate receptor

1 (S1PR1) and its effector STAT3 in myeloid cells, in response to B16 melanoma-derived factors, promoting myeloid cell proliferation and survival at the distant site [136].

Non-myeloid cells also compose the premetastatic niche. For instance, $CD4^+$ T cells in premetastatic bones increase osteoclastogenesis by secreting receptor activator of nuclear factor-kB ligand (RANKL), thus enhancing the metastasis of breast cancer cells to the bone [137]. Moreover, 4 T1 breast cancer cells systemically induce the expression of CCL22 in lung stroma, thereby inducing the recruitment of T_{Reg} cells to premetastatic lungs [138].

In addition to soluble tumor-derived factors generating the premetastatic niche, exosomes released from primary tumors also induce the mobilization of BM-derived cells which are then recruited to the secondary site to generate the premetastatic niche [139]. Metastatic B16 melanoma cells release exosomes that carry MET oncoprotein, transferring MET to BM progenitors, leading to their recruitment to premetastatic lungs and enhancing metastasis [140].

Organ Tropism

Correlations have been found between primary tumors and their preferred metastatic destination, and more recent studies have begun to identify mechanism of metastasis organotropism. This tropism, or preferential metastasis, of tumors to specific organs has been shown to be determined in part by cancer cell-intrinsic pathways, and as a consequence, gene signatures that mediate organ-specific metastasis have been described [141–143]. However, tumor non-cell-autonomous mechanisms have also been shown to play a necessary role in organ tropism. For example, chemoattractants in metastatic organs are able to recognize cognate chemokine receptors expressed on cancer cells that promote homing. Breast cancer cells expressing CXCR4 and CCR7 migrate toward SDF-1 and CCL21 chemokine gradients, respectively, in metastatic sites [144]. Signaling via CXCR4 and CCR7 mediates actin polymerization and pseudopodia formation, leading to chemotactic responses and invasion [144]. Moreover, blocking CXCR4/SDF-1 signaling suppresses metastasis of breast cancer cells to the lymph nodes and lungs [144]. CXCR4 expression is also required for human epidermal growth factor receptor 2 (HER2)-mediated breast cancer metastasis [145]. In addition to CXCR4 and CCR7, CCR10 expression on melanoma cells confers tropism to the skin [144].

Furthermore, chemokine (C-X3-C motif) receptor 1 (CX3CR1) expression on pancreatic cancer cells mediates their metastasis to chemokine (C-X3-C motif) ligand 1 (CX3CL1)-expressing peripheral neurons [146].

A major step after homing is adhesion of tumor cells at the distant site. During the early steps of pulmonary metastasis, disseminated breast cancer cells arrest in lungs via interactions between tumor α3β1 integrin and laminin 5 expressed on pulmonary vasculature basement membrane [147]. Furthermore, TNFα secreted by

primary tumors [119] and by myeloid cells in lungs [148] upregulates the expression of the adhesion molecules E-selectin, P-selectin, and VCAM-1, promoting tumor cell adhesion and migration [3].

Several types of tumors metastasize to the bones. Breast cancer causes osteolytic lesions in bones, stimulating the formation and activity of osteoclasts. Breast cancer cells express CSF1, which activates osteoclasts that break down bone. Breast cancer cells also express parathyroid hormone-related protein (PTHRP) and TNFα, which activate RANKL and inhibit osteoprotegerin synthesis, inducing formation and activity of osteoclasts [149, 150]. In melanoma, inhibition of RANKL reduced metastasis to the bone, but not to other organs [150].

Breast cancer cells that home to the bone express greater levels of CXCR4, osteopontin, MMP1, and IL-11 [141]. The bone stroma is rich in SDF-1, the ligand for CXCR4, which may mediate the tropism of breast cancer cells. Once in the bone, IL-11 activates osteoclasts, and MMP1 releases matrix-sequestered factors, enhancing tumor outgrowth and bone degradation [3]. When bone matrix is degraded, several sequestered factors are released, including insulin-like growth factor 1 (IGF1), TGFβ, and bone morphogenetic proteins (BMPs), which enhance metastatic survival and outgrowth and induce PTHRP synthesis, leading to further bone degradation [149]. This leads to a positive feedback loop of increased bone loss and enhanced metastatic tumor growth.

In the case of neuroblastoma that metastasizes to the bone, while some tumors secrete RANKL, others induce IL-6 expression in BM MSCs. IL-6 activates osteoclasts and is required for bone metastasis [151]. Furthermore, BM-derived IL-6 mediates survival and proliferation of IL-6R^{+} neuroblastoma cells [152].

Prostate cancer, on the other hand, generates osteoblastic lesions in the bone, characterized by disrupted bone deposition. Prostate metastatic lesions release endothelin 1, TGFβ2, FGF, and BMPs, all of which are osteoblastic and alter bone structure [3]. Moreover, prostate cancer cells also produce urokinase-type plasminogen activator (uPA) and prostate-specific antigen (PSA), which can release growth factors from the bone matrix, enhancing metastatic outgrowth [149].

More recently, tumor-derived exosomes were shown to be implicated in organ-specific metastasis. Exosomes carrying integrins α6β4 and α6β1 directed metastasis to the lungs, while exosomal integrin αvβ5 directed metastasis to the liver, in both cases by inducing the expression of S100 chemoattractants in the target organ [153].

Metastatic Outgrowth

After seeding in the secondary site, metastatic tumors establish vasculature in order to outgrow. This occurs via the production of angiogenic factors, such as VEGFA, and the recruitment of endothelial cells and pericytes. Metastasis-associated macrophages (MAMs) support tumor outgrowth at the metastatic site [111] via a TIE2-dependent mechanism that promotes angiogenesis [36]. In the MMTV-PyMT breast cancer model, BM-derived EPCs are recruited to metastatic lesions, where they

incorporate into nascent vessels and contribute to the angiogenic switch, promoting the progression of micrometastases to macrometastases [65]. EPCs express the transcription factor inhibitor of differentiation 1 (ID1), which is required for EPC mobilization and recruitment [65, 66]. Moreover, several proangiogenic genes are upregulated in recruited EPCs, suggesting an additional mechanism whereby EPCs promote angiogenesis and metastatic progression [65].

Furthermore, the contribution of neutrophils to metastatic progression was demonstrated in models of extrinsic lung inflammation, where the neutrophil-secreted serine proteases neutrophil elastase (NE) and cathepsin G (CG) degrade antiangiogenic Tsp-1, coinciding with increased lung metastasis [154]. Furthermore, neutrophils in the premetastatic lungs were shown to expand the metastasis-initiating cell population of breast cancer cells by expressing leukotrienes [155]. Neutrophils also secrete proangiogenic Bv8, which promotes metastatic progression [134].

As in earlier stages of tumor progression, myeloid cells also play a role in metastatic outgrowth. In the MMTV-PyMT breast cancer model, BM-derived $CD11b^{+}$ $Ly6C^{high}$ myeloid progenitor cells in premetastatic lungs secrete the extracellular matrix protein, versican, which promotes mesenchymal-to-epithelial transition (MET) of metastatic tumor cells via the TGFβ pathway, thereby increasing proliferation and accelerating lung metastatic outgrowth [156].

Studies have demonstrated that periostin (POSTN) secreted in the lung metastatic niche by stromal αSMA^{+} vimentin $(VIM)^{+}$ fibroblasts is required to maintain cancer stem cells (CSCs) and allow metastatic outgrowth of breast cancer cells by inducing Wnt signaling in the CSCs [157, 158]. Although this work did not determine the exact identity of the fibroblasts secreting POSTN, a previous study had shown that BM-derived MSCs are the source of POSTN [159], suggesting that BM cells in the premetastatic niche could be supporting metastasis by maintaining CSCs via POSTN secretion.

Recently, studies on metastatic dormancy have identified a mechanism whereby EC-derived Tsp-1 promotes breast cancer dormancy [160]. Interestingly, in another study, metastasis-incompetent primary tumors were shown to induce the expression of Tsp-1 in BM-derived myeloid cells recruited to premetastatic lungs [133], suggesting that myeloid-derived Tsp-1 could be similarly mediating metastatic dormancy.

Clinical Significance, Perspectives, and Future Directions

BM-derived cells contribute to various stages of cancer progression, and given their prevalence in patient tumors, BM-derived cells are being evaluated as prognostic tools and as therapeutic targets. Elevated levels of circulating inflammatory monocytes correlate with a poor prognosis in pancreatic cancer patients [161]. A high preoperative neutrophil to lymphocyte ratio is associated with poor prognosis after

resection in NSCLC [162]. In human breast cancer, a high TMEM score, i.e., the number of tumor cell, TAM, and endothelial cell interactions, is correlated with an increased risk of metastasis [163, 164]. Of note, TMEM score predicted the risk of distant metastasis in ER(+)/HER2(−) breast cancer [164]. This is in agreement with preclinical studies showing the role of macrophages in tumor cell egress from the primary site and intravital imaging revealing the direct contact between perivascular TAMs, endothelial cells, and tumor cells, forming the TMEM [87].

In NSCLC patients, high tumor islet $CD68^+$ macrophage density predicted increased survival, whereas high stromal macrophage density predicted reduced survival [165–167]. Further characterization of macrophage populations revealed that CD68+ M1 macrophages, defined as $HLA\text{-}DR^+$ $iNOS^+$ $TNF\alpha^+$ $MRP8/14^+$, were significantly increased in tumor islets of NSCLC patients with extended survival compared to patients with poor survival, whereas M2 macrophages ($CD163^+$ $VEGF^+$) were reduced [168].

Conversely, several reports demonstrated a correlation of TAMs with poor prognosis in lung cancer. Adenocarcinoma patients with high $CD68^+$ TAM density had significantly lower 5-year survival rates [169]. Furthermore, $CD68^+$ TAM density correlated with higher tumor expression of the angiogenic factor IL-8, higher microvessel density, and worse prognosis [170]. $CD68^+$ $CD163^+$ M2-like TAMs were significantly higher in patients whose disease progressed in the presence of EGFR tyrosine kinase inhibitor therapy [22]. M2 TAMs defined as $CD204^+$ were associated with poor outcome [171], and $CD206^+$ TAMs correlated with lymph node metastasis [172]. High levels of IL-10 expression by TAMs were significantly correlated with advanced tumor stage and predicted poor overall survival of NSCLC patients [173, 174]. Expression of MMP9 and VEGF by $CD68^+$ $IL\text{-}10^{hi}$ TAMs correlated with late stage of disease [175]. On the other hand, several studies have failed to find a correlation between macrophage density and NSCLC patient prognosis [25].

In patients with breast cancer, an immune signature consisting of $CD68^{high}/CD4^{high}/CD8^{low}$, denoting the infiltration of the different immune cell types into tumors, significantly correlated with reduced OS [21]. Interestingly, this immune signature predicted OS independently of histopathological grade or receptor status [21].

Immunosuppressive MDSCs, defined as $Lin^-HLA\text{-}DR^-CD33^+$ and $CD14^-CD11b^+CD33^+$ [176], were increased in patients with NSCLC and were associated with increased metastasis and a poor response to chemotherapy [177]. Increased levels of circulating and tumor-infiltrating MDSCs were also observed in patients with colon cancer and correlated with poor prognosis and increasing cancer stage [178]. Furthermore, the frequency of circulating monocytic MDSCs predicted patient response to the checkpoint inhibitor ipilimumab (anti-CTLA-4), where patients with low frequencies of MDSCs benefited more from ipilimumab treatment [179]. Similarly, glioblastoma patients showed an increase in circulating MDSCs, primarily of granulocytic lineage, which mediated immunosuppressive functions [180].

In addition to their prognostic role, the growing body of literature describing the contribution of the BM-derived microenvironment to tumor progression and metas-

tasis reveals potential therapeutic avenues that bypass the need to target highly mutagenic tumor cells and instead focus on the more genetically stable stromal cells that support them. In that respect, studies targeting BM-derived cells in the TME have shown some promise. For instance, inhibiting the recruitment of TAMs by blocking CSF1R signaling enhanced the cytotoxic effect of standard chemotherapy in a mouse model of breast cancer [21] and increased the efficacy of immunotherapy in a pancreatic cancer model [42]. Tumor resistance to anti-VEGF therapy was shown to be dependent on CD11b^{+} Gr1^{+} myeloid cell recruitment [54]. Blocking neutrophil recruitment to lungs by administering anti-G-CSF suppressed lung metastasis [134]. Moreover, inhibiting the myeloid cell-secreted angiogenic factor Bv8 reduced primary tumor growth, produced synergistic antitumor effects when combined with anti-VEGF treatment or chemotherapy [55], and reduced lung metastasis [134].

In a model of lung inflammation, deleting the neutrophil proteases, neutrophil elastase (NE), and cathepsin G (CG) in the BM compartment significantly suppressed metastatic outgrowth [154], suggesting that targeting neutrophil proteases could present a strategy to block metastasis. Furthermore, inducing the expression of Tsp-1 by myeloid cells in the lungs by administering a peptide derived from the protein prosaposin significantly reduced lung metastatic burden [133].

On the other hand, given the immunosuppressive function of MDSCs in cancer, strategies are being developed that promote the differentiation of MDSCs into mature, non-suppressive cells, decrease MDSC levels, or inhibit MDSC function [181]. Preclinical studies revealed that all-trans retinoic acid (ATRA) induced the differentiation of MDSCs and enhanced T-cell antigen-specific immune responses, but only induced an anticancer response when combined with a peptide vaccine [44, 182]. Administration of ATRA reduced MDSC levels in metastatic renal cell carcinoma (mRCC) patients that achieved a high plasma concentration of ATRA [183]. Sunitinib, an oral receptor tyrosine kinase inhibitor that targets PDGFR, VEGFR, and c-kit signaling and is FDA-approved for the treatment of advanced RCC, reduces MDSC levels in patients [184] and blocks the expansion of monocytic MDSCs while inducing apoptosis of granulocytic MDSCs in a mouse model of breast cancer [185]. Furthermore, the immunosuppressive activity of MDSCs was abrogated by the synthetic triterpenoid, CDDO-Me, which upregulated several antioxidant genes and decreased tumor growth in mice [176]. Moreover, CDDO-Me completely blocked the inhibitory function of MDSCs isolated from RCC patients [176]. Another way MDSC function is being targeted is by inhibiting IDO, the rate-limiting enzyme in tryptophan degradation, which leads to suppression of T-cell responses [48]. IDO inhibition using 1-methyl-tryptophan (1MT) retarded tumor growth via a T-cell-dependent mechanism in mouse models [186–188]. Furthermore, combining 1MT with therapies targeting immune checkpoints on T cells, such as CTLA-4 and PD-1/PD-L1, yielded a synergistic effect on the antitumor response in a mouse model of melanoma [189].

The BM-derived TME constitutes a relatively untapped resource of novel therapeutic targets. A major goal is to target only the “tumor-educated” BM cells and

spare normal counterparts so that side effects are drastically reduced. Consistent with this notion, analysis of enriched stromal compartments derived from human breast cancer revealed gene expression changes associated with cancer progression [190]. Similar analyses have led to the identification of activated stromal transcriptomes and tumor-stroma crosstalk pathways in human [191] and mouse [192] lung cancer. Future studies encompassing genomic, epigenetic, and proteomic analyses have the potential to provide insights into mechanisms that govern activation, expansion, mobilization, and recruitment of specific subsets of BM cells to the tumor bed leading to tumor growth and metastasis.

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Chapter 14
Neuropilin 1 and Neuropilin 2: Cancer Progression and Biomarker Analysis

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Abstract Neuropilins (NRP, human; Nrp, mouse) are a family of cell surface protein receptors originally named for their role in neuronal guidance during embryonic development. Over the past two decades, the expression, localization, regulation, and function of the NRP family have been intensely studied. The two-member family composed of neuropilin 1 (NRP1) and neuropilin 2 (NRP2) has now been shown to drive diverse processes including neuronal guidance, vasculogenesis, lymphangiogenesis, immunity, smooth muscle tone, epithelial cell migration and branching, epithelial-to-mesenchymal transition, and cancer progression. Although the two receptors share high sequence homology and domain structure, their unique ligand specificity, co-receptor nature, and disparate cell-specific expression patterns mediate pleiotropic functions in multiple tissue systems. Their abundant expression in a myriad of cancers and their location on the cell surface make them prime targets for antitumor therapies and potential use as surrogate biomarkers.

Keywords Neuropilin • Vascular endothelial growth factor • Cancer • Tumor Biomarker • Angiogenesis • Lymphangiogenesis • Semaphorin • Metastasis • Progression

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Neuropilin Structure

Neuropilins were first discovered in the neuropile of the Xenopus tadpole [1, 2]. Later, the neuropilin proteins were found in higher-order mammals as well and in a variety of tissues from neurons to epithelium to endothelium [3–6]. It is important to understand the structure of these two genes and proteins in order to appreciate their multifunctional role in various tissues. The human *NRP1* and *NRP2* genes span 120 and 112 kilobases on chromosomes 10p11.22 and 2q33.3, respectively [7]. Although the *NRPs* are on separate chromosomes, they may have originated from a gene duplication since 5 of the 17 exons are identical in size [8]. The NRP1 and NRP2 amino acid sequences are highly conserved (44% homologous) and encode proteins with similar overall domain structures [9]. Both NRP1 and NRP2 have multiple isoforms including transmembrane forms and soluble forms [10]. Table 14.1 compares characteristics between NRP1 and NRP2.

NRP1 and NRP2 are 130-kilodalton (kD) type I transmembrane glycoproteins, meaning they span the membrane in one pass and the N-terminal region of the protein is extracellular, and the C-terminal region is cytoplasmic. Approximately 80–90% of the protein is found outside the cell, leaving a small cytoplasmic region. The extracellular domains of both NRP1 and NRP2 are similar, consisting of two CUB motifs named domain a1 and a2, two domains homologous to coagulation factor V and VIII named b1 and b2, and a MAM (meprin, A5, μ) domain designated as domain c. The a and b domains are involved in ligand binding, while the c domain and transmembrane domains contain multiple conserved cysteine residues thought to be involved in adhesion or dimerization [11, 12]. The a

Table 14.1 Characteristics of neuropilin 1 (NRP1) versus neuropilin 2 (NRP2)

Characteristic	NRP1	NRP2
Chromosome location	10p11.22 (human)	2q33.3 (human)
Molecular weight	130–140 kDa	130–140 kDa
Receptor isoforms	NRP1, NRP1Δ16	NRP2a17, NRP2a22 NRP2b0, NRP2b5
Soluble forms	s_{11}NRP1, s_{12}NRP1, s_{III}NRP1, s_{IV}NRP1, s_{10}Nrp1	s_9NRP2
Cytoplasmic domain	Ends in SEA, binds PDZ-containing proteins	NRP2b does not end in SEA
Vascular localization	Arteries	Veins and lymphatic vessels
Angiogenic ligands	VEGFA, VEGFB, VEGFE, PGF, HGF	VEGFA, VEGFC, VEGFD
Inhibitory ligands	SEMA3A, 3B, 3C	SEMA3B, 3F, 3G
Knockout mouse	Lethal at E12.5-E13.5	Viable
Knockout mouse vasculature phenotype	Severely impaired blood vasculature	Fewer lymphatic capillaries, no edema

domain is involved specifically with binding to the amino-terminal sema domain in class 3 semaphorin (SEMA3) proteins. The crystal structure of NRPs shows that the a2b1b2 domain forms a tightly packed unit, while the a1 domain is only loosely associated [13]. Intact SEMA3 ligands also make contacts with the b domains of NRP via their C-terminal basic region [14, 15]. The angiogenic vascular endothelial growth factor (VEGF) family including VEGF-A, VEGF-C, and placenta growth factor (PGF) bind to the b1b2 domain of NRP1 and NRP2 [14, 16, 17]. The domains that other NRP ligands such as hepatocyte growth factor (HGF) or transforming growth factor beta (TGFβ) interact with have not yet been identified [18, 19].

There are two transmembrane receptor NRP1 isoforms, the full-length NRP1 (923 aa) that contains all 17 exons and an alternative isoform that lacks exon 16, NRP1(ΔE16) (907 aa) [8, 20]. The NRP1(ΔE16) receptor lacks 17 amino acids (replaced by an arginine) from an extracellular region near the transmembrane domain, but this receptor still binds VEGF ligand and presumably signals similarly to the full-length NRP1 [20]. Four NRP2 transmembrane isoforms exist: NRP2a(17) (926 aa), Nrp2a(22) (931 aa, insert of GENFK), NRP2b(0) (901 aa), and NRP2b(5) (906 aa, insert of GENFK) [8, 10, 21]. NRP2a and NRP2b isoforms are generated by alternative splicing and have identical extracellular domains, meaning that they can bind the same ligands. However, the cytoplasmic domain of NRP2a more closely resembles that of NRP1 in size and sequence and both end in a 3-amino acid sequence: serine-glutamic acid-alanine (SEA). This C-terminal SEA is reported to bind to proteins containing a PDZ (PSD95, Dlg1, Zo1) domain, which is a common structural motif in scaffold proteins that anchor membrane receptors to cytoskeletal components. NRP1 (and presumably NRP2a) binds to synectin, also called NIP (neuropilin-interacting protein) or GIPC (GAIP-interacting protein, C-terminus) [22–24]. The NRP2b isoform has a shorter cytoplasmic domain that is only 11% homologous to NRP2a [21]. NRP2b lacks the SEA cytoplasmic tail and therefore does not possess the capacity to bind synectin.

Soluble NRP (sNRP) proteins are truncated NRP isoforms with molecular weights ranging from 60 to 90 kD [10]. sNRP1 was first detected from a 2.2-kilobase (kb) mRNA species found after Northern blotting mRNA from human PC3 prostate carcinoma cells with radioactive probes to the a or b domain of NRP1 [25]. Subsequent cloning of this band resulted in the identification of a novel 644-amino acid (aa) isoform resulting from a read-through into intron 12 and a premature stop codon. As more isoforms began to be identified, this first isoform was later named s_{12}NRP1, which contained the entire a and b domains of NRP1 and a unique 28-base-pair (bp) sequence at its carboxy terminus, yet lacked the c domain, transmembrane domain, and cytoplasmic domain [25]. Three other soluble isoforms of human NRP1 were later identified: s_{11}NRP1 reads into intron 11 and is 704 aa, s_{III}NRP1 lacks exon 10–11 but includes intron 12 and consists of 551 aa, and s_{IV}NRP1 skips exon 11 but reads into intron 12 and consists of 609 aa [8, 26]. An additional sNrp1 isoform, s_{10}Nrp1, was cloned in the mouse and is 588 aa [27]. So far, the only human sNRP2 identified is s_9NRP2, a 555 aa isoform containing the a domains, b1 domain,

and part of the b2 domain [8]. Full-length NRP1 and sNRP1 share a common promoter, yet the expression and regulation of each isoform can be cell-type specific. For instance, in the liver, hepatocytes strongly express sNrp1, while sinusoidal endothelial cells express Nrp1 [25, 27, 28].

The sNRP1 isoform can bind and sequester VEGF-A [25, 29] or HGF [27], and sNRP2 can bind and sequester VEGF-A [14], VEGF-C [30], or semaphorin 3F (SEMA3F) [14]. Although sNRPs bind ligands, they do not form co-receptor complexes with other transmembrane receptors and do not transmit signals into the cytoplasm [25, 31]. sNRP1 was found to inhibit invasion of lung cancer cells in vitro [32], and a mutated sNRP2 which preferably binds VEGFA over SEMA3F [14] was shown to inhibit human melanoma growth in nude mice [31]. Therefore, sNRPs behave functionally in the opposite manner to full-length NRP receptors and can act in a stimulatory or inhibitory manner depending on the ligand interaction that they are antagonizing. sNRPs contain only a few novel amino acids that are unique and not found in NRP, and therefore no specific antibodies to any of the sNRPs are commercially available. Specific detection of sNRP requires either in situ hybridization with intron-specific probes in tissue sections or Western blotting of tissue lysates using N-terminal NRP-specific antibodies [25, 27]. The results of immunohistochemistry, which is routinely performed to detect NRP levels in tumor biopsies, must be scrutinized carefully since antibodies to the N terminus of NRPs will detect both membranous and soluble forms. C-terminal-specific antibodies are preferred to detect the full-length receptors with the distinction between NRP2a and NRP2b.

Neuropilin Ligands

Neuropilins were discovered in the neuronal system, and the first ligands identified for these receptors were the class 3 semaphorin (SEMA3) family of axonal guidance proteins [21, 33, 34]. Later NRPs were shown to be a unique class of receptors for the VEGF family of angiogenic molecules [35]. This link between the neuronal and vascular systems opened up an entirely new field comparing the molecular similarities in these two distinct yet similarly patterned systems [36]. Today it is now appreciated that NRPs can bind and mediate signals through many ligands including platelet-derived growth factor (PDGF), HGF, and TGFβ (reviewed by [37–39]). We will summarize each ligand family and its relation to NRP function below with particular interest in those processes involved in cancer progression such as angiogenesis, migration, invasion, and metastasis.

VEGF Family

VEGF is fundamental for the development and survival of vascular supply, and therefore VEGF signaling is pivotal for physiological functions and homeostasis by maintaining a functional network of blood vessels [40]. VEGF also plays an

essential role in inducing vascular permeability, which is important for angiogenesis associated with tumors and wound healing [41]. NRPs serve as receptors for growth factors in the VEGF family with differential binding affinities for each ligand. The VEGF binding sites reside in the b1 and b2 domains on the extracellular component of the NRP receptors [13, 14, 16, 42, 43]. Therefore, both membranous and secreted NRPs can bind VEGF, as they all contain intact b1/b2 domains.

The human *VEGF-A* gene encodes multiple isoforms that arise due to alternative splicing. The VEGF-A isoforms differ in their inclusion of exons 6 and 7, which are the domains responsible for heparin binding [44]. VEGF-A_{121} is a slightly acidic form that cannot bind heparin, while VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206} all contain exon 7 and bind heparin. NRPs were initially described as isoform-specific receptors for only heparin-binding isoforms of VEGF-A, in particular, VEGF-A_{165} [35, 45]. Later, crystal structures of NRPs indicated a VEGF-A exon 8a binding site [13, 46, 47]. Furthermore, a newly identified VEGF-A variant called VEGF-A_{165b}, which lacks exon 8a but includes exons 7 and 8b, did not bind to NRP1 [48]. Taken together, these data suggest that the b1 domain in NRP1 or NRP2 can bind VEGF-A via regions encoded by exon 7 and 8 [49]. The VEGF-A_{165} ligand can bind to both NRP1 or NRP2, but recent evidence shows that binding is 50 times stronger to NRP1 than NRP2 [49]—this may explain why the initial expression cloning of the putative VEGF-A_{165} receptor (now known as NRP) yielded six clones of NRP1 and only one clone for NRP2 [35].

VEGF-A isoforms bind to the canonical tyrosine kinase VEGF receptors, VEGFR1 or VEGFR2, via regions encoded by exon 4 (reviewed by [50]). Thereby, the VEGF-A_{165} protein can "bridge" between NRP receptors (via exon 7–8) and VEGFRs (via exon 4) at the same time forming a complex that enhances the output of the receptor tyrosine kinase (RTK) [35, 45]. As described above, the NRP cytoplasmic domain is small (40 aa) and does not contain kinase activity; therefore, NRPs are often referred to as "co-receptors" since the business end of the signaling is performed through VEGF RTKs. Both VEGFR1 and VEGFR2 have been shown to complex with NRP1 and NRP2 in the presence of VEGF-A [45, 51].

Nearly all members of the greater VEGF family, which share NH_2-terminal cystine knot domains, have been shown to interact with either NRP1 or NRP2. Specifically, two family members that most closely resemble VEGF-A in sequence and structure are VEGF-B and PGF. VEGF-B_{167} and PGF2 both contain basic COOH-terminal domains and sequences homologous to exon 7 of VEGF-A, and both have been reported to bind NRP1 [16, 52, 53] and signal through VEGFR1 [54].

Alternately, the lymphangiogenic members of the VEGF family, namely, VEGF-C and VEGF-D, primarily bind to NRP2 via its b1b2 domain (although they can also bind to NRP1) and transmit their signal through the VEGFR3 RTK [17, 55, 56]. The VEGF-C proprotein (uncleaved version) binds better to NRP2 than does the amino-cleaved version of VEGF-C. VEGF-C can bind to NRP2 in the absence of heparin, but only interacts with NRP1 in the presence of heparin [17]. VEGF-D protein requires heparin to bind to either NRP [17]. The presence of NRP2 enhances the phosphorylation of VEGFR2 in the presence of VEGF-A or VEGF-C [55].

Other ligands that are related to the VEGF superfamily include PDGF and TGFβ. Both of these proteins are capable of binding heparin and have cystine knot and beta-strand topology [57]. Although the precise binding sites on these ligands and NRP domains have not been mapped, it is clear that NRPs can form co-receptor complexes with the PDGF receptors (PDGFR) and TGFβ receptors (TGFβR). Knockdown of NRP1 in mesenchymal stem cells or vascular smooth muscle cells attenuated PDGFRα phosphorylation by PDGF-AA and/or reduced PDGFRβ activation by PDGF-BB [58, 59]. Additionally, NRP1 O-linked glycosylation at Ser612 regulated PDGF-induced smooth muscle cell migration [59].

NRP1 and NRP2 are able to bind to both the active and latent forms of TGFβ1 [19, 60]. TGFβ1 can compete with VEGF-A binding for NRP, suggesting that TGFβ also binds to the b1b2 domain of NRP. Moreover, Nrp1 expression can activate latent TGFβ1 [19]. NRP1/2 were shown to form co-receptor complexes with TGFβRI and RII. TGFβ is also a major inducer of the epithelial-to-mesenchymal (EMT) phenotype in cancer cells, and TGFβ has been shown to induce NRP2 expression in hepatocellular carcinoma cells [61].

HGF

NRP1 can bind to another heparan sulfate binding protein called hepatocyte growth factor (HGF) or scatter factor [62]. Active HGF is a heterodimeric, disulfide-linked protein composed of an alpha chain and a smaller beta chain [63]. HGF is secreted as a single-chain inactive protein and then cleaved by serine proteases to the active form. The N-terminal hairpin loop region of the HGF protein structure is strikingly similar to the heparin-binding C-terminal region of VEGF-A [64]. HGF binds and signals through the c-Met receptor tyrosine kinase. Classically, HGF stimulates mitosis in hepatocytes, but HGF also has potent angiogenic and lymphangiogenic properties in endothelial cells [65–67].

NRP1 and NRP2 both bind HGF, and HGF can compete VEGF-A binding to NRPs suggesting that HGF may bind to the b1b2 domain on NRPs [18]. NRP1 has been shown to act as a co-receptor for HGF with c-Met, and HGF likely binds to these two receptors independently or as a bridge via different domains since knockdown of NRP1 or NRP2 could not inhibit all signaling through c-Met [18]. sNRP1 also binds and sequesters HGF [27]. Since NRP1 is highly expressed in sinusoidal endothelial cells in the liver and the liver hepatocytes are a prime source of HGF, one may speculate that NRP1 in the liver is primarily an HGF receptor rather than a VEGF receptor. After hepatectomy, sNrp1 levels plummet in the liver and only increase after regeneration suggesting that this endogenous soluble receptor may regulate the bioavailability of ligands during the healing process [27].

SEMA3 Family

The semaphorin (SEMA) family is a large group of proteins (more than 20 vertebrate members) which share a common structure at the amino terminus called the "sema" domain that is folded into a β-propeller structure with seven blades homologous to alpha integrins (reviewed by [68]). The SEMA family is divided into eight classes (numbered 1–7, and a viral group) containing proteins that span the plasma membrane with the exception of the vertebrate class 3 SEMA (SEMA3) proteins that are secreted. There are seven different SEMA3 secreted proteins labeled A to G that share a cysteine-rich PSI (plexin-semaphorin-integrin) domain, an immunoglobulin domain, and a C-terminal basic-charged domain (in addition to the sema domain) (reviewed by [69]). SEMA3s were originally named "collapsins" for their ability to collapse axonal growth cones [70, 71]. Subsequently, NRPs were found to be high-affinity receptors for these secreted SEMA3 mediators of neuronal guidance [21, 33, 34]. All members of the SEMA3 subfamily bind to NRPs except SEMA3E, which binds directly to Plexin D1 [72, 73]. Although NRP1 and NRP2 extracellular domains are quite homologous, there is specificity within the SEMA3 family for binding to either receptor. SEMA3A binds specifically to NRP1 [33, 34], and full-length SEMA3G binds specifically to NRP2 [74], whereas SEMA3B, C, D, and F can bind to either NRP (reviewed by [75]). In the case of SEMA3F, its affinity to NRP2 is tenfold higher than to NRP1 [21], and SEMA3F appears to only function through its interaction with NRP2 [76], suggesting that NRP1 may be a lower-affinity decoy for SEMA3F.

Table 14.2 Structure to function relationship in SEMA3 proteins

SEMA3	Full-length protein	Protein cleavage products	
	p100/p95	p65	p30
SEMA3A	p95 active dimer, binds Nrp1, repulsion/collapse	p65, monomer, inactive, does not bind Nrp1	p30, inactive, does not bind Nrp1
SEMA3B	p95[a] active, binds Nrp1 and Nrp2, EC repulsion		
SEMA3C	p95[a] active, binds Nrp2, LEC repulsion	p65 active, Nrp dependency unknown, promotes survival in lung cancer	
SEMA3E	Mutant p95, binds Plexin D1, Inhibits EC survival and migration	p61 active, binds Plexin D1, promotes lung metastasis	
SEMA3F	p95 active, binds NRP2, competes with VEGFA, repels EC and LEC		c-furSema active, binds NRP1, blocks VEGF binding
SEMA3G	p100 binds NRP2 weakly p95 binds NRP2 strongly, binds NRP1 weakly	Inactive	

[a]SEMA3B,C have been tested as mutant proteins in which the cleavage site was mutated in order to get a p95 protein

The functions of SEMA3 proteins are regulated by proprotein convertases (PPC)—similar to that of other proproteins like MMP, VEGF-C, and TGFβ. All SEMA3 proteins contain conserved PPC recognition sites (KRRXRR) between the sema and Ig domains and in the basic C-terminal domain. The SEMA3 proteins are secreted as 100 kD proproteins that are initially cleaved at their C-terminus to 95 kD proteins and then subsequently cleaved upstream to create two protein fragments of ~65 kD and ~30 kD (Table 14.2). The second cleavage is important for function because some SEMA3 proteins bind to NRPs as dimers, making disulfide bridges at conserved cysteines, while others function as monomers. As an example, the affinity of the SEMA3A dimer (95 kD) is 10,000 times greater for NRP1 than the cleaved monomer (65 kD) [77], and dimerization is necessary for collapsing activity of SEMA3A [78].

Full-length SEMA3G (p100) binds to NRP2, processed SEMA3G (p95) binds strongly to NRP2 and weakly to NRP1, and cleaved SEMA3G (p65) does not bind to either receptor [79]. SEMA3B and SEMA3C (either endogenous or recombinant) found in the conditioned media from tumor cells are cleaved and inactive [80, 81], likely due to the high levels of PPC found in tumor cells [82]. However full-length uncleavable SEMA3B protein engineered with a mutant PPC recognition site binds NRP1 or NRP2 and induces endothelial cell repulsion [80]. Similarly, full-length mutant uncleavable SEMA3C binds NRP2 in lymphatic endothelial cells and induces repulsion [81]. Interestingly, the cleaved SEMA3C protein (65 kD) promoted the survival of lung cancer cells, although whether this action is NRP-dependent remains unclear [81]. SEMA3E proteins have dual functions depending on their PPC cleavage status [83]. In its naturally cleaved form, SEMA3E (p61) binds Plexin D1 and associates with ErbB2 to promote lung metastasis and growth [73, 84]. However, when engineered to an uncleavable form, mutant SEMA3E (p95) activated Plexin D1 and inhibited EC migration and survival [73].

The three-dimensional structure of the SEMA3F protein is important for its proper binding and function. The "sema" domain in SEMA3F binds to the a domain of NRP2, while the basic domain of SEMA3F binds to the b domain of NRP2 [85]; therefore, it may come as no surprise that SEMA3 proteins can compete with VEGF-A and VEGF-C binding to NRPs [14, 55, 86]. Additionally, furin cleavage in the C-terminal basic region of SEMA3F creates a fragment protein called c-furSema that can bind to both NRP1 and NRP2 and inhibit VEGFA binding to these receptors [87, 88]. It is currently unclear whether SEMA3 proteins can also compete with binding of PDGF, TGFβ, or HGF to NRP, but it would seem reasonable. Since growth factor signaling is generally stimulatory for proliferation, survival, or migration and SEMA3 proteins compete with the binding of these factors, then it is understandable why SEMA3 proteins are often termed "inhibitory" proteins.

Independent of their ability to compete with VEGF family members, SEMA3 proteins trigger a chemorepulsive signal through the interaction of NRPs and large transmembrane receptors called plexins (reviewed by [89, 90]). NRPs are the SEMA3 ligand-binding part of the complex, and plexins are the signal-transducing part of the complex. For instance, the activation of Plexin A1 by SEMA3A/NRP1 or SEMA3F/NRP2 leads to the inhibition of the RhoA pathway and the subsequent depolymerization of F-actin filaments [91, 92]. This "collapsing" phenotype thus inhibits cell motility, migration, and invasion.

Neuropilin Localization

Although the two NRP receptors were first found in neurons, their tissue expression pattern is now appreciated to be much more widely distributed [4, 5, 38, 93]. In the embryonic neuronal system, neural crest cells express both Nrps in a non-overlapping pattern such that Nrp1-expressing cells give rise to sensory and sympathetic ganglia, while Nrp2-expressing cells primarily give rise to sensory neurons [85, 94]. Melanocytes (and melanoma cells), which are derived from neural crest cells, express high levels of NRP2 [95]. In the embryonic vascular system, NRP1 is expressed in arteries, while NRP2 is found in veins and lymphatic vessels [96–98]. NRP receptors are also involved in the guidance and patterning of blood vessels. Thus, it is not surprising that numerous studies using transgenic and mutant mice have established that any of the following can result in abnormal vascular patterning during development: overexpression of *Nrp1* [99], constitutive knockout of *Nrp1* [100, 101], heterozygous knockout of *Nrp1* and *Nrp2* [102], or deletion of the cytoplasmic domain of *Nrp1* ($Nrp1^{cyto}\Delta/\Delta$) [103]. NRP expression in endothelial cells is plastic and can be affected by hemodynamic flow and ischemia/hypoxic conditions [9, 104, 105]. However, NRPs are dramatically downregulated in endothelial cells after birth when patterning is completed and homeostasis has been established [6].

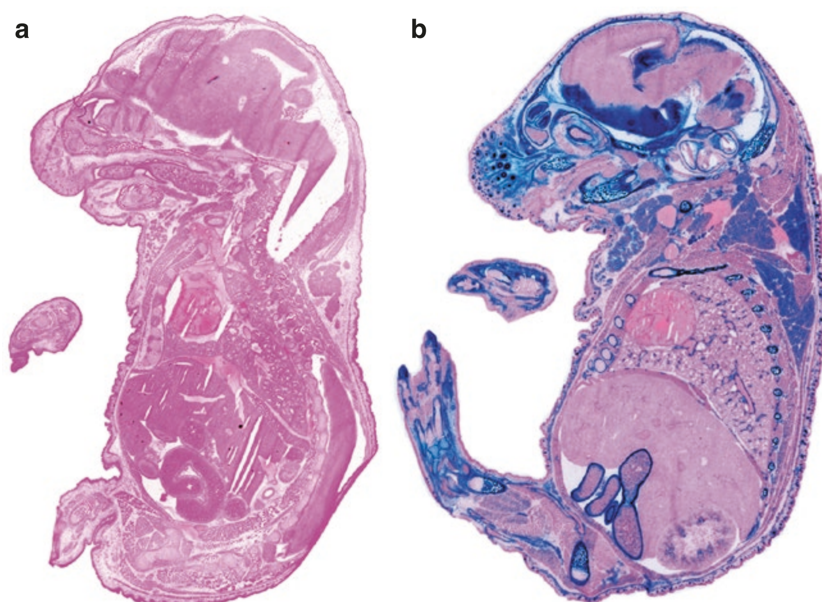

Fig. 14.1 Neuropilin 2 expression in a $Nrp2^{+/lacz}$ mouse. Cryosections of wild-type $Nrp2^{+/+}$ mouse (**a**) and heterozygous $Nrp2^{+/lacz}$ mouse (**b**) embryos (E18) were stained with X-Gal reagent to detect beta-galactosidase activity. *Blue color* denotes Nrp2 expression. Neurons in the brain, melanocytes in the skin, endothelial cells in the lung and kidney, and smooth muscle cells in the intestines are all visible

That being said, both NRP1 and NRP2 can be upregulated in activated adult capillaries during phases of remodeling or angiogenesis [106–109].

Although most published reports on NRPs have focused on their roles in blood vessels or neurons, NRPs are much more strongly expressed in epithelial cells and smooth muscle cells than in endothelial cells or neurons (see Fig. 14.1- *Nrp2*$^{+/lacz}$ embyro) [5, 29, 110, 111]. Nearly all epithelial cells express NRP1 including the epidermis (skin) [111, 112], mammary gland [25, 113], prostate gland, intestine, pancreas, and podocytes of the kidney [114]. Therefore, it should come as no surprise that the majority of all carcinomas, which are derived from epithelial cells, express NRP1 (discussed in more detail below). Interestingly, NRP1 expression in epithelial cells facilitates Epstein-Barr virus entry and promotes infection [115].

There is some degree of specificity between the expression of NRPs within smooth muscle subtypes such that vascular smooth muscle cells express Nrp1 [59] and visceral smooth muscle including cells from the GI tract and the bladder express predominantly Nrp2 [110]. NRPs are also strongly expressed in immune cells including T cells, macrophages, and dendritic cells [93, 116, 117]. Immunoregulatory CD4+ T cell subsets called Tregs highly express NRP1 [93, 118]. These NRP1+ Tregs may follow the gradient of VEGF in order to infiltrate a tumor. Evidence of this phenotype was seen when Nrp1 was knocked out in CD4^{+}Foxp3^{+} Tregs, and tumor infiltration was reduced [119].

Neuropilins in Cancer

As mentioned above, most epithelial cells express NRP1; therefore, most carcinomas express NRP1 including carcinomas of the skin (see Fig. 14.2a) [111, 120], tongue [121], breast [122], colon [123], stomach (gastric) [124], endometrium [125], ovary [126–128], prostate [129, 130], liver [28], pancreas [131, 132], kidney [133], and lung [32, 134]. NRP1 is normally found in differentiated epithelial cells but may be upregulated in basal cells in dysplastic tissues and is therefore an early marker of tumor progression [121]. Soluble NRP1 is an early diagnostic marker for cervical cancer and cervical intraepithelial neoplasia (CIN) [135]. NRP2, on the other hand, is not normally found in cells of epithelial origin (Fig. 14.1) but is reportedly upregulated in later stages of carcinogenesis especially in aggressive or metastatic carcinomas [136–138]. NRP2 is highly expressed in cancer cells of neuronal origin such as melanoma cells (Fig. 14.2b) [106], glioblastoma cells [4], neuroblastoma [139], and medulloblastoma cells [140], as well as in some sarcomas [141]. The topic of neuropilins in cancer has been reviewed previously [4, 75, 142–147]. Herein, we focus on the current theories explaining the function of NRPs in tumor cells.

The current paradigm of NRP1/2 function in endothelial cells is that they act as receptors of VEGF family members (VEGF-A, VEGF-C, VEGF-D) and enhance

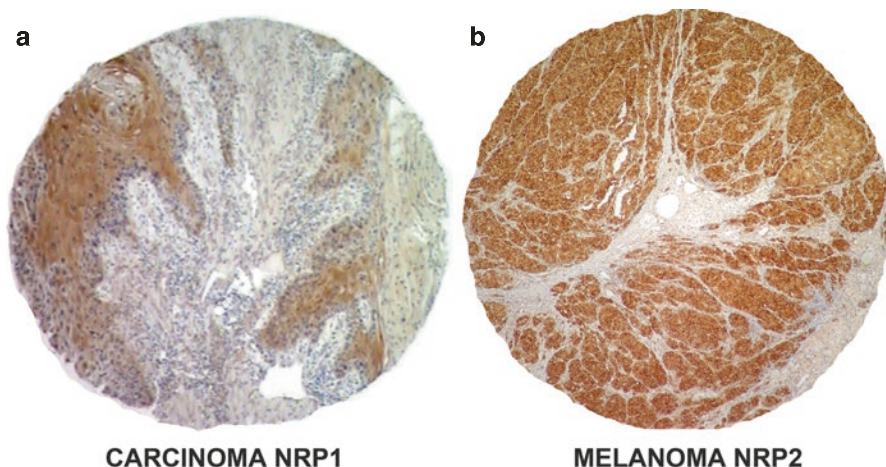

Fig. 14.2 Neuropilin expression in tumor biopsies. (**a**) The majority of carcinomas highly express NRP1 protein. Shown here is a human cutaneous squamous cell carcinoma biopsy (highly differentiated) stained with anti-human NRP1 antibody. *Brown color* denotes NRP1 expression, and *blue color* shows nuclei stained with hematoxylin. Section was photographed at 100×. Panel A is modified from [111]. (**b**) NRP2 is a marker for melanocytes and melanoma. Nearly all melanomas (and other neural crest-derived tumors) express NRP2. Shown here is a human melanoma biopsy stained with anti-human NRP2 antibody. *Brown color* denotes NRP2 expression, and *blue color* shows nuclei stained with hematoxylin. Section was photographed at 40×. Both panels were digitally enhanced for publication

VEGF receptor (VEGF-R1, VEGF-R2, VEGF-R3) tyrosine kinase signaling [17, 35, 45, 55]. The cytoplasmic portion of the NRP protein is small and does not have intrinsic kinase activity. Therefore, NRPs are ligand-binding proteins in the VEGF/NRP/VEGFR2 receptor complex but not signal-transducing proteins. Based on this information, it is unclear what role NRP is playing in carcinoma cells which typically lack expression of VEGFRs. Four potential scenarios have been suggested.

Tumor-Derived NRP Acts as a Reservoir for VEGF In this model, tumor cells secrete VEGF which can bind to NRP on the tumor cell surface, but this VEGF does not become internalized or signal in an autocrine fashion in the tumor cell since there are no VEGF receptor tyrosine kinases in these cells. The NRP protein acts somewhat like a heparan sulfate proteoglycan on the tumor cell surface in that it can bind VEGF, hold VEGF, and present the VEGF to juxtacrine cells such as endothelial cells. The NRP may help to increase the gradient of VEGF in the local tumor microenvironment as a way to recruit neovessels toward the hypoxic areas of the tumor. Several papers present findings that favor this model. In one report, recombinant VEGF protein given to the tumor cells in vitro had no effect on proliferation or migration of the tumor cells [148], yet when tumor cells are transfected with NRP1 and implanted in mice, the resulting

tumors stain strongly for VEGF, and tumor angiogenesis is increased [123, 148, 149]. Alternately, when NRP levels were diminished in tumor cells in vitro by shRNA and then implanted in vivo, tumor size and vascularity were reduced [138, 150].

VEGF Acts as an Autocrine Growth Factor for Tumor Cells That Express NRP and VEGFR1/2 Although it is rare, some tumor cells do upregulate VEGFRs, and in these tumor cells, signaling is similar to endothelial cells. Several studies have reported a VEGF autocrine signaling loop in tumor cells. In breast cancer cells, VEGF was shown to stimulate migration in vitro [122]. Highly metastatic cells that expressed NRP1 also upregulated VEGFR2 after cycling in mice from the primary organ to the metastatic site [130]. These tumor cells that expressed NRP1 and VEGFR2 showed increased proliferation and migration in response to VEGF [130]. In another study, merely repeatedly collecting cells that had invaded through Boyden chambers was enough stress to cause them to upregulate VEGFR2 and NRP1 [32]. Likewise, lung cancer cells that endogenously expressed VEGFR2 and were transfected with NRP1 showed increased growth in nude mice compared to control transfected cells [151].

VEGF Acts as an Autocrine Growth Factor for Tumor Cells That Express NRP via Synectin Signaling New studies suggest that NRPs may indeed "signal" but not in the canonical fashion of tyrosine phosphorylation (reviewed by [152]). As discussed above, the intracellular domains of both NRP1 and NRP2a proteins terminate in the same three amino acids, SEA, and bind to the PDZ domain of the cytoplasmic scaffold protein, synectin [22–24]. Yoshida and colleagues show that squamous cell carcinoma cells which express NRP1 but do not express any VEGFR can proliferate in the presence of VEGF and that this proliferative signal is mediated by the interaction of synectin and Syx, a RhoGEF [153]. This autocrine signaling through VEGF/NRP1 resulted in the activation of RhoA and the degradation of p27, a cyclin-dependent kinase inhibitor. When the authors deleted the cytoplasmic domain of NRP1 in these tumor cells, this signaling was lost [153]. VEGF/NRP1 direct interaction was also shown to be critical in epidermal stem cells for the initiation of skin cancer [120, 154]. Similar autocrine signaling was observed between PGF and NRP1 in medulloblastoma tumor cells [155].

NRP Is an HGF Receptor in Cancer Cells Although the majority of all research on NRP has focused on its role as a VEGF receptor, this may not be its main function in carcinoma cells. While carcinoma cells rarely express VEGFRs, they typically express high levels of c-Met, the tyrosine kinase receptor for HGF (discussed above). HGF is a potent oncogene and tumor promoter, and HGF-induced invasion in human glioma [156] and human pancreatic cancer [157] is dependent on NRP1. NRP1 depletion using shRNA in gastric cancer cells inhibited the VEGF/VEGFR, the EGF/EGFR, and the HGF/c-Met pathways [158]. Peptide N, a small protein corresponding to the N-terminal domain of human HGF that binds to NRP1 and inhibits the binding of endogenous VEGFA or HGF, inhibited hepatocellular carcinoma progression in a transgenic model [18, 28, 159].

Neuropilin as a Biomarker

As outlined above, NRPs are expressed in cancer cells, in tumor-associated endothelial cells that participate in neoplastic angiogenesis, in tumor-associated lymphatic endothelial cells that contribute to lymphangiogenesis and metastasis, and in tumor-infiltrating immune cells. NRP1 may be a potential biomarker in many different carcinomas. In some cases the expression of NRP1 merely increases in the early stages of transformation but is still found at a lower level in the surrounding epithelial cells [111, 123], in other cases the localization of the NRP1 protein may change within the epithelium from differentiated cells to basal cells [121], and in still other tissues, normal epithelial cells may lack NRP1 expression entirely but upregulate expression in carcinoma cells—such is the case in the liver [28]. NRP1 copy number gain (CNG) as analyzed by fluorescence in situ hybridization (FISH) in non-small cell lung cancer biopsies correlated with a worse overall survival (OS) and progression-free survival (PFS) [160]. Beyond carcinomas, NRP1 also serves as a novel biomarker associated with poor prognosis in acute myeloid leukemia patients [161].

In a screen of biomarkers for lung tumors induced by benzo[a]pyrene (coal tar), serum NRP2 was found to be highly expressed [162]. NRP2 has been shown to be a novel biomarker for aggressive human melanoma [95]. Furthermore, NRP2 gene silencing in human melanoma cells decreased tumorigenicity and metastasis in a preclinical trial [163].

Based on the aforementioned studies, NRPs are excellent candidates for targeted therapies in multiple forms of cancer. Potential strategies used to target NRPs have been reviewed previously [164–166]. Most successful have been antibodies targeting the VEGF-binding domain of NRP1 or NRP2 [107, 108]. When used in combination with bevacizumab (anti-VEGFA antibodies), anti-NRP1^{B} antibodies were shown to inhibit tumor size, tumor angiogenesis, and tumor progression [107]. These antibodies were later humanized and tested in a phase Ib clinical trial with bevacizumab and chemotherapy [167]. Unfortunately, this combination therapy caused a high degree of proteinuria in patients—likely due to the off-target effect of anti-NRP1 on podocytes in the kidney [114]. Antibodies to NRP2 were also shown to be efficacious at blocking lymphangiogenesis and metastasis in preclinical trials [108] and have not been tested to date in human clinical trials.

Researchers are continually investigating parameters that may predict whether a potential therapy will be beneficial to select cancer patients. For instance, anti-VEGF therapy with bevacizumab has been approved since 2003, yet we still cannot predict which patients will benefit from this approach [168]. Often, retrospective studies are performed to determine whether a potential growth factor or growth factor receptor can predict a patient's response to therapy. In a surprising number of trials with different drugs, tumors that express either NRP1 or NRP2 have been shown to correlate with a worse prognosis.

In a randomized phase II clinical trial in stage IV metastatic colon carcinoma (BATON-CRC), low-serum NRP1 levels correlated with a better progression-free

survival in patients treated with tivozanib, a tyrosine kinase inhibitor (TKI) that targets VEGFR1–3, in combination with the mFOLFOX6 chemotherapy (folinic acid, fluorouracil, and oxaliplatin) [169]. Patients with high NRP1 levels progressed more quickly on this drug regimen than patients with low NRP1, and future trials using tivozanib plan to use NRP1 as a biomarker to predict response to therapy. Biomarkers associated with disease progression were also evaluated from metastatic breast cancer patients ($n = 462$) treated with bevacizumab plus capecitabine (AVF2119g phase III trial). Tumors with low scores for NRP1, Dll4, and VEGFC all trended toward improvements in PFS but did not reach significance (NRP1: $p = 0.07$) [170]. The opposite result was found in the AVAGAST trial (global, randomized, double-blind, phase III study in advanced gastric cancer that compared bevacizumab and chemotherapy to placebo plus chemotherapy) where expression of NRP1 was correlated with survival in the placebo group—meaning that patients with high NRP1 lived longer [171]. On the other hand, patients with low NRP1 levels at baseline experienced a better outcome from bevacizumab treatment in both OS and PFS [171].

NRP2 has also been shown to be an important predictor in some trials. In a retrospective study, NRP2 expression was evaluated in tumors from renal cell carcinoma (RCC) patients treated with TKIs targeting the VEGF pathway after interferon-alpha failure. NRP2 overexpression was found to negatively correlate with PFS and OS in these RCC patients [172]. Specifically, the RCC patients that lacked NRP2 expression gained 7 months in PFS and 13 months in OS compared to patients with high NRP2. Archival tumor samples from patients with advanced, metastatic esophagogastric adenocarcinomas that received the chemotherapy XELOX (oxaliplatin and capecitabine) plus bevacizumab showed a worse PFS in NRP2 high-expressing tumors than in NRP2 low-expressing tumors ($p = 0.05$) [173]. However, in astrocytomas, none of the VEGF receptors including neuropilins correlated with the lack of patients' response to bevacizumab [174].

Mechanistically, it may make sense that patients with high NRP-expressing tumor cells may not respond to bevacizumab because bevacizumab is an antibody which targets the antigen of VEGF in its domain encoded by exon 4. This is the portion of the VEGFA protein that binds to VEGFR2 but not the exon 7–8 region that binds to NRP1/2 [165]. It is anticipated that therapies that target NRPs directly would show added benefits to anti-VEGF therapy. As described above, NRPs are found on numerous cells in the tumor microenvironment; therefore, drugs that target NRPs may inhibit diverse processes including tumor cell invasion, angiogenesis, lymphangiogenesis, and tumor immunity and inhibit several pro-angiogenic factors including VEGFA, VEGFC, and HGF.

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Chapter 15
The Role of Axl Receptor Tyrosine Kinase in Tumor Cell Plasticity and Therapy Resistance

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Abstract In spite of the advances in cancer treatment over several decades, resistance to antitumor therapy continues to confound current treatment strategies. Recent insights into the epigenetic heterogeneity of cancer have emphasized a need to address the underlying mechanisms driving tumor cell plasticity. Epithelial-to-mesenchymal transition (EMT)-related transdifferentiation programs are prevalent in aggressive tumors displaying a drug-resistant, invasive, and immune-evasive phenotype. Novel therapeutically actionable targets are needed in order to disable tumor plasticity mechanisms. The Axl receptor tyrosine kinase has a remarkably broad association with aggressive and therapy-resistant cancers, and the understanding that Axl is not a traditional oncogenic driver as first envisioned, but rather involved in regulating tumor cell plasticity related to the EMT program has provided a framework to understand the role of Axl-mediated signal transduction in cancer. Accordingly, a growing number of studies have demonstrated that Axl signaling is required to maintain tumor plasticity and resistance to cytotoxic and targeted anticancer agents. Novel Axl-targeting agents are emerging, facilitating clinical translation of novel combination approaches dedicated to reverse the plasticity-mediated resistance mechanisms and potentiate current anticancer treatments. In this chapter, we describe the unique roles of the Axl receptor tyrosine kinase in tumor cell plasticity and therapeutic resistance and provide an update on Axl-targeting agents entering clinical trials.

Keywords Axl • EMT • EMP • Plasticity • Drug resistance • Targeted therapy Clinical trials • Biomarker • Tumor microenvironment • Tyrosine kinase inhibitors

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Abbreviations

AML	Acute myeloid leukemia
BMDSC	Bone marrow-derived stem cells
CML	Chronic myeloid leukemia
DKK3	Dickkopf-homologue 3
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor/ErbB-1
EMP	Epithelial-to-mesenchymal plasticity
EMT	Epithelial-to-mesenchymal transition
GISTs	Gastrointestinal stromal tumors
HER2	Human epidermal growth factor receptor 2/ErbB-2
HER3	Human epidermal growth factor receptor 3/ErbB-3
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-inducible factor 1α
HNC	Head and neck cancer
HUVECs	Human umbilical vein endothelial cells
MET	Mesenchymal-to-epithelial transition
MMPs	Matrix metalloproteinases
NSCLC	Non-small cell lung cancer
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cells

Introduction

Cancer remains a leading cause of death worldwide. More than half of the adults born after 1960 are expected to be diagnosed with cancer at some point during their lifetime [1]. Our understanding of the molecular basis of cancer has evolved remarkably during the past two decades, and massive parallel sequencing endeavors performed by The Cancer Genome Atlas program, the International Cancer Genome Consortium, and numerous individual investigators have identified a broad range of recurrent genetic mutations and structural rearrangements driving tumorigenesis. In concert with this, the pharmaceutical industry has developed a wide range of molecularly targeted therapeutics. However, in spite of this progress, most cancer patients with advanced disease do not experience durable clinical responses [2].

The confounding reality for anticancer drug development is the heterogeneity of tumors [3, 4]. The overall landscape of inter-tumor and intra-tumor heterogeneity comprises both genetic and epigenetic components evolving from the founding clone. In concert with genomic instability, the breakdown of normal tissue structure during malignant progression exposes tumor cells to numerous biophysical challenges, nutritional deprivation, and a hostile nonnative microenvironment comprising different matrix proteins and a variety of stromal cells. How this mutational landscape influences reciprocal tumor-stroma interactions is less well understood. The tumor microenvironment triggers adaptive, cellular plasticity programs related to stem cell differentiation and transdifferentiation, characteristic of adult tissue homeostasis and repair. This endows tumor cells with a remarkable phenotypic and functional flexibility engendering malignant attributes of stemness, invasiveness, chemotherapeutic resistance, immune evasion, metastasis, and poor prognosis. Hence, delineating the molecular mechanisms necessary to induce and sustain the cellular plasticity and how the resulting phenotypic diversity contributes to therapy resistance is a key health challenge [5].

Tumor Plasticity and Therapeutic Resistance

Malignant tumors are appropriately considered “quasi-organs” [6], an abnormal tissue comprising tumor cell hierarchies and a dynamic microenvironment, which interacts tightly with the body’s healthy cells and organs and evades an immune response against its oncogenic mutant proteins. Within this shifting landscape, the tumor cell population displays a significant phenotypic variation or plasticity [7]. Importantly, these acquired malignant traits are in part attributed to cellular plasticity programs governing normal embryonic development, wound healing, and adult organ homeostasis [8]. For example, carcinomas, which are epithelial-derived tumors, encompass nearly 80% of human malignancies and display a remarkable phenotypic diversity reflective of the normal epithelial cell hierarchies. Such a cellular hierarchy provides the carcinoma cells with a repertoire of cellular functions, similar to those required to form and maintain adult organs [9–11].

This epithelial cell plasticity is engendered by the epithelial-to-mesenchymal transition (EMT) program, a complex embryonic transdifferentiation program whereby nonmotile, polarized epithelial cells within cohesive planar barrier-cell sheets degrade their cell-cell junctions and convert into solitary migratory mesenchymal cells with enhanced cell survival attributes [12, 13]. Epithelial-to-mesenchymal plasticity (EMP), comprising EMT and the reverse, mesenchymal-to-epithelial transition (MET), is regulated by several dedicated developmental transcription factors that act in concert with epigenetic mechanisms including DNA methylation, histone modifications, and microRNAs to affect the expression of hundreds of genes in concert [14]. Interestingly, epithelial plasticity has been shown to be governed largely by microenvironmental cues from the local “niche,” comprised of growth factors, cytokines, extracellular matrix (ECM), oxygen tension, and tensile forces that control cellular signal transduction systems [15].

The process of EMT is well established as a critical component of successful embryonic development [16]. EMT regulators also induce epithelial plasticity during mammary gland development, and evidence suggests that EMT-related gene expression is a primary component of adult mammary epithelial stem cells [17]. Epithelial-to-mesenchymal plasticity is apparent in adult epithelial cell hierarchies mediating conversions between stemlike and more differentiated progeny [18, 19]. It has been shown that induction of EMT in differentiated adult epithelial cells may induce stem cell traits consistent with dedifferentiation [20, 21]. This indicates that aspects of the embryonic EMT gene program have been assimilated into the adult epithelial cell hierarchies to mediate cell state conversions. In parallel, carcinomas have the ability to assume an intermediate or hybrid EMT state along an epithelial-to-mesenchymal continuum providing an expanded functional repertoire [22].

Phenotypic plasticity within tumors is considered the main source of stem cell-like traits driving tumor initiation capacity and drug resistance and underpinning recurrence and metastasis [5]. EMT gene signatures correlating with stem cell-like traits were shown to predict poor patient survival in several malignancies [23, 24]. The EMT transcriptional program was initially associated with drug resistance and later invasiveness and metastasis. Induction of tumor plasticity is critical for the invasive behavior of malignant tumors, and blockade of EMT can inhibit metastasis [25]. Epithelial plasticity in the metastatic microenvironment allows metastatic cells to suppress cell migration while enhancing stem cell traits during metastatic site colonization to reestablish cellular hierarchies [11, 26]. In this respect, metastatic cells resemble multipotent epithelial progenitors found in adult epithelial cell hierarchies.

While recent reports challenge the notion that EMT is required for all metastasis, these studies concluded that EMT is required for resistance to chemotherapy [27, 28]. The ability to dynamically switch between different phenotypic cellular states is closely linked to acquired drug resistance [29]. Tumor cells that can readily alter gene expression programs and assume new phenotypes in response to therapeutic challenge are more likely to survive, and thus it is of importance to understand the molecular basis of tumor cell metastable EMT states [5]. The dynamic nature of tumor plasticity has important clinical implications for the design of anticancer therapeutic strategies, and the identification of novel druggable therapeutic targets that can impact the fundamental mechanisms underpinning cellular plasticity is crucial to overcome acquired therapy resistance [30]. In this context, the receptor tyrosine kinase Axl has emerged as a promising candidate to target tumor cell plasticity.

Axl Is Associated with a Wide Range of Malignancies and Poor Clinical Outcome

Axl belongs to the TAM family of receptor tyrosine kinases (RTKs), comprising Tyro3, Axl, and Mer. The TAM family of RTKs is the youngest family of RTKs, and the relatively late evolutionary development of this tyrosine kinase family underscores its important role as immune and homeostasis regulators [31]. The TAM

family of RTKs shares a common unique molecular structure and is activated via the ligands Gas6 and protein S [31]. Gas6 has the highest affinity for Axl, while protein S is a ligand for Tyro3 and Mer. Vitamin K-dependent carboxylation of the ligands is essential for kinase activation. Gas6 activation of Axl has been associated with varying cellular functions in different cell types, including growth, proliferation, migration, aggregation, and survival, through different downstream signal transduction pathways (Fig. 15.1). For a comprehensive review of Axl activation and downstream signaling, see [31].

Axl is correlated with a remarkably wide range of solid tumor types and myeloid malignancies. In spite of a current dearth of substantiated Axl activating mutations or genetic amplifications, transcriptional upregulation and increased ligand-induced activation is frequently associated with neoplasia [32, 33]. Axl was first isolated and described as a putative oncogene from two patients with chronic myeloid leukemia (CML) in 1988 [32, 34, 35] (see [36] for review). During the ensuing three decades, Axl expression has been associated with most cancer types (Table 15.1).

Axl is linked to poorer prognosis in several cancer types [42, 83, 98], and furthermore to acquired drug resistance [61, 99, 136], and increased invasiveness [137]. Table 15.1 provides an overview of the correlation of Axl with poor outcome in the different cancer forms.

The Role of Axl in Tumor Plasticity

In spite of Axl's prevalence in cancer, there are only sparse reports of activating Axl gene mutations or amplifications common among receptor tyrosine kinases [32, 33]. This indicates that maintaining Axl signaling dynamics in tumors is important and that Axl tumor-specific functions may be incompatible with constitutive activation. Congruently, a recent study shows that constitutive Axl overexpression blocks lung metastasis [138]. Thus Axl may be particularly important in settings requiring adaptive survival to altered or foreign microenvironments, such as during metastatic dissemination or following therapy [57, 108].

The demonstration that Axl expression is induced by EMT transcription factors including Slug, Snail, Twist, and Zeb2 and required for metastasis in breast cancer models solidified the notion of Axl signaling as a regulator of plasticity during malignant progression [42, 139]. This unique relationship between Axl and EMT was bolstered by similar observations in several different cancers [24, 68, 90, 99, 109, 136, 140]. Inhibition of Axl signaling affects EMT transcription levels and reverses tumor plasticity features in carcinoma cells supporting the notion of a maintenance role for Axl signaling in epithelial plasticity [107, 109, 137].

Carcinoma metastases often exhibit histopathologically similar traits to the primary tumor, lacking a mesenchymal phenotype and supporting the requirement of an epithelial phenotype via MET [141–143]. However, studies indicate that this may not be absolute and that the requirement of MET for the outgrowth of metastases at distant sites may depend on the specific organ site [144]. This is consistent

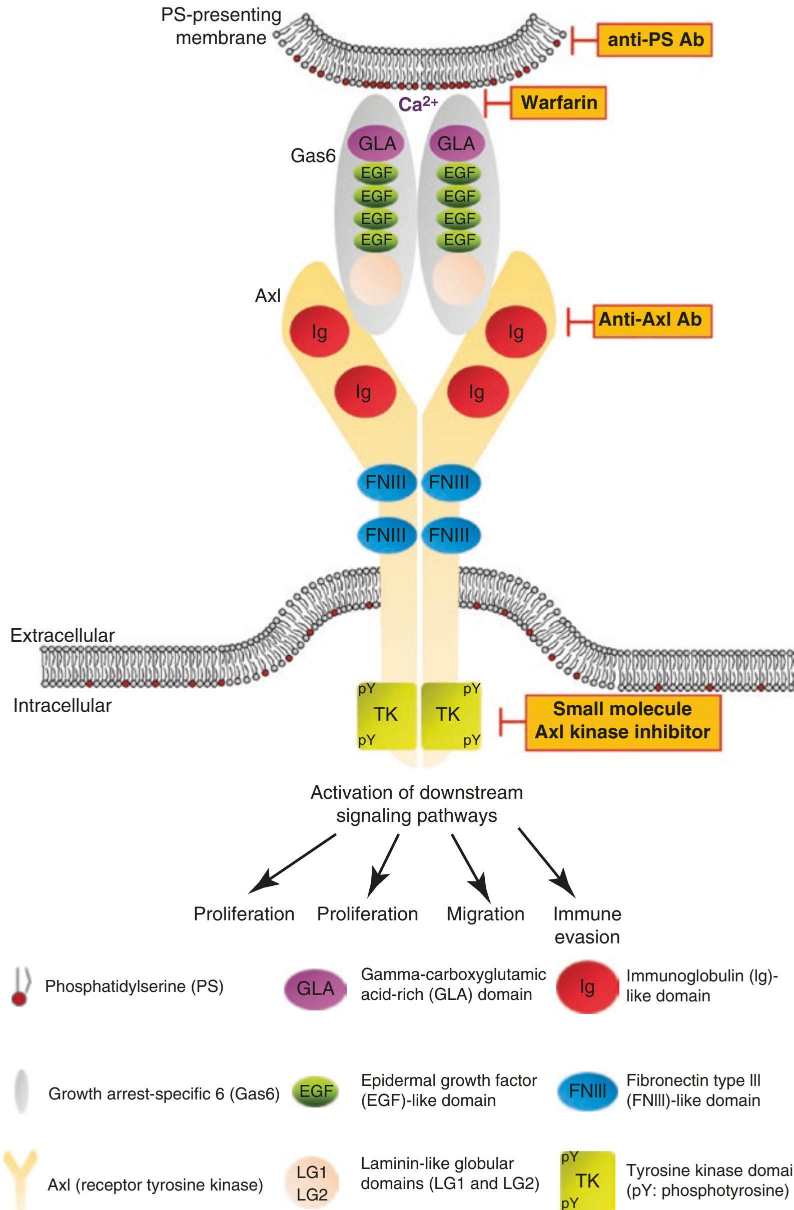

Fig. 15.1 Gas6-Axl activation and therapeutic targeting. The Axl receptor tyrosine kinase is activated by a single protein ligand, Gas6. Gas6 binds phosphatidylserine-containing membranes (e.g., apoptotic cells) via an N-terminal vitamin K-dependent GLA (gamma-carboxyglutamic acid) domain and the Axl receptor through its C-terminal LG domains. This unique activation mechanism results in autophosphorylation of tyrosines on the Axl kinase domain and downstream signaling. Axl signaling can be targeted at several levels including blocking of Gas6-PS interactions with anti-PS antibodies, inhibition of Gas6 posttranslational gamma-carboxylation with vitamin K antagonists (e.g., warfarin), disruption of the Gas6-Axl interaction with compounds or antibodies that recognize epitopes within the Axl Ig-like domain, or Axl-targeting small-molecule kinase inhibitors

Table 15.1 A summary of publications linking Axl expression to various cancer forms and poor prognosis

Malignancies	Upregulation	Human tumor samples	Poor prognosis	Independent prognostic factor
Astrocytic brain tumors	[37–41]	[38, 40, 41]	[38]	[38]
Breast cancer	[42–52]	[42, 46–53]	[42, 52]	[42]
Gallbladder cancer	[54]	[54]	[54]	
GI cancers				
– Colon cancer	[55–60]	[58, 59]	[58]	
– Esophageal cancer	[61–63]	[61, 63]	[63]	
– Gastric cancer	[64, 65]	[64, 65]		
Gynecological cancers				
– Ovarian cancer	[66–70]	[66–70]	[66, 69]	[66]
– Uterine cancer	[71, 72]	[71, 72]		
HCC	[73–76]	[74]	[74]	[74]
HNC	[77–81]	[78, 81, 82]	[78, 81, 82]	[81]
Leukemias				
– AML	[83–86]	[83–86]	[83, 84]	[83, 84]
– CLL	[87–89]	[87–89]		
– CML	[32, 86, 90]	[32, 86]		
Melanoma	[91–94]	[94]		
Mesothelioma	[95–97]			[95]
NSCLC	[24, 55, 57, 98–106]	[98, 99, 104–106]	[103–105]	
Pancreatic cancer	[107–109]	[107, 108]	[107, 108]	[108]
Sarcomas				
– Ewing sarcoma		[110]	[110]	
– Kaposis sarcoma	[111]	[111]		
– Liposarcoma	[112, 113]	[112, 113]	[112]	[112]
– Osteosarcoma	[114–116]	[114]	[114]	[114]
Skin SCC	[117, 118]	[117, 118]		
Thyroid cancer	[119–122]	[119, 120, 122]		
Urological cancers				
– Bladder cancer	[123–125]		[123]	
– Prostate cancer	[126–129]	[127, 129]		
– RCC	[130–135]	[130–132, 134, 135]	[132, 133]	[132]

AML acute myeloid leukemia, *CLL* chronic lymphatic leukemia, *CML* chronic myeloid leukemia, *GI* gastrointestinal, *HCC* hepatocellular carcinoma, *HNC* head and neck cancer, *NSCLC* non-small cell lung cancer, *RCC* renal cell carcinoma, *SCC* squamous cell carcinoma

with measurable gene expression differences between metastases recovered from different organs that also maintain plasticity traits required for establishment of cellular hierarchies [11]. Indeed, in a cohort of breast cancer patients, we noted that Axl expression was maintained in the metastatic lesions when compared to their matched biopsy samples from the primary tumor site, suggesting a requirement of maintained plasticity in the evolving metastatic lesions [42].

A comprehensive proteomic analysis of different EMT signaling states reveals key changes in different cell signaling pathways [145]. Using distinct epithelial, metastable EMT, and "epigenetically fixed" mesenchymal lung tumor cells in an isogenic background, this systems-view study highlights the metastable (hybrid) EMT state. In this metastable state, cancer cells are not epigenetically fixed and are the most aggressive and therapy-resistant cancer cells. This is congruent with separate reports based on transcriptional analysis [22]. During EMT, epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and c-Met phosphorylation are reduced, indicating a loss of signaling via these receptors. Concomitantly, pro-survival IL11/IL6-JAK2-STAT and Ax1/Tyro3/PDGFR/FGFR signaling was increased. Phosphorylated Axl receptor was correlated with acquisition of EMT-related plasticity. This study further demonstrated that Axl was specifically upregulated and activated by EMT induction by Snail in a non-small cell lung cancer (NSCLC) cell system [145]. These emerging signaling landscapes associated with the metastable EMT state provide a novel venue for future targeting strategies against EMT-derived cancer cells and indicate a prominent role for Axl signaling.

Axl-Related Tumor-Stroma Cross Talk

An early indication that Axl is not exclusively a driver of tumor cell proliferation came as the result of a functional genetic screen to identify regulators of cell migration in response to gradients of extracellular matrix, also known as haptotactic cell migration [146]. Strikingly, dominant negative regulators of both Gas6 and Axl were isolated in this screen and independently validated by RNA interference. These results suggest that Gas6-Axl signaling mediates pro-invasive microenvironmental cues consistent with a role of Axl in regulating tumor plasticity and metastasis.

The anticoagulant warfarin, a drug that has been used clinically for more than 50 years for the prevention of thrombosis, blocks gamma-carboxylation of Gas6, which is necessary to effectuate the ligand-induced activation of Axl. In a study of pancreatic ductal adenocarcinoma, inhibiting Gas6-dependent Axl activation with low-dose warfarin, or with tumor-specific Axl-targeting agents, efficiently blocks the tumor-stroma cross talk-mediated progression and spread of pancreatic cancer in vitro and in vivo. The authors concluded that Gas6-induced Axl signaling is a critical driver of pancreatic cancer plasticity and progression and suggest that inhibition with low-dose warfarin or other Axl-targeting agents may improve outcome

in patients with Axl-expressing tumors [109]. These findings are consistent with those made in the 1960s, which reported that warfarin exerts anticancer effects [147]. Kirane and colleagues demonstrate that the molecular mechanism underlying the antitumor effects of warfarin is unrelated to its effect on coagulation, but due to the inhibition of the Axl receptor tyrosine kinase on tumor cells [109].

In the environment of a tumor, the conditions are often hypoxic compared to the surrounding tissue. Hypoxia increases the expression of hypoxia-inducible factor 1α (HIF1α), which in turn promotes increased Axl transcription [133]. Indeed, Axl expression is prevalent in myeloid leukemia where it has been shown to play a key role in mediating cytokine cross talk with the hypoxic bone marrow niche. A study from Ben-Batalla and colleagues [83] demonstrated that acute myeloid leukemia (AML) cells educated the bone marrow-derived stem cells (BMDSCs) to secrete its ligand Gas6, which then mediated proliferation of the Axl-positive AML cells and induced therapy resistance.

Axl is not only regulated by the microenvironment; this communication is a two-way cross talk and Axl can also regulate factors in the tumor microenvironment. Malignant tumors have the ability to invade tumor-surrounding tissues. A crucial trait required for invasion is the capacity to produce matrix-degrading enzymes including matrix metalloproteinases (MMPs). MMP-9 is a type IV collagenase, an important structural component of the basement membrane and the extracellular matrix. It has been shown that Axl enhances the expression of MMP-9, by regulating its promoter activity via the MAP kinase pathway [148].

A study from Martin et al. [138] demonstrated that metastatic mammary carcinoma cells require Axl to maintain a mesenchymal phenotype and metastasis initiating capacity in the lung, consistent with previous reports [42, 137]. Notably, Axl signaling in the lung metastatic mammary carcinoma cells is required for activation of cancer-associated fibroblasts (CAFs) and secretion of thrombospondin 2. Importantly, these CAFs exert a reciprocal effect on carcinoma cell plasticity by downregulating Axl expression and reverting the carcinoma cells into a more proliferative epithelial phenotype. This demonstrates a key role for dynamic Axl signaling in mediating tumor-stromal cross talk in the metastatic niche.

Axl also plays a role in regulating angiogenesis [146, 149]. Studies show that Axl expression is present not only in tumor cells but also in surrounding vascular cells of tumors [38, 106]. Vascular smooth muscle cells (VSMCs) express Gas6, and exogenous application of Gas6 stimulates proliferation and mobility of VSMCs [150]. Axl influences angiogenesis through modulation of signaling, via angiopoietin/Tie2 and dickkopf-homologue 3 (DKK3) pathways [57]. Axl knockdown together with anti-vascular endothelial growth factor (anti-VEGF) therapy blocks in vitro tube formation more efficiently than anti-VEGF therapy alone [57]. Furthermore, a report by Ruan et al. shows that Axl is essential for activation of PI3K/Akt via VEGF-A [151]. Notably Axl knockdown in human umbilical vein endothelial cells (HUVECs) impaired regenerative blood vessel formation in an in vivo tissue engineering model [146], and pharmacological inhibition of Axl reduced angiogenesis in the corneal micropocket and tumor models [139, 146], supporting a role of Axl in angiogenesis.

Axl RTK in Resistance to Cytotoxic Therapies

Axl was identified as one of several genes upregulated in ovarian cancer cell lines with acquired resistance to cisplatin [152]. Since this first indication, Axl expression has been shown to be prevalent in therapy-resistant cancers and a key regulator of acquired drug resistance to a variety of different anticancer agent classes (Table 15.2). This was initially attributed to enhanced anti-apoptotic signaling apparent in several different cancer systems. Axl is a potent activator of the PI3K pathway [162], and activation of PI3K and subsequently Akt and NF-κB is correlated with increased

Table 15.2 Axl in resistance to cytotoxic therapy

Treatment	Malignancy	Reference
Radiation	HNC (SCC)	[78]
	Pancreatic cancer	[108]
Platinum compounds: – Cisplatin – Carboplatin	AML	[153]
	Astrocytoma	[39]
	Esophageal adenocarcinoma	[61]
	HNC (SCC)	[78]
	Neuroblastoma	[154]
	NSCLC	[106, 140]
	Ovarian cancer	[152]
Anthracyclines: – Doxorubicin	AML	[153]
	Breast cancer	[155]
	CML	[155]
	NSCLC	[106, 156, 157]
	Skin cancer (SCC)	[158]
Alkylating agents: – Temozolomide	Astrocytoma	[39]
Tubulin inhibitors: – Taxanes: - Paclitaxel - Docetaxel	Breast cancer (TNBC and BCSC)	[155, 159, 160]
	CML	[155]
	NSCLC	[140, 157, 160]
– Vinca alkaloids: - Vincristine	Breast cancer	[155]
	CML	[155]
	Neuroblastoma	[154]
	NSCLC	[157]
Topoisomerase inhibitors: – Etoposide	AML	[153]
	Breast cancer (BCSC)	[159]
	NSCLC	[106]
	Skin cancer (SCC)	[158]
Antimetabolites: – Fluorouracil	Breast cancer (TNBC)	[47]
	Colon cancer	[161]

AML acute myeloid leukemia, *BCSC* breast cancer stem cells, *CML* chronic myeloid leukemia, *HNC* head and neck cancer, *NSCLC* non-small cell lung cancer, *SCC* squamous cell carcinoma, *TNBC* triple-negative breast cancer

expression of multiple anti-apoptotic proteins (e.g., Bcl-2, Bcl-XL, and Puma) and inactivation of pro-apoptotic factors such as caspase-3 [37, 154]. Also, Akt-mediated phosphorylation of Bad blocks the inhibitory interaction between Bad and the anti-apoptotic proteins Bcl-2 and Bcl-XL. The cumulative effect is the prevention of apoptosis and increased cell survival [36, 163].

Overexpression of the plasma membrane efflux pump *P*-glycoprotein (*P*-gp) is another common mediator of multidrug resistance in cancer cells. In adriamycin-paclitaxel-vincristine-resistant breast cancer and CML cell lines, Axl and P-gp are co-upregulated, and siRNA knockdown of Axl results in decreased P-gp expression and decreases resistance to these agents in vitro and in vivo [155]. Drug-resistant Axl-overexpressing cell lines are also more invasive in vitro, which can be attenuated by Axl silencing [155]. Axl expression also blocks apoptosis by inhibiting c-Abl/p73 signaling in response to DNA damage in p53-deficient esophageal adenocarcinoma cells [61]. In pancreatic cancer, Axl downregulation increases apoptosis following radiation in vitro, as measured by PARP cleavage [108]. In head and neck squamous cell carcinoma, Axl inhibition sensitizes Axl-expressing cells to radiation, with data indicating that Axl mediates DNA double-strand break repair. In HNC xenograft and patient-derived xenograft models, intrinsically radioresistant tumors have high Axl expression and phosphorylation [78]. Inhibition of Axl in putative cancer stem cell populations sensitizes these inherently chemoresistant cells to cytotoxic chemotherapy, suggesting a role for Axl in protecting stem cell-like populations of cells [158, 159].

An analysis of 643 human cancer cell lines found a strong correlation between Axl expression and a mesenchymal, drug-resistant phenotype [160]. Mesenchymal NSCLC cells are more resistant to chemotherapeutic agents, and Axl inhibition sensitizes cross-resistant EGFR-mutated NSCLC cells to taxanes and also to other anti-mitotic agents such as aurora kinase inhibitors, which activates the mitotic spindle checkpoint. Co-treatment also resulted in dephosphorylation of the cyclin-dependent kinase-1(CDC2), which regulates mitotic entry [160]. Acquisition of resistance to cisplatin is shown to impair sensitivity to subsequent gefitinib treatment through induction of EMT, and Axl is found to be responsible for increased motility of cisplatin-resistant cells [164].

Axl RTK in Resistance to Molecularly Targeted Therapies

Upregulation of Axl has been demonstrated to be a key mechanism of acquired drug resistance to several molecularly targeted anticancer therapies, including several compounds directed toward the ErbB family of RTKs, including EGFR, human epidermal growth factor receptor 2 (HER2/ErbB-2), and human epidermal growth factor receptor 3 (HER3/ErbB-3). In addition to regulating phenotypic plasticity, Axl is reported to heterodimerize with other RTKs and diversify downstream signal transduction that circumvents molecularly targeted kinase inhibitors [79, 165]. Table 15.3 provides an overview of Axl in resistance to targeted therapies.

Table 15.3 Axl in resistance to targeted therapeutics

Target	Drug	Malignancy	Reference
ALK	Crizotinib	NSCLC	[166]
ALK^{F1174L}	TAE684, ceritinib	Neuroblastoma	[167]
Bcr-Abl	Imatinib, nilotinib	CML	[90, 168, 169]
c-Kit	Imatinib	GIST	[170, 171]
EGFR	EGFR-TKI (erlotinib, gefitinib)	NSCLC (EGFR mutated)	[24, 99, 100, 103, 140, 172–174]
		NSCLC (EGFR wild type)	[140, 157]
		Breast cancer (TNBC)	[165]
		HNC (SCC)	[82]
		Colon cancer	[175]
		Liver cancer	[175]
		Tongue cancer	[175]
	Irreversible EGFR-TKI (CO-1686)	NSCLC	[176]
	Cetuximab	HNC (SCC)	[77, 78]
		NSCLC	[77]
FLT3	PKC412, AC220	AML	[177]
HER2	Lapatinib, trastuzumab	Breast cancer, including TNBC	[136, 178]
		Ovarian cancer	[178]
	AZD8931 (EGFR/HER2/HER3 inhibitor)	Breast cancer	[179]
IGF-IR	MAB39 (IGF-IR-blocking Ab)	Rhabdomyosarcoma	[180]
MAPK pathway in BRAFV600E	BRAF inhibitors (PLX4720, vemurafenib, dabrafenib) MEK inhibitors (AZD6244/ selumetinib, trametinib) ERK inhibitor (SCH772684)	Melanoma	[94, 181]
MEK	MEK1 inhibitor	Pancreatic cancer	[182]
PI3Kα	BYL719	Esophageal SCC	[79]
		HNC (SCC)	[79]
TRAIL	Recombinant TRAIL	Esophageal adenocarcinoma	[183]
VEGF	Bevacizumab	Colon cancer	[184]
	Anti-VEGF	Breast cancer	[140]
		NSCLC	[140]
	Sunitinib	RCC	[135]

The Role of Axl in Resistance to ErbB Family Targeted Therapy

NSCLC tumors with EGFR-activating mutations are currently treated with EGFR small-molecule tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib. Although initially effective, acquired resistance to these targeted agents

represents a significant obstacle to clinical efficacy. Several explanations have been postulated to explain the lack of response to EGFR-targeted therapy, including mutations in the binding sites of the targeted drug, as well as secondary effector mutations affecting downstream signaling molecules in the EGFR-activated pathway [185]. In addition to secondary mutations in EGFR (T790M) and upregulation of the c-Met kinase, the presence of Axl RTK has been shown to limit the response to EGFR-targeted inhibitors in NSCLC [99]. Additionally, EMT has been recognized as an important mechanism of non-mutational resistance to EGFR inhibitors [186]. EMT signatures derived from patient samples and cell lines with EGFR-TKI resistance highlight a potential role for Axl [24], and independent estimates establish Axl expression in approximately 50% of NSCLC samples correlated with advanced stages and poor clinical outcome [98, 104, 187].

In an analysis of matched human samples before and after EGFR-TKI treatment, Axl was found to be upregulated in 20% of resistant specimens [99]. Axl expression and activation is also detected in NSCLC tumors with EGFR-TKI resistance mutations [99, 187, 188]. In acquired resistance to third-generation mutant-selective EGFR-TKIs, resistant cells displayed an EMT gene signature and upregulated Axl [176]. A study using the cell line H820 harboring both the T790M mutation and c-Met amplification validated Axl overexpression as an important contributor to EGFR-TKI resistance [172]. Furthermore, in patients with primary resistance, Axl expression was found in 22% of tumors [173]. Analysis of HCC827 NSCLC xenograft tumors with in vivo-acquired resistance to erlotinib showed Axl upregulation present in 88% of tumors [99]. Importantly, Axl knockdown effectively reversed erlotinib resistance in this system.

EGFR is frequently overexpressed in triple-negative breast cancer (TNBC), but a response to EGFR inhibitors in this aggressive, inherently chemoresistant disease is lacking. TNBC often expresses high levels of Axl [160], and Axl has been found to be trans-activated by EGFR through a physical clustering interaction, leading to downstream signaling diversification that impacted migration and proliferation in response to EGF [165]. It has been shown across several cancer cell lines (breast, colon, tongue, liver, kidney) that EGFR inhibitor resistance is mediated through hepatocyte growth factor (HGF)/c-Met signaling. EGFR is inactivated by HGF/c-Met, leading to resistance to EGFR inhibition and facilitating interaction of the EGFR with receptors, including Axl [175]. Reduced degradation of Axl has also been identified as a mechanism for gefitinib-induced Axl overexpression [100].

EGFR is targeted in the clinic by the anti-EGFR antibody cetuximab, but primary and acquired resistance is common. Axl expression and activation is increased in cetuximab-resistant cell clones and xenograft tumors from NSCLC and HNC. Inhibition of Axl signaling decreases proliferation, migration, and invasion and increases sensitivity to cetuximab in Axl-expressing HNC cell lines [78]. Axl and EGFR are physically associated in resistant cells and tumors, and overexpression of Axl confers resistance to cetuximab in vitro. Furthermore, EGFR directly regulates the expression of AXL mRNA through MAPK signaling and the transcription factor c-Jun [77, 78].

In breast cancer, Axl upregulation was identified as a resistance mechanism to the HER2 inhibitor lapatinib by mass spectrometry-based peptide sequencing of a protein detected by a phosphotyrosine antibody in cell lines with acquired lapatinib

resistance. Downregulation of Axl by siRNA and inhibition of Axl using small-molecule inhibitors of c-Met, VEGFR, and Axl restored sensitivity to the HER2-targeting agents, lapatinib and trastuzumab [136].

The Role of Axl in Resistance to c-Kit/PDGFR/Bcr-Abl Inhibitors

Gastrointestinal stromal tumors (GISTs) are driven by c-Kit and/or αPDGFR mutations and respond to the c-Kit/PDGFR/Bcr-Abl inhibitor imatinib. A kinase switch from c-Kit to Axl was identified as a novel mechanism of resistance in imatinib-resistant cell lines as well as patient samples [170]. The presence of Axl upregulation was confirmed by immunohistochemistry of human patient samples, and inhibition with the dual Axl/c-Met inhibitor amuvatinib acted synergistically with imatinib, erlotinib, and the covalent EGFR and HER2 inhibitor afatinib in a panel of GIST cell lines [171]. Pan-genomic microarrays of CML cell lines with acquired resistance to Bcr-Abl inhibitors also found increased expression of Axl [189] as a possible mechanism of resistance, and Axl knockdown resensitized the imatinib-resistant CML cells [90]. Furthermore, resistance to the second-generation Bcr-Abl inhibitors, like nilotinib, has been shown to be promoted by a tyrosine kinase (TK) network where Axl and non-receptor TKs Syk and Lyn form a complex. These tyrosine kinases were verified to be upregulated in nilotinib-resistant tumor cells from CML patients [168]. The ubiquitin ligase CBL was identified as crucial in regulating the expression of these TKs, by regulating their degradation. Interestingly, Axl induced resistance in the absence of kinase activity, indicating a scaffolding role for this model [169].

The Role of Axl in Resistance to MAPK and PI3K Pathway Inhibitors

Axl is overexpressed in a subset of melanomas lacking the microphthalmia-associated transcription factor (MITF) and correlates with a more invasive phenotype [91]. Cell lines sensitive to MAPK pathway inhibitors strongly express MITF, and intrinsically resistant lines show low MITF expression and express Axl [94]. However, extrinsic overexpression of MITF can increase resistance to BRAF and MEK inhibitors. Cell lines resistant to BRAF and ERK inhibitors can be divided into two categories: one that maintains high MITF expression upon resistance and one in which MITF expression is lost. These findings were supported by the same pattern in human melanoma samples. The resistant cells that maintained high MITF expression were not cross-resistant to MEK or ERK inhibition, while the resistant cells that lost MITF expression were cross-resistant to a full panel of MAPK

pathway inhibitors. The MITF null cells were more invasive and displayed properties of EMT. Cells with endogenous low MITF expression were intrinsically resistant to BRAF, MEK, and ERK inhibition. Axl was again confirmed to be inversely correlated with MITF and consistently upregulated in cell lines that lost MITF expression during acquired resistance to BRAF inhibition. Targeting Axl increased sensitivity to MAPK pathway inhibition in resistant cells [181].

A recent study from Elkabets and colleagues shows that head and neck and esophageal squamous cell carcinomas refractory to PI3Kalpha inhibition express Axl [79]. Axl dimerization with EGFR results in PLCγ-PKC signaling and subsequent PI3K/AKT-independent mTOR activation. Importantly, inhibition of Axl kinase activity reverses PI3K inhibitor resistance.

For the majority of pancreatic cancer patients, mutated KRAS is a key oncogenic driver, and targeting KRAS directly has so far not been feasible. Targeting downstream pathways with combined PI3K and MEK inhibitors has resulted in high toxicity [190], and MEK inhibition lacks clinical benefit in KRAS-driven tumors. Inhibition of MEK1 drives activation of the PI3K-AKT-mTOR pathway through feedback loops with recruitment of Axl, PDGFRa, and ErbB receptors EGFR, HER2, and HER3. However, inhibition of any single RTK activated by MEK inhibition was shown in a KRAS-mutated mouse model to have no additional benefit, and to achieve antitumor effects, all RTKs activated by MEK inhibition had to be targeted simultaneously [182]. Whether such an approach will be tolerated clinically remains to be explored. In ovarian cancer, simultaneous activation of multiple RTKs has also been established in cell lines and patient samples, and inhibition of Axl, EGFR, HER2, and c-Met by HSP90 inhibitors led to inactivation of these receptors, which inhibited proliferation to a greater extent than single RTK inhibition [67].

Taken together, Axl is upregulated in response to cytotoxic chemotherapy as well as targeted therapies and is upregulated under challenging microenvironmental conditions like serum starvation [83], acidification [191], oxidative stress [192, 193], and laminar shear stress [194]. This suggests that Axl serves a broad function in protecting cells under particularly challenging conditions.

Axl-Targeted Agents in Clinical Development and Clinical Trials

The accumulating evidence supporting involvement of Axl RTK activity as a key mediator of therapeutic resistance has spurred interest in developing Axl-targeting agents for clinical translation [195, 196] (Fig. 15.1). Table 15.4 provides an overview of current experimental Axl-targeting agents in clinical trials registered at *clinicaltrials.gov*. The majority of these are small-molecule kinase inhibitors originally identified as potent c-Met inhibitors. BGB324 (R428) is the only small-molecule inhibitor specifically developed to target Axl kinase currently in clinical trials [197]. Several companies report ongoing preclinical development of selective Axl

Table 15.4 Axl-targeted kinase inhibitors in clinical development

Company	Compound	Target	Indication	Clinical trial	Phase
BerGenBio AS (Bergen, Norway)	BGB324 (R428)	Axl	AML	NCT02488408	I
			NSCLC	NCT02424617	I/II
Betta Pharmaceuticals Co., Ltd. (Beijing, China)	BPI9016M	c-Met, Axl	Advanced solid tumors	NCT02478866	I
Astellas Pharma Global Development (Tokyo, Japan)	ASP2215 (gilteritinib)	FLT3, Axl	AML	NCT02014558	I/II
				NCT02310321	I
				NCT02236013	I
				NCT02181660	I
				NCT02421939	III
			NSCLC	NCT02495233	I/II
Bristol-Myers Squibb (New York, USA)/ASLAN Pharmaceuticals (Singapore)	BMS777607 (ASLAN002)	c-Met, Axl, Ron, Tyro3	Advanced or metastatic solid tumors	NCT00605618	I/II
				NCT01721148	I
Servier (Neuilly-sur-Seine, France)	S49076	c-Met, Axl, FGFR1/2/3	GBM	2013–003079-37	I/II
Mirati Therapeutics Inc. (San Diego, USA)	MGCD265	c-Met, Axl, VEGFR	NSCLC	NCT02544633	II
			Advanced malignancies	NCT00697632	I
				NCT00679133	I

AML acute myeloid leukemia, *GBM* glioblastoma, *NSCLC* non-small cell lung cancer

inhibitors. The results from ongoing trials with specific Axl inhibitors are of particular interest to provide proof-of-principle evidence for the clinical efficacy of Axl inhibition in the therapy-resistant setting.

Conclusions and Future Perspectives

Despite the steady introduction of improved molecularly targeted compounds and tailored treatment regimens, inherent and acquired therapy resistance remains a confounding reality for cancer patients and a significant obstacle to successful cancer therapy. Cellular plasticity is increasingly recognized as a major mediator of therapy resistance and metastatic dissemination of cancer cells, although the underlying molecular mechanisms are still largely unexplored. The Axl receptor has emerged as a prominent mediator of cellular plasticity, and as summarized in this chapter, upregulation and activation of Axl RTK is shown in a wide range of both solid and liquid malignancies with a poor prognosis. Accumulating evidence has further demonstrated Axl expression and signaling as a central mechanism of acquired resistance to both cytotoxic and molecularly targeted therapies. Thus, the use of Axl as a predictive biomarker in this context deserves further exploration in

clinical studies. Due to its possible role as a mediator of cancer cell plasticity, Axl is an exceptionally promising molecular target for contemporary treatment regimens under evaluation in ongoing clinical trials with specific Axl inhibitors.

Conflict of Interest Statement J.B.L. has ownership interest in BerGenBio AS. The remaining authors do not declare any potential conflicts of interest.

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Part II
Organ Related Biomarker Studies

Chapter 16
Gene Expression Signatures of the Tumor Microenvironment: Relation to Tumor Progress in Breast Cancer

Elisabeth Wik and Lars A. Akslen

Abstract Cancer cell invasion and progression toward the metastatic stage are biological processes that have been studied for a long time. There is not one all-inclusive model that encompasses the complete picture of the different conditions and pathways operating in human tumors. Application of gene expression signatures is one way of mining the complex tumor landscape, and this has been proposed to represent a robust method to reflect the many signaling systems.

This chapter gives an update on gene expression signature studies related to breast cancer progress. Signatures reflecting cancer-associated stroma, in particular tumor fibroblasts, parts of the vascular system, and signature profiles pointing to immune-related alterations, are in focus. Several signature studies support that a combination of extracellular remodeling, activated vascular biology, and immune-related signaling takes place during breast cancer progress. Stromal alterations and processes are likely to represent a wide spectrum of novel biomarkers and companion treatment targets.

Keywords Breast cancer • Tumor progress • Tumor microenvironment • Gene expression signatures • Tumor-associated stroma • Cancer-associated fibroblasts • Vascular biology • Immune-related signatures • Extracellular matrix

Introduction

Tumor cell invasion and metastasis are multistep processes that are immensely detrimental to the organ in which they grow and the organism as a whole. The route to cancer dissemination is suggested by distinct steps: local infiltration, intravasation, and transport of cancer cells in the lymphatic or hematogenous systems, followed by

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extravasation of tumor cells from the vessels into the tissue parenchyma of the new site where micrometastases may form and grow to macroscopic lesions [1, 2]. The English surgeon Stephen Paget postulated "the seed and soil hypothesis" in 1889, suggesting that tumor cells (denoted "seeds") have affinity for specific tissue environments (denoted "soil") in certain organs [3]. Paget literally seeded a hypothesis followed by many researchers studying cancer invasion and the metastatic process the next century.

Before setting off on the invasion-metastasis cascade, it is crucial that tumor cells fulfill prerequisites such as the ability to detach and move from the original colony, with unlimited proliferative potential and a capacity to evade from destruction [4]. The underlying effectors in the invasion-metastasis cascade are suggested to be classified as *metastasis initiating*, *metastasis progressing*, and *metastasis virulent* [5]. Metastasis-initiating genes generate a supportive environment that facilitates tumor infiltration to surrounding tissue. Expression of such genes, in the epithelial cells or the microenvironmental compartments, may promote angiogenesis, vascular invasion, epithelial-mesenchymal transition (EMT), and evasion from immune destruction with important implications to the processes involved in cancer metastasis.

The microenvironment is regarded to play a crucial role both in embryonic organ development and in cancer invasion, two processes with several similar features [6]. Cellular and molecular interactions between the epithelial cells and the microenvironment, and between elements within the microenvironment, are demonstrated in functional differentiation of the normal mammary development. Exploiting the normal microenvironment programs, by a form of "hacking" these pathways, is suggested as potential ways of promoting cancer invasion [7] and may be reversely exploited when targeting the metastatic processes in the therapy setting. For example, in 2015, it was demonstrated in a xenograft model of breast cancer that neutrophils within the lung microenvironment were identified as drivers of establishing lung metastases and to support metastatic initiation [8]. Inhibiting a specific enzyme (Alox5) abolished the pro-metastatic neutrophil activity in the lung microenvironment and reduced the occurrence and growth of lung metastases.

Genes supporting *metastasis progression* promote extravasation and survival of the cancer cells outside of their original environment [5]. Cancer cells that have entered the circulation may subsequently extravasate and infiltrate distant organs. For colonization to occur, where the disseminated cancer cells reside in their new microenvironment and grow into macro-metastases, adaptation of the tumor cells to this new environment is required. Specific cancer cell gene expression has been implicated to direct organ-specific tropism. One example is the expression of IL-11, which facilitates breast cancer metastases to the bone [9]. The establishment of a "receptive" environment at the future metastatic location before the colonization of tumor cells (the *pre-metastatic niche)* is suggested as a mechanistic model explaining metastatic organotropism [10]. Cancer-specific factors released from the primary tumor promote changes in the future metastatic microenvironment before the tumor cells arrive to this location. Also, bone marrow cells may migrate to the pre-metastatic niche in response to the systemically released factors, facilitating the environment for the cancer cells to "thrive" [11, 12]. A recent study demonstrated that tumor-derived exosomes with specific cargo prepare the microenvironment at future metastatic sites [13]. The increasing focus of research on the tumor microen-

vironment, in the preinvasive lesions, in primary tumors, in the pre-metastatic niches, and in the metastatic lesions, coupled with the role of the microenvironment in embryonic development, strongly supports an important role for the stroma both in normal processes and in cancer development and progression. Moreover, the components of the tumor microenvironment have been regarded as genetically more stable than the tumor cells. This is another factor that should render the stromal components a strategic focus when searching targets for therapy.

Since the discovery of cell signaling, researchers have debated how to best reflect alterations of pathways and levels of pathway activation in different model systems. One major trend in cancer research has been to undertake relatively simple approaches (e.g., measuring one protein or one specific mutation) when searching for markers of deregulated pathways as prognostic and potentially predictive markers. One important question to as then, is what is the effect of this strategy on the clinical translation of the research findings?

Global gene expression data may have a stronger potential to reflect the complexity of cancer biology as compared to the detection of single gene alterations. Additionally, it may be a more powerful platform for identifying markers for more complex biological processes taking place in the cancer cells. When taking the global expression pattern into account, we somehow compensate for the lack of knowledge regarding "the complete picture" of specific signaling pathways and the phenotypic consequences including potential compensatory mechanisms derived from their deregulation.

Beginning in the early part of this century, gene expression arrays have been increasingly applied in translational cancer research. Some of the first array studies within this research field demonstrated that gene expression data could identify known and novel cancer subclasses with similarities in terms of biological behavior [14, 15]. In addition to identifying molecular phenotypes in various cancer types [16–19], transcriptional alterations have demonstrated to be powerful tools for creating classifiers predicting cancer recurrences [20–23] and to identify alterations in functional pathways, thereby suggesting relevant targets for therapy [24].

Improved Understanding of Cancer Biologic Processes

Oncogenic and non-oncogenic alterations underlie and support the cancer biological processes leading to cancer progress and metastatic disease. High-throughput techniques such as DNA microarrays and RNA sequencing measure the expression of large numbers of genes in a single experiment. From a gene expression perspective, this enables multifaceted views of the phenotypes being studied and also allows the associations between complex gene expression alterations and phenotypes to be examined.

In the era of global gene expression studies, two hallmark reports in the field introduced the potential of exploring biological function via studies of gene expression alterations [25, 26]. By studying how the gene expression pattern changed when altering the conditions from fermentation to aerobic metabolism in the yeast *Saccharomyces cerevisiae*, deRisi and colleagues characterized this metabolic

reprogramming at a functional genetic and biochemical level [25] and were among the pioneers in applying large-scale gene expression data to biological questions. deRisi also demonstrated how the gene expression pattern changed according to deletion or overexpression of specific transcription factors and proposed applying DNA gene expression microarrays for examination of the "signature pattern" accompanying for instance DNA mutations, and to translate this information into drug screening. deRisi stated: "Perhaps the greatest challenge now is to develop efficient methods for organizing, distributing, interpreting, and extracting insights from the large volumes of data these experiments will provide" [25]. And he was right: although such "global analyses" have assisted in some of the major progresses made in translational cancer research, the issues deRisi raised are still a major challenge in translating "omics" analyses into biological-relevant information.

Hughes and colleagues published one of the earliest reports taking into account the signaling complexity when relating gene expression data to genetic and phenotypic alterations [26]. Here, the functions of uncharacterized genes were identified through mapping of gene expression alterations induced by specific gene deletions to transcriptional profiles of known perturbations. A few years later, Huang and colleagues demonstrated that the expression pattern from several genes included in a "metagene", characterized and predicted the neoplasm classes under study [27]. These were among the early "precursor studies" to the many reports on gene expression signatures that followed the next decade.

Several studies have since been conducted that support the assertion that multigene markers better reflect the complexity in the signaling of multiple pathways [28–30], as demonstrated by Huang et al. for *MYC* and *HRAS* pathways [27].

Gene Expression Signatures as Biomarkers

The Biomarkers Definitions Working Group defines "a biomarker" as: "A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." [*Biomarkers Definitions Working Group*, [31]]. D. Hanahan and R.A. Weinberg describe in their two *Hallmarks of Cancer* reviews tumor biologic processes and enabling characteristics that are essential for tumor initiation and progression to take place [32, 33]. Gene expression signatures might reflect such hallmark characteristics of a tumor as well as specific biological processes, and may as such function as biomarkers. Gene expression signatures have been published as prognostic markers in cancer, have assisted in identifying targets for therapy, and have in this context also been suggested as predictive markers for specific cancer therapies.

In a study by Bild and colleagues, the integration of gene expression characteristics of multiple elements into a signature demonstrated to reflect the activation level of signaling pathways following specific oncogenic alterations (e.g., *MYC* and *RAS* activation) [28]. Further, the signature clusters were associated with patient outcome, demonstrating a prognostic effect of these signatures. Bild suggested that oncogenic signatures may reflect the oncogenic phenotype and point to tumor bio-

logical processes underlying the phenotypic alterations. Moreover, measures of pathway deregulation in this study were linked to therapy response to drugs targeting components of specific pathways. In this manner, Bild suggested a potential for gene expression signatures as markers guiding therapy selection.

Lamb and colleagues defined in one of their Connectivity Map papers "the ultimate objective of biomedical research": *To connect human diseases with the genes that underlie them and drugs that treat them* [34]. He regarded this "a daunting task," but aimed for a solution. The tool Connectivity Map was developed, aiming to reveal functional connections in diseases and linking these to genetic perturbations and drug actions [34]. As part of this endeavor, a reference bank of gene expression signatures derived from the effects on cultured human cells treated with small molecules (e.g., approved drugs and other bioactive compounds) was established. Bioinformatic analyses were integrated in a publicly available (online) tool, making it possible to match any other signatures (also the "homemade" ones) to drug signatures, thereby enabling researchers to pattern-match the specific gene expression profiles under study with gene expression profiles reflecting effects of the small molecules tested as part of the Connectivity Map database [34, 35]. In the primary publication of the Connectivity Map, the authors demonstrated this tool as a powerful resource to link gene expression patterns to functional effects and biophysiological processes as well as targets for therapy in various diseases. However, the Connectivity Map is suggested as a hypothesis-generating tool, and the importance of validating the findings in other model systems is stressed by the authors.

Gene Expression Signatures in Breast Cancer

In the era since microarray analyses entered the cancer research field, many breast cancer gene expression signatures have been published. Perou and colleagues explored global gene expression data in breast cancer and identified molecular classes [17]; these were further demonstrated with clinical relevance in follow-up studies [18, 36]. In the same decade, van 't Veer described a "poor prognosis gene expression signature" [21]. Subsequently, gene expression signatures like MammaPrint, Oncotype Dx, PAM50, and Genomic grade Index (GGI) have been approved by the FDA and have demonstrated prognostic value for breast cancer patients in general as well as within subgroups (e.g., stage I/II, ER-positive breast cancer in postmenopausal women, for the Oncotype DX [37]).

These early signatures were primarily derived as part of a "whole-tissue approach," focusing on enrichment of the epithelial component of the tissue samples included. This implies a lower expression signal to the signature scores from the stromal cells as compared to the epithelial component [38]. Thus, it is important to determine whether there is a potential benefit of also studying the stromal gene expression contribution. For prognostication, adding data about the stromal components could add important information. To better understand the tumor microenvironmental processes taking place in cancer development and progression, the

microenvironment definitely needs to be specifically focused, as part of an integrated approach to gain knowledge about epithelial-microenvironmental interactions.

Gene Expression Signatures Reflecting the Tumor Microenvironment

As the role of the tumor stroma came on to the stage in discussions of the mechanisms for cancer progression, researchers stepped aside from gene expression analyses of "whole-tissue" material to focusing on specific tumor compartments. Gene expression changes related to the tumor microenvironment (TME) in cancer have been increasingly studied in many types of cancer, especially breast cancer. Cell-specific alterations (e.g., gene expression changes in immune cells, endothelial cells, cancer-associated fibroblasts, adipocytes) have been described and gene expression signatures generated. Such microenvironmental signatures might help elucidate biological processes critical for the progression of cancer and may thereby show glimpses of light at the still so blurred path of tumor progression and development of metastatic disease. In the following sections of this chapter, thematic groups of gene expression signatures are elucidated, reflecting biological processes and acting as prognosticators and predictors of therapy response.

Allinen and colleagues were among the first to define the "bulk tumor" approach in gene expression analyses as problematic when exploring stromal features in cancer [39]. They aimed to elucidate cellular interactions along with paracrine regulatory modules in breast cancer and reported the transcriptional and genetic alterations in various cell types in invasive breast cancer, ductal carcinoma in situ, and normal breast tissue. All cell types were purified, and the gene expression pattern of the cell types such as the epithelial cells, myoepithelial cells, myofibroblasts, fibroblasts, endothelial cells, and leukocytes was described. Among several novel descriptions in this study, the identification of an upregulation of CXCL14 and CXCL12 specifically in tumor myoepithelial cells and myofibroblasts was demonstrated to cause epithelial cell proliferation and invasion via the binding of these ligands to their cognate receptors on epithelial tumor cells [39]. This study uniquely examined cell type-specific gene expression programs and additionally validated the functional consequences of these alterations and, as such, enlightened a novel method to study tumor-stroma interactions.

Gene Expression Signatures Reflecting the "Unspecified" Cancer-Associated Stroma

As the cancer stroma is composed of several cellular components, examination of general stromal gene expression alterations may again bring us into problems of low specificity with regard to which cell type generates the different expression signals. However, the literature on general stromal signatures demonstrates new information as compared to what was derived from the studies on "whole-tissue approaches," as elucidated in the following section.

Several gene expression signatures derived from the tumor stroma have been published, with some of them also analyzed with regard to disease progress. Two studies explored the differences in the tumor stroma by assessing preinvasive ductal carcinoma in situ lesions and invasive breast carcinomas. Ma and colleagues assessed the global expression alterations specifically in the stromal and epithelial compartments [40] and demonstrated comprehensive gene expression changes in the tumor-associated stroma during progression from normal to the preinvasive and invasive states. A gene expression signature reflecting histologic tumor grade was identified in the stromal compartment. This study added support to the hypothesis that tumor-stromal-related changes contribute to tumor progression, specifically in the step from preinvasive to invasive disease.

In a similar manner, Roman-Peréz and colleagues compared the expression pattern of tumor-adjacent tissue from invasive carcinomas and ductal carcinoma in situ and identified breast cancer subtypes defined by extra-tumoral expression patterns [41]. Two distinct "microenvironmental subtypes" were identified, denoted as "active" and "inactive" types. Tumors with "active signature" shared features of claudin-low breast cancer and were associated with TGF-β-induced activation score. The "active signature" also correlated with tumor aggressiveness and clinical outcome in ER-positive breast cancer.

In supervised analyses of global gene expression data, gene expression patterns between different predefined groups have been examined. What would be the best groups to compare when investigating the microenvironmental alterations that support or drive tumor progression? Normal versus cancer? Normal versus preinvasive in situ lesions? The preinvasive cases versus cancer? Or simply (although a more complex analytical approach) the whole sequence from normal through preinvasive and eventually invasive carcinomas? In the following section, studies approaching this challenge in different ways are summarized.

Troester and colleagues compared global expression patterns of normal breast tissue from reduction mammoplasty resections and normal breast tissue adjacent to tumor tissue. A 155-gene "cancer-adjacent normal tissue" signature was derived [42]. Genes reflecting constituents of the extracellular matrix, and remodeling of this, as well as genes of inflammation were enriched in this signature (Fig. 16.1). Further, some of the signature genes were known to be involved in cell adhesion, angiogenesis, and reepithelialization such as keratins. Interpreting these transcriptional findings in a functional manner, similarities to wound healing were seen, and this signature was regarded to reflect an in vivo "wound response." The signature strongly associated with breast cancer survival, indicating that tumor-related microenvironmental responses might be of importance in the progression of breast carcinomas.

Finak and colleagues applied tissue laser-capture microdissection (Fig. 16.2a) to assess the gene expression pattern of tumor stroma in primary breast cancer [43]. Several gene expression signatures identified in this series associated with disease course. The 26-gene signature denoted "stroma-derived prognostic predictor" pointed to contrasting immune responses and angiogenic and hypoxic responses in different tumors. This signature also predicted prognosis, as validated in multiple breast cancer data sets (Fig. 16.2b, c). Based on clustering of the 26-gene signature, the authors suggested stroma-dependent breast cancer subtypes. The stroma signa-

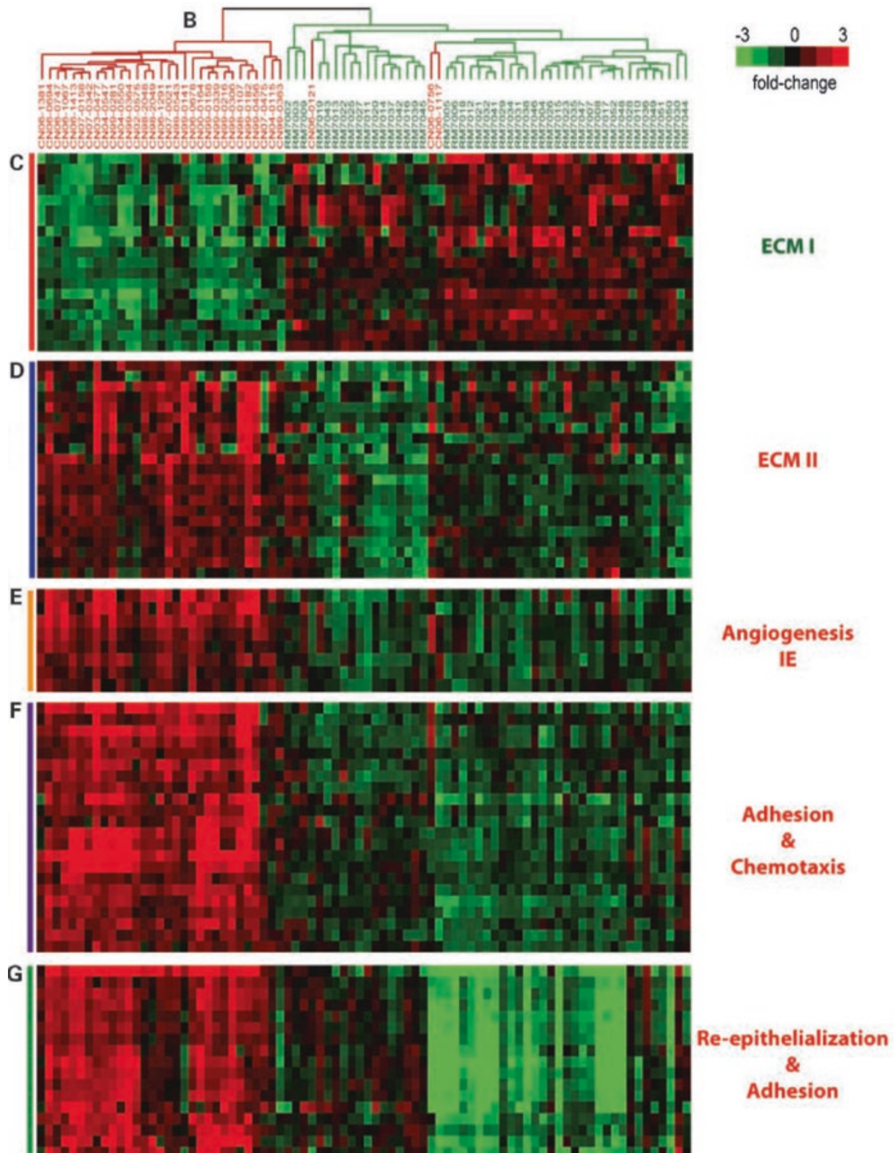

Fig. 16.1 A wound response signature of genes differentially expressed between tissue from reduction mammoplasty and histologically normal tissue of cancer patients. The dendrogram in cancer-adjacent normal samples (*red*) and reduction mammoplasty samples (*green*) in (**a**). The clusters show that various biological processes involved in wound repair are differentially expressed between the two groups, including ECM alterations (**b**, **c**), immediate early (IE) genes involved in angiogenesis (**d**), reepithelialization and cellular adhesion (**e**), and cellular adhesion and chemotaxis (**f**). (Adapted from Clinical Cancer Research, 2009, 15/22, 7020–8, Troester et al., "Activation of Wound Host Responses in Breast Cancer Microenvironment," with permission)

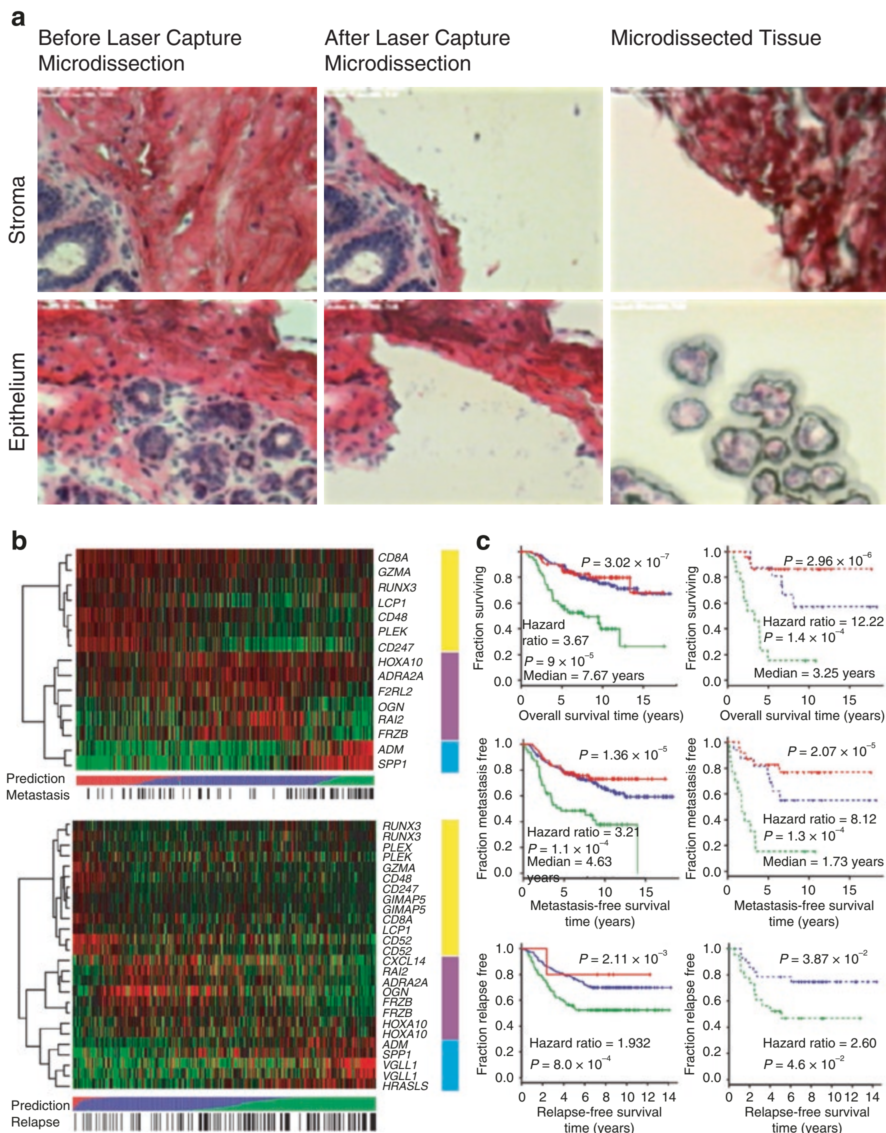

Fig. 16.2 Laser-capture microdissection of breast carcinomas to assess gene expression pattern of tumor stroma (**a**). The "stroma-derived prognostic predictor" predicted relapse and metastatic disease and pointed to contrasting immune responses and angiogenic and hypoxic responses in different tumors (**b**). The signature also predicted prognosis, as validated in multiple breast cancer data sets (**c**). (Figure 16.2a is reprinted from Breast Cancer Research, copyright 2006, **8**:R58, Finak G. et al., with permission from BioMed Central Ltd. Figure 16.2b, c are reprinted by permission from Macmillan Publishers Ltd.: Nature Medicine 14(5):518–27, copyright 2008)

ture of Finak predicted clinical outcome independent of other signatures, which were also associated with prognosis, indicating that the "stroma-derived prognostic predictor" mirrors specific biological processes taking part in directing the clinical disease course. In this study, Finak and colleagues demonstrated an independent stromal impact within the tumor, showing that genes of their "stroma-derived prognostic predictor" did not predict prognosis when assessed in the epithelial component.

When combining the stroma signatures identified by Finak with other signature scores of prognostic value, the prediction of metastatic disease improved, suggesting an even better reflection of the stroma-related processes when merging signatures developed in different analytical approaches.

How do the stromal and epithelial cells communicate? Are we able to reflect the interplay between these two compartments by the use of gene expression data? To address these questions, Casey and colleagues examined the transcriptomic expression pattern of epithelial and stromal cells, both in normal breast tissue and in invasive breast cancer [44]. Cell type-specific interactions were also assessed. A "motile phenotype" was identified in the epithelial compartment and a "reactive phenotype" in the stromal compartment, with genes reflecting remodeling of the extracellular matrix in a proteolytic manner in the invasive cancer. Also, genes promoting epithelial-mesenchymal interaction, such as fibroblast activation protein alpha (FAP), were identified. This study interestingly supports a molecular crosstalk between the epithelial and stromal cell compartments and suggests that alterations facilitating invasion are one of the features of cancer-associated stroma.

By examining global gene expression alterations relating to specific tumor microenvironment elements that are microscopically assessable, it might be possible to identify underlying alterations of the histopathologic phenotype. Van den Eynden examined fibrotic tumor foci and associated gene expression patterns [45] and demonstrated Ras signaling and HIF1α-pathway activation along with other hypoxia- and angiogenesis-related genes in the large fibrotic foci. Also, fibrotic foci correlated with an activated wound healing signature [46] and with earlier development of distant metastases.

What would be the model system best fit to capture ongoing microenvironmental processes promoting tumor progression? Marchini and colleagues examined the transcriptomic alterations in A17 mouse mammary carcinoma cells [47]. Three gene expression signatures reflecting stroma-related features and processes were identified: One "stemness signature," one "angiogenesis signature," and one "signal transduction signature." These signatures are associated with mesenchymal stem cell signatures, ER-negative breast cancer, a basal-like phenotype, and breast cancer bone metastases. In post treatment assessment of breast cancer xenograft models, the A17 angiogenesis and signal transduction signatures were more highly expressed after hormonal therapy. This study indicates a linkage between mesenchymal features, tumor progression, and therapy resistance, directing an interpretation of these findings toward epithelial-mesenchymal transition (EMT). EMT is regarded as having critical importance in tumor progression [48], and recent studies indicate that epithelial-mesenchymal plasticity contributes to stemlike tumor features and generates cancer stem cells [49–51].

Are the tumor microenvironmental changes in cancer progression common or specific across tumor types? Planche and colleagues examined this question by laser microdissecting stromal cells of invasive breast and prostate carcinoma. These two tumor types displayed distinctly different stromal gene expression patterns [52]. The expression alterations of the cancer type-specific stromal genes clustered both breast and prostate cancer samples into groups with different disease courses. Of note, genes of extracellular matrix constituents and proteolytic enzymes were upregulated in the invasive breast cancer stroma, in line with the observations done on the tumor histology sections.

Most mRNA expression studies extract RNA from tissue that is snap frozen in the surgical theater, as the RNA is best preserved for quantitative analyses in this manner, as compared to that extracted from formalin-fixed paraffin-embedded (FFPE) tissue. However, as of the writing of this chapter, FFPE patient-derived tissue is generally more widely available, as it is stored in pathology labs worldwide. Winslow and colleagues made a critical step forward in this field when they succeeded in studying gene expression alterations from laser-dissected tumor epithelial and stromal compartments from FFPE-invasive breast cancer samples. This study found that stroma-specific gene expression signatures segregated into three major thematic groups: (1) extracellular matrix- and fibroblast-related genes, (2) vascular-related genes, and (3) immune cell-related genes. Strikingly, the immune-related signature is associated with basal-like breast cancer subtype [53]. As the results from this study were in line with other similarly designed studies on fresh frozen tissue, the study gave new hope for RNA studies on FFPE tissue.

A few studies have related global gene expression data to specific molecular microenvironmental alterations. Specifically, a relationship between CD10+ stromal cell expression and breast cancer progression was previously reported [39], and Desmedt and colleagues followed up on this by exploring gene expression alterations related to CD10+ stromal cells [54]. A “CD10+ stroma signature” of 12 genes was generated by comparing the gene expression patterns of CD10+ cells isolated from breast carcinomas and normal breast tissue. In co-culture experiments, the CD10+ cells were characterized as specific cell populations: fibroblasts, myoepithelial cells, and mesenchymal stem cells. As seen in many of the stroma- and CAF-derived signatures, the CD10+ signature was composed of genes related to matrix remodeling. Interestingly, genes related to osteoblast differentiation (e.g., osteopontin) were also upregulated in the CD10+ signature. All the different CD10+ cell types contributed to this stroma-related signature; however, the highest CD10+ stroma signature score was found in mesenchymal stem cells. Of clinical value, the signature was able to differentiate in situ and invasive breast cancer lesions. Also, the CD10+ signature demonstrated a potential to predict response to chemotherapy, and high CD10+ stroma score associated with reduced survival in HER2-positive breast cancer cases. This study is a good example of how to combine in vivo and in vitro studies, specifically with respect to validating the functionality of a gene expression signature.

In another study describing gene expression alterations reflecting specific molecular alterations, Rajski and colleagues identified a signature associated with IGF-I-

stimulated stromal cells [55]. Among the IGF-I signature genes, there was enrichment of proliferation-associated genes. This signature clustered the cancer samples in two major groups: those with upregulated IGF-I and those without. Cases in the cluster with genes upregulated by IGF-I experienced shorter survival.

An example of a signature related to specific histopathologic tumor features is one necrosis-related signature derived from gene expression alterations between endometrial carcinomas with and without tumor necrosis [56]. In this case, tumor necrosis was found to be associated with gene expression programs of hypoxia, angiogenesis, and inflammatory responses.

The cancer biology underlying phenotypic features of various cancer types may be cancer specific but also share commonalities with other diseases and noncancerous conditions. West and colleagues exploited the potential of approaching the research question from a different angle, when postulating that fibroblasts present with different activation states. Their approach to this question was to distinguish fibroblast populations in noncancerous samples [57]. They found that solitary fibrous tumors and desmoid-type fibromatosis exhibited different expression patterns. In particular, the expression of growth factors and extracellular matrix genes were differentially expressed. When assessing the gene signature separating solitary fibrous tumor from desmoid-type fibromatosis in a series of invasive breast cancer, two groups of breast carcinomas were identified, and patients in these two groups presented with different survival. The cases with an expression pattern similar to the desmoid-type fibromatosis showed more favorable outcome, while the other group showed variable expression of genes enriched in solitary fibrous tumors and was observed with poorer prognosis. These findings supported the hypothesis that tumor stromal response varies among carcinomas of different aggressiveness.

Gene Expression Signatures Reflecting Cancer-Associated Fibroblasts

Most of the studies mentioned above have investigated tissue stroma and thereby potentially reflect expression contribution from the combination of different stromal cell types. Many of the stromal signatures correlate with clinicopathologic features and disease course, and seem to reflect underlying stroma biology. Still, it is tempting to ask: What is the contribution to the signatures from each of the specific stromal cell types?

Chang and colleagues were among the first to generate a pure fibroblast signature, where the expression alterations were generated by exposure of fibroblasts to serum [46]. The signature was denoted a “core serum response.” Functional analyses revealed involvement of the signature genes in myofibroblast activation, matrix remodeling, and cell motility. All these processes contribute to wound healing. Based on the expression of the “wound signature,” breast cancer samples segregated into two groups. The group with activated signature pattern was associated with increased risk of metastatic disease and death from breast cancer. Further, the signa-

ture pattern was consistent in paired samples of locally advanced breast carcinomas, biopsied before and after chemotherapy, indicating stability of the biological program reflected in this signature [46]. Interestingly, the basal-like molecular breast cancer subtype significantly associated with the expression pattern of the "wound healing signature," suggesting that the signature points to intrinsic properties of the basal-like phenotype. The signature was also examined in gene expression data sets of various tumor types, and the findings were striking: The expression pattern of this signature separated the cases in two groups, with significantly increased risk of metastatic disease in the group with the activated signature pattern. Harold F. Dvorak suggested in a review in 1986 the wound as an analog to the stromal processes observed in tumors [58]. The gene expression signature by Chang might have captured some of the alterations observed by Dvorak.

Also, Tchou and colleagues demonstrated subtype-specific stromal gene expression patterns in breast cancer [59]. In this analysis, the expression profiles of CAFs from breast cancer samples of the HER2-positive subtype, triple-negative cases, and ER-positive cases were distinctly different. In particular, pathways linked to the cytoskeleton and integrin signaling were differentially enriched in the different CAF groups. The results from this study add to the arguments of specific "stroma subtypes" in breast cancer and support the hypothesis that fibroblasts participate to the disease biology underlying clinically relevant breast cancer subtypes.

Two projects, exploring transcriptional alterations in tumor-associated fibroblasts compared to normal mammary fibroblasts, demonstrated an increased expression of genes involved in tumor progression in the CAFs. Cytokines, genes related to remodeling of the extracellular matrix, and genes reflecting paracrine or intracellular signaling as well as cell-matrix interactions were upregulated in the tumor-associated fibroblasts [60, 61]. In the study by Singer, it was noted that these gene expression alterations take place also in the isolated cell culture state, in the absence of adjacent malignant epithelium [61]. In the study by Bauer, the CAF-associated genes were incorporated into a 31-gene signature that was validated by qPCR. Some of the genes upregulated in CAFs were validated by immunohistochemistry, with respect to location and quantitation [60]. Taken together, the findings from these two studies are supportive with respect to indicating a fibroblastic subpopulation of the tumor stroma that facilitates tumor progression.

By comparing global gene expression patterns of platelet-derived growth factor (PDGF)-stimulated human fibroblasts and resting fibroblasts, Frings and colleagues identified a 113-gene expression signature reflecting PDGF-activated fibroblasts [62]. This signature had the potential to identify breast cancers with a stroma of PDGF-stimulated fibroblasts. The signature correlated with high expression of the PDGF receptor β (PDGFRβ) and its ligands, and was enriched for genes related to angiogenesis and regulation of the extracellular matrix. Signature analyses in several breast cancer data sets demonstrated associations between the PDGF signature score and clinicopathologic features reflecting aggressive tumors, such as large tumor size, high histologic grade, and HER2-positive and ER-negative tumors. Moreover, signature activation correlated with the HER2-positive, basal-like, and luminal B subtypes of breast cancer. In line with these observations, the signature

demonstrated a robust association with survival. High signature score correlated with reduced survival, specifically in multivariate analyses when adjusted for other stroma signatures and a proliferation signature.

Siletz and colleagues assessed transcription factor signatures and activity (by array method) specific for mammary CAFs versus normal mammary fibroblasts [63]. A transcription factor activity signature included activation of reporters for ELK1, GATA1, retinoic acid receptor, serum response factor, and vitamin D receptor (VDR). An increased activation of reporters for HIF1, and several STAT and proliferation-related transcription factors, was seen after induction of fibroblasts by conditioned medium from breast cancer cell lines. These transcription factor activity profiles indicate CAF subtype-specific signaling that promotes tumor progression through a pro-invasive stroma.

Validating the potential functionality of gene expression signatures is a challenging task. A study by Navab and colleagues analyzed how the gene expression patterns in matched CAFs and normal fibroblasts promote tumor progression in lung cancer cell lines [64]. A 46-gene signature was enriched for genes encoding proteins regulated in particular by the TGF-β signaling pathway. The involvement of TGF-β was confirmed by additional network analyses. Additionally, a subset of the signature genes was induced by TGF-β. Eleven of the signature genes predicted prognosis in several non-small cell lung cancer data sets. This study demonstrates one example of functional validation with good study design.

Woelfle and colleagues derived a signature of 86 genes differentially expressed between primary tumors with and without bone marrow metastases [65]. Although the tumor microenvironment was not the focus of this study when deriving this signature, the majority of the signature genes were related to extracellular matrix remodeling, cytoskeleton plasticity, and cell adhesion. Also, RAS- and HIF1α signaling were enriched in tumors with bone marrow metastases. The many similarities between this signature and the stroma- and CAF-related signatures described above lead to an intriguing perspective on this signature. In addition to facilitate invasive growth and tumor progression, perhaps the tumor stroma is heavily involved in directing tumor metastases to different locations? Another interesting perspective of this signature was that 77 of the 86 signature genes were downregulated in primary tumors with bone marrow metastases, indicating transcriptional repression as part of the picture in tumor progressive processes.

A few studies have examined transcriptional alterations related specifically to the extracellular matrix in breast cancer. Bergamaschi and colleagues set out to classify breast carcinomas based on constituents of the extracellular matrix (ECM), selecting 278 ECM-related genes from the literature [66]. These ECM-related genes segregated the breast cancer samples into four ECM classes with different clinical courses. The ECM group that associated with best survival showed upregulation of protease inhibitors of the serpin family. The ECM group associated with poorest survival presented with overexpression of integrins and metallopeptidases and low expression of laminin chains. In a follow-up study, Triulzi and colleagues demonstrated that one of the ECM groups consistently predicted one cluster in several independent breast cancer data sets [67]. The 58-gene signature of this ECM-subset

contained 43 genes encoding structural ECM proteins. Investigation of gene expression data sets on separate cancer epithelial and stromal cells demonstrated that genes of this ECM signature were expressed both by the epithelial and stromal compartments. In vitro experiments showed induction of signature genes, in particular in fibroblasts and in ER-negative breast cancer cells. Single genes and gene sets reflecting EMT were significantly associated with this ECM signature.

Gene Expression Signatures Reflecting Vascular Biology

Various measures of histologically verified tumor vasculature (e.g., mean vessel density, vascular proliferation) are related to tumor progress and metastatic disease in solid cancer types. The vasculature is viewed as a target for therapy, as exploited in therapeutic programs in several tumors. Studies on genomic programs measuring the transcriptional alterations have a strong potential to reveal novel aspects of vascular biology in malignant tumors.

With this in mind, Wallgard and colleagues sought to elucidate the transcriptome and molecular processes specific to endothelial cells [68]. Fifty-eight genes specifically linked to microvascular expression were identified, many of them not previously described in relation to functions of endothelial cells. Wallgard suggested several genes, in particular those expressed on the cell surfaces, to be further explored in relation to drugs targeting the microvasculature.

The vasculature is regarded as the main route for breast cancer metastases, and comparing the global transcription pattern of primary tumors and distant metastases might point to vascular-related biology. Hu and colleagues applied this approach and identified an in vivo hypoxia signature reflecting VEGF activation and also predicting poor clinical outcome in breast cancer and other tumor types [69]. This 13-gene signature was composed of several angiogenesis-related genes, and 8 of the 13 genes contained binding sites for the hypoxia-related transcription factor HIF1α and had been demonstrated to be regulated by HIF1α.

Pepin and colleagues identified two tumor vasculature types by analyzing global transcription patterns across laser-capture microdissected tumor-associated and matched normal vasculature [70]. The two tumor vasculature types demonstrated specific gene expression signatures. One of these was related to anti-angiogenic signaling. Samples enriched for this signature demonstrated lower mean vessel density as compared to the group enriched for the gene signature associated with active vascular remodeling and reduced vascular shear stress. Reduced vascular shear stress is suggested to reflect reduced vessel flow rate and may reflect an inappropriate tumor perfusion. Significantly, several therapeutic targets with potential relevance in anti-angiogenic treatment (e.g., MET, PDGFRβ, ITGAV) were differentially expressed between the vasculature subtypes.

When studying alterations in vascular gene expression, different study design may reveal different layers of the full picture. In a supervised manner, in an analysis of genes known to be angiogenesis related, Bender and colleagues demonstrated

that the gene expression of the VEGF and semaphorin families was altered in pro-angiogenic manners [71]. A signature of these genes was associated with triple-negative breast cancer and also with reduced survival in this tumor subtype.

Wallace and colleagues approached angiogenesis-related biology in a more indirect manner. In an analysis of genes and pathways mediating fibroblast contribution in cancer progression [72], the authors studied how Ets2 function varied between mammary stromal fibroblasts and epithelial cells. In HER2-positive breast cancer mouse models, Ets2 inactivation in fibroblasts reduced tumor growth. The same effects were not seen when inhibiting Ets2 in epithelial cells. An Ets2-dependent gene signature was derived, enriched in genes related to remodeling of the extracellular matrix, cell migration, and angiogenesis. Supportive to these functional interpretations, Wallace found fewer functional blood vessels in tumors lacking Ets2 in the fibroblasts. The Ets2-dependent gene expression signature was able to segregate human breast cancer stroma and normal stroma. This study indicated a link between Ets2 and the fibroblast-endothelial cross talk and points to a contribution of Ets2 in the angiogenic process.

Xiao and colleagues [73] have developed in vitro models studying breast cancer-specific endothelial cells and have identified multiple subpopulations of tumor-associated endothelial cells, each population with distinct gene expression patterns. For several of the genes, a relationship with tumor-associated endothelial cells had not previously been established. For example, the genes *Irx2* and *Zfp503* were highly upregulated in tumor endothelial cells but had no previous known relevance to vascular biology. These genes are known to regulate neuronal patterning and developmental differentiation [74, 75] and may point to new information on vascular-related mechanisms and co-regulatory circuits in vascular biology.

Mannelqvist and colleagues published an 18-gene expression signature related to vascular invasion in endometrial carcinomas, also relating to features of aggressive disease and disease outcome [76]. In a follow-up study on multiple breast cancer gene expression data sets, the vascular invasion signature was associated with tumor progression and clinical course in breast cancer [77]. Also, a high signature score associated with the basal-like phenotype and response to neoadjuvant chemotherapy. The signature was composed of genes related to angiogenesis, immune response, and extracellular matrix biology. Further, the vascular invasion signature identified by Mannelqvist correlated with other gene expression profiles of vascular biology, hypoxia, EMT, immune response, and tumor progression.

The same research group later published a 32-gene signature reflecting tissue-based vascular proliferation. Microvessel proliferation was assessed by dual endothelial immunostaining of factor VIII/Ki67, and global gene expression data as well as copy number information were explored in supervised manners [78]. Several genes in the signature had previously been linked to processes such as neovascularization, endothelial cell migration, and adhesion, supporting this signature as relevant for tumor angiogenesis. Also, amplification of the region 6p21, potentially harboring VEGF, associated with high microvessel proliferation.

Harrell and colleagues sought to determine whether tumor-associated vascular properties could identify mechanisms contributing to the different risks of meta-

static disease across the intrinsic subtypes of breast cancer [79]. They found that claudin-low and basal-like tumors were enriched for transcriptional programs reflecting vascular quantity, vascular proliferation, and a VEGF/hypoxia signature. Incorporating several of the vascular gene signatures described above added information about risk of metastatic disease. Furthermore, experimental studies demonstrated that claudin-low cells exhibited endothelial-like morphology, and claudin-low xenograft tumors were highly perfused through intercellular spaces and nonvascular tumor cell-lined channels. This study combines the transcriptional studies with experimental validation in an interesting manner and demonstrates both endothelial-like characteristics of cancer cells and how the vasculature in conceptually new manners may contribute to breast cancer progression. Also, the gene expression signatures were suggested as predictive markers to anti-angiogenic therapy.

Pitroda and colleagues explored how vascular inflammation influences cancer prognosis [80]. A gene expression signature reflecting inflammation in tumor-associated endothelial cells was developed. The endothelial-derived 6-gene inflammatory signature predicted reduced overall survival in breast cancer and other tumor types. Also, inflammatory pathways activated in endothelial cells linked to tumor progression in mice, supporting a vasculo-immunogenic link contributing to tumor progression in breast cancer.

Gene Expression Signatures Reflecting Immune-Related Alterations

The role of the immune system is one of the emerging areas in cancer research and has become important in many cancer-related discussions: its role in cancer prevention, as part of therapeutic strategies, as a prognosticator and predictive marker, and not least its role in tumor progression. The last part is not yet well understood. It is probably more realistic to state that per today (2016) we have likely reached the beginning of this field, seeing the picture as T.S. Eliot reflected: “What we call the beginning is often the end. And to make an end is to make a beginning. The end is where we start from.”

Perou and colleagues had in their early breast cancer classification study touched upon the transcriptional heterogeneity of ER-negative breast cancer [17]. Teschendorff and colleagues followed up on this and demonstrated transcriptional alterations associated with the clinical course of ER-negative breast cancer [81]. Distinct subclasses among ER-negative tumors were shown based on transcriptional patterns. One of the classes consisted of basal-like tumors with upregulation of genes related to immune response and complement activation. This subset of ER-negative samples demonstrated better survival pattern as compared to the rest of ER-negative tumors. Based on this study, a seven-gene immune response signature was derived. Downregulation of this module associated with increased risk of advanced disease.

Rody and colleagues followed up on this study and focused on the clinically and prognostically heterogeneous triple-negative breast cancer subtype [82]. The basal-like and claudin-low subtypes were described by metagenes reflecting angiogenesis, inflammation, and nonneoplastic cell types like immune cells, adipocytes, and fibroblasts. High immune cell score associated with improved survival, and high inflammation and angiogenesis scores correlated with reduced survival. Rody defined a ratio of the B-cell and IL-8 metagenes, and a subgroup (32%) of triple-negative cases with high B-cell and low IL-8 scores experienced improved outcome.

Further, two other breast cancer studies have underpinned the association between an immune response and tumor subsets with milder disease courses [83, 84]. In the study by Alexe and colleagues, a HER2-positive subtype with low recurrence rate associated with high expression of lymphocyte-associated genes [83]. Also, a prominent lymphocytic infiltration was seen by histologic examination of these tumor cases. In the study by Schmidt and colleagues, high B-cell metagene score associated with metastasis-free survival in node-negative cases with high proliferation, as validated both in high-grade cases and in young breast cancer patients [84].

Schmidt and colleagues [85] followed up on this study and aimed to identify one single immune system marker for cancer progression. Immunoglobulin κC (IGKC) demonstrated similarly predictive and prognostic value as the entire B-cell metagene [84], (Fig. 16.3). IGKC gene expression associated with improved survival across different molecular subtypes in node-negative breast cancer. Also, levels of IGKC measured by immunostaining in a series of FFPE breast cancer tissues correlated with clinical outcome. Tumor-infiltrating plasma cells were identified as the source of the protein. The findings suggest further exploration of the humoral immune response and its relevance in the therapeutic setting.

One such study specifically examined genes related to TH1-mediated adaptive immunity in breast cancer [86] and demonstrated that inflammation and immune suppression predicted tumor subsets with different clinical outcomes. Data sets on various tumor types were analyzed, and Hsu showed that upregulation of the TH1-mediated adaptive immunity genes correlated with good prognosis in young breast cancer patients (<45 years).

Two other studies demonstrated better survival in cases of high immune signature score in breast cancer [87, 88]. In the study by Bianchini, high expression of a B-cell/plasma cell signature associated with improved survival in ER-positive cases with high proliferation, specifically when adjusting for standard prognostic variables and other transcriptional scores [87]. In the study by Nagalla, a cluster of cases without distant metastases associated with genes related to immunological functions. These genes could be clustered into three major "immune metagenes," one cluster reflecting B-cells and/or plasma cells, another cluster reflecting T-cells and natural killer cells, and a third cluster reflecting monocytes and/or dendritic cells [88]. In tumors of high proliferation, high immune metagene score associated with reduced risk of metastasis. Cases with low immune metagene scores associated with poorer outcome.

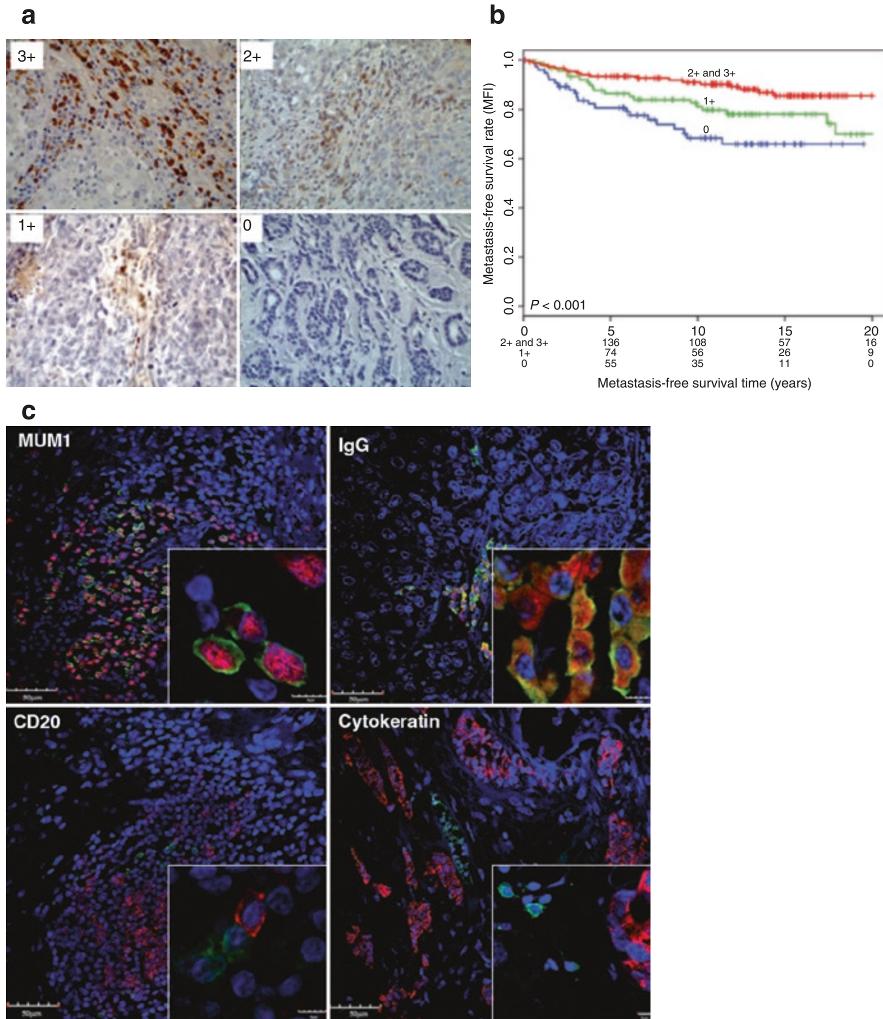

Fig. 16.3 IGKC immunostaining intensities in ductal breast carcinomas; note the expression within the desmoplastic stroma in-between tumor cell nests (**a**). Low IGKC immunostaining intensity associates with shorter metastasis-free survival (**b**). In (**c**), coexpression by immunofluorescence of IGKC with a marker for plasmablasts and plasma cells [MUM1/IRF4]; IgG coexpression illustrates the isotype switch; B-cell marker CD20; epithelial cell marker cytokeratin shows tumor cells. IGKC is visualized by green fluorescence, whereas MUM1/IRF4, IgG, CD20, and cytokeratin emit a red signal. (Reprinted from Clinical Cancer Research, 2012, 18/9, 2695–703, Schmidt et al., "A Comprehensive Analysis of Human Gene Expression Profiles Identifies Stromal Immunoglobulin kappa C as a Compatible Prognostic Marker in Human Solid Tumors," with permission)

A few studies of immune-related signatures have suggested therapy strategies based on the findings. Ascierto and colleagues [89] elucidated how immune function networks related to tumor-infiltrating immune cells were more highly expressed in cases without recurrent disease. These network genes were related to B-cell development, interferon signaling, and autoimmune reactions as well as antigen presentation pathways. The results indicated cross talk between the adaptive and innate immune systems. Five B-cell response genes predicted relapse-free survival (>85% accuracy), also validated by qPCR. The authors thus suggested immunotherapy, in the neoadjuvant setting, to patients with high risk of recurrent disease, potentially by inducing genes of immune function.

Iglesia and colleagues aimed to elucidate transcriptional alterations related to the cancer immune response of breast and ovarian cancers with high lymphocyte infiltration and improved survival [90]. RNAseq data and a microarray data set were applied to identify signatures reflecting the adaptive immune response. The B-cell signatures predicted improved survival in the basal-like and HER2 subtypes. Further, analyses of B-cell receptor sequences were assessed through RNAseq data. It was previously shown that a clonal expansion of the B-cells and somatic hypermutations in B-cell tumor-infiltrating lymphocytes in breast tissue represent an antigen-directed response [91–93], and the response of antigen-specific B-cell populations actively demonstrate features of clonal expansion. A part of the basal-like and HER2-enriched cases with shorter survival showed upregulation of BCR gene segments with low diversity, indicating lack of B-cell clonal expansion, and was also indicative of an ineffective antigen-directed response in these cases, potentially contributing to their poorer prognosis. More and varied BCR segments with increased expression associated with improved prognosis. The results indicate a limited B-cell antitumor response in a subset of basal-like breast cancer. Also, immunomodulatory therapies were suggested, and supporting B-cell responses may be one relevant approach in B-cell-infiltrated carcinomas.

Perez and colleagues developed a transcriptional signature of immune-related genes predicting clinical benefit in a clinical trial of adjuvant trastuzumab in combination with chemotherapy in HER2-positive breast cancer [94]. Signature enrichment associated with increased recurrence-free survival only in the study arms receiving trastuzumab. Cases in the trastuzumab study arms without immune signature enrichment did not benefit from trastuzumab, suggesting interactions between immune-related genes and therapy response.

As we see, immune-related signatures associate with improved survival in several studies. However, when it comes to immune responses, the picture is not black and white. Rody and colleagues elucidated how the transcriptional changes of immune metagenes related to clinical outcome [95]. An IgG metagene, which was found to be a marker for B-cells, did not associate with prognosis. However, high expression of a T-cell-/lymphocyte-specific kinase signature associated with survival in ER-negative cases and cases of concurrently ER and HER2 positivity. This study also suggests inhibition of the IL-8 pathway as a potential therapeutic strategy in breast cancer. Adding to the complexity, a link between the EMT program and immune evasion seen in cancer has been suggested recently [48, 96–98].

Methodological Aspects of Gene Expression Signatures

When exploring biological characteristics of the tumor microenvironment and the ongoing processes underlying cancer development and progression, we may feel like Mr. Jones in the song of Bob Dylan (1941–): "… something is happening here, but you don't know what it is. Do you, Mr. Jones?" How can we best capture "what is going on" in the microenvironment surrounding the tumor? When using global gene expression data, is there a "perfect" way of picturing the stromal activities? The statistician George E.P. Box (1919–2013) stated that "All models are wrong, but some are useful," indicating that not one single model is able to catch the complete picture, and combining different and complementary approaches is probably one way out.

In dealing with gene expression analyses as one model, we most likely assess relevant information about the processes and pathway signaling taking place in the tumor microenvironment. But the results from our studies are, as always, dependent on the input and analytic strategies. Microarray analyses can be divided into *unsupervised* and *supervised* analyses. The former requires no supplementary information to the expression data. The latter is driven by sample characteristics, typically in two groups, e.g., "positive" versus "negative" molecular phenotype and high versus low tumor stage.

Unsupervised Analyses and Class Discovery: Unbiased Exploring

By unsupervised analyses, without guidance by additional data except for the gene expression information itself, the aim is to find patterns in the expression profiles where no predefined class is presented. *Hierarchical clustering* is one example of unsupervised analysis. This method aims to group together objects based on measures of similarity and dissimilarities between them [99]. Hierarchical clustering requires specification of *similarity metrics* and *linkage*. The *similarity metric* describes how similar two samples are, by reflecting the distance between two samples. Additional information for the distance between clusters is needed, and this is in the hierarchical cluster analyses reflected by the *linkage method* (single, average, or complete linkage). Complete linkage is demonstrated to be superior for clustering genes [100], while for clustering of samples, both average and complete linkages are proven useful [101]. Validation of the identified clusters is crucial, including validation of both biological and clinical plausibility, and the level of statistical evidence.

Supervised Analyses: Genes Differentially Expressed Between Groups

Identifying genes with known functions that are differentially expressed between two groups may provide better understanding of biological differences between the predefined groups [101]. If the genes identified are of unknown function, the

analyses have the potential to provide novel insight into new gene functions. Supervised analyses require supplementary information about the groups, such as clinicopathologic data or molecular phenotypic data. An increased risk of false positive findings due to *multiple testing* occurs as we run, e.g., 20,000 tests simultaneously on the same data, when searching for genes differentially expressed between classes. There are various methods to adjust for multiple testing, all of them with the aim to provide greater certainty that the genes in our analysis output are truly differentially expressed between the groups we examine and not listed due to chance. Being very strict in the multiple testing adjustments might mask true biological effects. The adjustments will thus be a "trade-off" between too few and too many genes correctly identified as differentially expressed between classes. It is generally accepted that applying filters that results in no false positive genes in the output is a too stringent approach. When searching for single genes differentially expressed between classes, the genes identified should nevertheless be further validated, and elimination of false positive candidate genes or biomarkers occurs at these stages. In the search for the optimal cutoff on the output lists, it is important to remember that "statistical significance does not imply biological relevance."

The number of genes differentially expressed between classes might be reduced to a limited number of genes with specific biological and/or prognostic information and presented as *gene expression signatures*. Such signatures (i.e., gene sets) might be regarded as *metagenes* with respect to expression value, and a *signature score* is calculated to evaluate the *metagene expression value* [27]. Such signature scores have been derived in various ways [27, 102–104]. One simple approach is to generate a "sum score" or "average score" (the score value of one sample equals the sum or the average of the expression values of the genes in the signature). One potential way of better preserving the biological information in a signature score is an algorithm where each sample is given a score value by subtracting the sum of downregulated genes from the sum of upregulated signature genes. More complex algorithms for derivation of gene expression signatures exist [105], and which algorithm to select depends on what you want to demonstrate by use of the signature and how the signature gene list is derived.

Gene Sets Differentially Expressed Between Classes

Gaining further insight into biological mechanisms involved in a given process is a major challenge when working on high-throughput gene expression data. Subramanian et al. pointed to a few highly relevant obstacles in how to interpret the single-gene lists into new and/or relevant biological information [104]: We may miss information about pathway alterations by single-gene analyses, as the interpretation of these are heavily dependent on the researcher's preexisting knowledge of the field. Pathway signaling may involve large gene networks and thus should not be too focused on "large enough" fold changes of single genes in the search for biological information in our data output. Minor changes in all genes known to be

involved in a signaling pathway may be of higher importance than large fold changes of a few genes. *Gene Set Enrichment Analysis* (GSEA), an online freely available tool (*www.broadinstitute.org/gsea*), is a method that determines whether an a priori defined set of genes shows statistically significant differences between two classes (e.g., phenotypes). The Molecular Signatures Database (MSigDB) is a publically available collection of seven major classes of annotated gene sets (*www.broadinstitute.org/gsea/msigdb*) and is implemented in the GSEA. The gene expression signatures applied in GSEA/MSigDB are generated in various ways, and caution needs to be drawn when interpreting the results. To draw conclusions on gene set analyses, it is crucial to understand how the gene sets and signatures in question are generated and evaluate whether the gene set as generated is relevant for the current study. Also, when analyzing gene set alterations between classes, as in GSEA, it is important to adjust for multiple testing.

In a context-dependent view, when analyzing the microenvironmental alterations and the interplay between the epithelial and microenvironmental compartments in tumor progression, integrating multiple levels of data will likely add information [106]. Large breast cancer studies have aimed at such integrative analyses [107, 108], although a similar "all-level approach" not has been done with the microenvironment in focus.

Future Perspectives: Example Studies

Virtual microdissection of gene expression data is a novel approach, potentially revealing new information from the gene expression data that we have not fully exploited. Moffitt and colleagues published a study on pancreatic ductal adenocarcinoma where, by deconvolution of gene expression data (by nonnegative matrix factorization), they managed to glean information on normal-, tumor-, and stroma-specific gene expression signatures [109]. Two different stromal subtypes were identified (normal and activated subtypes), each with different prognostic information. The activated stroma was seen with more diverse groups of genes, among other genes associated with macrophages and genes with a role in tumor progression. Further integration of the epithelial- and stroma-specific gene expression data, classified the pancreatic tumors into four subtypes with different clinical courses.

Lawson and colleagues pointed to the importance of understanding how metastases are initiated and how they progress, and aimed to elucidate the properties of metastasis-initiating cells in human breast cancer. By single-cell analyses from early-stage metastatic lesions, Lawson demonstrated that cells from these lesions are characterized by a gene expression signature reflecting stemness [110]. Strikingly, the gene expression signature patterns in metastatic cells from tissues in early and advanced stage metastatic disease (patient-derived xenograft models) were distinctly different. The early-stage metastatic cells demonstrated increased expression of stem cell markers, epithelial-mesenchymal transition, and pro-survival and dormancy-associated genes. The metastatic cells from the advanced stage were

more heterogeneous and displayed an expression pattern more similar to the primary tumor. This study adds important information about the role of stemlike cells to the picture of the early stages of the metastasis process.

Two elegantly designed studies, linking information about tumor-stroma interactions, pointed at integrin signaling as being of major importance in tumor progression and in the organotropism of the metastatic lesions. Reuter and colleagues profiled gene expression data of both epithelium and stroma at specific time points during tumor progression in an experimental 3D tumor model [111]. A "core cancer progression signature" was identified, and data indicated extracellular matrix-interacting network hubs as essential in tumor progression. Blocking the β1-integrin hub inhibited tumor development. A recent study on the role of exosomes in the metastatic process demonstrated that tumor-derived exosomes prepare the pre-metastatic niche in organ-specific cells [13]. Lung and liver metastases were associated with specific integrin expression patterns. Targeting these integrins decreased the exosome uptake as well as lung and liver metastases, and Hoshino suggests that exosomal integrins have a potential role in directing metastatic cells in organotropic manners.

Conclusion

The tumor-associated stroma may exhibit both tumor-promoting and tumor-inhibiting effects. Analysis of gene expression alterations may likely reveal novel aspects of these processes, as supported by the studies reviewed here. Capturing gene expression alterations in multigene signatures may better reflect the complex biological programming both driving and supporting tumor development and progression. Stroma-related alterations and processes are probably exploitable, with respect to treatment identification. As underlined from many of the studies on transcriptional alterations of the tumor-associated microenvironment, interplay between extracellular remodeling, vascular biology, and immune-related signaling appears to be critically important features of tumor subtypes and their associated outcomes. How to best reflect the interactions between the functional compartments is a daunting task. Integrating, interpreting, and validating results from global gene expression analyses are still major challenges, as deRisi stated in the very beginning of the "omics" era [25]. But with the words of Albert Einstein (1879–1955), we still aim for further progress: "It's not that I'm so smart, it's just that I stay with problems longer."

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Chapter 17
MR-Derived Biomarkers for Cancer Characterization

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Abstract Magnetic resonance (MR) can be exploited in a variety of ways to obtain a wide range of anatomical and physiological information in a safe and noninvasive manner. This makes MR imaging (MRI) and spectroscopy (MRS) valuable tools in cancer research and clinical oncology, among other fields. This chapter provides a basic introduction to MR physics and describes how different in vivo MR techniques are used to noninvasively characterize tumors and the tumor microenvironment. Two of the most commonly utilized techniques are contrast-enhanced MRI and diffusion-weighted MRI. Contrast-enhanced MRI methods are used to evaluate *tumor vascularization and vascular function* by measuring the kinetics and distribution of intravenously administered contrast agents. Diffusion-weighted MRI is sensitive to the diffusion of water molecules in the tissue, from which inferences about *tumor cellularity and tissue microstructure* can be made. Blood-oxygen-level-dependent MRI can distinguish between oxygenated and deoxygenated blood as a proxy to *tumor oxygenation*. In addition, efforts have been made to develop targeted contrast agents to directly image hypoxia. MRS can be used to measure the levels of various metabolites such as lactate and choline that are involved in *metabolic reprogramming in cancer*. Both endogenous and exogenous pH-sensitive indicators enable spectroscopic measurement of *tumor pH*. While this chapter does not provide an exhaustive overview of the MR methods used for cancer characterization, it discusses both clinical and experimental techniques that highlight the versatility of MR as a tool for exploring some key aspects of the tumor microenvironment.

Keywords Magnetic resonance imaging • Magnetic resonance spectroscopy Dynamic contrast enhanced • Susceptibility contrast • Vessel size imaging • Diffusion-weighted imaging • Restriction spectrum imaging • Diffusion tensor

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imaging • Intravoxel incoherent motion • ^{1}H MRS • ^{31}P MRS • Hyperpolarized ^{13}C MRS • Blood oxygen level dependent • Perfusion • Vasculature • Cellularity • Extracellular matrix • Metabolism • Warburg effect • Hypoxia • pH

MR Basics

Magnetic resonance (MR) signals arise from the intrinsic magnetic moments possessed by certain atomic nuclei in the body. MR imaging (MRI) and MR spectroscopy (MRS) are sensitive to nuclides with an odd number of protons and/or neutrons. The most commonly utilized is the ^{1}H nuclide (i.e., proton) due to its high intrinsic sensitivity and 99.99% natural abundance. Others include ^{31}P phosphorus, ^{13}C carbon, and ^{19}F fluorine.

In an MRI scanner, these nuclear magnetic moments align with each other and precess at a specific resonance frequency, the Larmor frequency, to produce a net magnetization pointing in the direction of the scanner's main magnetic field, B_0. This equilibrium magnetization can be perturbed by applying a radiofrequency (RF) excitation pulse at the Larmor frequency—the nuclei absorb this RF energy, causing the magnetization to tilt away from the B_0 axis. Conceptualizing the magnetization as a vector, this reduces the component parallel to B_0 (longitudinal magnetization) and produces a component perpendicular to B_0 (transverse magnetization). When the RF pulse is turned off, the nuclei reemit the energy they absorbed as the magnetization returns, i.e., relaxes, to its equilibrium state. This emitted RF energy is detected by an RF receiver coil tuned to the Larmor frequency of the excited nuclei.

The rate at which excited nuclei return to their equilibrium state is characterized by the longitudinal relaxation rate R_1, which can be measured with MRI. There is a concomitant decay (relaxation) in the transverse magnetization caused by two distinct phenomena—R_2 refers to the transverse relaxation rate due to microscopic magnetic field fluctuations created by random molecular motion, and R_2' is the transverse relaxation rate due to static magnetic field inhomogeneities. R_2* is the sum of R_2 and R_2' and can be measured with a gradient echo sequence. The effect of static field inhomogeneities can be reversed by a spin-echo sequence, allowing the measurement of R_2. The reciprocals of the relaxation rates are called the relaxation times T_1, T_2, and T_2*. Intrinsic MR image contrast can be manipulated by exploiting the different relaxation properties of different tissues, e.g., in T_1-weighted (T_1w) images, tissues with shorter T_1 appear brighter.

There are many intrinsic MR contrast mechanisms that can be exploited to investigate a wide range of anatomical and functional characteristics. Also, exogenous contrast agents can be administered to increase tissue relaxation rates and enhance image contrast. This inherent versatility allows investigation of various aspects of the tumor microenvironment using MR. This chapter will discuss MR techniques for characterizing tumor vasculature, cellularity and tissue microstructure, metabolism, hypoxia, and pH.

Imaging Tumor Vasculature

For tumors to grow and metastasize, a vascular network is required to deliver oxygen and nutrients, remove waste products, and disseminate cancer cells. In many cancers, hypoxia and genetic alterations induce increased expression of vascular endothelial growth factor (VEGF), which is the primary mediator of tumor angiogenesis [1]. VEGF-driven angiogenesis produces structurally and functionally abnormal vessels that are characteristically hyperpermeable [2]. Increased vascularization and vessel leakiness can result in increased delivery of an intravenously (i.v.) injected contrast agent to tumors, making contrast-enhanced imaging methods like dynamic contrast-enhanced (DCE)-MRI a potentially useful method for in vivo characterization of tumor angiogenesis.

DCE-MRI is a commonly used technique in clinical oncology for cancer detection, diagnosis, and characterization. It involves the serial acquisition of T_1w images before, during, and after i.v. administration of a gadolinium-based contrast agent (GBCA) in order to capture the dynamic signal enhancement caused by the T_1-shortening effect of the GBCA as it extravasates from the blood vessels to the extravascular extracellular space (EES). Calculating contrast agent concentration from the signal enhancement is possible with an additional scan to measure pre-contrast T_1 values. DCE-MRI data can be analyzed by (1) qualitative inspection of signal intensity-time curves, (2) semiquantitative characterization of signal intensity or concentration-time curves, or (3) pharmacokinetic (PK) modeling of concentration-time curves.

PK modeling allows quantification of physiological parameters, e.g., the widely used Tofts model provides estimates of the EES volume fraction (v_e) and the volume transfer constant between the intravascular space and the EES (K^{trans}), which depends on blood flow, vessel permeability, and vessel surface area [3]. However, PK modeling requires high temporal resolution and the additional measurement of an arterial input function (AIF), which is the time-dependent contrast agent concentration in the blood plasma of the vessel that supplies the tissue of interest. This is not a trivial task, and population-averaged AIFs are often used instead of measuring individual AIFs.

DCE-MRI gives indirect measures of tumor angiogenesis, and the interpretation of these measurements is not straightforward. Studies have reported correlations between DCE-MRI parameters and microvessel density (MVD) [4, 5]. However, other studies have reported that DCE-MRI does not correlate with MVD or VEGF expression [6, 7]. MVD measures the number of blood vessels in a given area, whereas DCE-MRI measures vascular function and perfusion. It is not surprising that the two do not always correlate, especially in tumors, which have characteristically abnormal and dysfunctional vessels. Still, DCE-MRI has been demonstrated to provide useful diagnostic and prognostic indicators.

DCE-MRI signal enhancement depends on perfusion, vessel surface area, and vessel permeability; these in turn reflect angiogenic activity, which is associated with tumor aggressiveness and metastatic potential [8]. Studies have shown that pretreatment DCE-MRI examinations can predict breast cancer patient survival. For example, several semiquantitative parameters such as relative signal enhancement and area under

the enhancement curve (AUC) correlated with disease-free survival and overall survival (OS) of breast cancer patients who received neoadjuvant chemotherapy (Fig. 17.1a) [9]. Another study reported significantly faster enhancement kinetics (measured by the maximum enhancement in the first minute and the steepest slope of the enhancement curve) in breast cancer patients who developed local recurrence or distant metastases after surgery compared to those without recurrence or metastases [10].

The shape of DCE-MRI signal intensity-time curves has been shown to have diagnostic value in breast cancer [11]. Enhancement curves are typically classified as one of three types—persistent (continuous enhancement over time), plateau (enhancement reaches a plateau), or washout (initial enhancement followed by signal decrease). Most benign breast tumors (83.0%) displayed persistent enhancement, whereas malignant lesions were characterized by plateau- (33.6%) or washout-type (57.4%) curves (Fig. 17.1b) [11]. Such qualitative classification of enhancement curves may still be the most common form of DCE-MRI analysis, but efforts have been made to automate this classification to eliminate intra- and interobserver variability [12, 13].

Quantitative parameters are desirable in the context of treatment monitoring and drug trials as they enable better assessment of longitudinal changes and comparison between different centers and studies. It has been recommended that K^{trans} or the initial area under the contrast agent concentration-time curve (IAUC) should be used as primary end points in early-phase cancer drug trials [14]. Anti-angiogenic therapies are expected to decrease vascularization, perfusion, and/or vessel permeability, which would lead to decreases in K^{trans} and IAUC. Many clinical and preclinical studies have utilized DCE-MRI for monitoring response to various anti-angiogenic and vascular disrupting agents, with most reporting significant reductions in K^{trans} and IAUC (Fig. 17.1c) [15, 16]. However, some studies showed no significant change, which may simply indicate drug resistance or point to the complexity of the therapeutic mechanisms of action and of the physiological meanings of the DCE-MRI readouts. It is still not fully clear how anti-vascular agents work, and this uncertainty is also present in the interpretation of K^{trans} and IAUC, which are dependent on several factors (perfusion, vessel permeability, and surface area) that may change and affect these parameters in different ways after treatment. There are other, more generalized PK models that provide separate estimates of blood flow and the vessel permeability surface area product, which are reviewed by Sourbron and Buckley [17]. But they are also more complex and computationally expensive, require higher temporal resolution, and have not been widely adopted in clinical practice.

Susceptibility contrast MRI utilizes (super)paramagnetic contrast agents to measure vascular function and morphology. The difference in the magnetic susceptibilities of the i.v.-administered contrast agent and biological tissue locally enhances transverse relaxation rates ($R_2^{(*)}$) in and around blood vessels. Pre- and post-contrast images are acquired to measure the increase in relaxation ($\Delta R_2^{(*)}$), which is dependent on contrast agent concentration and distribution.

In the clinic, dynamic susceptibility contrast (DSC)-MRI is used to measure perfusion, primarily in the brain. A series of pre- and post-contrast T_2w or $T_2{*}w$ images are acquired with high temporal resolution to capture the first pass of a GBCA bolus

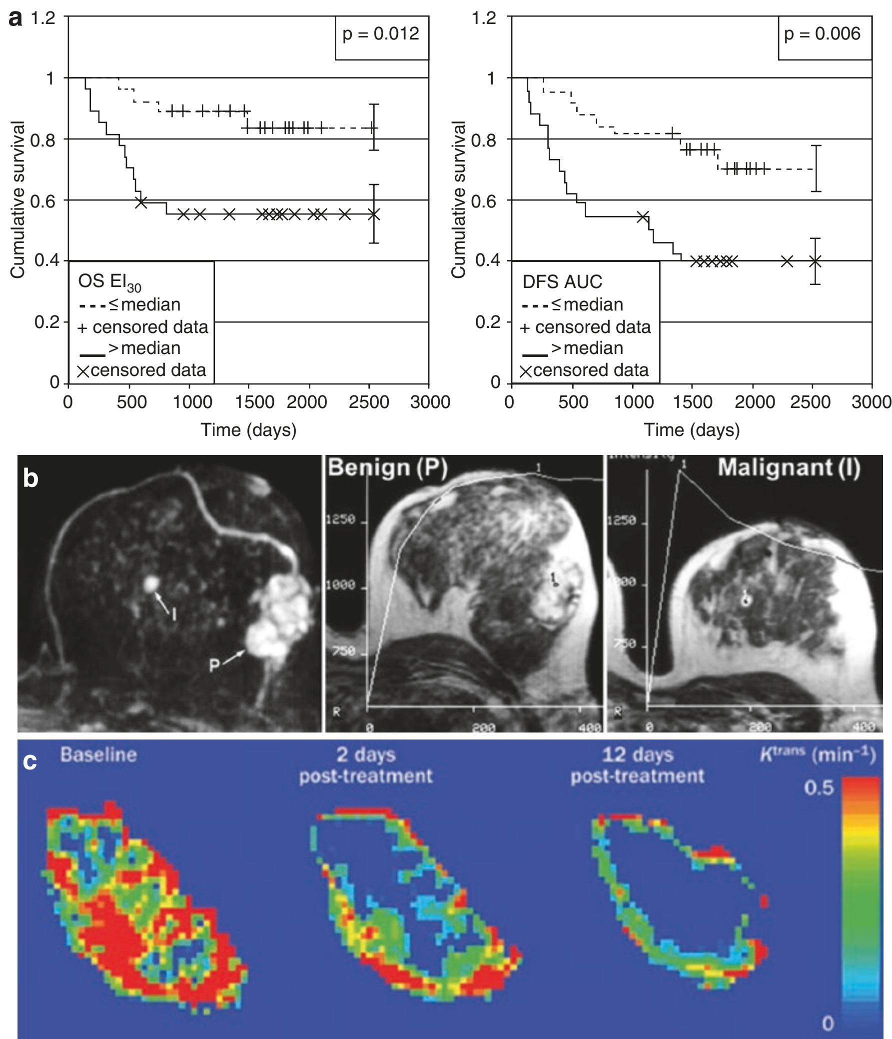


Fig. 17.1 (**a**) Kaplan-Meier survival plots for baseline DCE-MRI parameters in breast cancer patients scheduled to undergo neoadjuvant chemotherapy. *Left*: overall survival plot for the relative signal enhancement at 30 s after enhancement onset (EI_{30}). *Right*: disease-free survival plot for the area under the enhancement curve (AUC) (Reprinted from [9], Copyright 2009, with permission from Elsevier). (**b**) *Left*: axial maximum intensity projection of a DCE-MR image of a breast with a palpable mass (P) and a nonpalpable, incident lesion (I). *Center*: the palpable mass (benign fibroadenoma) displayed a persistent-type signal intensity-time curve. *Right*: the incident lesion (invasive ductal carcinoma) displayed a washout-type signal intensity-time curve. (**c**) K^{trans} maps from a patient with a colorectal liver metastasis showing decreased perfusion, vascularization, and/or vessel permeability after bevacizumab treatment (Reprinted by permission from Macmillan Publishers Ltd.: Nature Reviews Clinical Oncology [15], Copyright 2012). (**d**) Conventional T_2-weighted (T_2w) images (*left*) and DSC-MRI-derived rCBV (*center*) and rCBF maps (*right*) from a patient with grade IV glioblastoma (*top*) and a patient with grade II astrocytoma (*bottom*). The differences in rCBV and rCBF between the high- and low-grade gliomas are readily apparent (Reprinted from [20], Copyright 2005, with permission from Elsevier)

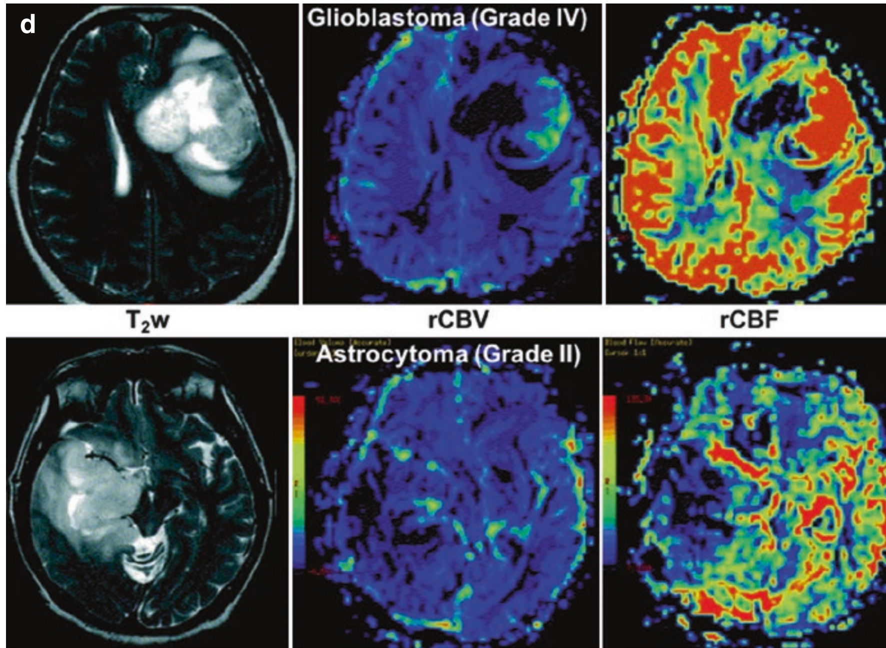


Fig. 17.1 (continued)

through the vasculature. Relative cerebral blood volume (rCBV) can be estimated from the signal intensity-time curve [18]; an AIF in addition to complex mathematics is required for quantification of absolute CBV and cerebral blood flow (CBF) [19]. DSC-MRI has been shown to be able to distinguish between high- and low-grade gliomas, with the high-grade lesions having significantly higher rCBV and rCBF (Fig. 17.1d) [20]. Similarly, Schmainda et al. showed that rCBV is predictive of OS in patients with recurrent high-grade glioma who received bevacizumab treatment, with OS being significantly longer if the rCBV of the lesion was below a certain threshold [21]. DSC-MRI is also sensitive to therapeutic response, but differentiating tumor progression from pseudoprogression and, in the case of anti-angiogenic therapy, response from pseudoresponse can be challenging [22, 23].

Intravascular contrast agents with long circulation times such as ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles are used preclinically for steady-state susceptibility contrast (SSC)-MRI (i.e., vessel size imaging). Pre- and post-contrast spin and gradient echo images are acquired to measure steady-state ΔR_2 and ΔR_2*, respectively. These can be used to calculate various parameters that estimate mean vessel density [24], fractional blood volume, and mean vessel diameter [25]. SSC-MRI parameters have been shown to correlate with vascular measurements from histology [26] and high-resolution micro-CT [27, 28]. A review by Emblem et al. discusses the potential use of vessel size imaging parameters as clinical biomarkers of treatment response [29].

To summarize, there are multiple MRI techniques that are widely used preclinically and in clinical oncology for characterization of the vascular phenotype,

diagnosis, and treatment evaluation. But a better understanding of the underlying biophysics that affect the MRI measurements and of the mechanisms of action of anti-angiogenic drugs and other therapies is needed for the development of clinically validated MRI-based biomarkers of tumor angiogenesis.

Imaging Tissue Cellularity and Microstructure

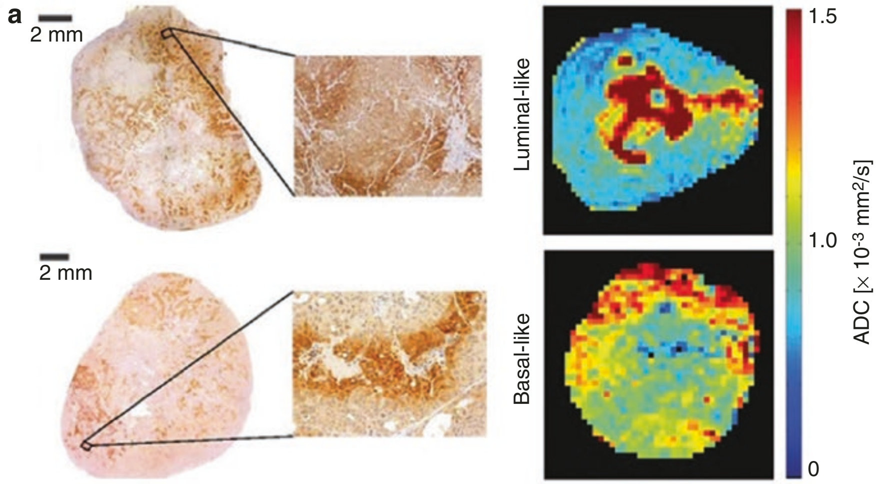
Diffusion-weighted MRI (DWI) is currently one of the fastest developing MRI-based techniques in oncology. DWI allows the mapping of water diffusion due to the Brownian motion of water molecules in vivo. The water diffusion is measured indirectly as a signal loss induced by diffusion-sensitizing magnetic field gradients applied during the MRI pulse sequence. The diffusion can be quantitatively assessed by calculating the apparent diffusion coefficient (ADC) value. This assessment is done by acquiring several (at least two) images with different diffusion sensitization, from which the ADC value can be derived by exponential fitting. High ADC values indicate relatively free diffusion, while low indicate restricted diffusion. The distribution of ADC values is commonly illustrated in parametric maps (Fig. 17.2a).

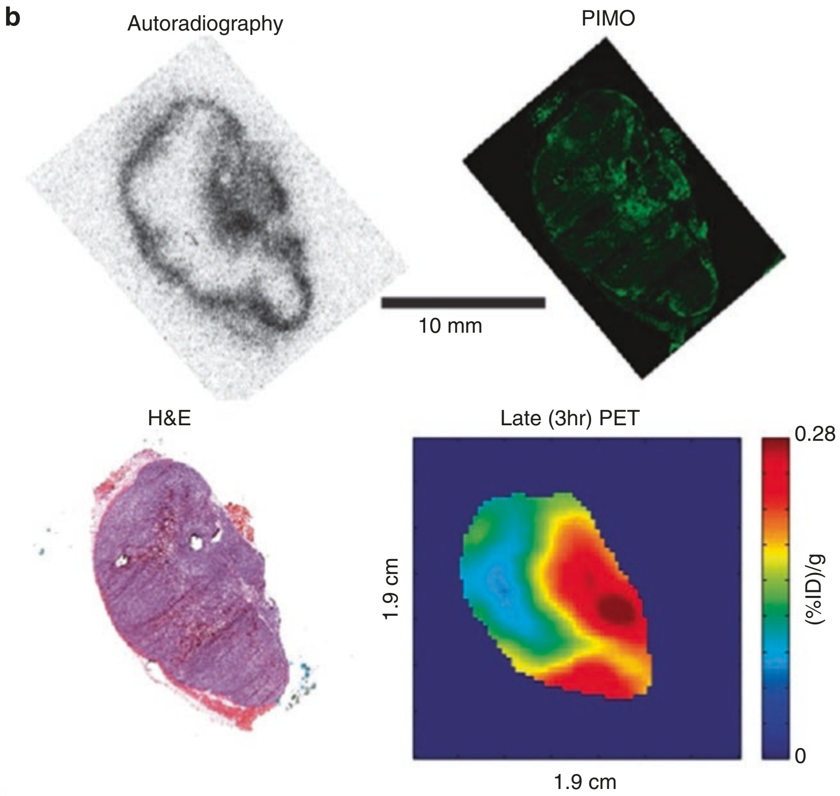
The contrast in DWI arises from the different compositions of biological tissue, such as cell membranes, macromolecules, fibers, or other tissue components, all of which restrict water diffusion. Diffusion is also affected by water exchange between intracellular and extracellular compartments, the shape of the extracellular space, and tissue cellularity. Diffusion patterns can therefore reveal microscopic details about the tissue architecture. Due to this complex mixture of contributions to the measured DWI signal, the complete biophysical interpretation is still not fully clear.

Several studies have been performed to better understand the association between the microenvironment and the obtained diffusion properties. Importantly, tumor tissue is usually characterized by low water diffusivity, which is most likely related to higher cellular density [30] and proliferation [31]. Low ADC values have also been associated with high hypoxic fraction (Fig. 17.2a), interstitial hypertension, and elevated metastatic propensity in melanoma xenografts [30].

Epithelial-to-mesenchymal transition (EMT) is important for tumor metastasis. It has been proposed that an increasing ADC value can be detected in tumor cells undergoing EMT [32]. Water diffusion is altered during the transition between epithelial and mesenchymal phenotypes due to changes in cell-cell contact and the volume of extracellular space. Subcutaneous xenograft tumors with epithelial-like phenotypes showed significantly lower ADC values compared to those with mesenchymal-like phenotypes.

Exploiting the diffusion properties by histogram analysis of ADC values across a tumor volume enables a better description of intratumoral heterogeneity compared to using mean or median ADC. Such analyses have, for example, been utilized to differentiate gliomas [33]. Moreover, histogram analysis of ADC values of glioma contributed to distinguish between isocitrate dehydrogenase gene mutation-positive and mutation-negative high-grade gliomas, which could be relevant for patient management since the mutation-positive patients have a favorable prognosis [34].


a
2 mm
2 mm
Luminal-like
Basal-like
1.5
1.0
0
ADC [× 10-3 mm2/s]


b
Autoradiography
PIMO
10 mm
H&E
Late (3hr) PET
1.9 cm
1.9 cm
0.28
(%ID)/g
0

Restriction spectrum imaging (RSI) is a new extension of the DWI methodology [35]. Restricted diffusion is a term used to describe the trapping of water molecules within an enclosed compartment, for example, as defined by the cell plasma membrane. RSI requires the use of high diffusion sensitization in addition to directionality and enables quantitative estimates of tissue microstructure based on modeling of tissue properties such as cell size, density, and orientation as a function of diffusion sensitization. The calculated cellularity index, an in vivo measure of spherically restricted water, has shown promising clinical results in improving the tumor conspicuity of high-grade brain tumors [36], capturing the treatment response of anti-angiogenic treatment [37], and improving visualization of white matter pathways through regions of peritumoral edema [38]. More recently, the RSI-derived cellularity index was found to be associated with the aggressiveness of prostate cancer (Gleason score) [39] and significantly contributed to prostate cancer staging based on accurate detection of extraprostatic extension of the tumor [40].

Anticancer treatment will change structural features of the tumor tissues and cells. Treatment-induced cell death is usually reflected by increased ADC values, due to loss of cell membrane integrity and decreased cell density caused by both necrosis and apoptosis, and this may be observed prior to any significant reduction of the tumor volume [41]. Increased diffusion due to treatment response has been shown after standard cytotoxic treatment [42, 43], targeted treatment [43], anti-angiogenic treatment [44], and radiation therapy [45]. A comprehensive review of DWI in oncologic applications can be found in [46].

The relationship between diffusion and the tumor stroma has been investigated in breast cancer [47], where ADC values were negatively correlated with tumor/stroma ratio, most likely because stroma-poor tumors have higher cellularity. However, diffusion was observed to be lower in collagen-dominant stroma types compared to fibroblast- or lymphocyte- dominant types [47]. This is in agreement with findings in patient-derived luminal-like breast cancer xenografts, where a low ADC value was attributed to the high content of collagen and fibronectin in the

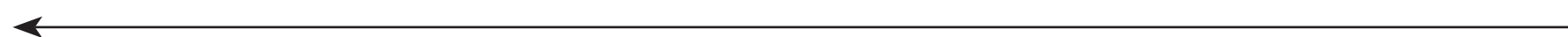


Fig. 17.2 (**a**) Pimonidazole staining of luminal-like breast cancer xenografts (*top left*) demonstrates that these tumors are more hypoxic than basal-like breast cancer xenografts (*bottom left*). ADC values were significantly lower in the luminal-like (*top right*) compared to the basal-like tumors (*bottom right*), which demonstrates the inverse correlation between hypoxia and ADC (Reprinted with permission from [48], Copyright 2011 Wiley Periodicals, Inc.). (**b**) Autoradiography (*top left*) demonstrates high accumulation of ^{18}F-MISO (*dark areas*) in regions positive for pimonidazole (*top right*, *green areas*), whereas minimal tracer accumulation is seen in areas negative for pimonidazole. The difference between these tissues is difficult to recognize in an H&E section of the tumor (*bottom left*). Accumulation of ^{18}F-MISO in hypoxic regions is also seen using in vivo PET imaging (*bottom right*) (Reprinted from [111], Copyright 2009, with permission from Elsevier)

stroma [48–50]. A recent work in prostate cancer also demonstrates that ADC correlates with the tumor tissue composition, and a positive association between ADC and the volume of the luminal space was identified [51].

An interesting extension of DWI is diffusion tensor imaging (DTI), which adds information about tissue microstructure by addressing diffusion direction. In DTI, diffusion-sensitizing gradients are applied in many (at least six) different directions, and the diffusion profile is fitted to a tensor model. The tensor model assumes that one dominant direction of diffusion is present and that the diffusion anisotropy can be described by an ellipsoidal symmetry [52]. DTI has been suggested as a new approach for detection of breast cancer based on tracking the mammary architectural elements [53]. The breast's fibroglandular tissue is orientated along tubular ducts and ligaments, and the diffusion properties of the mammary fibroglandular tissue change during malignant transformation. The sensitivity of DTI to detect breast cancer was found to be high, particularly in dense breasts. A challenge in the management of breast cancer is the detection of early response to therapy. DTI could potentially aid in this, as changes in diffusion anisotropy are expected to reflect changes in tissue structure induced by neoadjuvant therapy. Fractional anisotropy (FA) describes the degree of anisotropy of the diffusion process. FA and ADC were found to correlate with collagen fiber density in breast cancer xenografts [54]. The hypoxic regions of the same xenografts contained lower density of collagen fibers and simultaneously exhibited lower FA and ADC, suggesting that ADC and FA could serve as clinically relevant, noninvasive markers of fiber density as well as hypoxia.

Intravoxel incoherent motion (IVIM) imaging is another advanced DWI approach that was first described nearly three decades ago but is now gaining huge interest in oncology [55]. This technique takes into account the fact that the motion of water molecules contributing to the diffusion signal not only arises from extra- and intracellular diffusion but also from intravascular blood flow (perfusion). This is especially apparent for images acquired with low diffusion sensitization [56]. IVIM allows for the separation of motion of water molecules due to microcirculation from motion due to diffusion, which is promising for response measurement in treatment studies targeting both vasculature and cell proliferation [57]. Importantly, this reflection of tissue diffusivity and microcapillary perfusion is obtained without contrast agent injection.

In conclusion, DWI is now frequently used as one of the sequences in multiparametric MRI for preclinical and clinical oncological applications. The DWI signal, and the derived ADC value, largely depends on the tissue cellularity. To establish ADC as a robust biomarker, standardization of the DWI acquisition (how to apply the diffusion sensitization) and subsequent post-processing and analysis is necessary [58]. Importantly, preclinical studies have shown that absolute ADC values are comparable between sites and equipment, provided standardized protocols are employed [59]. Advanced extensions of DWI, such as DTI, RSI, and IVIM, broaden the applicability of the methodology. DWI offers promising biomarkers for both cancer detection and evaluation of treatment response and opens a window for in vivo, noninvasive characterization of the tumor microenvironment.

Investigating Cancer Metabolism

During cancer progression, molecular changes are associated with metabolic reprogramming [60, 61], which is a hallmark of cancer [62]. Metabolic changes can be measured by MRS at the molecular level, providing insights into the causes of altered metabolism in oncogenesis. This technique can be performed on current clinical MR systems in vivo or within cell/tissue extracts and intact tissue samples in laboratory settings using high-resolution MRS [63, 64].

MRS exploits the fact that the Larmor frequency is different for every nuclide and also depends on the magnetic field strength. Different nuclei experience slightly different magnetic fields depending on their molecular environment and chemical shielding and therefore precess at slightly different frequencies. These resonance offsets can be described on a field-independent dimensionless scale called chemical shift (δ), which is expressed in parts per million (ppm). Individual molecular properties can be characterized by a single or multiple resonances in MR frequency spectra, thereby allowing detection and quantification of the relative concentrations of different metabolites and other molecules via MRS.

One of the most common metabolic anomalies observed in cancer is the Warburg effect. Partly due to hypoxia and partly due to direct metabolic regulation through oncogenic signaling, most cancer cells exhibit high glycolytic activity and convert a substantial fraction of their glucose to lactate even in the presence of adequate oxygen levels [65]. The lactate dehydrogenase (LDH) enzyme catalyzes the reversible conversion of pyruvate to lactate. LDH expression and high tumor lactate are required for the progression of many tumors. MRS-measured lactate concentration can be a good indicator of the metabolic adaptation in cancer cells, and many studies have revealed its correlation with prognosis, treatment efficacy, and clinical outcome in a variety of human cancers [66]. Some other commonly measured metabolites in ^{1}H MRS include choline-containing compounds, lipids, *N*-acetyl aspartate, creatine, glutamate, glutamine, GABA, myoinositol, citrate, and 2-hydroxyglutarate.

Altered membrane choline phospholipid metabolism is associated with malignancies, oncogenesis, and tumor progression [67]. Changes in choline-containing metabolite concentrations have been shown to be potential early biomarkers of targeted antitumor therapies. Increased levels of choline-containing metabolites, referred to as total choline (tCho), in cancer cells has been interpreted to be associated with cancer cells' demands for increased proliferation, upregulation of choline kinase activity, and oncogenic cell signaling such as overactivity of PI3K signaling [67]. ^{1}H MRS can detect tCho noninvasively on clinically available MR scanners. ^{31}P MRS is another useful tool for noninvasive investigation of phospholipid metabolism in vivo. Compared to ^{1}H MRS, this technique is less prone to water and lipid contamination; however, it is less sensitive and requires an additional dedicated phosphorus coil. A number of key metabolites involved in phospholipid metabolism are detected using this method, such as phosphocholine (PCho), phosphoethanolamine (PE), glycerophosphocholine (GPC), and glycerophosphoethanolamine

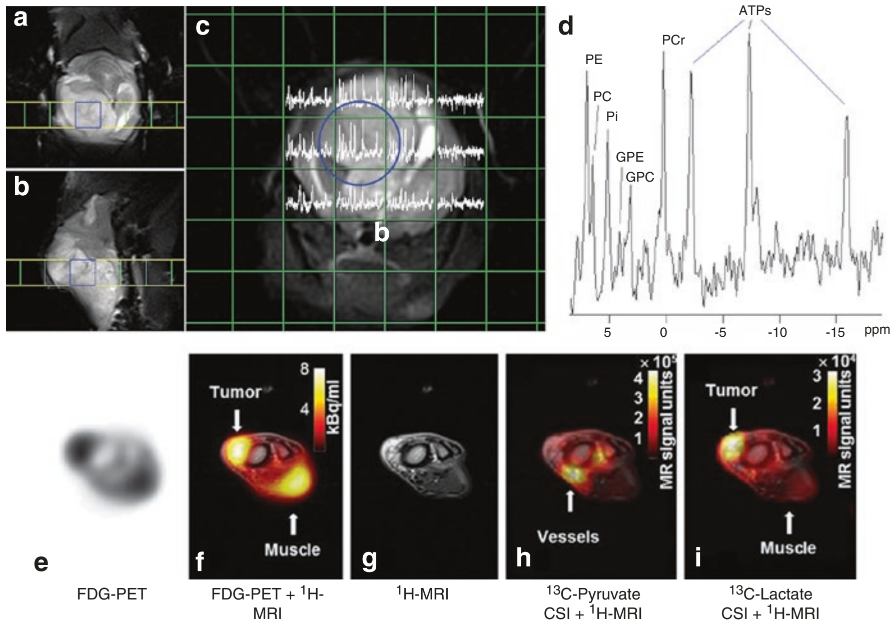


Fig. 17.3 (**a**–**c**) ^{31}P MRSI voxels (27 mm^3 nominal resolution) overlaid on orthogonal T_2-weighted MR images of a tumor-bearing mouse brain. (**d**) Corresponding ^{31}P MR spectrum of the voxel outlined in *blue* from the 3D ^{31}P MRSI data. The assigned peaks in (**d**) are (from *left* to *right*) *PE* phosphoethanolamine, *PC* phosphocholine, *Pi* inorganic phosphate, *GPE* glycerophosphoethanolamine, *GPC* glycerophosphocholine, *PCr* phosphocreatine, and *ATP* adenosine triphosphates (Reprinted with permission from [112]). (**e**–**i**) Axial images of the right front leg of a canine cancer patient with liposarcoma. Note the high concentration of ^{18}F-FDG in the muscle (*arrow*, panel *F*, ^{18}F-FDG-PET overlaid on ^{1}H-MRI) and of ^{13}C-pyruvate in the large vessels (*arrow*, panel *H*, ^{13}C-pyruvate CSI + ^{1}H-MRI), as well as increased level of ^{13}C-lactate in the tumor (*arrow*, panel *I*, ^{13}C-lactate CSI + ^{1}H-MRI). *CSI* chemical shift imaging (Reprinted with permission from [76])

(GPE) (Fig. 17.3a–d). Other potential applications of ^{31}P MRS include the evaluation of high-energy phosphates: phosphocreatine (PCr), adenosine triphosphate (ATP), and adenosine diphosphate (ADP) (Fig. 17.3d).

With 1.1% natural abundance of the ^{13}C isotope, ^{13}C MRS of endogenous metabolites is less sensitive than ^{1}H MRS. However, exogenous ^{13}C MRS is feasible following administration of a ^{13}C-labeled substrate, including ^{13}C-labeled glucose, and subsequent incorporation of the ^{13}C label from the exogenous substrate into other molecules. For instance, glycolytic rates can be measured by investigating the uptake and metabolism of [^{13}C]-glucose in vivo. However, the utility of ^{13}C MRS is limited due to its relatively low sensitivity and the fact that most commercial MR systems are only capable of ^{1}H MRS. One of the most important innovations in recent years has been the development of dynamic nuclear polarization (DNP, or “hyperpolarization”) of ^{13}C-labeled metabolic substrates, enhancing the sensitivity of the ^{13}C MRS experiment dramatically (> 10,000-fold higher than non-hyperpolarized ^{13}C) [68]. This improvement allows the analysis of several

metabolic pathways and metabolic fluxes through select enzyme-catalyzed steps. Due to its excellent polarization properties, rapid transport into important metabolic pathways, and longer relaxation time, [1-^{13}C]pyruvate has been the most widely studied substrate to date. In addition to indicating the presence of the Warburg effect in tumors, [1-^{13}C]pyruvate has been employed to investigate the response to anticancer therapies by noninvasively evaluating real-time flux of pyruvate to lactate and LDH activity [69, 70]. [1,4-$^{13}C_2$]fumarate has been used to investigate cell necrosis and treatment response in tumors [71]. Hyperpolarized [1-^{13}C]pyruvate and [1,4-$^{13}C_2$]fumarate have also been used for detection of early changes in tumor metabolism following administration of a vascular disrupting agent [72].

Positron emission tomography (PET) is a sensitive and quantitative method for measuring the uptake and trapping of different radiolabeled PET substrates, such as ^{18}F-fluorodeoxyglucose (^{18}F-FDG), a radioactive form of glucose. This technique allows noninvasive molecular imaging of cancer cell metabolism, heterogeneity, and metastases in systems ranging from advanced tumor models to patients in the clinical setting. When combined with computed tomography (CT), PET/CT provides both anatomical localization and functional information. There is a wide range of novel and established PET radiotracers, which can be used to investigate various aspects of cancer, including carbohydrate, amino acid, and fatty acid metabolism. ^{18}F-FDG-PET by far is the most successful tracer in in vivo cancer studies. The reduction in ^{18}F-FDG uptake has been used to detect treatment response in some cancer subtypes [73].

Integration of ^{18}F-FDG-PET and MRS may potentially increase the sensitivity and accuracy in tumor localization [74] and specificity of tumor detection [75, 76] (Fig. 17.3e–i). The increased availability of clinical PET/MR scanners has recently raised the interest in simultaneous DNP-MRS and PET imaging [76, 77]. PET provides relatively higher sensitivity than DNP-MRS, detecting in the range of nano- to picomolar compared to the millimolar range sensitivity of MRS. Due to low sensitivity, the concentrations of DNP substrates that have to be administered may exceed that of physiologic levels, which may perturb normal metabolism. The short half-life (in tens of seconds) is another notable limitation of DNP-MRS, which calls for improvement in fast MRS data acquisition. But unlike PET, DNP-MRS does not employ ionizing radiation and can detect injected substrate and its metabolic products simultaneously. The latter enables the observation of both the uptake of the targeted molecule and its downstream metabolic products.

Multimodal imaging techniques have provided novel opportunities for cancer treatment by providing comprehensive cancer metabolomic information. In clinical cancer management, there has been a great tendency toward personalized therapies—including targeting specific metabolic pathways, enzymes, and/or oncogenes—and away from aggressive or cytotoxic treatments. Integration of anatomical information of MRI and metabolic information provided by multivoxel MRS imaging (MRSI) or PET can significantly improve the assessment of cancer location, extent, aggressiveness, and response to treatment.

Imaging Tumor Hypoxia

Hypoxia can be defined as subnormal levels of oxygen in tissues and is a frequent phenomenon in solid tumors. A functional mismatch between cell proliferation and vascularization often leads to poor oxygenation of tumor regions—either because the cancer cells are located so far from the nearest blood vessel that oxygen supply through diffusion is insufficient or because the tumor vasculature is dysfunctional and cannot provide oxygen to meet the demands of the surrounding tumor tissue [78, 79].

During the life span of a tumor, there will be both temporal and spatial variations in the degree of hypoxia. This will in turn induce adaptive changes in the biology of the cancer cells. These changes are predominantly mediated through the hypoxia-inducible factors (HIFs) and hypoxia response elements, which transcriptionally regulate genes that are relevant for cancer cell growth and disease development [80].

A well-documented clinical consequence of low partial oxygen pressure in tumors is resistance to radiotherapy caused by insufficient production of free oxygen radicals [81]. However, it has also been shown that hypoxia is associated with poor prognosis in several cancers [82]. Furthermore, it has been demonstrated that hypoxia promotes local invasion and metastatic dissemination of cancer cells [83, 84].

Since hypoxia is a driving force for cancer progression and since the outcome of radiotherapy is strongly associated with oxygenation of the target tissue, measuring the level of hypoxia in solid tumors has potential clinical implications. There is therefore significant interest in development of noninvasive imaging methods that can report on the degree of hypoxia in solid tumors, preferably with high spatial resolution. This can be achieved either through direct approaches using oxygen-level-sensitive contrast agents or through indirect approaches using functional proxy markers of hypoxia.

MRI cannot directly measure the partial pressure of oxygen in the tissue. Therefore, hypoxia has traditionally been imaged using indirect markers of oxygen concentration. Oxygen delivery and consumption depend on vascular perfusion and cellular density, which can be imaged using DCE-MRI and DW-MRI, respectively. While these methods do not provide information on actual tissue oxygenation, several reports describe relationships between standard DCE-MRI and DW-MRI readouts and hypoxia in preclinical model systems. For example, an inverse correlation between K^{trans} and ADC and the fraction of hypoxic cells has been demonstrated in several experimental model systems (Fig. 17.2a) [30, 48, 85, 86]. However, this may represent an indirect association because hypoxia can arise from the imbalance between oxygen supply and demand created by high cellular density. Interestingly, these studies have also demonstrated associations between these functional MRI parameters, hypoxia, and metastatic potential, emphasizing the potential clinical value of imaging the hypoxic tumor microenvironment.

Using blood-oxygen-level-dependent (BOLD) MRI, the ratio of oxygenated to deoxygenated blood can be measured through differences in intrinsic magnetic susceptibility between oxy- and deoxyhemoglobin—the presence of paramagnetic deoxyhemoglobin will increase the R_2* relaxation rate of water protons. In cancer, it

has been suggested that changes in R_2* in response to inhalation of hyperoxic gas can be used to identify hypoxic tumor fractions [87]. However, changes in R_2* are not directly proportional to changes in tissue oxygenation levels, and this technique has therefore not yet found clinical use [88, 89]. The same problem applies to R_1 relaxivity-based tissue-oxygen-level-dependent (TOLD) MRI, which recently has been suggested as a tool for mapping regional oxygenation in tumors [90]. The role of BOLD and TOLD MRI in assessment of tumor hypoxia therefore remains unclear.

The direct approach of assessing hypoxia is predominantly based on 2-nitroimidazole derivatives, a group of compounds that form covalent bonds with cellular macromolecules at oxygen levels below 10 mmHg pO_2 [91]. Nitroimidazole adducts can be detected ex vivo using immunohistochemistry [92], but contrast agents for in vivo labeling and imaging have also been developed. Several fluorinated nitroimidazole derivatives have been tested clinically, using ^{19}F MRS to detect accumulation of contrast agent in hypoxic tumor regions [93]. Recently, a gadolinium-labeled nitroimidazole contrast agent ($GdDO_3NI$) was found to accumulate in poorly perfused regions of xenografted tumors, suggesting that T_1-based MRI of hypoxia may be a possibility [94]. However, efforts to develop hypoxia-targeted MRI contrast agents are hampered by the inherent low sensitivity of this imaging modality.

In contrast, PET imaging is highly suitable for quantitative imaging of contrast agents present in low concentrations in the tissue. Several PET probes, such as ^{18}F-fluoromisonidazole [^{18}F-MISO] and ^{18}F-flortanidazole [^{18}F-HX4], bind to viable hypoxic cells in vivo, thereby allowing direct imaging of hypoxia in cancer (Fig. 17.2b). Using quantitative readouts such as tumor/blood or tumor/muscle signal intensity ratios, it has been shown that ^{18}F-MISO can accurately and reproducibly image regional insufficiencies in pO_2 across a wide range of cancers [95, 96]. In several trials, pretreatment ^{18}F-MISO uptake predicted the outcome of radiation therapy, demonstrating the value of noninvasive hypoxia assessment [97, 98]. The technique has been cross-validated against DCE-MRI, demonstrating that high ^{18}F-MISO uptake correlates with low K^{trans}. Based on the current clinical evidence, the FDA has granted an Investigational New Drug (IND) status for ^{18}F-MISO.

In summary, the clinical implications of noninvasive assessment of tumor tissue oxygen levels are significant, predominantly due to its predictive value in radiotherapy. Currently, PET imaging with ^{18}F-MISO is the most widely used imaging approach. As the BOLD and TOLD MRI techniques do not require the use of exogenous contrast, further understanding of how they reflect tumor tissue oxygenation would make them attractive for clinical use.

Probing Intratumoral pH

As mentioned above, most cancer cells have high glycolytic rates, and the resulting pyruvate is converted to lactate instead of being oxidized in the mitochondria, even under normoxic conditions. To maintain a sustainable intracellular pH (pH_i), excess lactate is transported out of the cells via the monocarboxylate transporter system.

This contributes to acidification of the extracellular compartment, which has been associated with tumorigenic transformation, decreased genetic stability, induction of growth factors and proteases, and, ultimately, increased migration and invasion leading to increased metastatic potential [99, 100]. The extracellular pH (pH_e) in solid tumors can be as low as 6.0, in contrast to normal tissues where pH_i (7.2–7.4) normally is slightly lower than pH_e [101].

Noninvasive pH measurement is therefore of clinical interest—both since pH may provide independent prognostic/predictive information and since pH measurements may be relevant for early response monitoring as well as guiding development of pH-sensitive drug delivery systems [102, 103].

Imaging pH in vivo has been a challenge for the scientific communities for several decades, partly because it requires an exogenous or endogenous pH indicator that has a pKA in the relevant range, as well as the ability to provide sufficient signal at nontoxic/endogenous concentrations. One approach has been to develop self-quenching fluorescent probes that are activated at low pH [104]. This approach, however, is only semiquantitative by nature. Using quantitative MRS for simultaneous measurement of the protonated and ionic fractions of weak acids through differences in chemical shift is therefore a more accurate method for noninvasive determination of pH.

The chemical shift of inorganic phosphate (Pi) is pH dependent, and ^{31}P MRS can therefore estimate pH based on the resonance frequency of Pi within 0.05 pH units [105]. However, it has been confirmed that Pi primarily represents the intracellular pH, which is neutral/slightly alkaline even in highly acidic tumors [106, 107]. This led to development of membrane impermeant phosphonate-based probes, such as 3-aminopropylphosphonate (3-APP), which can be used to measure pH_e. Despite the limited sensitivity of ^{31}P MRS, these compounds unequivocally demonstrated that the acidic pH in tumors is due to the low extracellular pH.

Attempts to develop more clinically relevant extracellular NMR-detectable agents also include several imidazole-based pH indicators for ^{1}H MRS [108, 109]. Preclinical experiments have demonstrated that MRSI of these agents can describe pH heterogeneity in tumors with differences as large as 0.5 pH units within less than a centimeter in distance. Co-registration studies suggest that the extracellular pH correlates to regional lactate concentration but also that low pH is associated with poor perfusion.

An intriguing new approach to in vivo pH measurements is the use of hyperpolarized ^{13}C bicarbonate, allowing real-time assessment of HCO_{3-} and dissolved CO_2 concentrations with ^{13}C MRS, from which pH can be calculated using the Henderson-Hasselbalch equation [110]. Preclinical studies with this tracer confirmed the presence of a pH gradient across cell membranes ($pH_i > pH_e$) and that the method reports on pH across the physiologically relevant range.

In summary, MRI/MRS offers several approaches for measurement of tissue pH. Depending on the need for accuracy and spatial resolution, as well as access to specialized equipment, several methods may be of value in clinical cancer management.

Conclusion

It is increasingly recognized that tumor heterogeneity, both with respect to cellular clonality and regional microenvironmental variability, has an impact on the aggressiveness and response to therapy in cancer. Solid tumors are inherently dynamic systems, and their characteristics change over the course of the disease. Methods that allow noninvasive, longitudinal studies of tumor heterogeneity are therefore essential for development of personalized cancer therapy. MRI and MRS are extremely versatile techniques that provide a wide array of readouts of various aspects of the tumor microenvironment. Some elements of the MR toolbox, such as DCE-MRI and DWI, are already integral parts of clinical practice. The ability to obtain intrinsically co-registered, complementary anatomical and physiological information through multiparametric imaging is one of the greatest strengths of MR; indeed, multiparametric MR has already proven to significantly improve diagnostic accuracy (e.g., in prostate cancer) and represents the direction in which the field of cancer imaging is headed.

Microenvironmental parameters are functionally interconnected, and interpretation of their biological and clinical significance therefore benefits from a multimodal approach where several biomarkers are determined simultaneously. This is made possible by the recent emergence of novel technologies such as hybrid PET/MRI. This instrumentation allows simultaneous acquisition of PET and MR images, and the examinations can be tailored to provide complementary information from these modalities from careful selection of radiotracers and MRI protocols. An interesting extension of this technique is the combination of PET/MRI and hyperpolarized MRS (hyperPET), which in principle can provide coverage of vascular function (through DCE-MRI), cellularity (through DW-MRI), hypoxia (through PET), metabolism (through [1-13C]pyruvate MRS), and pH (through $H^{13}CO_3^-$ MRS). However, the future role of multimodal assessment of the tumor microenvironment depends on concurrent development of analytical tools. A multivariate statistical approach to information extraction will allow combination of multiple image-based biomarkers into 3D probability maps and thereby greatly facilitate clinical interpretation of image-based biomarkers.

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Chapter 18
The Influence of Tissue Architecture on Drug Response: Anticancer Drug Development in High-Dimensional Combinatorial Microenvironment Platforms

Chun-Han Lin and Mark A. LaBarge

Abstract Successfully predicting how anticancer compounds will function clinically, based on preclinical studies, remains a significant challenge. High rates of phase II clinical trial failures indicate that many candidate compounds satisfy minimal safety requirements but lack efficacy in patients. Following the discovery of oncogenes and tumor suppressors, essentially the de facto demonstration that DNA mutations are at the heart of cancers, huge investments have been made in developing technologies to enable exploration of the total complexity of genomes and proteomes that are intrinsic to cells. One important, and wholly unexpected, outcome of those massive investments to understand cancer as a cell-intrinsic problem is the undeniable conclusion that mutations do not explain everything. Indeed, the fact that frankly malignant cells can be phenotypically normal, when held in check by a normal microenvironment, suggests that there is a dominant role of the microenvironment. Tumor microenvironments are known to modulate the malignant phenotype of cells and impact drug responses. Conventional 2-D plastic dishes are the substrate of choice for most drug screening, and rodent and other animals are used as in vivo models, but these modalities lack context in a way that is relevant to predicting drug activity. Alternatively, combinatorial microenvironment microarray platforms provide a high-throughput means of exploring cell-based functional responses in diverse microenvironmental milieus. Data from these techniques are single-cell

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resolution and encapsulate cell-cell heterogeneity, which provides direct linkages between cellular phenotypes, such as drug responses, and microenvironments. Here, we focus on the applications and analytic approaches used for functional cell-based exploration of combinatorial microenvironments using microarray technology.

Keywords Microenvironment • Cancer • MEMA • Combinatorial microenvironment microarray • Drug development • Tissue architecture

The Challenge of Predicting Efficacy

In anticancer drug development, a suboptimal ability to predict how molecules will perform in humans based on preclinical drug screening often delays the progress of drug discovery. Tremendous resources have been invested in translating preclinical anticancer compounds into drugs used clinically in the last few decades. However, any given candidate is more likely to fail rather than succeed in clinical trials, which further elevate the cost of development and make drugs less affordable [1].

Advancements in cell and molecular biology, as well as engineering, have ushered in the modern era of pharmacology, which aspires to identify compounds with potentially selective activity against tumors in cell-based high-throughput screening (HTS), and then further validate the drug's efficacy in animal model systems, rodents being the most popular mammalian system. Indeed, our knowledge of the molecular underpinnings of tumor biology has accelerated remarkably over the past two decades. For instance, the Cancer Genome Atlas program has identified a broad range of recurrent gene mutations and structural rearrangements that putatively drive tumorigenesis, and a number of drugs have been selected to target protein changes resulting from those specific genetic mutations. These candidates often show promising effects in small animals but have much less success in patients [2]. Almost 70% of new drugs fail despite meeting safety standards established in phase I trials but exhibit no efficacy in phase II [3]. Studies have started to address the possible mechanisms involving the different expressed genomes of mice and men, and there are other significant differences that arise at the level of physiology and tissue architecture that can impact drug responses as well [4].

The tumor microenvironment, i.e., the sum of cell-cell, cell-extracellular matrix (ECM), cell-soluble factor interactions, and the physical properties and geometry, has been shown to impact cancer progression, drug responses, and a number of other tumor properties [5, 6]. Thus, an important challenge that is being undertaken in drug development is to identify preclinical screening modalities that take microenvironment into account and that are generally more reflective of the relevant biology in human tissues in order to provide a more predictable clinical outcome.

Tumors Are Heterogeneous "Organs," and Tumor Microenvironments Are Important Determinants in Therapeutic Responses

The natural inter- and intra-tumor heterogeneity is thought to be a major component of drug resistance. A nuanced view of tumors has begun to emerge such that they are not just a homogeneous expansion of neoplastic cells, but they are viewed as abnormal organs, comprising multiple cell types within dynamic microenvironmental ecologies [7]. These "organs" interact with the body via unique vascular systems and changes in immune homeostasis that lead to evasion of immune responses as well as cancer treatments [8]. The combinations of ECM, growth factors, cytokines, tensile force, and oxygen tension contribute to control the malignant progression, metastasis, and drug responses [6, 9–11].

The cancer stem cell (CSC) hypothesis offers attractive explanations for generation of heterogeneity within tumors, metastatic dissemination, and resistance to therapy. The underlying logic is modeled on normal developmental hierarchies that are delineated for a number of adult tissues. Undifferentiated stem cells give rise to less potent progenitors, which produce the most specialized cells of a given tissue. Analogously, only CSCs are thought capable of self-renewal, of initiating tumors at primary and distant locations, and of giving rise to more differentiated daughters that are incapable of reestablishing the tumor. Normal stem cell activity is maintained in niches; therefore, employing the same logic used for developmental hierarchies, niches that maintain CSCs, should also exist (reviewed in [12–14]). Niches are specialized microenvironments, wherein stem cells reside (reviewed in Refs. [15, 16]), which exert control over cell function. It was shown that progenitors both in skin and skeletal muscle could adopt residency in vacated stem cell niches, where they reacquired stem cell traits [17–19]. Impressively, testis and neural stem cells from male mice were shown to give rise to lactating mammary glands when transplanted into the mammary fat pads of female mice [20, 21]. And in true reductionist models that used defined microenvironments, embryonic and adult stem and progenitor cell fate decisions were shown to be quantifiably flexible in response to combinatorial microenvironments [22–25]. The ability of the niche to determine the functional spectrum of stem cell activities led us to hypothesize that stem cell niche microenvironments beget stem cell functions [26]. Due to their role in maintaining stem cell activity, disrupting CSC-niche interactions may be crucial for overcoming barriers to therapeutic resistance [27]. Thus, understanding the interactions between tumor microenvironments and cancer cells is important for the identification of druggable mechanisms (e.g., proliferation, differentiation, quiescence, etc.) regulated by tumor microenvironments and for improving drug efficacy in humans.

Deconstructing Tumor Microenvironments into Experimentally Tractable Combinations

Tissues are collections of cells and ECM knit together into unique spatial configurations that collectively carry out specialized functions. Remarkably, tissues with an intact architecture can maintain many basic functions in spite of the presence of gene mutations that cause dysfunctions when introduced into cells on tissue culture plastic [28]. Studies showing that wound-healing microenvironments unleash malignant potential demonstrated the principle that tissue architecture confers the resilience of normal function in the face of mutations and other external perturbations [29]. Organized asymmetry is therefore an important basic feature of metazoan tissues; there must be distinctive topologies on which receptors assemble in order to correctly integrate the signaling patterns associated with tissue-specific functions. Tumor microenvironments should as well possess combinatorial signaling asymmetries, though the microenvironments may be less obviously organized. One hypothesis is that the normal and tumor microenvironments integrate the signaling apparatuses differently. Accordingly therapeutic targets could be identified to selectively harm the tumor cells, with microenvironment composition functioning as a determinant of drug efficacy. Those potential differences in signal integration can be revealed by technologies that recapitulate aspects of in vivo microenvironments, using defined physical, geometric, and molecular elements, and allowing one to assess the contribution of each attribute to emergent properties of tissues.

The complexity of microenvironments is a major impediment to understanding their impact on cells. A majority of our understanding of biological mechanisms in human cells has been built upon studies on two-dimensional (2-D) plastic plates or dishes. Since the first human cell line, HeLa, was established on cell culture dishes, 2-D cell culture has been a mainstay of biological research. However, as the dominant nature of the microenvironment over physiological processes has become increasingly appreciated, engineered 2-D and 3-D culture platforms that better recapitulate the molecular and physical nuances of tissues in vivo are being developed.

It is an oversimplification to distinguish 2-D and 3-D culture platforms based on dimensionality; the details of the culture microenvironments need to be considered and delineated with care to understand how each property effects cell physiology. Although 2-D tissue culture plastic has been used extensively for biological research, they are far from physiological representations of tissues. In addition to the synthetic polymer composition of the plastic, cells in conventional 2-D culture systems adhere to surfaces that are nonphysiologically rigid (>2 GigaPascals Young's Elastic Modulus (GPa)) as opposed to the rigidity of normal tissue (hundreds of Pa in soft tissues to tens of thousands of Pa for stiffer tissues like cartilage and bone) [30, 31]. As the importance of microenvironment in therapeutic response has become more widely accepted, the urgency to identify tractable organotypic culture systems for studying human tissues in vitro has manifested.

Matrigel, HuBiogel, HuMatrix, and a number of other commercially available laminin-rich ECM are widely used to provide 3-D cell growth environments, and these gels are used increasingly to study the impact of drugs on cells grown in

3-D. Matrigel, which is harvested from a rodent sarcoma cell line, is comprised of hundreds of proteins that can vary significantly in their exact composition between production lots [32]. Adoption of 3-D culture to HTS systems is an important advance, the use of 3-D gels in HTS studies is now a less daunting prospect, and achieving good quality imaging of cells in 3-D seems to be the major rate-limiting step [33]. Biopolymers used for 3-D culture systems such as Matrigel are around 400 Pa to 1 kPa elastic modulus, and type I collagen gels can range from 500 Pa to over 12 kPa depending on collagen concentration. However, placing human cells in an undefined rodent sarcoma 3-D context may not mimic the intended in vivo microenvironment, and variability in the molecular components may confound interpretations and reproducibility of the results. Synthetic 3-D culture hydrogels, such as polyethylene glycol-based systems, offer precision tunability of the elastic modulus, which tends to cover a range similar to collagen gels and allows control over molecular compositions [34].

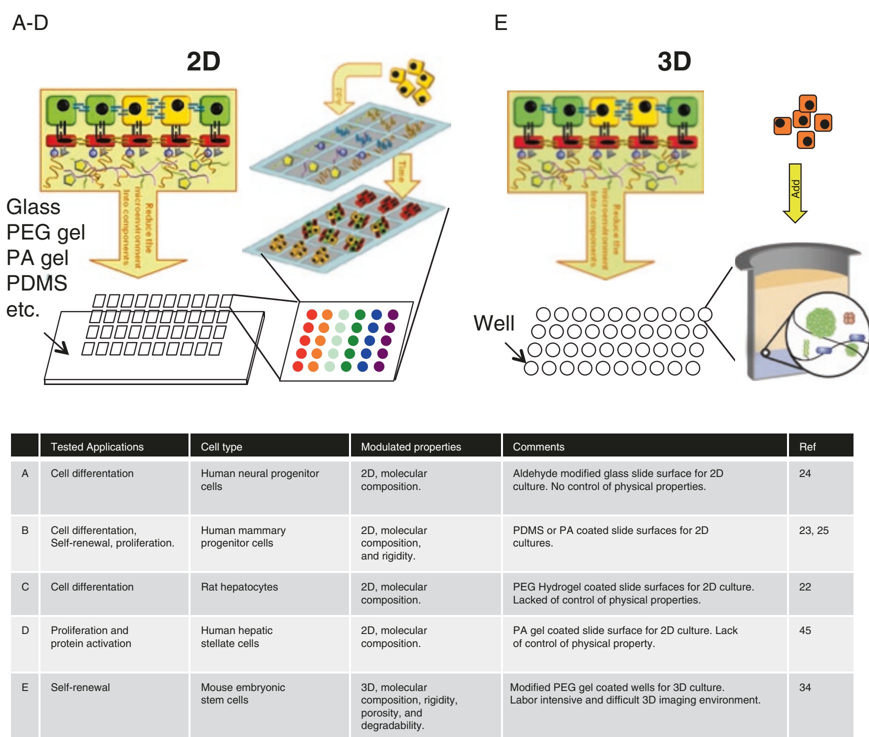
Every in vitro system for studying tissue microenvironment sacrifices important aspects of the in vivo situation, but there is merit in studying microenvironmental properties in isolation. Although engineered and biopolymer-derived systems necessarily over simplify tumor microenvironments, they can reveal important mechanistic elements of cellular responses by winnowing down the possible candidate pathways involved in a given functional response. The microenvironment can be dissected into biophysical (e.g., rigidity, shear force), biochemical (e.g., ECM, growth factors, cytokines), and architectural (e.g., dimension and geometry), and each property plays a role in regulating various cellular functions. For instance, by examining normal mammary epithelial cells in the context of matrix rigidity, in isolation from many other microenvironment properties, we discovered age-dependent regulation of the mechanotransducing YAP and TAZ transcription factors [35]. We focused on mechanobiology from the outset which made it possible to test hypotheses that revolved around pathways known to be involved in mechanobiology, and it revealed important information about age-related functional changes that also happen in vivo. Similarly, by using engineered polymer surfaces, we showed that substrate rigidity is a determinant for HER2-targeted therapeutic efficacy via YAP and TAZ signaling, both in vitro and in vivo [36]. While the microenvironments are deconstructed and different properties are studied individually or in defined combinations, the knowledge that we accrue over time allows us to form a portrait that models, and possibly explains, microenvironment affects on cellular functions.

Combinatorial Microenvironment Platforms Mimic Diverse and Defined Milieus and Meet Needs for Higher Throughput

Established human cell lines and primary cells propagated in 2-D culture are amenable to high-throughput experimentation. Potentially powerful tools for performing drug design in microenvironmental contexts are being developed by merging together the flexibility of functional cell-based screening with the highly parallel

nature of microarray-type experiments. A microarray is a device that contains thousands of functionalized probes immobilized on a substrate. Microarray-type tools provide both complexity and high-throughput ability and are used to explore diversity in various biological systems. Broadly speaking, the technology can be classified into protein arrays, gene chips, or carbohydrate microarrays, depending upon what probes are immobilized on the substrate [37]. An interesting innovation in this technology space has been to fabricate microarrays in 2-D and 3-D contexts, printing proteins that support adhesion of cultured cells. These types of combinatorial microenvironment microarrays (MEMA, or formerly called MEArrays) facilitate highly parallel cell-based functional screening. Indeed, using different ECM and growth factor, pathway-blocking or pathway-activating antibodies in various combinations as printed probes, enables molecular dissection of more complicated 3-D microenvironments (Fig. 18.1; reviewed in Refs. [22–25, 34, 38]).

While these array platforms create caricatures of in vivo microenvironments, they enable researchers to functionally define molecular components that maintain adult and embryonic stem cells, thus revealing molecular regulators and pathways of the stem cell state. We predict this type of functional cell-based dissection of combinatorial microenvironments will have particular high impact in understanding normal and malignant human stem cells, because in vivo experiments are essentially impossible. For instance, putative niche proteins and other tissue-specific proteins have been identified using MEMA, and validated in vivo in some cases,



	Tested Applications	Cell type	Modulated properties	Comments	Ref
A	Cell differentiation	Human neural progenitor cells	2D, molecular composition.	Aldehyde modified glass slide surface for 2D culture. No control of physical properties.	24
B	Cell differentiation, Self-renewal, proliferation.	Human mammary progenitor cells	2D, molecular composition, and rigidity.	PDMS or PA coated slide surfaces for 2D cultures.	23, 25
C	Cell differentiation	Rat hepatocytes	2D, molecular composition.	PEG Hydrogel coated slide surfaces for 2D culture. Lacked of control of physical properties.	22
D	Proliferation and protein activation	Human hepatic stellate cells	2D, molecular composition.	PA gel coated slide surface for 2D culture. Lack of control of physical property.	45
E	Self-renewal	Mouse embryonic stem cells	3D, molecular composition, rigidity, porosity, and degradability.	Modified PEG gel coated wells for 3D culture. Labor intensive and difficult 3D imaging environment.	34

Fig. 18.1 Examples of MEArray platforms

that were relevant to human embryonic [22, 39], neural [24], mammary [23, 25], and hepatic stem cells [38]. MEMA also were used to profile cell-ECM adhesion biases [40] and to optimize growth conditions of cultured cells [41]. Taking a combinatorial approach, relative to a candidate-based approach, allows screening combinations of multiple tissue-specific microenvironment proteins to identify extracellular cues that are the basis for emergent cell behaviors. Functional roles for a number of molecules known to be expressed in human mammary gland and brain, but hitherto had not been ascribed respective roles for mammary or neural stem and progenitor cell regulation, were discovered using this type of approach. The successful application of MEMA requires managing a number of technical details that are, in many cases, on the edge of discovery themselves. The remainder of this chapter will elaborate on some of the issues that arise most often when producing MEMA on 2-D substrates and provide some discussion of how we are managing them. There are relatively fewer examples of MEMA-type platforms in 3-D, perhaps because some of the high-throughput liquid handling and 3-D imaging requirements raise the barrier to entry; however, an excellent example of 3-D MEMAs is available in Ranga et al. [34].

Selecting the Printing Substrate: It Depends on the Biological Questions Being Asked

There are numerous materials used to immobilize proteins, but the primary objective remains the same where MEMA fabrication is concerned: a suitable surface coating for printing proteins upon should provide high adsorption capacity, low cell attachment in areas not printed with proteins (i.e., non-fouling), and low spot-to-spot variation. Other important considerations include the capacity to retain protein structure, functionality, and binding sites.

The most commonly used approaches are to chemically modify surfaces of glass slides, e.g., with aldehydes or epoxies, or to coat them with very thin layers of polymers such as polydimethylsiloxane (PDMS). Slides with these surfaces adsorb proteins with either covalent bonds or strong electrostatic interactions, respectively. Covalent modifications provide irreversible attachment; however, protein 3-D structures may not be well maintained. Unintended cell attachment also can be problematic with the chemically modified glass and with the hydrophobic PDMS without the addition of non-fouling coatings, like Pluronics F108 or bovine serum albumin. Another option is to coat glass surfaces with polyacrylamide (PA) or poly(ethylene glycol) (PEG) hydrogels. These hydrogels physically absorb proteins through relatively weak electrostatic interactions, which retain most of the native protein conformation, but there is higher variation in protein-binding capacity [42]. One of the most convenient properties of PA and PEG gels is their native non-fouling character, which removes any problems of nonspecific cell attachment.

Rigidity of the substrate is another important property to consider. PDMS is inexpensive, and its elastic modulus is easy to manipulate by altering the cure: polymer ratio, covering a range of elastic modulus similar to cartilage, skin, and tendon (0.6–3.5 MPa). PEG represents a range of elastic moduli from 500 kPa to 1.6 GPa. PA is another inexpensive substrate, which can be tuned from 150 Pa to 150 kPa, which is closer to the biological microenvironment for soft tissues like brain and breast [43]. Which substrate for protein immobilization should be used ultimately depends upon the characteristics of the cells used, the tissue being mimicked, and the outcomes being measured.

MEMA Data Analysis: Seeing the Forest for the Trees

A main goal of MEMA-type experiments is to provide causal links between cellular responses and specific microenvironments. Both inter- and intra-microenvironment heterogeneity of cellular responses are to be expected and can be instructive about the continuum of phenotypic plasticity within the experimental system. Measuring heterogeneity of drug responses in a diversity of contexts may result in more realistic expectations of drug responses in vivo. By incorporating sufficient numbers of replicate features into the design of a MEMA, significant associations between microenvironments and cell phenotypes can be identified, but the high dimensionality of the data is a hindrance to extraction of meaningful information. Most MEMA platforms use fluorescent probes to visualize biochemical and functional phenotypes and fluorescent and phase microscopy to capture morphological and colorimetric phenotypes. There are no specialized high-throughput imaging systems for this type of work currently available; however, microarray scanners and programmable, motorized epifluorescence or laser scanning confocal microscopes have been successfully used to acquire the necessary images [23, 34]. Micrographs of cells attached to the arrayed microenvironments can be treated as ensemble data, i.e., averaging the signal from many cells on one spot in a manner similar to DNA arrays, or as single-cell data when used in combination with cell segmentation algorithms. Even in cases where MEMA are designed to have fairly low complexity, e.g., 100 or fewer unique microenvironment combinations, the analytical challenges are significant. The complexity of the information space generated from MEMA experiments increases rapidly when taking into consideration multiple microenvironmental properties such as rigidity, geometry, and molecular composition. In practice, the statistical analysis of MEMA experiments is a rate-limiting step for this technology, and there are multiple solutions for addressing this challenge. The basic data processing workflow for MEMA experiments includes: signal normalization, identifying functionally similar microenvironments by clustering, dimension reduction, data visualization, and further pathway analysis. Table 18.1 shows some suggested software packages that aid with analyses of MEMA-type data, with comments on specific strengths and weaknesses.

Table 18.1 Software for processing microarray data

Software	Application	Advantages	Limitations
ImageJ	Image processing	Easy to use, batch processing	Needs Java to improve automation
Fiji (ImageJ 2)	Image processing	Built-in plugins specifically for biological data, batch processing	Needs Java to improve automation
Cell Profiler	Image processing	No coding needed and better native automation compared to ImageJ or Fiji	Less customization compared to Matlab
Matlab	Image processing	Highly customizable for image processing	Needs intensive coding
Excel	Data processing	Easy to use and very limited coding needed	Difficult to process large data sets. Limited visualization choices
R	Data processing	Handles very large data sets	Needs intensive coding
Python	Data processing	Easier to use compared to C ++ and can be integrated with other software, such as R	Needs intensive coding

Data Normalization

All microarray-like data contain some useful information and a significant degree of noise; thus, proper normalization is crucial. The data analysis begins with measuring fluorescence intensity or colorimetric density of each target protein in cells on each array feature. In this context, intensity typically reflects the relative abundance of the target protein. Intensities are impacted by factors such as the characteristics of the dye (antibody), spatial location, and uneven surfaces of the slides that cause inconsistent background [44]. Unlike DNA microarrays, which load the same amount of cDNA onto the array and then uses total intensity as an internal reference, the number of cells attached on MEMA features varies by microenvironment. Thus, we may use the average of the total signal from all cells on all array features as a reference for normalization of arrays of the same treatment condition. A signal emanating from cells on a control microenvironment, which is known a priori to reproducibly bias toward a given phenotype, can be used as a reference [23]. An alternative is to use spots that contain the same amount of fluorescence molecules and should have the same intensity as an internal control printed on each array.

Statistical Considerations

The main purpose of MEMA experiments is to identify the specific microenvironments that modulate certain cellular functions by comparing cellular phenotypes between treatments and controls. Table 18.2 shows some methods, which have been

Table 18.2 Data analysis and visualization techniques used with MEArray-type data

Methods	Type	Advantages	Limitations	References
Z-score	Normalization	Easy to implement even in excel	Sensitive to outlier values, and less accurate in cases with few cell numbers	[45]
Φ-score	Normalization	Overcomes the limitations of Z-score	Needs specialized software for implementation, such as R	[46]
Dunnett's test	Statistical test	Overcomes problems with type I errors (false positives) due to multiple comparisons to a single control	Does not make all pair-wise comparisons	[23]
PCA	Dimension reduction	A simple method to PCA to identify patterns due to variance	Only reflects linear relationships	[47]
ICA	Filter noise and data separation	An alternative method to identify patterns and filter noise	Data needs to be non-Gaussian distribution and independent to each other	[48]
IPCA	Filter noise and dimension reduction	A method combined PCA and ICA to identify patterns	Similar to ICA, certain assumptions are needed	[49]
SPADE	Visualization	Identifies patterns in high dimensional data	Lower resolution compared to ViSNE, needs further statistical tests for validation	[50]
ViSNE	Visualization	Similar to SPADE but has higher resolution. Can reflect nonlinear relationships	Needs further statistical tests for validation	[51]

used for processing MEMA data. Compared to using Student's t-test, a widely used statistical test in biological research, Dunnett's test is a better option for correcting false P values due to multiple comparisons and identifying microenvironments that impose phenotypes that are significantly different from the control [23]. The Z-score standardization is a simple method used to identify meaningful groups that are distinct from the global mean. Z-scores have been used successfully to identify and optimize better culture conditions for rare cell populations [45]. However, the Z-score has several limitations, like skewing of values due to outliers within a data set as well as decreased accuracy when cell numbers are reduced. Moreover, the Z-score is based on the assumption that the data fit a Gaussian distribution, which is

not the case in many biological systems. Thus, Guyon et al. proposed the Φ-score as a cell-to-cell phenotypic scoring method for selecting the hit discovery in cell-based assays. The Φ-score ranks cells instead of averaging them and shows performance that surpasses the Z-score for coping with the above limitations. Indeed the Φ-score can be more sensitive (more true hits) and more specific (fewer false positives) compared to other conventional methods [46].

Clustering methods commonly used for DNA microarray data sets, such as hierarchical or k-means clustering, also are used with MEMA data to separate meaningful groups. Konagaya et al. interrogated a relatively small number of growth factor combinations to optimize neural progenitor cell culture microenvironments and then used hierarchical cluster analysis to reveal three major clusters of microenvironment combinations that facilitated growth versus astrocyte or neuron differentiation [41]. Although these analyses can reveal the meaningful groups within simple data sets, like traditional two-color DNA microarray data, the difficulty and challenge of data clustering arise rapidly in multidimensional data sets [52]. The phrase, "the curse of dimensionality" [53], described the general phenomenon that data analysis techniques, which work well at lower dimensions, are often unable to perform as well when the dimensionality of data are increased. To overcome some of these difficulties, dimension reduction techniques have been developed.

Dimension Reduction and Data Visualization

Due to improvements in computational processing power, we now are able to better deal with high-dimensional data and with algorithms that do not make painful compromises in the name of efficiency. Dimension reduction essentially distills vast amounts of information into snap shots that are emblematic of the underlying biology.

Principal component analysis (PCA) is used for dimension reduction and can reveal the most variable factors that contribute to certain phenotypes [47]. However, not all biological questions are related to the variables with highest variance in the data set, and in these cases, PCA is less able to identify the contributing factors. Thus, independent component analysis (ICA) is an alternative to PCA, particularly when some certain characteristics of the data are known, allowing the assumption that the observed data are separated into groups that are independent of each other [48]. An example of a case where ICA has been applied is the cocktail party problem, describing the human ability to selectively recognize speech sounds that are often assumed to be independent from each other in noisy environments [54]. However, the need to make assumptions about the data and to choose the number of components analyzed is a limitation of ICA, particularly in high-dimensional data sets where we may not fully understand the relationships between variables. Due to this limitation, Yao et al. proposed independent principal component analysis (IPCA) combining the advantages of PCA and ICA, where they applied PCA as a preprocessing step to extract components for subsequent analysis and then applied

ICA to filter out noise [49].They assumed that microarray-based gene expression measurements that follow a Gaussian distribution represented noise (i.e., most of the genes are not expected to change at a given condition), and they showed that IPCA was better able to reveal patterns within those biological data [49]. All of these approaches are used in microarray analysis, but they often suffer from preserving important information during data reconstruction when trying to analyze high-dimensional single-cell data. Linear techniques such as PCA focus on separating dissimilar data points far away in low-dimensional representations after data transformation. However, biological data is often nonlinear, and for high-dimensional data, it is usually more important to keep similar data points close together in low-dimensional representations, which is typically not feasible with linear mapping techniques [55].

MEMA data are of a similar level of dimensionality to the data generated by techniques like mass cytometry (CyTOF). CyTOF is being developed as a new single-cell analysis technique that combines flow cytometry and transition element isotope labeling. Accordingly, CyTOF allows one to simultaneously measure up to 100 protein markers inside and on the plasma membranes of single cells [56]. CyTOF and MEMA experiments generate very high-content information, incorporating information about multiple protein expression levels, multiple cellular morphology parameters, and other image-based measurements among different treatments, all at the single-cell level. Qiu et al. developed spanning-tree progression analysis of density-normalized events (SPADE) to mass cytometry data and demonstrated the ability of SPADE to recapitulate known patterns of hematopoiesis and to identify previously unknown subpopulations of cells [50]. Based on the t-SNE technique, Amir et al. developed a nonlinear approach called viSNE, for high-dimensional data visualization. Using this approach they were able to identify a rare leukemia population when comparing leukemia diagnosis and relapse samples [51]. These two approaches take into account the major and minor sources of variance within a data set and represent them on a lower dimensional surface. Once the meaningful information has been extracted, the data collected from MEMA needs to be connected to the existing body of knowledge in order to perform further biological validation.

Conclusions

Following the discovery of oncogenes and tumor suppressors, essentially the de facto demonstration that DNA mutations are at the heart of cancers, huge investments were made in developing technologies to enable exploration of the total complexity of genomes and proteomes that are intrinsic to cells. One important, and wholly unexpected, outcome of the massive efforts to understand cancer as an entirely cell-intrinsic problem is the undeniable conclusion that mutations do not explain everything. Indeed, the fact that frankly malignant cells can be phenotypically normal, when held in check by a normal microenvironment, suggests that there is a dominant role of the microenvironment. New investments need to be made in

technologies that facilitate the dissection and exploration of tissue microenvironments. MEMA-type platforms, and their successors, will provide opportunities to gain a comprehensive understanding of how the microenvironment modulates drug responses in human cells and will provide functional cell-based data for preclinical drug screening that is ultimately more predictive of in vivo biology.

These platforms are amenable to high-throughput scale-up using a number of imaging modalities for quantification. The main challenges of this approach are access to purified extracellular proteins, managing the combinatorial complexity to minimize cost and maximize the combinatorial space that is evaluated, data visualization, and statistical analysis to identify microenvironment components that contribute to a given outcome. An important component that is still in its infancy is robust network analysis that can provide a systematic understanding of how microenvironments are linked to activity in specific signaling pathways, which underlie cell phenotypes, and reveal candidates for further investigation. Tapping into the accumulated knowledge, represented in public databases and tools for pathway mapping like GO (Gene Ontology) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes), will increase the possibility that we can connect microenvironment-imposed phenotypes to known signaling pathways and, hence, to cellular functions. Different microenvironment components, such as ECM or substrate rigidity, are the input, and the measurements, such as morphometrics and other protein markers, are the output. The major object of pathway analysis is to delineate the relationship between input and output.

There is an obvious need to improve preclinical drug discovery and evaluation. Overall, MEMAs are meant to address the shortcomings of experimentation that uses standard human cell culture models (i.e., the nonphysiological contexts), rodent models (i.e., the nonhuman context), and human beings (i.e., the intractable model). One of the approaches is to take into account the microenvironmental impact on drug responses during the earliest design stages of therapeutics. The combinatorial nature of MEMAs provides the advantages of exquisitely controlling microenvironmental properties and enabling high throughput. MEMA data are high-content, single-cell resolution and can capture cell-to-cell heterogeneity; hence, it may provide a more realistic picture of drug performance. However, it remains to be seen whether data from MEMA-type experiments are capable of building in vivo response models. Improved knowledge of microenvironmental impact on drug responses will economize and hasten drug development by making the preclinical stage more predictive and aid in the deployment of more precision therapeutics.

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Chapter 19
Models of Tumor Progression in Prostate Cancer

Waqas Azeem, Yaping Hua, Karl-Henning Kalland, Xisong Ke, Jan Roger Olsen, Anne Margrete Øyan, and Yi Qu

Abstract Human prostate cancer is initiated in a benign prostate epithelial cell which gains the potential to progress to metastatic disease. The exact cell of origin of prostate cancer has been debated in recent years based upon different models. Primary prostate epithelial cells have restricted life-spans in culture, but can be immortalized. Prostate cancer cell lines have been difficult to establish and new ones are desirable. Attempts to transform benign prostate epithelial cells in vitro have proved difficult without the use of strong carcinogens or oncogenes in processes not likely to mimic closely carcinogenesis in the aging human prostate. Models of epithelial-to-mesenchymal transition (EMT) and cancer stem cells in prostate carcinogenesis have become available, and advances in three-dimensional organoid culture technology represent a breakthrough in prostate cancer research. Organoids may recapitulate multiple features of prostate cancer and have the potential to replace costly and laborious animal experiments. Still, animal models are needed to investigate and validate molecular mechanisms and to develop therapeutic principles in the pipeline between in vitro experiments and clinical applications. Although mice represent the most common experimental animal in prostate cancer research, species like rat, dog, and zebrafish may have advantages depending upon the hypothesis or question. Animal models can generally be categorized into spontaneous or induced development of cancer, immunodeficient animals with xenografts, and genetically engineered animals. In prostate cancer, neuroendocrine differentiation and bone metastases are prevalent in the final stages of cancer progression and animal models that recapitulate these processes are available.

Keywords Prostate cancer • Animal model • Cell culture model • Prostate cell

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Origin of the Prostate Cancer Cell

The prostate gland consists of multiple small glandular elements embedded in a vascularized connective tissue stroma (Fig. 19.1). Each small glandular element is defined by an outer basement membrane on which a layer of basal epithelial cells is situated (Fig. 19.1). In these basal cells, the androgen receptor (AR) is silenced. One prevailing view regarding normal prostate epithelial differentiation is that when AR is induced by unknown mechanisms in the presence of androgen, basal cells differentiate into luminal cells and a minor population of neuroendocrine cells. These events may be reproduced in in vitro cultures of immortalized basal cells which can be propagated as transit amplifying (TA) cells in growth media with low-calcium concentration [2] (Fig. 19.1). The lineage relationships between basal and luminal cells, and in particular which one is the cell of origin of prostate cancer and of putative prostate cancer stem cells (CSCs), have been vigorously debated [3, 4]. The bulk of prostate adenocarcinoma cells retain mostly luminal cell expression patterns, but evidence has been provided to support both basal cells and luminal cells as the cell of origin of prostate cancer. Recent work has revealed considerable plasticity in the differentiation pathways and suggested that it may be more fruitful to focus on the activated regulatory networks and mechanisms [4]. There are, however, strong clues that the key regulatory mechanisms in normal prostate epithelial differentiation are retained in a perverted form in advanced prostate cancer. This notion is exemplified by the importance of the AR transcription factor during prostate cancer progression, including in castration-resistant prostate cancer (CRPC) [5], and by the neuroendocrine differentiation [6, 7] in end-stage prostate cancer. The unknown activation status of AR in putative prostate CSCs remains an important unresolved question with significant therapeutic consequences [8].

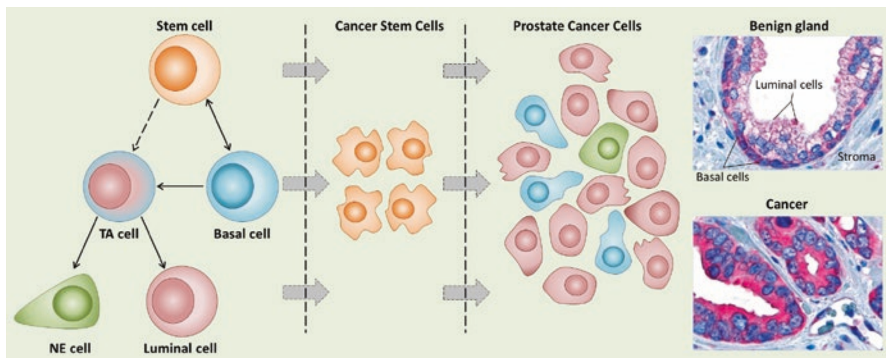
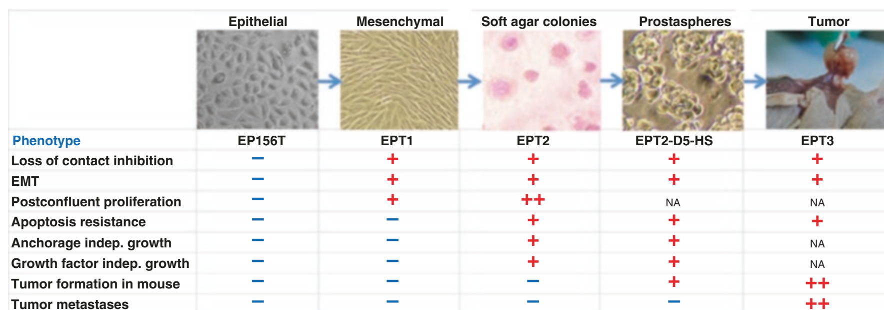


Fig. 19.1 Possible normal differentiation pathways from prostate stem cells to epithelial basal cells, luminal cells, and neuroendocrine (NE) cells and possible transformation pathways to prostate cancer stem cells and cancer cells (TA = transit amplifying cell). Stained histological sections of prostate benign (*upper*) and cancer (*lower*) tissue are from publication [1]

Cell Culture Modeling of Prostate Carcinogenesis

Primary prostate epithelial cells (PrECs) can be obtained from biopsies and surgical material, as well as commercially, and can be propagated for a limited number of passages in monolayers. PrECs have been immortalized using either hTERT (human telomerase reverse transcriptase) or the transforming elements of DNA viruses [9–11]. The 957E/hTERT cells [12, 13] and EP156T cells [14] were immortalized by exogenous expression of hTERT. PZ-HPV7, CA-HPV10, and RWPE-1 cells were immortalized by human papilloma virus (HPV) transforming elements [15]. Immortalization of PrECs has been achieved without exogenous gene expression [4, 16–18], but there is no model available of PrECs that spontaneously have transformed into malignant cell lines in vitro.

Many attempts have been made to study malignant transformation of benign prostate cells in culture, but the use of strong carcinogens or oncogenic viral elements was necessary to achieve transformation [19]. Forced transformation may be useful for many purposes but is suboptimal when physiological mechanisms of transcriptional reprogramming during prostate carcinogenesis are investigated. Physiological selection pressure was applied to EP156T cells by keeping the cells in a confluent monolayer with regular replacement of fresh growth medium. After several months progeny EPT1 cells with reduced cell-to-cell contact inhibition dominated the culture. EPT1 cells had undergone EMT but were not tumorigenic [19]. EMT turned out to be the first step in the accumulation of malignant traits in a succession of progeny cells, eventually resulting in tumorigenic EPT3 cells (Fig. 19.2) [20, 21]. This model encompasses benign transit amplifying epithelial cells (EP156T), benign (EPT1) and pre-malignant (EPT2) mesenchymal type cells, and tumorigenic (EPT3-N04/EPT3-PT1) and metastatic (EPT3-M1) cells in mice (Fig. 19.2). The very different phenotypes share a common genotype. Forensic grade DNA microsatellite, karyotype, and copy number breakpoint anal-



Phenotype	EP156T	EPT1	EPT2	EPT2-D5-HS	EPT3
Loss of contact inhibition	−	+	+	+	+
EMT	−	+	+	+	+
Postconfluent proliferation	−	+	++	NA	NA
Apoptosis resistance	−	−	+	+	+
Anchorage indep. growth	−	−	+	+	NA
Growth factor indep. growth	−	−	+	+	NA
Tumor formation in mouse	−	−	−	+	++
Tumor metastases	−	−	−	−	++

Fig. 19.2 Overview of the EPT prostate stepwise tumorigenesis model. EPT1 cells were selected for loss of cell contact inhibition. EPT2 cells were selected from foci of confluent EPT1 cells and cloned in soft agar. EPT2-D5-HS was selected in protein-free medium. EPT3 cells were selected following subcutaneous injection. Cells were recovered from the EPT3 tumor. The progressive accumulation of malignant hallmarks is summarized [20]

yses verified progeny authenticity [20]. Each of the different cell types can be passaged indefinitely and to high cell numbers in sub-confluent monolayers. Subpopulations of tumor-initiating EPT3 cells (TICs) show activation of the WNT pathway and an autocrine IL6/STAT3 loop and show increased resistance to apoptosis and anoikis [21]. Genome-wide analyses revealed that epigenetic promoter patterns at different steps of the model corresponded strongly with coordinated expression changes of regulatory gene modules, such as HOX and microRNA genes, and structural gene modules, such as desmosome and adherens junction genes [19, 20, 22]. The model demonstrates, however, an absence of gene expression pattern characteristic of the bulk cellular population of prostate adenocarcinomas. Prostate luminal gene expression is strikingly absent, and the model is more likely to represent features of mesenchymal type cells in prostate cancer progression. In fact, evidence of EMT in the progression of primary prostate cancer has been shown in patient tissue [23]. However, the role of EMT, and its significance, in early prostate carcinogenesis, in metastasis, and in the development of resistance to androgen deprivation treatment (ADT) and other prostate cancer therapy requires further investigation in available cell cultures, preclinical models, and patient samples [24]. A particular pressing issue is the mounting evidence that ADT and highly potent inhibitors of AR function, such as enzalutamide, might induce EMT and more aggressive cancer, possibly involving prostate CSCs [25–27]. Alternative hypotheses have been discussed, such as the existence of a common progenitor prostate cancer stem cell that gives rise to both the neuroendocrine-like and adenocarcinoma components, and both these components continue to evolve and respond to selective pressures in parallel [26]. A negative feedback loop between AR and ZEB1 wherein ADT upregulates ZEB1 expression toward induction of EMT has been demonstrated [28]; ZEB1 in turn is indirectly able to induce stem cell factors through repression of miR200 family members which themselves repress stemness factors including SOX2 and KLF4 [29].

Prostate Cancer Cell Lines

LNCaP, PC-3, and DU-145 and their metastatic derivatives are still the most widely used human prostate cancer cell lines despite the length of time these “classical” cell lines have been in culture since isolated from human metastases [11, 30–33]. It has proven to be difficult to establish stable cell lines from primary prostate cancer. The Prostate Cancer Cell Lines Database provides an updated online overview of registered prostate cancer cell lines (http://www.capcelllines.ca/). LNCaP cells are androgen responsive in contrast to the AR-negative PC-3 and DU-145 cell lines, but are less effective in forming tumors and metastatic colonies in mouse xenografts. Reviews summarize in vitro models of AR signaling in prostate cancer [30] and useful cell lines for mouse xenografting [33]. Among additional prostate cancer cell lines, the VCaP and DuCaP cell lines

express AR and the androgen-responsive TMPRSS-ERG fusion, and the 22Rv1 cell line is considered an in vitro model of CRPC [30]. 22Rv1 cells express the androgen-independent AR splice variant denoted AR-V7 [34]. These and additional prostate cancer cell lines have provided important information on prostate cancer but also have many limitations. With their origin in metastatic tissue and lack of exact passage history, they cannot be used to recapitulate prostate carcinogenesis, and it is difficult to estimate which genetic changes are due to in vitro culture selection. Thus, in one genome-wide ChIP-seq study, only 3% overlap in AR-binding sites were found between prostate cancer cell lines and prostate cancer tissue prior to treatment [35]. Since most cell lines were isolated from patients who had undergone treatment, this could also be a factor in the differential gene expression. In prostate cancer research, isolation of additional prostate cancer cell lines is desirable, in particular from primary cancer tissue, in addition to more complex and emerging models.

In Vitro Modeling of the Prostate Cancer Microenvironment and Three-Dimensional (3D) Growth Conditions

Cancer cells develop, proliferate, and invade in crosstalk with a microenvironment consisting of fibroblasts, immune cells, vessels, and nerves embedded in a connective tissue matrix. The simplest experimental approximation to the in vivo situation is to co-culture prostate cancer cells and stromal cells in monolayer or double layers. Several techniques are available to culture thin tissue slices of the cancer tissue as ex vivo explants [36, 37]. The advantage of this system is that it preserves most of the features of the tissue architecture as well as its heterogeneity, although it is difficult to maintain and propagate the cultures for more than a few weeks. Ex vivo explants can also be useful for hormone and drug testing. This model also has potential usefulness in designing personalized medicine and in experiments that assay morphological or signal pathway changes in a tissue context. Many models recapitulate selected aspects of cancer growth in 3D or microenvironment conditions. Gels consisting of extracellular matrix substances, such as Matrigel, Geltrex, and collagen, or alternatively synthetically bioengineered scaffolds may support 3D growth of both benign and malignant prostate cells. Several techniques are available to support 3D spheroid growth of prostate cells with or without extracellular matrices. When grown on surfaces with ultralow attachment, prostate cells tend to form spheroids or prostaspheres resulting in the enrichment of cells with stem cell features [21]. Spheroids grown either in extracellular matrix or in ultralow attachment plates or as hanging drops may all reproduce the nutrition, oxygen, and pH gradients that are found in cancer tissues that outgrow their blood supply [37].

Organoid Cultures

The basis of organoid cultures is the availability of a matrix that supports 3D growth in vitro and an essential cocktail of compounds that modulate defined signal transduction pathways. In this way adult stem cells have been able to differentiate and self-organize into organoids that retain many features of the organ of origin [38, 39]. Organoid culture technology has significantly improved the success rate of establishing in vitro cultures of cancer cells [40]. Organoid technology has successfully generated benign epithelial prostate cultures [17, 41] and cultures that represent different subtypes of prostate cancer [42]. Prostate organoids have been established from metastatic cells, though establishment of in vitro cultures of primary prostate cancer cells remains a challenge [18, 42]. Organoid cultures have the additional advantage of being able to be propagated indefinitely and can be stored in liquid nitrogen as a living biobank. The experimental potential of prostate organoids has only begun to be exploited. Compared to monolayer cultures of stable cancer cell lines, organoid cultures may recapitulate more features of the original cancer although more experience needs to be gathered regarding the extent and for how many passages essential aspects of the original tumor can be preserved. The outcome will be very important for the use of organoids in personalized medicine in order to test drug sensitivity in vitro and to have an expandable antigen source that may be exploited in individualized immunoassays and dendritic cell-based vaccine development in the expanding immunotherapy field. In prostate cancer experimental research, the availability of organoids established from distinct cancer subtypes should facilitate investigation of critical molecular signaling pathways. Presently, microenvironment and immune interactions are not established in organoid methodology. Organoids may find their place between traditional cell cultures and animal models [40] (Fig. 19.3). In the design of animal experiments, it should be considered whether organoids could replace traditional cell lines for ethical, cost, and capacity reasons. The CRISPR-Cas9 genome editing system has recently transformed genome editing by its efficiency to knock out or knock in genes in cells and animals [43–45]. When used in combination with organoid technology, CRISPR-Cas9 technology may generate attractive experimental systems with systematic manipulation of single cancer relevant genes or combinations of genes [17, 46].

Animal Models

Animal models are needed for better understanding of how cancer cells interact with the tumor microenvironment and with the entire organism during metastasis. Spontaneous development of prostate cancer is relatively common in dogs and some rat strains, but less common in mouse strains. Mouse models can be broadly divided into xenograft models and genetically engineered models [47]. While immunodeficient mouse strains are necessary for xenograft models, the current interest in immunotherapy has increased the demand for immunocompetent (syngeneic) mouse models and humanized mouse models.

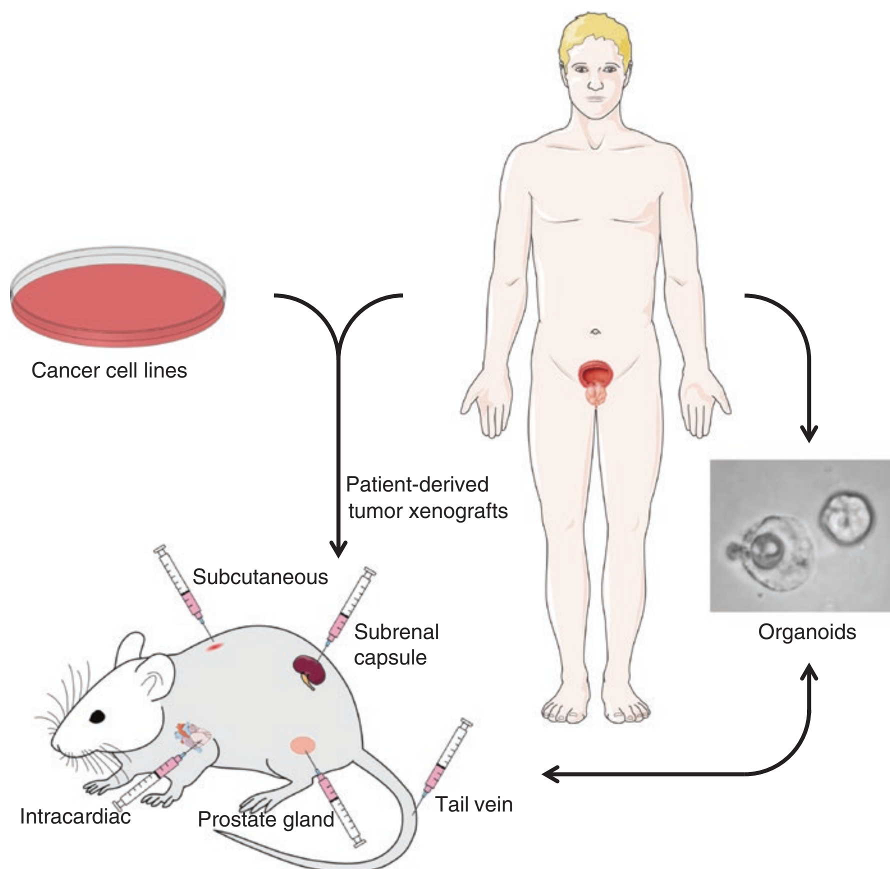


Fig. 19.3 Prostate cancer cell lines have most commonly been used for mouse xenograft models. Patient-derived xenografts (PDXs) have several advantages in retaining heterogeneity and features of original cancer tissue. Organoids can be established directly from prostate cancer tissues or via PDXs and vice versa. Patient-derived organoids (PDOs) have advantages when it comes to capacity and biobanking and several experimental types. In the panel to the right are shown short-term organoids grown from a primary prostate cancer core biopsy obtained at our Haukeland University Hospital from a patient in a Phase I clinical trial of cryoimmunotherapy against metastatic castration-resistant prostate cancer

Mouse Xenograft Models

Xenografts can be grown from any tumorigenic prostate cell culture or pieces of tissue. LNCaP, PC-3, and DU145 are the three most commonly used prostate cancer cell lines in xenograft models and have provided significant insight into disease biology [48]. Technically, the simplest approach is to inject tumorigenic cells subcutaneously with or without an intercellular matrix support, such as Matrigel. Tail vein injection or technically more demanding orthotopic injection into the prostate gland may be advantageous to answer questions related to metastasis and stromal invasion. Subrenal capsular injection of cells that are otherwise difficult to graft

may be successful in part due to the high vascularization at this site. Tissue recombination models in which dissociated adult prostate cells are combined with embryonic urogenital sinus mesenchymal cells and implanted under the renal capsule have been useful for cell differentiation studies and epithelial-stroma interactions [49].

The choice of mouse strain, and in particular the extent of immunodeficiency, may also affect the efficiency of xenograft formation. The "nude" mouse was first established more than 40 years ago, and the advancement of immunodeficient mice to model human tumor growth has been reviewed recently [50].

Patient-Derived Xenografts

Although cell culture-based xenografts may provide useful information on cancer biology, these models have important limitations. In recent years the cancer research field has become highly aware of the importance of cancer cell heterogeneity which cannot be recapitulated by available cell culture-based xenografts. Patient-derived tumor xenografts (PDX) have emerged as a powerful technology: capable of retaining the molecular heterogeneity of their originating sample [51] and have been shown to exhibit genomic clonal dynamics reminiscent of their originating tumor sample [52]. In contrast to cell-based xenografts, PDXs have original tumor morphology [53]. PDXs have the potential to improve basic research on cancer subtypes with specific genomic lesions and could provide mouse avatars in personalized medicine drug evaluation and co-clinical trials [54]. Relevant stromal or immune drivers of malignant progression could, however, be missing [51] when immunodeficient mice are used as recipients.

Genetically Engineered Mouse Models (GEMMs)

Genetically engineered mouse models (GEMMs) allow the overexpression or deletion of selected genes in order to study the effect of defined pathways on carcinogenesis and tumor progression [47, 55–60]. Advantages compared to xenografted mouse models are that GEMMs are compatible with intact immune systems and stromal microenvironments of the same species as the tumor. The limitation of GEMMs is related to differences between mouse and men, regarding prostate architecture, cancer propensity in rodents, and small size of mice compared to humans. The TRAMP model is one of the most commonly used early transgenic models [61]. The model was generated by the introduction of a gene construct with the minimal rat probasin promoter driving expression of the SV40 virus early region. Androgen-responsive expression of the SV40 large T antigen inhibits p53 and Rb, and the small t antigen inhibits protein phosphatase 2A [57]. C57BL/6 TRAMP mice develop prostatic intraepithelial neoplasia (PIN) by 3 months of age. PIN typically progresses to neuroendocrine carcinoma within half a year with the lymph nodes

and lungs as metastatic predilection sites. The model has been extensively used in preclinical testing and studies on carcinogenesis and tumor progression, but several limitations exist [57, 58]. DNA virus oncoproteins, such as SV40 large T antigen or human papilloma virus E6 or E7, have a special power to force cancer development, but these viruses have not been shown to induce prostate cancer. Furthermore, neuroendocrine differentiation is a feature of end-stage human prostate cancer and is seen in less than 2% of primary human cancer [6]. Furthermore, TRAMP mice rarely develop bone metastases, a common event in human patients.

The LADY model provides a modification compared to the TRAMP model by using a larger region of the rat probasin promoter to drive the SV40 large T antigen expression without small t antigen. Thereby a panel of less aggressive tumor lines, collectively referred to as LADY, was generated to study cancer-preventing factors and synergistic effects of different oncogenes [47, 55–57, 59].

A number of transgenic mouse models have since been generated to study signal transduction pathways involved in prostate carcinogenesis and progression in humans. Overexpression of the transcription factor MYC is prevalent in early prostate cancer [62], overexpression of which immortalizes primary prostate cells, induces PIN in normal prostate tissue, and stimulates growth of both early stage and CRPC [63, 64]. Mouse models of prostate cancer based on c-Myc have been reviewed [47, 55–58, 65].

The most frequently mutated single genes in primary prostate cancers are *SPOP*, *TP53*, *FOXA1*, and *PTEN* [66]. In a recent analysis of 333 primary prostate carcinomas, 15% harbored homozygous deletions spanning the *PTEN* locus [67]. The homozygotic knockout of *Pten* is lethal in mouse embryos, while heterozygotic knockout results in a spectrum of prostate phenotypes that, combined with other genetic lesions, such as $p27^{Kip1-/-}$ or $Nkx3.1^{-/-}$ mice, result in the progression to PIN and invasive prostate cancer [55]. The health problems associated with *Pten* knockout mice and the value of this genetic background in the study of additional genes and pathways in prostate cancer have encouraged the development of several conditional *Pten* knockout mouse models [47, 55–59, 65].

Genomic Editing of Mouse Models

Traditional GEMMs have exploited genetic engineering and homologous recombination of embryonic stem cells followed by injection of the manipulated stem cells into wild-type blastocysts. Selected chimeric mice are then crossed to generate single-gene knockout or double-mutant mice [68]. Genetic elements that allow inducible gene expression [69], such as tetracycline inducible element, or conditional knockout [70], such as the Cre-Lox system, or knock-in [71], have further expanded the utility of GEMMs [72]. The generation of these useful models has, however, been costly and time-consuming. The CRISPR-Cas9 genome editing system may lead to a breakthrough in fast, and effective generation of precision mouse cancer models [68].

Prostate Neuroendocrine Tumor Models

Small-cell neuroendocrine carcinoma is a rare form of primary prostate cancer [6]. When the common acinar adenocarcinoma has reached the stage of CRPC, it can often still be efficiently targeted by the AR-inhibiting compounds enzalutamide and abiraterone. Tumor relapse is, however, the eventual outcome and often in the form of aggressive neuroendocrine cancer. Neuroendocrine transdifferentiation may provide important clues to the nature of putative prostate CSCs. Thus, models of neuroendocrine prostate cancer, such as xenograft and genetically engineered mouse models, are of increasing importance [6, 7, 26].

Bone Metastasis Models

The bone is a predilection site for metastases of prostate cancer resulting in high morbidity associated with late stages of this disease [73–75]. Dogs spontaneously may develop benign prostate hyperplasia and prostate cancer with osteoblastic bone metastases similar to the natural course of prostate cancer in men [76]. DPC-1, Ace-1, Leo, and Probasco represent four dog cell lines available for research on bone metastases, including xenograft models. The human prostate cancer cell line PC-3 also forms bone metastases in xenograft models. Available prostate cell lines and xenograft models regarding prostate bone metastases have been reviewed [75, 77]. A mouse model of bone metastasis was generated by grafting human lung and bone tissue followed by tail vein injection of LNCaP cells. The LNCaP cells preferentially metastasized to the human bone tissue [78].

Spontaneous Cancer Development

In general, mice do not develop spontaneous prostate cancer with an incidence that makes them useful prostate cancer models. ApcMin/+ mice, which were originally selected from randomly mutagenized mice, develop multiple intestinal neoplasia (min), presumably as a consequence of β-catenin activation due to *Apc* gene inactivation [79]. It has been shown that up to 40% of male ApcMin/+ mice developed histological features of both PIN and prostate carcinoma at 5 to 6 months of age, thus mimicking the early stages of prostate cancer in aging men [80] making this an interesting model not only for intestinal tumors, but also for prostate cancer [81].

Several rat models are prone to spontaneous or chemically induced prostate cancer [47, 77]. Almost one third of Lobund-Wistar rats develop spontaneous androgen-sensitive metastatic prostate adenocarcinomas at a mean age of 26 months. These tumors subsequently become androgen independent and metastasize primarily to the lung. The model has been useful in studies of chemical and dietary effects on carcinogenesis [82].

Dog Models

Many breeds of domestic dogs are prone to develop spontaneous age-dependent benign prostatic hyperplasia (BPH), high-grade prostatic intraepithelial neoplasia, and invasive prostate cancer [77, 83–85]. Development of bone metastases with mixed osteoblastic and osteolytic lesions and the emergence of new woven bone in the later stages of prostate cancer are similarities shared between dogs and humans [86]. The use of next-generation sequencing and a greater level, and depth, of information generated by large-scale sequencing of human prostate cancers [67] (Cancer Genome Atlas Research Network, Electronic address 2015) could increase the utility of dog models in studies of the relevance of selected genes in cancer development and progression. Dog breeding records would facilitate association analysis and family-based linkage studies [83].

Pet dogs could be valuable in preparing for Phase I clinical trials of novel targeted therapies, immunotherapies, and personalized innovative combination therapies.

When considering the dog model, the potential advantages, such as animal size and propensity of skeletal metastases, should be balanced against potential limitations. Specifically, in contrast to what has been observed in castrated humans, castration of dogs does not seem to protect against development of prostate cancer. Additionally, the effect of androgen deprivation therapy may differ between human and dog prostate cancers [77].

Model Organisms

The zebrafish and fruit fly models have become useful in cancer research [87]. These model organisms are particularly useful in the study of defined oncogenes and signal transduction pathways. The zebrafish has become a widely used model organism for prostate cancer research with several advantages regarding optical clarity, fecundity, rapid embryo development, and absence of immune system development until 14 days post-fertilization. Genomic tools have made possible disease modeling and large phenotype-based screens in zebrafish models. The zebrafish model offers a rapid and inexpensive means of evaluating the metastatic potential of prostate cancer cells. By injection into the perivitelline space of 2-day-old embryos, DU145 prostate cancer cells can be found throughout the body after only 24–48 h, and knockdown of WASF3 led to suppression of metastasis in zebrafish [88]. In addition, the zebrafish model can be used for identification of prostate tumor-initiating cells from cultured cells and primary prostate cancer cells and shows advantages over mouse models in prediction of therapy response, because its translucent nature allows noninvasive observation of tumor progression in real time [89].

Zebrafish might also provide an excellent vertebrate tool to accelerate cancer drug discovery and development, including high-throughput screening, toxicology,

and target identification. In our group we have evaluated compounds that inhibit Wnt/β-catenin signaling in vivo using a transgenic zebrafish harboring the Tcf/Lef-miniP:dGFP reporter [90]. Imaging of the fluorescent protein reporter allows real-time determination of drug potency, targeting specificity and body toxicity in vivo.

Models in Drug Discovery and Development

Mice represent the most commonly used animal model in drug discovery and preclinical development [91]. The small size of mice is advantageous in order to save money on expensive compounds in preclinical testing of their toxicity and pharmacokinetic and pharmacodynamic properties. For absorption, distribution, metabolism, excretion, and toxicity tests (ADME/T), common mouse strains are robust and easy to breed, such as the inbred C57B/6 congenic mouse strain or the BALB/c strains. Genetically engineered mouse strains can also be utilized for specific purposes.

The small size of the mouse may have its disadvantages for use as a tumor model. Even large tumor masses in mice could have a volume 1000-fold smaller than a human tumor of the same stage. Consequently, the cancer cell number would be proportionately lower in mice tumors, and the cancer cell heterogeneity problem could be underestimated. This could be one reason why mouse tumors often have a higher cure rate than what is found in subsequent clinical testing in patients.

Finally, the utility of in silico models should not be discounted for drug screening, target identification, and drug development [92]. Multiple experimental and preclinical models are available and under development for prostate cancer research. Ultimately, the choice of model should be carefully evaluated during experimental design in order to achieve optimal scientific results with due attention given to statistics, ethics, capacity, and costs.

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Chapter 20
Tumor-Host Interactions in Malignant Gliomas

Lina Leiss, Ercan Mutlu, Mohummad Aminur Rahman, and Per Øyvind Enger

Abstract Malignant gliomas are infiltrative tumors arising in the brain, characterized by degradation of the extracellular matrix and tumor cell migration along white matter tracts. The most aggressive form displays florid angiogenesis and recruitment of host vessels. In a reciprocal fashion, host-derived factors exert modulatory effects on the glioma cell compartment. Thus, tumor-stroma interactions regulate critical aspects of brain tumor progression. These interactions are shaped by the structural organization of the central nervous system (CNS) and involve multiple cell types, extracellular matrix (ECM) components, and host cell-derived soluble factors that are unique to the CNS. Here, we will first provide an overview of the CNS microenvironment, followed by a review of how these elements contribute to the brain tumor-host interplay.

Keywords Gliomas • Central nervous system • Blood-brain barrier • Brain tumor-stroma interactions • Tumor-associated glial cells (TAGs) • Brain tumor immunity • Invasion • Angiogenesis

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Constituents of the Central Nervous System

Tumor-stroma interactions are heavily influenced by the histoarchitecture of the host tissue and structural organization of the CNS. The main cell types found in the CNS are neurons, astrocytes, oligodendrocytes, ependymal cells, neural stem cells, progenitor cells, microglia, immune cells, endothelial cells, and pericytes. The extracellular matrix comprises hyaluronic acid, proteoglycans, and glycoproteins and accounts for 10–20% of the volume of the CNS tissue [1]. Following is a description of the aforementioned individual components.

Neurons

These cells exert the core function of the central nervous system by propagating information coded as electric impulses, action potentials [2]. Through cellular extensions, neurons establish contacts, synapses, with other neurons. As a result, neurons are interconnected in a functional network that enables the flow of information between different CNS regions. Across the synapses, information is transmitted in the form of chemical substances called neurotransmitters. The brains' neuronal cell bodies are mostly located underneath the brains' surface where they form the gray matter or cerebral cortex. Action potentials are generated near the cell body and transmitted to other neurons along fibers, axons. These axons are often coated with a lipid-rich sheet called myelin that allows electric pulses to be transmitted more rapidly along the nerve fibers [3]. The brain parenchyma underneath the cerebral cortex contains bundles of these myelinated fibers that appear white, referred to as white matter.

Glial Cells

These cells are the most numerous in the CNS and can be grouped into different subtypes based on morphology and function [4]. They do not generate action potentials but have multiple roles linked to homeostatic functions and maintenance of structural support and integrity of the parenchyma. Astrocytes represent a major subgroup of glial cells that are present in both gray and white matter. They regulate ion concentration and osmotic pressure in the extracellular space and also express transporters for uptake of neurotransmitters. Astrocytes express intermediate filaments, typically glial fibrillary acidic protein (GFAP), which provides a robust cytoskeleton, consistent with their role in structural support [5]. These cells form cellular protrusions with enlargements at the distal ends, so-called foot processes or end feet, which establish contacts with cerebral capillaries, neurons, as well as the surface of the brain. Around the capillary network, these cellular extensions form a structural element of the blood-brain barrier [6, 7]. Around neurons, astrocytic processes provide an insulation that is essential to neuronal function.

Oligodendrocytes are glial cells whose primary function is to produce the myelin sheets that insulate nerve axons in the CNS, enabling faster propagation of action potentials [8]. The myelin sheets comprise multiple layers of cell membranes concentrically wrapped around the nerve axons. Oligodendrocytes produce myelin basic protein (MBP), which is part of the myelin sheet and essential for its function and structure [9]. Due to their role in nerve fiber myelination, oligodendrocytes are abundantly present in areas containing nerve fibers, such as the white matter of the brain. However, they also exist in the gray matter where myelin lamellae surround fibers traversing the cerebral cortex.

Immune Cells

The immune surveillance of the CNS differs markedly from other organs, due to the blood-brain barrier that limits the access of blood-borne pathogens and the absence of lymphatic vessels. However, peripheral immune cells including macrophages, dendritic cells, T cells, and NK cells are able to enter the CNS upon activation [10]. In the healthy state, mesenchymal cells are clustered in the perivascular space, and dendritic cells are present in proximity to the meninges and in the choroid plexus. These cells may detect and phagocytose foreign antigens and present these to circulating T cells. Upon activation, T cells migrate across the BBB into the brain parenchyma.

Microglia are smaller than astrocytes and oligodendrocytes; they account for 5–20% of all glial cells and mediate the CNS' innate immunity toward harmful events. They are derived from the yolk sac and enter the CNS as monocytes during fetal development [10]. Residing in the brain parenchyma, they develop cellular processes and enter a resting state in the mature healthy brain, although they retain their ability for phagocytosis.

Studies suggest that microglia constantly monitor their environment for abnormalities through their cellular extensions and that detection of such changes activates them. This response state is accompanied by proliferation, migration to the area of pathology, phagocytosis, antigen presentation, and release of proinflammatory factors. Thus, resident microglia constitute the local and primary immune response, but they are also able to stimulate the adaptive immune system [10].

Endothelial Cells and the Blood-brain Barrier

The CNS is highly vascularized, reflecting the neurons' high consumption of oxygen and vulnerability to oxygen deprivation. Moreover, the cerebral capillaries in the CNS have unique features that markedly restrict the exchange of chemical substances between blood and nervous tissue compared to other organs. These capillaries prevent extracellular levels of ions and neuroactive substances from being affected by varying concentrations of these compounds in the blood stream. Notably,

endothelial cells in the CNS are not fenestrated and are tightly held together by aptly named tight junctions that effectively block transport between the cells. These cells are surrounded by a continuous basal lamina that again is covered by pericytes. Astrocytic end-feet form an outer layer that insulates the capillaries and which induces the formation of tight junctions between the endothelial cells. Collectively these structural elements are referred to as the blood-brain barrier, and it is crucial for normal neuronal functioning [7].

Extracellular Matrix (ECM)

Apart from its cellular constituents, the CNS comprises an extracellular matrix that accounts for 10–20% of the brain volume. In the adult CNS, this matrix provides structural support and regulates synaptic plasticity and function. The ECM is organized into a looser neural interstitial matrix of ECM molecules occupying the extracellular space between the cells, the basement membrane which supports and separates endothelial cells from the brain parenchyma, and perineuronal nets which are mesh-like structures that surround the neuronal cell bodies and dendrites [11]. The interstitial matrix consists of proteoglycans, glycoproteins with one or more side chains of unbranched glycosaminoglycans covalently bound to a core protein [12]. In addition it has a high content of hyaluronan, a glycosaminoglycan without a core protein, tenascin, and linker proteins. Glycosaminoglycans are negatively charged and thus bind water molecules and cations. The basal lamina is a specialized ECM structure built from fibrous proteins including collagen, fibronectin, and laminin. These molecules have physical properties distinct from glycosaminoglycans and create a firmer structure than the neural interstitial matrix. The basal lamina contributes to the integrity of the blood-brain barrier, and it forms the glia limitans together with astrocytic end feet that is the outer surface of the CNS, underneath the pial covering. The perineuronal nets are condensed matrix structures lying around the neuronal cell bodies in areas such as the cerebral cortex and the hippocampus. Its chemical composition includes elements such as the chondroitin sulfate proteoglycans, hyaluronan, tenascin R, and link proteins. The perineuronal nets regulate synaptic function and stabilize the microenvironment around the neuronal cell bodies.

Gliomas: A Brief Overview

Gliomas are the most common malignant primary brain tumors and are classified according to the WHO based on their resemblance to glial cell types and graded based on their degree of malignancy according to histopathological criteria [13] (Fig. 20.1). The most common tumors are astrocytomas, oligodendrogliomas, and tumors with mixed differentiation. Grade II–IV gliomas are highly infiltrative and

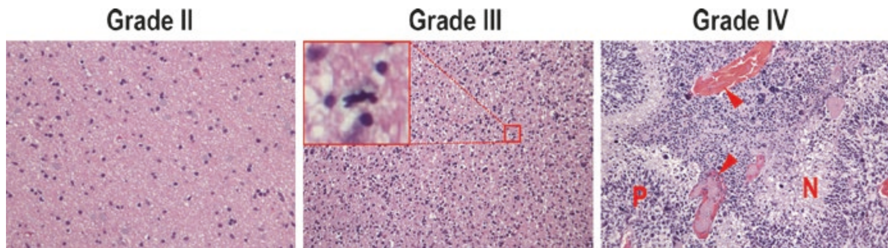

Fig. 20.1 Shown are H/E stainings of astrocytomas, WHO grade II–IV as indicated. Grade II astrocytoma displays a slight hypercellularity and moderate cellular pleomorphism. Grade III, or anaplastic astrocytoma, shows pronounced hypercellularity, nuclear atypia, and occasional mitotic figures (*insert*). Grade IV tumors or glioblastoma exhibits hypercellularity, striking nuclear atypia, as well as necrotic regions (N) surrounded by pseudopalisading cells (P) with microvascular proliferations and enlarged vessels (*red arrowheads*). *H* hematoxylin, *E* eosin, magnification: ×100

incurable malignancies that never metastasize outside the CNS. Lower-grade tumors have a strong propensity to become more malignant over time. Grade II tumors tend to occur in younger individuals aged 20–30, whereas GBMs mostly occur in patients over 50 years. Median survival is reportedly 6–12 years and 3 years for grade II and grade III gliomas, respectively. Grade IV glioblastomas are highly vascular, necrotic, and infiltrative tumors. Median survival for GBMs is around 15 months if standard multimodal therapy is administered. This involves surgery followed by fractionated radiotherapy and temozolomide administered concomitantly and then adjuvantly in repeated cycles [14, 15]. GBMs can arise de novo or from conversion of lower-grade tumors and are classified based on mutational status, gene expression profile, and epigenetic changes into prognostic subgroups [16].

Brain Tumor Angiogenesis

The onset of angiogenesis is a key event in the malignant progression of gliomas and marks the transition from anaplastic gliomas to glioblastomas, coinciding with a drastically shortened survival [17]. Various cellular processes and biological mechanisms involved in the vascularization of brain tumors have been implicated [18], including sprouting from existing vessels, co-option of the host vasculature by invading cells, vascular mimicry, vessel intussusception, recruitment of bone marrow-derived endothelial precursors, contribution from M2-polarized macrophages, and transdifferentiation of cancer stemlike cells into endothelial cells [17].

Among these, several studies strongly suggest that vessel formation by endothelial cell proliferation and sprouting from the host capillaries is a main mechanism of angiogenesis in malignant gliomas [18]. Histopathologically, GBMs exhibit microvascular proliferations with endothelial hyperplasia, forming nonluminated structures referred to as glomeruloid bodies or vascular tufts, due to their resemblance

with glomeruli in the kidneys. The proliferation index of tumor endothelial cells has been estimated to be 22–29% in GBMs, higher than in lower-grade gliomas, whereas proliferation of endothelial cells in normal brain tissue is largely absent [19]. Furthermore, VEGF expression is particularly upregulated in palisading tumor cells around necrotic regions [20]. In addition, the VEGF receptor is abundantly expressed in these endothelial cells but hardly detectable in the normal brain vasculature. These findings also explain the striking presence of endothelial cell proliferations surrounding necrotic areas.

Gliomas are hallmarked by their infiltrative growth into the surrounding brain parenchyma. During invasion, glioma cells migrate along vessels of the existing host vasculature – a process referred to as co-option [21]. Holash et al. reported that co-option facilitates vascularization of experimental gliomas in early stages. Subsequently, vessels in the tumor center regressed, accompanied by upregulation of angiopoietin-2 (Ang-2). Simultaneously, however, newly formed capillary sprouts at the tumor periphery also expressed Ang-2. Their data suggested coordinated roles for angiopoietins. Upregulation of Ang-2 in the absence of VEGF induced endothelial cell death, whereas it mediated angiogenesis in the presence of VEGF. Apart from this experimental study, however, limited data are available regarding vessel co-option, and it is not documented that similar processes take place in human tumors.

Animal studies also suggest that GBM cells with a stemlike phenotype had the ability to integrate into the vessel wall and transdifferentiate into endothelial cells [22]. Others, however, have reported that endothelial cells carrying mutations found in the GBM cells occur at a very low rate mostly outside the endothelial lining of the vessel wall [22]. Thus, the experimental findings regarding this phenomenon are conflicting, and no clinical data have established a role for cancer stem cell transdifferentiation in human glioma angiogenesis.

Several studies implicate bone marrow-derived cells in brain tumor angiogenesis. In particular, macrophages may acquire an M1 tumor-inhibitory phenotype or an M2 tumor-promoting phenotype. M2 macrophages exert their effects in vivo by releasing cytokines that promote tumor cell growth and angiogenesis. In experimental studies, integration of bone marrow-derived cells into the vessel wall seems to occur at a low rate and has not been demonstrated in human gliomas [18].

Intussusception refers to invagination of the endothelial vascular wall, leading to extravascular tunnels with tissue traversing the vascular lumen. Although this phenomenon has been described in experimental tumors, no studies have demonstrated a role for vascular intussusception in human gliomas.

Brain Tumor Angiogenesis from a Therapeutic Perspective

Glioblastomas have been considered attractive candidates for anti-angiogenic therapy, due to their highly vascular nature. However, two prospective multicenter trials randomizing more than 1500 patients with newly diagnosed GBMs to treatment

with the humanized monoclonal anti-VEGF antibody, bevacizumab, or placebo, failed to demonstrate a survival benefit after bevacizumab [23, 24]. The escape mechanisms mediating brain tumor progression during bevacizumab treatment are incompletely characterized. However, both experimental studies and autopsy analyses of tumors from GBM patients receiving bevacizumab suggest that other angiogenic factors are upregulated, including FGF2, and that tumors undergoing anti-angiogenic treatment acquire a more invasive growth pattern, possibly by co-opting the host vasculature [25, 26].

Brain Tumor Immunity

It has been well established through histopathological studies that immune cell tumor infiltration is prevalent in glioma patients. Whereas a high degree of lymphocytic infiltration in the perivascular space was reported to be associated with up to 4 months longer survival in one study [27], others found that lymphocytes infiltrating the tumor correlated with a poor prognosis [28]. A predictive value of tumor-infiltrating lymphocytes is not yet established, consistent with the presence of functionally distinct classes of lymphocytes. Furthermore, early studies also reported that glioma patients displayed reduced peripheral cellular and humoral immunity [29]. Since then, numerous experimental and clinical studies have shown that glioma cells interact extensively with the immune system. These interactions involve both the innate and adaptive components of the immune system, as well as local and peripheral immune cells. Despite the presence of the blood-brain barrier and the absence of lymphatic vessels, peripheral immune cells gain access to the brain parenchyma and the tumor bed via multiple routes. The BBB is typically disrupted in malignant gliomas due to reduced pericyte coverage, gaps between the endothelial cells and basement membrane, and defects in the brain tumor vasculature [30, 31], allowing peripheral immune cells to enter the CNS. Moreover interstitial fluid drains to the perivascular space, enabling tumor antigens in the brain parenchyma to reach antigen-presenting cells around the meninges and in the subarachnoid space [32]. It has also been demonstrated that CSF communicates with deep cervical lymphatic drainage and that antigens in the ventricles may induce antibody-producing cells in cervical lymph nodes [33]. T lymphocytes are the main cell type involved in adaptive antitumor immunity. These cells belong to different subclasses with different roles in the host-tumor interplay and can be distinguished by their expression of cell surface markers. CD8+ T cells are cytotoxic and become activated in the presence of antigen-presenting cells and CD4+ helper T cells. Several studies show that the glioma-infiltrating effector cells correlate positively with tumor grade as well as survival in glioblastomas [34, 35]. However, it has also been shown that the glioma microenvironment is immunosuppressive due to tumor-derived factors and regulatory cells, Tregs, that impair the function of these effector cells [36]. Tregs are CD4+ T cells that express the transcription factor Foxp3, and numerous studies have consistently reported their presence in the glioma

microenvironment at higher, although varying rates compared to normal brain [37–39]. However, data regarding a correlation between Treg recruitment and survival in glioblastoma patients are conflicting [38, 39].

Moreover, numerous studies have clearly demonstrated that malignant gliomas are heavily infiltrated with microglia [40] that are recruited by glioma-derived chemoattractants such as MCP-1 and CSF-1 [41]. Importantly, microglia exert immunosuppressive effects through multiple mechanisms. In the presence of glioma cells, microglia display impaired MHC class II antigen presentation [42] and produce anti-inflammatory IL-10, which inhibits cytotoxic T cell function [43]. Moreover, tumor-associated microglia express FASL and upregulate the immunosuppressive molecule B7-H1, both capable of inducing T cell apoptosis [44, 45].

Recently, myeloid-derived suppressor cells (MDSC) have been identified both in peripheral blood and tumor tissue of glioma patients [46]. These cells have the ability to suppress T cell function through depletion of amino acids that are critical for T cell function and through production of reactive oxygen species [47]. Notably, an association between tumor-infiltrating granulocytic MDSCs and CD4+ T effector memory function has been reported [48].

Immunotherapy for Gliomas

The strategies explored to overcome glioma-related immunosuppression have largely been cancer vaccines, cellular therapies, immune checkpoint therapies, or combinations of these. Cellular therapies have involved adoptive T cell transfer with T cells immunized against tumor antigens, including genetically modified T cells with chimeric antigen receptors that activate T cells upon antigen binding [49]. Patient trials have demonstrated bioactivity and acceptable safety [50], although no survival benefit has been shown so far. Tumor vaccines involve tumor-associated antigens that are preferentially expressed by tumor cells but also expressed by normal cells or tumor-specific antigens that are confined to the malignant cell pool. Tumor-specific vaccines have been developed against the epidermal growth factor receptor variant III (EGFRvIII), a mutation occurring in 30% of GBMs, and *Cytomegalovirus* (CMV), since CMV-encoded proteins are present in most GBMs. Both vaccines have triggered potent responses, and a randomized phase III trial validating the EGFRvIII vaccine is ongoing for patients with newly diagnosed EGFRvIII-positive GBMs [49]. Immune checkpoints serve to ensure an appropriate T cell response toward foreign antigens while maintaining self-tolerance. Inhibitory checkpoints suppress T cell function. Blockage of two of these, the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1) or its ligands PD-L1 and PD-L2, has successfully prolonged overall and progression-free survival in patients with metastatic cancers [49, 51]. Although combinatorial blockage of CTLA-4 and PD-L1 has demonstrated antitumor activity in animal glioma models [52], their efficacy in glioma patients has yet not been validated.

Tumor-Associated Glial Cells

Glial cell types including astrocytes and oligodendrocytes represent the most abundant cell types in the CNS. These cells provide structural support and maintain homeostasis in the normal brain but are also present in the brain tumor microenvironment (Fig. 20.2), where they can influence multiple aspects of glioma growth and sensitivity to treatment (Table 20.1).

Reactive astrocytes are abundantly present in the tumor bed among infiltrating glioma cells, and brain tumor growth is accompanied by astrogliosis [53]. Activated astrocytes secrete various factors [54] and have been shown to increase the proliferation of malignant cells in vitro [55]. Moreover, they also secrete fac-

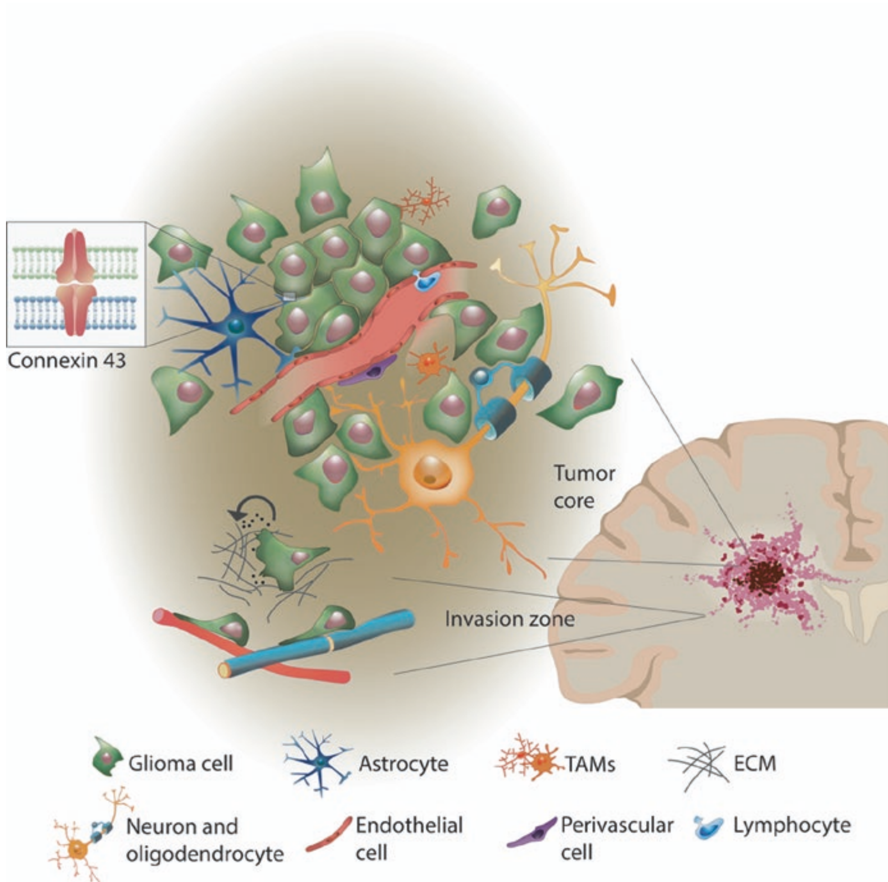

Fig. 20.2 Illustration of the main cellular interactions in different regions of malignant gliomas. Existing blood vessels serve as a substrate for glioma cell migration and are promoted by astrocytes. Glioma cells secrete factors that break down extracellular matrix to facilitate invasion. *TAM* tumor-associated macrophage/microglia (Illustration: Lina Leiss)

Table 20.1 Overview of published experiments investigating the effect of stromal cells on glioma cells

Experiment type	Cell type	Effect	Reference
In vitro	Astrocytes	Reduce tumor proliferation	[63]
In vitro	Astrocytes	Induce neuroprotection and reduce tumor growth	[64]
In vitro or in vitro and in vivo	Astrocytes	Promote tumor invasion	[54, 57, 58, 65–67]
In vitro or in vitro and in vivo	Astrocytes	Induce tumor drug resistance	[61, 62]
In vitro	Astrocytes	Decrease tumor radiosensitivity	[68]
In vitro	Astrocytes	Protect tumor against chemotherapy	[69]
In vitro	Endothelia	Promote tumor growth and invasion	[70]
In vitro and in vivo	Endothelia	Promote tumor invasion	[71]
In vitro or in vitro and in vivo	Endothelia	Promote tumor angiogenesis	[72–75]
In vitro and in vivo	Endothelia	Promote tumorigenicity	[76]
In vitro and in vivo	Endothelia	Protect against radio- and chemotherapy	[77]
In vitro	Microglia	Promote tumor growth	[78–80]
In vitro and in vivo	Microglia	Promote tumor progression	[81]
In vitro	Microglia	Promote tumor proliferation and invasion	[82]
In vitro or in vitro and in vivo	Microglia	Promote tumor invasion	[83–89]
In vivo	Microglia	Promote tumor growth and angiogenesis	[90]
In vitro or in vitro and in vivo	Microglia	Promote tumor angiogenesis	[91–93]
In vitro or in vitro and in vivo	Microglia	Induce immune suppression	[94, 95]
In vitro	Microglia	Induce secretion of IL-8 and MCP-1	[96]
In vitro and in vivo	Neurons	Promote tumor growth	[97]
In vitro	Neurons	Decrease tumor invasion	[98]
In vitro and in vivo	Oligodendrocyte progenitor cells	Promote tumor growth and angiogenesis	[60]
In vitro and in vivo	Tumor stromal cells	Promote tumor growth and angiogenesis	[99]

tors that may promote the proliferation of glioma cells, including EGF, IGF-1, GDF-15, and TGF-β [55–57]. However, in vitro or in vivo studies directly investigating the effects of astrocytes or oligodendroglia on glioma cell growth have not been published.

Conversely, several studies have clearly demonstrated a proinvasive effect of tumor-associated astrocytes on glioma cells both in vitro [54, 57] and in vivo [58, 59], involving multiple mechanisms: Astrocytes have been shown to secrete an inactive preform of matrix metalloproteinase-2, a proteolytic enzyme linked to cancer cell invasion, which is converted into an active form by glioma cells [54]. Moreover, connective tissue growth factor (CNTF), which has been implicated in cancer metastasis, has been shown to be secreted from reactive astrocytes surrounding infiltrative gliomas and bind to tyrosine kinase receptor type A (TrkA). Furthermore, targeting either CNTF or TrkA both reduced glioma cell infiltration [59]. Of note, it was reported that the gap junction protein connexin 43 (CX43) is overexpressed by astrocytes in the tumor bed [58]. Furthermore, gliomas in CX43 knockout mice had more circumscribed margins suggesting that CX43 promotes glioma cell detachment from the tumor core.

One study also suggests that oligodendrocyte progenitor cells may enhance angiogenesis in gliomas by disrupting the blood-brain barrier, thereby abrogating the effect of perivascular pericytes and promoting vessel sprouting and tubule formation [60].

Gliomas are characterized by chemoresistance, and several studies show that astrocytes can modulate the response of the tumor cell compartment to chemotherapy. Co-cultures of astrocytes and a panel of glioma cell lines showed that the presence of astrocytes increased glioma cell survival after treatment with temozolomide and doxorubicin in several cell lines [61]. However, the gap junction channel inhibitor CBX as well as CX43 siRNA knockdown abolished this protective effect suggesting a major role for gap junction channels between glioma cells and astrocytes in mediating chemoresistance [62].

Concluding Remarks

Malignant gliomas are characterized by dynamic interactions with the immune system, glial cells in the tumor bed as well as recruitment of host vasculature. Since these interactions are critical to tumor progression, they may be attractive targets for glioma therapy. Unlike some other cancer types, however, neither anti-angiogenic nor immune-based therapies have demonstrated any survival benefit in glioma patients, suggesting that the mechanisms that regulate tumor-host interactions in the CNS are in many ways unique to gliomas. Thus, therapeutic progress in this field requires that those mechanisms be explored in further detail.

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Chapter 21
The Tumor Microenvironment in Cutaneous Melanoma: Friend or Foe

Oddbjørn Straume and Cornelia Schuster

Abstract Malignant melanoma is one of the most aggressive and lethal cancers. Even a primary tumor of 1 mm in thickness can metastasize and kill the patient. However, without the interactions with a supporting microenvironment, the tumor cannot grow and thrive. Most of the time, the microenvironment imposes an inhibitory effect on melanoma growth, and the vast majority of mutated neoplastic cells occurring during life will be destroyed. But how is the tumor microenvironment (TME) sometimes co-opted to support tumor growth? Does it not recognize the tumor lesion as a potential threat? Does the TME perceive the tumor as "*a wound that needs to heal*"? This chapter will describe some important players in the melanoma microenvironment. In addition to the biology of the melanocyte, the different roles played by keratinocytes, fibroblasts, endothelial cells, and immune cells will be discussed.

Keywords Melanoma • Microenvironment • Stem cells • Tumor plasticity • Phenotype switch • Keratinocytes • Fibroblasts • Endothelial cells • Immune cells • Ulceration

Introduction

One unexplained phenomenon observed in melanoma patients is that ulcerated primary melanomas have a poorer prognosis in comparison to non-ulcerated primaries. The question then emerges as to whether the ulcer on the surface of the tumor reflects an underlying aggressive tumor biology per se, or is it the ulcer itself, and the persistent wound healing events in the environment enclosing the tumor, that drives the cancer cells into a more aggressive and more lethal phenotype? These questions give rise to a larger, more central question as to whether stressful conditions, such as

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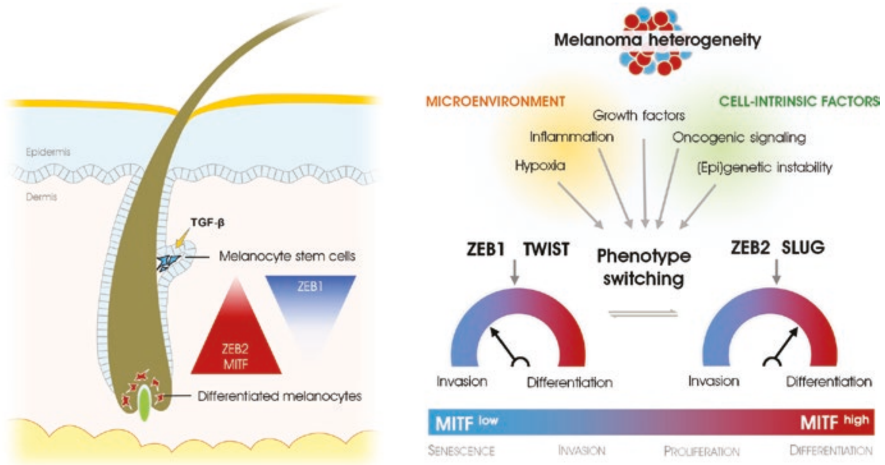

Fig. 21.1 Nodular malignant melanoma. Cutaneous melanoma of the nodular subtype. Please note the red halo around the lesion representing an inflammatory immune response against neo-antigens present in the tumor (with permission from © 2016 BMJ Publishing Group Ltd. All rights reserved)

hypoxia, frequently found in rapidly growing and ulcerated tumors, initiate a phenotypic switch enabling escape of tumor cells from the primary focus to distant sites. These clinically relevant questions may not be so simple at all and might define how we perceive and interpret melanoma with respect to its interactions with the host. Realizing that cancer in general, and melanoma in particular, is a distorted and corrupted reflection of our own body tissue, and that the nature of the malignancies basically are co-opted normal, out of control, physiologic processes, makes it easier to grasp the core of the challenges clinicians and researchers are up against.

Cancer is, in its essence, a genetic disease. Still, a sick cell is not on its own sufficient to generate a lethal tumor let alone metastatic dissemination to kill the host. Growth and spread do not happen without some contribution from a supportive microenvironment. Clearly, the tumor cell is not an island. Only by taking control over the interactions with normal cells and the extracellular matrix can melanoma cells thrive, grow, and spread. These interactions are essential in melanoma biology, and they can be targeted therapeutically Fig 21.1.

Embryology and Phenotypic Heterogeneity

To better understand the behavior of melanoma cells in host tissues, it is necessary to consider the normal developmental biology of the melanocyte. Many of the features of melanoma cell behavior can also be observed during melanocyte development, with the exception that the latter is under a strict control and fine-tuned during evolution. For melanoma cells, these features include returning to a proliferative,

dedifferentiated, and migratory phenotype similar to is observed during epithelial-to-mesenchymal transition (EMT) in epithelial cancers. In fact, many of the same genes involved in melanoma progression and metastasis are active during normal development (Table 21.1).

During embryonic development, the melanocyte originates from pluripotent cells in the dorsal edge of the neural tube and further differentiates in the neural crest. These stem cells have the ability to differentiate into a variety of specialized cells like neurons, glial cells, cardiac cells, as well as pigment cells [1]. The developing melanocyte will undergo four important steps before it arrives at the final destination and differentiates into mature melanocytes: specification, migration, survival, and proliferation. These four stages are regulated by genetic programs defined by specific sets of genes outlined in this section (for a detailed review, see [2]). During early gestation, cells destined to become melanoblasts begin expressing genes encoding transcription factors that further push the cells toward differentiation into specialized pigment cells. Of special importance are PAX3, LEF1, FOXD3, as well as genes involved in the Wnt signaling pathway [3–7]. In addition, the transcription factors SOX9 and SNAI2/Slug play a central role in initiating EMT and in activating MITF, thereby preparing the melanoblast for migration [8, 9]. Microphthalmia-associated transcription factor (MITF) is regarded as the master regulator of melanocyte differentiation [10, 11] and is frequently amplified or overexpressed in melanoma [11–13]. In addition to the melanoblast intrinsic expression pattern of genes, paracrine factors produced in the local environment are also involved in regulating the migrating melanoblast. For example, stem cell factor (SCF) is able to regulate gene expression programs, like MITF expression, through interaction with its cell surface receptor c-Kit and the downstream MAP kinase pathway [14]. Also, the cell-cell adhesion molecule β-catenin has been shown to activate MITF [15]. Cell-cell and cell-matrix interactions are also important for the migrating melanoblast. The melanoblasts need to escape the tissue of origin (neural crest) and not get stuck in any structures before the destination is reached. Up- and downregulation of various proteins, including cadherins, ensure the appropriate interaction with components of the extracellular matrix (ECM), such as integrins, laminin, lectins, and fibronectin [16, 17]. The melanoblasts also need to force themselves through biologic barriers and basement membranes to get to their destination tissues, for instance, by upregulating the expression of metalloproteinases like ADAMTS20 [18].

Interestingly, melanoblasts proliferate extensively before and after their migration but are more quiescent during the migration phase [19], again similar to the EMT process in epithelial tumors. This points to the importance of cellular plasticity and phenotypic switching in melanocytes, as well as in their malignant counterparts, melanoma cells. The phenotype-switching model of melanoma postulates that melanomas switch between proliferation and invasion (“growing or going”), but they rarely do both simultaneously [20, 21]. As BRAF inhibition and chemotherapy attack melanoma cells during the proliferative state, there will always be a subset of cells in a nonproliferative state that are less sensitive to these drugs [7]. There is even data to suggest that these migrating cells are evading an immune attack [22, 23]. In melanoma, Snail-induced EMT accelerates cancer metastasis

Table 21.1 A selection of genes involved both in normal melanocyte development and melanoma development [147]

Gene	Function	Specification	Migration	Proliferation	Survival	Differentiation	Melanoma
PAX3	Transcription factor	X		X	X		X
LEF1	Transcription factor	X				X	X
SNAI2/SLUG	Transcription factor	X	X				X
SOX9	Transcription factor	X	X			X	X
WNT1	Wnt signaling	X		X		X	X
β-catenin	Intracellular signaling, transcription	X				X	X
Cadherins	Surface binding proteins		X				X
Integrins, laminin, fibronectin	Extracellular matrix proteins		X				X
SOX10	Transcription factor	X	X	X	X	X	X
MITF	Transcription factor	X	X	X	X	X	X
KIT	Tyrosine kinase	X	X	X	X	X	X
SCF	Stem cell factor, ligand for c-KIT	X	X	X	X	X	X
FGFR	Fibroblast growth factor receptor			X		X	X
bFGF	Basic fibroblast growth factor			X		X	X
MC1R	G protein receptor			X		X	X
BCL-2	Apoptotic protein				X		X
MET	Tyrosine kinase			X	X	X	X
HGF	Hepatocyte growth factor. C-met ligand			X	X	X	X

through both enhanced invasion and induction of immunosuppression due to induction of regulatory T cells, immunosuppressive cytokines, impaired dendritic cells, and direct cytotoxic T-lymphocyte resistance. EMT-reversal by Snail blockade simultaneously inhibited both cancer invasion and multiple immunosuppressive mechanisms, resulting in efficient inhibition of cancer metastasis [24].

Considering the relative small size of primary tumors at the time metastases are observed, no other cancers can compete with the melanoma in eagerness to invade and metastasize. This aggressive feature of melanomas does in part rely on their ability to rapidly switch between phenotypes. The significant cellular plasticity observed in melanoma is reflected in the switching between a differentiated and an invasive phenotype, under the control of MITF and several different EMT-associated transcription factors [10]. Phenotypic heterogeneity is a result of (epi)genetic changes as well as of changing conditions in the tumor microenvironment. Growth factors and transcription factors inducing a more mesenchymal-like phenotype, i.e., motile and invasive, like TGFβ, HIF1α, ZEB1, SNAIL, and TWIST, are upregulated under stressful conditions such as hypoxia, UV-radiation, and inflammation [25–27]. These stress-responsive cellular programs are beneficial for melanoma cells under harsh conditions in hostile environments like the blood stream or other refractory tissues. Cellular stress responses like hypoxia have been extensively studied in melanoma. In a study by O'Connel and coworkers, an adaptive phenotype shift was described in response to hypoxia in the tumor microenvironment [7]. A rapid shift in expression, between Wnt5A receptors ROR1 and ROR2, was observed in response to hypoxia. Hypoxia thus induced a switch from a proliferative ROR1-positive phenotype to a more invasive ROR2-expressing phenotype. Of significant importance, this switch led to a tenfold decrease in sensitivity to BRAF-inhibitors in clinical use.

Going back to normal melanocyte development, once the migration is completed and the melanocyte arrives at the intended destination, another cellular program takes over. To ensure proliferation, maturation, and survival, the melanocyte relies on growth factors such as basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) to support the final steps in their development into functional melanocytes, i.e., in the dermal-epidermal junction of the skin [28].

The close relationship between melanocytes and the basal keratinocytes, which they protect from the damaging UV-light of the sun, is both quiet and balanced. These cells (basal keratinocytes and melanocytes) are highly stable. Similarly to the malignant counterpart, once the melanoma cell has survived the journey from the primary to a distant site, regrowth, differentiation, and escape from a micrometastatic state, is initiated by regaining MITF, SLUG, and ZEB2 [10, 11, 29]. The balance between the stem cell-like, differentiated, and invasive melanoma phenotype is illustrated in Fig. 21.2 (from [10]).

Taken together, the processes at work during melanocyte differentiation, migration, and colonization can be also observed when the cell becomes malignant. Both the genetic programs controlling these processes and the interactions with the neighboring cells and matrix play a decisive role during progression. We have learned that the malignant melanoma cell can reactivate and co-opt the programs necessary for melanocyte development and thereby travel to distant organs. With

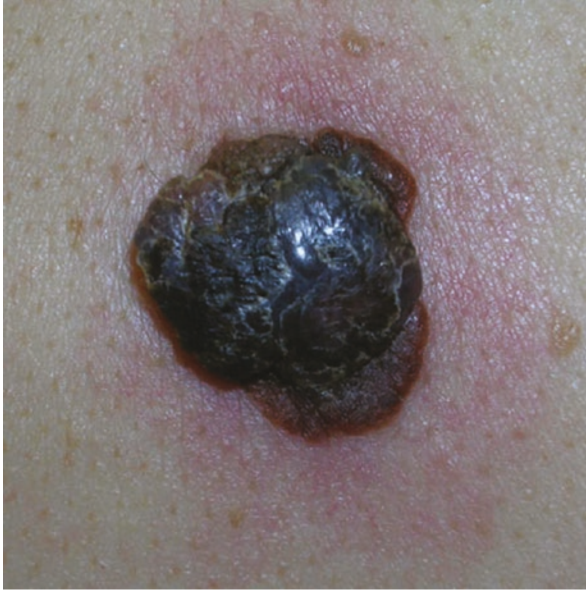

Fig. 21.2 Epithelial-to-mesenchymal transition (EMT) inducing transcription factors in physiological and pathological development of the melanocyte lineage. EMT-inducing transcription factors regulate stemness and differentiation in melanocytes (*left*), whereas they determine the oscillation between differentiated vs. invasive cancer cells in melanoma (*right*). Phenotype switching that accounts for melanoma heterogeneity depends on a signaling switch of different EMT-inducing transcription factors and is regulated by microenvironmental cues, (epi)genetic instability, and oncogenic signaling (reproduced with permission from Vandamme & Berx (11))

this in mind, the next paragraphs will review the supporting and inhibiting interactions at play between melanoma cells and their microenvironment.

Interactions with Other Cells and Tissues One by One

Keratinocytes and the Skin

Normal melanocytes reside at the dermal-epidermal junction of the skin between basal keratinocytes and directly on top of the basement membrane. Their role is to produce melanosomes. Packed with melanin and through a complex network of dendrites, melanosomes are distributed among the adjacent keratinocytes and, once in place, protecting them from damage, e.g., by UV-light, from the external environment. The stability of this intimate relationship between melanocytes and keratinocytes is ensured through cell-cell adhesion molecules and paracrine growth factors. A well-functioning "melanin unit" (melanocyte and basal keratinocyte) is necessary to create a sanctuary for the skin cells living under otherwise stressful conditions. The most important known cause of melanoma is the damaging effects of UV-light. UV-light

can induce specific mutations driving oncogenesis [30], but UV-light can also directly disturb the fine interactions between keratinocytes and melanocytes, reducing the anti-proliferative and anti-invasive influence from keratinocytes on melanocytes [31]. This critical homeostasis between pigment cells and their microenvironment is disrupted during melanoma initiation. Following genomic damage, oncogene overexpression, and subsequent reprogramming of the melanocyte, the melanocyte escapes the control previously imposed on it by keratinocytes and initiates independent growth [31].

A significant part of the current knowledge about melanoma biology and the role of the complex microenvironment is the result of work performed by Dr. Meenhard Herlyn and his team at the Wistar Institute in Philadelphia [32]. Since most basic research on melanoma cells was performed on isolated cells growing in monocultures on plastic, an artificial condition that does not factor in the importance of an appropriate microenvironment, new and more sophisticated experimental models were needed. Three-dimensional organotypic cultures and collagen-implanted spheroids now offer new insight on how melanoma cells behave and respond to physiological stress as well as applied drugs in a more relevant setting, reflecting the behavior of melanoma cells in patients [33].

Upon malignant transformation, melanoma cells escape the control imposed by the keratinocyte by downregulation of cell adhesion molecules like E-cadherin, P-cadherin, desmoglein, and connexins [17, 34]. As illustrated in Fig. 21.3, the extracellular regions of cadherins create a homotypic interaction with cadherins on neighboring cells. The cytoplasmic domain of these transmembrane molecules is linked to the cytoskeleton and interacts with protein complexes including β-catenin. As we know from embryology, EMT, and stress responses described above, E-cadherin is downregulated, and N-cadherin is upregulated, making melanoma cells capable of interacting with other N-cadherin-expressing cells, like endothelial cells and fibroblasts, residing in the dermis. This critically important *cadherin switch* is one of the earliest steps in the creation of the invasive vertical growth phase of melanoma. Loss of E-cadherin expression, as part of resetting the cellular program to an invasive and stress-responding phenotype, also results in reduced inhibition of β-catenin signaling including upregulation of stress response genes such as c-Myc, cyclin D1, and MITF [35, 36] and silencing of the p16 tumor suppressor gene [37]. Loss of this specific inhibition will, in turn, stimulate the melanoma cells to proliferate. On the other hand, increased survival of transformed melanoma cells is supported by repression of proapoptotic factors like Bad as a consequence of increased expression of N-cadherin [38]. Basically, these tumor-promoting processes are not only independent changes in individual proteins happening by chance in a genetically unstable and chaotic tumor cell but rather a dramatic result of a cellular program with striking similarities to normal biological processes, such as stress responses, wound healing, and embryonic development.

Although little is known of what controls the cadherin switch, the evidence points to an alteration in the cellular programming discussed above. It has been shown that the EMT inducers Slug and Snail inhibit E-cadherin at the transcriptional level [39, 40]. Li and coworkers showed that autocrine HGF decouples melanomas from keratinocytes by downregulating E-cadherin and desmoglein 1 [41], indicating an important role of C-met/HGF. In turn, this interaction is counteracted by the activation of

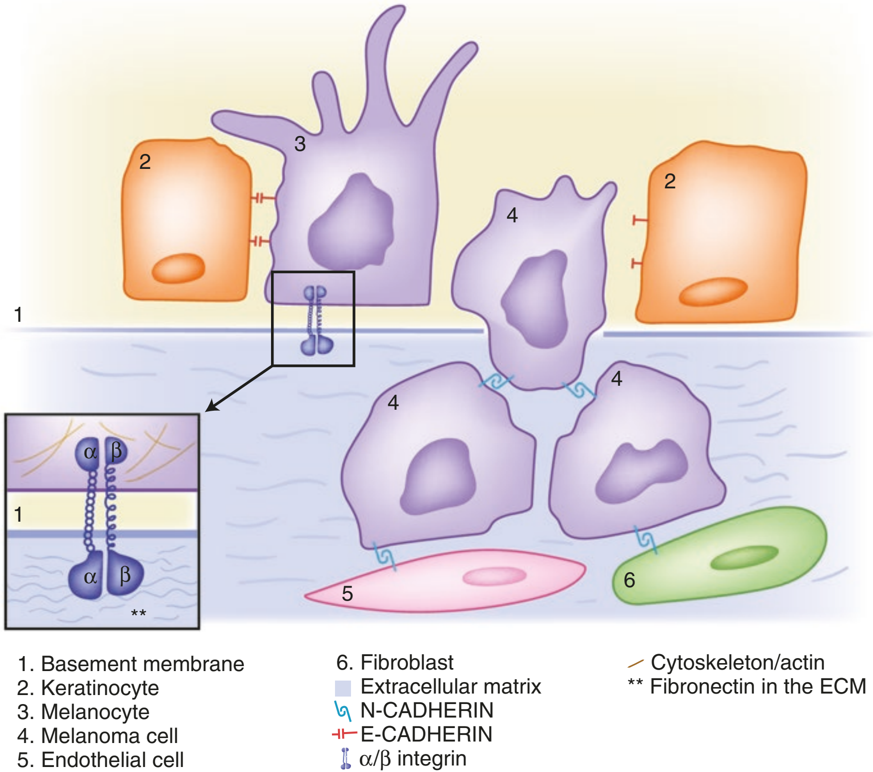

Fig. 21.3 Keratinocytes and melanoma cells. The extracellular regions of cadherins create a homotypic interaction with cadherins on neighboring cells. During melanoma development, E-cadherin is downregulated and N-cadherin is upregulated, making melanoma cells capable of interacting with other N-cadherin-expressing cells, like endothelial cells and fibroblasts, residing in the dermis. This critically important *cadherin switch* is one of the earliest steps in the creation of the invasive vertical growth phase of melanoma

the semaphorin 4D/Plexin B1 ligand/receptor complex, which again is downregulated by UV-light [42, 43]. Interestingly, Plexin B1 is a downregulated, downstream target of the frequently activated B-Raf/Mek/ERK pathway and seems to have a tumor-suppressing function in early melanoma development [44]. Taken together, this illustrates how the reprogrammed transformed melanoma cell can actively influence its relationship with the ECM and thereby increase its likelihood of succeeding in invading and metastasizing. As a net outcome, the cadherin switch ensures a program change in the transformed melanoma cell from a dormant, nonproliferating cell under strict control by the keratinocyte into an invasive, proliferating, apoptosis-resistant melanoma cell able to escape from the site of origin and to metastasize.

Whereas the cadherins ensure appropriate cell-cell adherence, the integrins anchor the melanocytes to the ECM (Fig. 21.3). In addition, the integrins are involved in controlling proliferation, invasion, immune response, angiogenesis, and

survival [45]. Integrins are transmembrane heterodimers composed of one α- and one β-subunit. Binding of a ligand to the extracellular domain leads to a conformational change inducing signaling cascades in the cells, such as MAPK, PI3K, and NFκB. Importantly, conformational changes in the extracellular region of integrins can also be induced by the binding of signaling molecules to the intracellular part, thus leading to changes in how the cell responds and interacts with the microenvironment [32]. Integrins play an important role in modulating the transformation of signals mediated by various growth factor receptors in response to ligation of growth factors produced in the microenvironment. Receptor tyrosine kinases like epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptor (PDGFR), and focal adhesion kinase (FAK) [32, 45, 46] have major influence over the ability of tumor cell to proliferate, migrate, invade, and survive. Thus, the activity and composition of the integrin apparatus in the cell have an important role in modulating these processes. Especially important for the transition from horizontal to vertical growth of melanoma cells is the $\alpha_v\beta_3$ integrin. Increased expression of this specific transmembrane heterodimer results in increased production of the antiapoptotic factor bcl-2 as well as ECM degrading proteins like MMP-2 [47–49].

The melanoma cell has now escaped the suppressing influence of the keratinocyte and entered the vertical growth phase. There, it will encounter cells that its ancestors, the melanocytes, have not interacted with since the time of migration from the neural crest to the skin during embryonal development. These interactions can be supportive or suppressive depending on how the new host tissue responds to the intruder.

Seed, Soil, and Melanoma Stem Cells

More than a century ago, after studying 735 cases of fatal breast cancer, the surgeon Stephen Paget formulated his famous "*seed and soil*" hypothesis. He observed that some environments were more receptive for metastatic growth than others and concluded that secondary tumor growth at distant sites is both dependent on properties of the "seed" (cancer cell) and of the "soil" (microenvironment) [50]. Acknowledging the properties of the melanocyte during embryonic development to migrate, invade, and colonize distant organs (i.e., skin, eye, mucosa), it is of no surprise that circulating melanoma cell can find fertile soil and establish metastases at almost any site.

The acquisition of more mesenchymal-like attributes together with the downregulation of epithelial traits during epithelial-to-mesenchymal transition (EMT) and the reversion of this process, mesenchymal-to-epithelial transition (MET), is important for the metastatic process [51]. Progenitor cells, cancer stem cells, and metastatic cells can be "seeds" evolved from the primary tumor that may establish metastasis in proper niches or organ microenvironments ("soil") [52].

Melanomas metastasize mainly through the lymphatic system but also via hematogenic dissemination to more distant sites like the lungs, liver, and brain. The metastatic process includes local invasion into blood vessels or lymphatic channels,

transportation of multicell aggregates to distant sites, arrest in the capillary bed and subsequent extravasation into organ parenchyma, and, finally, establishment in the new microenvironment and further proliferation and tumor expansion [52]. These processes can be interrupted by the antitumor host response at many different levels and are therefore fully completed only by a very limited number of cells. Nowell suggested that acquired genetic variability during tumor progression together with selection pressure results in more aggressive tumor cells [53]. This concept of *clonal origin of metastases* has been verified by others and may explain heterogeneity in response to chemotherapy [52].

The role of cancer stem cells (CSCs) in the pathogenesis of melanoma metastasis is still under investigation, but there is evidence for an association between the presence of CSCs and disease progression, increased potential for metastases, and worse prognosis [54]. CSCs in melanoma are also named malignant melanoma-initiating cells (MMICs) [54]. CSCs are defined by three characteristics: the ability to initiate tumor growth, the capacity of self-renewal, and the ability to differentiate into tumor cells. ABCB5 and CD271 are markers characterizing MMICs in melanoma; others are under investigation. Increased expression of CD271 which promotes immune evasion and expression of ABCB5 is a characteristic in chemo-resistant cells. Furthermore, MMICs may promote invasion and metastasis in part reflected by EMT and MET [54]. Other melanoma stem cell markers are CD20, CD133, and ABCG2 [54].

Fibroblasts and Connective Tissue

During tumor progression and invasion, the transformed melanoma cells secrete growth factors to modulate the new microenvironment in a paracrine manner and to stimulate proliferation and survival in an autocrine manner. One important growth factor is basic fibroblast growth factor (bFGF), and most melanomas produce and secrete bFGF [55, 56]. Whereas the normal melanocyte does not produce bFGF and relies on paracrine bFGF from fibroblasts and keratinocytes, melanoma cells can co-express bFGF and the FGF receptor [55–57]. Interestingly, in both melanomas and melanocytic nevi, the expression of bFGF seems to be highest in the dermal tumor areas [55]. Most cells in the tumor microenvironment are able to express and secrete bFGF and bFGF with different consequences. In addition to increased proliferation and survival of melanoma cells [58], bFGF secretion by invading melanoma cells stimulates growth of endothelial cells and fibroblasts and consequently promotes angiogenesis and fibrous stroma formation [59].

The fibroblasts in the tumor microenvironment are also stimulated by platelet-derived growth factor (PDGF) secreted by the transformed melanoma cells. In response to PDGF, the fibroblasts produce ECM proteins, such as collagen, fibronectin, and laminin [60], as well as growth factors like bFGF, IGF-1, and TGF-β [32]. These ECM proteins convert the neoplasm from a soft mass into a firm tumor. Similarly, paracrine secretion of transforming growth factor beta (TGF-β) by melano-

mas also has a multitude of consequences in the tumor microenvironment, including increased deposition of ECM proteins, angiogenesis, and immunosuppression [61].

Normal dermal fibroblasts suppress growth and progression of premalignant melanoma lesions at early stages [62]. In part, normal dermal fibroblasts exert this effect by mobilizing immune cells via the secretion of cytokines, such as interferon γ, interleukin 6, and TNFα [63]. In addition, normally functioning dermal fibroblasts create a physical barrier for the melanoma cells. To penetrate the dense dermis, invading melanoma cells need to modulate the ECM and do this by expression of several different matrix metalloproteinases. In healthy tissue, normal dermal fibroblasts are able to regulate and restrain these changes, thereby preventing degradation of the basement membrane and blocking invasion (reviewed in [64]). In contrast, cancer-associated fibroblasts (CAFs) are believed to be heterogeneous with similar properties as the activated myofibroblasts frequently found under inflammatory conditions and wound healing. CAFs express α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (FSP-1), fibroblast-activating protein (FAP), PDGF, and α11β1 integrin [64, 65]. CAFs in the tumor stroma originate from different sources, such as from bone marrow mesenchymal cells, EMT of resident epithelial cells, or by recruitment and activation of resident normal dermal fibroblasts [64, 66]. The interaction between melanoma cells, actively trying to break down the basement membrane, and fibroblasts residing in the dermis is probably the major cause of the recruitment of CAFs in the invading melanoma (Fig. 21.4).

Once stimulated and established in the ECM, CAFs can promote tumor growth in different ways. Following the E-cadherin to N-cadherin shift in melanoma cells,

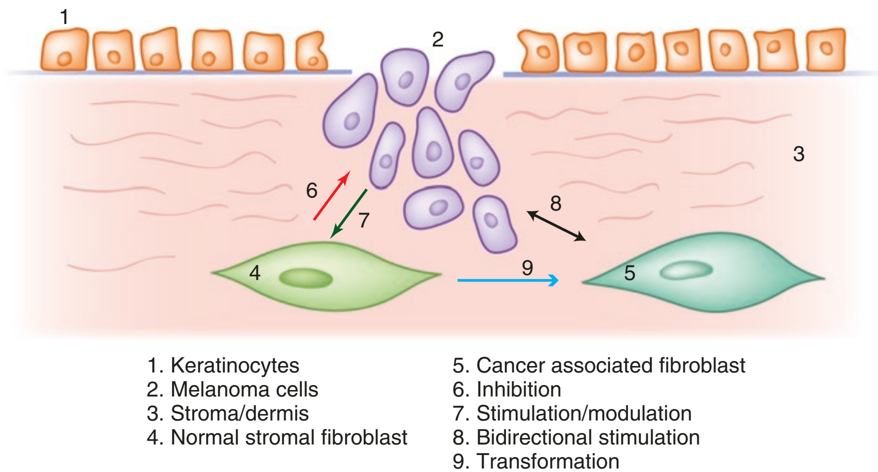

Fig. 21.4 Melanoma-fibroblasts-CAFs. Initially, invading melanoma cells are inhibited by normal fibroblasts acting as a physical barrier in the dermal stroma. Melanoma cells can interact with the fibroblasts in the ECM by expressing several different factors, such as bFGF, PDGF, and TGF-β, and thereby contribute to the transformation of the dermal fibroblasts into cancer-associated fibroblasts (CAFs). Once CAFs are established around the invading tumor border, they further stimulate the progression of the melanoma cells

CAFs can interact through direct cell-cell contacts, mediated through N-cadherin, and help melanoma cells to migrate away from the restraining effects of keratinocytes and invade into the underlying dermis. CAFs produce a number of ECM proteins, such as laminin and fibronectin, which are structural components that make up the connecting tissue and contribute to the dense fibrous nature of solid tumors [67]. In addition, CAFs are able to remodel the ECM through secretion of MMPs [68] and fibroblast activation protein(FAP)-α, thus facilitating further growth and migration by melanocytes. Hypoxic stress in the TME induces a stress response in melanoma cells, which in turn can send signals to the surrounding CAFs to secrete growth factors such as vascular endothelial growth factor (VEGF), stromal-derived factor-1 (SDF-1), IL-6, as well as a number of chemokines, thus favoring angiogenesis, chemotaxis, and invasion [64, 69]. Taken together, after leaving the suppressive partnership with the keratinocytes, the promiscuous melanoma cell now interact with CAFs as their new supporters, and together they can overcome any obstacle they meet in the dermis on the way to metastasizing.

Endothelial Cells and Vasculature

In 1971, Dr. Judah Folkman postulated that tumor growth beyond the size limit of about 1–2 mm, the maximum diffusion limit oxygen in tissue, is dependent on angiogenesis [70]. Angiogenesis is the establishment of new vessels by sprouting from preexisting vasculature. After the melanoma has entered the vertical growth phase and penetrated the basement membrane, angiogenesis is required for further tumor growth [71]. Without the capability to induce angiogenesis, the melanoma will linger on in dormancy as a microscopic lesion for years. Following an angiogenic switch [72, 73], the dormant tumor will enter a phase of rapid growth followed by metastasis, morbidity, and death. The triggering factor for the angiogenic switch is not yet completely identified and will be discussed later when discussing ulceration and wound healing.

As mentioned earlier, melanoma growth in a hostile site (local or metastatic) is associated with a tremendous stress on the melanoma cells. To survive such stressful conditions, stress response programs are initiated in the cell. Hypoxia is such a major stress factor, caused by excessive tumor growth in the absence of a sufficient vascular supply. The transcription factor HIF1-α [74] is rapidly induced following hypoxia and activates transcription of a plethora of stress response genes encoding angiogenesis-related growth factors such as VEGF, bFGF, PDGF, and TGF-β [75–77].

Algire was the first to observe vascularization in melanoma transplanted to the mouse in a transparent chamber in 1943 [78]. Preclinical models and clinical investigations have characterized primary melanomas and metastases as highly vascularized, and increased angiogenic capacity implies impaired patient prognosis [79–81]. The most prominent of the angiogenesis-related growth factors is vascular endothelial growth factor (VEGF). As a response to stressful conditions such as hypoxia in a tumor, VEGF expression in melanoma cells is increased. Also, other cells in the

tumor microenvironment, such as endothelial cells, CAFs, and inflammatory cells, express and secrete VEGF [82]. The total angiogenic response is dependent on many different growth factors (and inhibitors). In addition to VEGF, bFGF, PDGF, IL8, and PlGF act in concert with increased vascular supply as a result [82].

In 1983, vascular permeability factor (VPF) was purified [83] and later renamed as vascular endothelial growth factor A (VEGF-A) [84]. The VEGF-family consists of five members, VEGF-A to VEGF-D and placenta growth factor (PlGF), and belongs to the platelet-derived growth factor supergene family [85]. VEGF-A plays a key role in physiological and pathological angiogenesis, activates proliferation and migration of endothelial cells, and induces vascular leakage and vasodilatation [86]. Alternative splicing of the VEGF-A gene results in several pro-angiogenic isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183,}$ $VEGF_{189}$, $VEGF_{206}$) as well as anti-angiogenic VEGF-xxxb isoforms that have a different C` terminus [87]. $VEGF_{165}$ is the most common isoform and is primarily heparin bound upon secretion [86]. Hypoxia, several cytokines, and growth factors as well as oncogenes and tumor suppressor genes regulate VEGF expression [86]. VEGF binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 on endothelial cells and tumor cells; binding to VEGFR-2 is crucial in angiogenesis and activates further downstream signaling via phospholipase C_y and PI3K pathways [88]. Conversely, binding of $VEGF_{xxx}$b to VEGFR-2 seems to inhibit the key functions of VEGF-A [87]. VEGF-A expression is described in primary melanomas as well as metastases [79].

A specific neovascular phenotype associated with VEGF has been identified in subgroups of melanoma, as well as other cancers, and is associated with aggressive tumor biology. Glomeruloid microvascular proliferations (GMPs) are focal aggregates of small vessels resembling a renal glomerulus. In mice, GMP has been induced by local injection of an adenovirus vector directing VEGF-A expression [89, 148]. A human parallel appears to be the POEMS syndrome [89], where increased VEGF-A is associated with glomeruloid hemangiomas. In humans, GMP is a defining histologic feature of glioblastoma multiforme and a prognostic factor in several other tumors [90, 91]. The presence of GMP's in primary tumors has been associated with impaired prognosis [90].

Previously it was shown that inhibition of bFGF- and FGFR-1-mediated signaling abolished angiogenesis in human melanoma xenograft tumors [92]. In addition, bFGF expression was associated with higher microvessel density in primary melanomas [56]. There is evidence for a cross talk between FGF and VEGF signaling in angiogenesis [93].

Interestingly, not only endothelial cells have functional receptors for angiogenic factors. Melanoma cells have been observed to induce expression of growth factor receptors such as VEGFR as well as bFGF receptors [56, 79, 82]. This suggests the existence of autocrine and intracrine loops, in which melanoma cells produce VEGF in order to self-stimulate through its own VEGF receptors. VEGF secreted from melanoma cells also stimulates nearby endothelial cells in a paracrine fashion. Interestingly, in contrast to the paracrine stimulation by VEGF, the autocrine and intracrine stimulation was not inhibited by anti-VEGF-specific targeting by bevacizumab (an anti-VEGF antibody) [94]. This can, in part, explain why VEGF targeting drugs like

bevacizumab can reduce microvessel density (MVD, quantification of angiogenesis in biopsies) yet still have only limited effects in clinical trials of melanoma [95–97].

As a rapid response to stress occurring in the microenvironment of melanomas, such as hypoxia, UV-light, as well as the omnipotent EMT inducer, TGF-β1, production of interleukin 8 (IL8) is induced. Increased level of IL8 in melanoma patients is associated with advanced disease and overall survival and is a potent stimulator of tumor angiogenesis, tumor progression, and metastasis [82, 98]. As mentioned above, the integrin $\alpha_v\beta_3$ is of particular significance in melanoma progression. VEGF- and bFGF-induced angiogenesis can be modulated by $\alpha_v\beta_3$ integrin in concert with its ligands vitronectin and osteopontin [99].

The pro-angiogenic role of heat-shock protein 27 (HSP27) was proven in cell-line studies, xenografts, and patient samples [100]. HSP27 belongs to the family of small heat-shock proteins that maintain cell survival under stressful conditions [101, 149]. Downregulation of HSP27 in an angiogenic breast cancer cell line resulted in decreased secretion of VEGF-A and bFGF and a non-angiogenic phenotype in vivo. Additionally, weak expression of HSP27 in human melanoma and breast cancer samples was associated with less aggressive phenotype and better outcome [100]. As many of the processes discussed in this chapter relate to normal cellular stress responses co-opted by melanoma cells, rapidly induced stress response proteins, like HSP27, might serve as promising treatment targets as well as biomarkers for prognosis and treatment response.

In 1999, Maniotis and colleagues presented a concept of vasculogenic mimicry (VM) in melanoma [101]. VM refers to the plasticity of aggressive cancer cells forming functional vascular networks, contributing to tumor perfusion by connecting to the normal endothelial-lined vasculature [102]. Although the very existence of VM as a functional fluid transporting network of channels has been heatedly debated and questioned [103], these studies have brought the field of angiogenesis forward and shed new light on the importance of tumor cell plasticity in aggressive tumors. VM has been validated as a prognostic marker in several tumor types, including melanoma [102], but can also be used as a marker of increased cellular plasticity and aggressiveness. Drugs to target cellular plasticity by inhibiting multiple signaling pathways simultaneously have shown some promise in preclinical studies. Combination treatment with anti-VEGF antibodies in conjunction with the Notch ligand anti-delta-like ligand 4 (Dll4) resulted in significant tumor growth inhibition in comparison to monotherapies [104], and anti Dll4 treatment might prevent resistance to anti-VEGF therapies [105]. Potentially, VM may serve as a relatively easily available marker of response to such treatment strategies.

Immune Cells and the Immune System

As soon as melanoma cells migrate into the wrong side of the basement membrane, they encounter the cells of the immune system. These cells are constantly on the look for pathogens and foreign elements. There has been increased interest in the interactions between melanoma cells and the immune system, both in the local

tumor microenvironment and systemically. The immunogenicity of cancer, and especially melanoma, has long been recognized (Reviewed in [106]). At the beginning of the twentieth century, Paul Ehrlich postulated the tumor immune surveillance theory. He proposed that cancer cells spontaneously arise in the organism and that immune responses could effectively eliminate them. Still, for a long period of time, most approaches to stimulate the immune system to combat the growing melanoma failed. Following the paradigm shift from trying to stimulate the immune system to inhibiting the immune checkpoints, a whole set of new treatment opportunities have emerged. The first drug ever to show increased patient survival in a randomized phase III clinical trial was the checkpoint inhibitor ipilimumab (anti CTLA-4) [106]. The identification of the inhibitory effect of the PD-1 ligand [107], frequently expressed on growing tumors including melanoma, unleashed a highly potent strategy to fight cancer. These immune checkpoints (CTLA-4 and PD-1) modulate the immune system independently from each other, and their inhibition results in enhanced antitumor immune response and increased overall survival. Whereas CTLA-4 regulates T-cell proliferation and migration to the tumor, the PD-1 and PD-L1 interaction takes place at the tumor site [108]. Immune checkpoint inhibition with anti-CTLA4 antibodies and anti-PD-1/PD-L1 antibodies represents a major breakthrough in the treatment of melanoma [109, 110], as well as other cancers with a similar mutational load [111, 112]. Many mutations indicate many distorted proteins, many potential antigens, and ultimately increased response to immune-modulating treatments. Previously, patients suffering from these cancers were historically the most hard to treat with conventional treatments like chemotherapy and radiation, but the advent of checkpoint inhibitors has opened up a whole new era with regard to prognosis and symptom relief.

There are three phases of interaction between tumor cells and the immune system from the onset of the first tumor cells through metastatic dissemination [113]. First, the innate and adaptive immune system recognizes and eliminates a portion of the tumor cells. Then, equilibrium is generated between the remaining tumor cells and the immune system; the resultant tumor can exist in dormancy for many years. During this period, there are however ongoing interactions between the surrounding host immune system and the genetically unstable tumor cells. This process is defined as “immune editing.” Finally, the tumor cells can escape from immune surveillance and initiate uncontrolled proliferation [113]. Escape mechanisms include secretion of cytokines that inhibit or mediate immune response, induction of an immune suppressive environment, and alteration of important antigens and molecules on the surface of the tumor cells themselves. Interference of dendritic cell maturation, inappropriate presentation of tumor antigens, deficiency of costimulatory molecules involved in T-cell activation, and recruitment of immune-suppressive myeloid cells (MDSCs) are other approaches of the melanoma to escape the host immune system. Most importantly, cancer cells are in fact the organism’s own cells, and the immune system is, during development of self-tolerance in the thymus, by default set to *not* attack the organism’s own cells.

Melanomas have a high mutational load, one of the highest among all cancers [30], which has been linked to the pro-mutational and carcinogenic effect of UV-light. Most of these mutations are not drivers of tumor progression, but rather passengers.

Nevertheless, these non-driving mutations can cause expression and secretion of atypical and misfolded proteins, which again can be recognized by the constantly patrolling antigen presenting cells (i.e., dendritic cells) and the T cells as foreign antigens. Once a tumor's antigens are recognized by the immune system, an antitumor response can be activated. Unless the reaction is stopped by the aforementioned immune checkpoints, e.g., PD-1/CTLA-4, meant to prevent autoimmunity, an effective tumor destruction can occur [114, 115]. Actually, these processes are constantly ongoing, and the immune system can effectively keep a microscopic primary tumor as well as an occult micrometastasis dormant throughout the lifetime of a person [116].

Vascular endothelial growth factor (VEGF) is not only a potent angiogenic factor but also plays a role during inflammation. As a response to cellular stress, like hypoxia, acidosis, and wound healing, VEGF is secreted by cancer cells and CAFs, recruits MDSCs and macrophages to the tumor, and inhibits the maturation of dendritic cells [86, 117]. Thus, high pretreatment levels of VEGF are associated with decreased OS in melanoma patients treated with ipilimumab [118]. Other mechanisms of melanoma cells that are associated with immune escape are expression of FasL [119] and inadequate expression of HLA class I molecules [120].

Lymphocytes

In 1863, Rudolph Virchow observed lymphoid cells within tumors and hypothesized a connection between inflammation and cancer [121]. Presence of tumor-infiltrating lymphocytes (TIL) has been correlated with patient outcome in many different cancer types, including melanoma [122]. T-lymphocytes are the most important effectors of cellular immune response against cancer. Spontaneous regression of large primary melanomas is observed in the clinic, leaving only a flat scar with a depigmented halo. Although rare, even spontaneous regression of metastatic lesions has been reported. The T-lymphocyte attack on malignant tumors is extremely potent when not inhibited by immune checkpoints, such as programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4). These immune checkpoints are meant to prevent autoimmunity. Being one of the bodies "*own*" cells, melanoma cells send signals through these checkpoints, identifying them as "*not to be touched*," thus escaping the immune attack. Inhibition of these checkpoints with antibodies against PD-1 and CTLA-4 has become a major breakthrough in the treatment of cancer. TILs can also be modulated ex vivo to increase their potency against melanoma antigens, and adoptive T-cell therapy shows great promise in clinical trials (reviewed in [123, 150]).

Cells of the Innate Immune System

The effector cells of innate immunity include granulocytes, macrophages, and natural killer cells. Tumor-associated neutrophils (TANs) seem to be of importance and will be discussed in the next chapter. Tumor-associated macrophages (TAMs) are also important antigen-presenting cells (reviewed in [124, 125]). The TAM content in melanoma tumor samples ranges from 0 to 30%, and their density increases with increasing tumor thickness. The melanoma cells and TAMs seem to interact with each other through the release of soluble factors that either prevent or enhance tumor growth. TAMs are derived from circulating precursors and are key regulators of the link between inflammation and cancer. There are two different phenotypes of TAMs, M1 and M2, with opposing effects on tumor-specific immune reactions and tumor progression [125]. Melanoma-derived cytokines, such as monocyte chemotactic protein-1 (MCP-1), attract TAMs and control their differentiation from a tumoricidal M1 phenotype to an M2 phenotype, which favors growth and tissue remodeling [126]. TAMs, once controlled by the tumor, deliver important growth factors to the tumor vascular supply, such as VEGF and IL8 [127, 128].

Tumor Ulceration

According to pathologists, a primary melanoma is recorded as ulcerated if there is an absence of an intact epidermis overlying a portion of the primary melanoma based on microscopic examination [129, 130] (Fig. 21.5). Ulceration has been

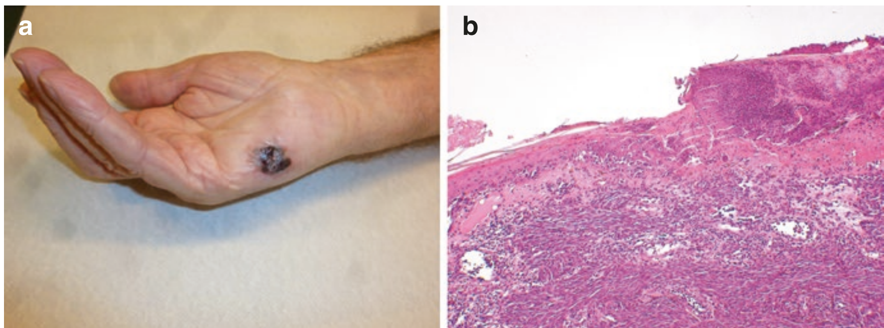

Fig. 21.5 Ulcerated malignant melanoma. (**a**) Primary malignant melanoma of the hand with a central ulceration (picture provided by Dr. H. L. Svendsen, Haukeland University Hospital, Norway). (**b**) H&E-stained histologic section of a large nodular malignant melanoma (not the same as in (**a**)) with a central ulceration. There is an absence of an intact epidermis overlying a portion of the tumor. Ulceration is defined by the combination of the following features: first, full thickness epidermal defect; second, evidence of a host response (i.e., fibrin deposition and neutrophils); and third, thinning, effacement, or reactive hyperplasia of the surrounding epidermis

described in more detail by Spaz et al. [131] and is defined by the combination of the following features: first, full thickness epidermal defect; second, evidence of a host response (i.e., fibrin deposition and neutrophils); and third, thinning, effacement, or reactive hyperplasia of the surrounding epidermis. This indicates that ulceration is much more than just the loss of the epithelial lining and suggests a distinct biologic process [132]. Presence of ulceration has major prognostic impact and is therefore included in the TNM and the AJCC staging systems to create subgroups with different prognoses within each T-category [133]. After inclusion of ulceration in the TNM staging, ulceration as an independent and important biomarker has been much more focused upon in the pathology reports. Additionally, research has revealed that ulcerated tumors have increased vascularity [134], more lymphovascular invasion [135, 136], and more lymph node metastases [137]. Perhaps even more relevant, in a study evaluating the immune status by analyzing the dendritic cell (DC) population in sentinel nodes, Elliott et al. were able to show that sentinel lymph nodes of ulcerated primary tumors were severely immunosuppressed even in the absence of tumor cells in the lymph node [138]. This interesting observation provokes speculation around the relation between ulceration, wound healing processes, immunosuppression, and cancer progression. It is unknown whether tumor ulceration can directly lead to a decreased DC response in the primary melanoma and therefore participates in the creation of an immunosuppressive environment or whether ulceration occurs stochastically in a phenotype that is immunosuppressed [138]. In several reports, including large population-based studies, it has been shown that ulceration is dependent on mitotic activity [139]. In addition, the gene expression profile in ulcerated melanoma was found to be completely different when compared with non-ulcerated cases [140]. Finally, and not less impressive, adjuvant interferon immunotherapy did not show any significant benefit with regard to survival, except in cases with ulcerated primaries [141], again linking ulceration and the antitumor immune response.

From all these results, it has been concluded that ulceration is dependent on increased angiogenesis, increased proliferative capacity, reduced immune responses, and changes in gene expression. Or could it be the other way around? Is it possible that ulceration, and all the associated active physiologic processes that go on during wound healing, per se causes increased angiogenesis, increased proliferation, immunosuppression, and alters the gene expression programs in all cells in the microenvironment, including melanoma cells? Perhaps ulceration is not just a passive marker of aggressive tumor biology but rather an active player in the malignant progression. To further discuss these unsolved and potentially relevant questions, we will take a quick look at what is known about the similarities and differences between tumors and wounds.

Under acute inflammation, such as after tissue damage, ulcerations, or infections, the inflammatory response is self-limiting, and immune cells resolve by apoptosis or return to the circulation [142]. In malignant tissues, however, pro-inflammatory signals continue to intensify to support the needs of the tumor. Hence, the inflammatory response never resolves, and tumors have been likened to "wounds that do not heal" [143]. In the tumor microenvironment, tumor-associated

macrophages (TAMs) and neutrophils (TANs) can constitute a large proportion of the tumor mass (Condeelis and Pollard 2006) and are associated with poor prognosis [144].

In a study by Antonio et al., the mechanisms underlying the association between ulceration, wound healing, and inflammation in melanoma were studied [145]. The authors had previously shown that neutrophils and macrophages interact with preneoplastic cells before these cells divide to form clones [146]. They also showed that these immune cells, when initially attracted to a wounded site, are rapidly drawn away from the wound by competing signals from preneoplastic cells, which in turn are stimulated to form tumors. This indicates that by induction of ulceration (wounding), the tumor biology changes due to physiological wound healing processes, such as by influx of innate immune cells. In a series of clinical melanoma samples, the amount of tumor-infiltrating neutrophils and macrophages are closely correlated with tumor cell proliferation and prognosis [145]. The detrimental impact of ulceration in melanoma is thus no surprise, considering the hyperproliferative microenvironment associated with a rapidly growing granulation tissue found in wounds. The defining loss of self-limitation in cancer, in contrast to normal wounds, makes the "wounds that do not heal" analogy by Dvorak highly relevant. In the case of cancer, ulceration seems to act as fuel for the fire.

In the title we posed the question of whether the tumor microenvironment (TME) is a friend or foe. Most likely, it is neither. The TME just responds automatically like it is supposed to do in "normal" stressful or pathologic conditions, such as hypoxia, heat shock, starvation, UV-light, tissue wounding, infection, and inflammation. The melanoma cells, on the other hand, driven by their huge number, plasticity, and genomic instability, exploit and co-opt these normal responses and utilize this to their advantage. Thereby, the normally helpful cells in the TME unwillingly become supporters of disease progression.

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Chapter 22
Biomarker Panels and Contemporary Practice in Clinical Trials of Targeted Therapy

Nina Louise Jebsen, Samantha Scarlett, Bergrun Tinna Magnusdottir, and Bjørn Tore Gjertsen

Abstract Development of cancer therapy follows three main veins: mutation-driven drug development, immunomodulatory therapy, and evolution of conventional chemo- and radiotherapy. All of these therapeutic modalities require more precise biomarkers, not only for increasing precision and enhancing efficiency but also to avoid unnecessary toxicity for the patient and costs for the society. In clinical trials, there is an increasing use of biomarker panels for risk stratification and therapy guidance. Single biomarkers, in particular genetic mutations, have been tested to optimize therapy with only limited success. So far, only a small fraction of the patients may benefit from state-of-the-art diagnostics and biomarker determination, although predictive factors have successfully been implemented in treatment of, for instance, breast cancer and colorectal cancer. We will exemplify and illustrate the use of biomarkers in late- and early-phase clinical trials, in which biomarker panels employed on acute leukemia or sarcoma assisted in pivotal decision-making. Clinical trials in acute leukemia and sarcoma often include biomarkers based on combinations of cytogenetics, gene mutations, gene expression, and protein detection. Acute leukemia and sarcoma are suggested to originate from progenitor or stem cells of hematopoietic or mesenchymal origin, respectively. The different biology of these diseases, based on cancer cell context and in relation to healthy tissue and tumor stroma, leads to their clinical manifestations and may provide guidance on the direction of future biomarker-tailored therapy.

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There is no fast track to establishing biomarker-based targeted therapy. However, combining biomarkers of different nature may provide clinically relevant and robust biomarker panels that can be used in late-phase clinical trials in acute leukemia and soft tissue sarcoma. For future prospects of simplifying biomarker approaches, functional subdivision of particular cancers into defined subsets may be the most promising path to provide molecular personalized therapy that optimally benefits the patient.

Keywords Biomarker panels • Mutational analysis • Design of clinical trials • Functional genomics tests

Introduction

Treatment of broad cancer patient populations with therapy that provides little benefit to the majority is no longer economically sustainable. Specifically, expensive, molecularly targeted therapeutics are less likely to be successful on large disease populations. Rather, a tailored approach is required [1]. This is a reality for the three main fields of cancer therapy development: (1) mutation-driven drug design, (2) immunomodulatory therapy, and (3) further improvement of conventional cancer surgery and chemo- and radiotherapy [2, 3]. Meta-analysis of selected oncology trials has indicated that a personalized, targeted therapy approach to treatment has better outcomes than non-personalized and cytotoxic agent regimes [3]. We see indications that advanced pathway analyses with identification of master regulators or functional genomics may represent alternative avenues for therapy individualization [4]. However, these methodologies need to be more mature and robust before they can be applied on a larger scale [5–7]. In parallel with the emerging precision medicine development, we also see improvement of conventional therapy principles including novel chemotherapeutics, antimetabolites, and improved radiation therapy [8, 9].

It is clear that the molecular heterogeneity of human cancers and the ability to characterize this heterogeneity through next-generation sequencing techniques present new opportunities for the development of more effective treatments as well as challenges for the design and analysis of clinical trials. Simultaneously, understanding the importance of the tumor microenvironment has been accelerating. The microenvironment must be considered an intrinsic part of a malignant tumor [10]. Stromal fibroblasts and leukocytes are manipulated by cancer cells to facilitate critical steps in infiltration and metastasis by degrading extracellular matrix and providing a nurturing environment as well as to avoid attacks from the immune defense [11, 12]. There are indications that stromal cells activate embryologic transcription factors in cancer cells resulting in production of secretory proteins necessary for adhesion, motility, intercellular communication, angiogenesis, and invasion [10]. Moreover, release of microvesicles (containing both proteins and nucleic acids)

plays an important role in cell-to-cell communication necessary to oncogenic transformation [13]. Improved knowledge about the fine-tuned cross talk between cancer cells and the microenvironment may provide better characterization of the multistep disease progression. Stromal cell chemokines involved in differentiation and tumor proliferation may serve as novel targets for oncologic treatment. Similar to the multiclonality of cancer cells, there are dramatic dynamic variations throughout the landscape of the tumor microenvironment. Abundance of stromal cells or proteinases has been shown to correlate with tumor aggressiveness [14]. Variations in the tumor vasculature and immune suppression contribute to this topographic heterogeneity and may explain differences in response or resistance to cancer therapy.

The use of biomarkers in oncology trial design has been well documented [15–17]. In this review, we will present selected examples from acute leukemia and sarcoma, which are two rare and aggressive malignancies characterized by distinct molecular and biological heterogeneity. Based on these examples, we will discuss the use of multiple biomarkers, or biomarker panels, in the development of targeted cancer therapy. The question that remains unanswered is whether the perfect methodology exists on how to deploy biomarker panels in early-phase clinical trials.

Design of Biomarker Panels

A biomarker represents characteristics that are objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention [18]. In addition to risk biomarkers foreseeing potential disease, there are at least three types of biomarkers relevant in clinical trials: diagnostic, prognostic, and predictive biomarkers. Diagnostic biomarkers assess the absence or presence of a disease. Prognostic biomarkers predict the natural progress of a disease and can be used to decide whether a patient should be treated or not. Predictive biomarkers are used to identify a treatment regime that is effective for a subgroup of patients [19]. This review will focus on predictive biomarkers and surrogate endpoints.

A clinically significant biomarker is associated with treatment of a patient subpopulation that has historically shown a differential and substantial clinical response, e.g., based on epidemiologic, pathophysiologic, therapeutic, or molecular evidence. Biomarkers include chromosomal aberrations and genomic alterations, such as deletions, insertions, mutations, or polymorphisms of DNA, as well as proteins, metabolomic patterns, histology, imaging, clinical observations, or even self-reported patient surveys. In some cases, a biomarker represents a bridge between understanding the mechanisms of preclinical findings and the observed clinical findings.

An important implication of the human genome project followed by the genomics projects in cancer is the mapping of recurrent mutations in cancer, where maybe 50% of the patients may comprise actionable mutations in their tumor [16, 20]. Enrolment in clinical trials may reduce this to 20%, and as few as 5% may respond

to a genomic approach to targeted therapy [16, 21]. However, some of the most important discoveries following next-generation sequencing are not necessarily identifying targeted therapy but provide an improved understanding of cancer development, e.g., germ line mutations and genetic susceptibility to pediatric cancer [22], mutations associated with external exposures such as smoking [23], and, most importantly, the clonal evolution seen in cancers [24, 25]. However, a tempting clinical use of knowledge about these mutations is to direct experimental therapy. Unfortunately, several reports indicate that this has been of limited success in solid cancer, with therapy responses below 10% [16, 21]. Use of molecularly targeted agents outside their indications in patients with solid cancer failed to improve progression-free survival compared with standard treatment regimes.

Biomarker panels may consist of clinical parameters only [26], mutations [21], gene expression profiles [27], or protein expression panels [28]. In cases where the therapeutic impact is dramatic, such as in pediatric leukemia and in the decision-making of allogeneic hematopoietic stem cell transplantation (HSCT), the panel is composed of a mix of clinical and molecular markers. Similarly, categorizing the mesenchymal-derived gastrointestinal stromal tumors (GIST) into different risk groups to determine adjuvant treatment with tyrosine kinase inhibitors depends on macroscopic, morphologic, immunohistochemical, and molecular tumor features [29]. In soft tissue sarcoma, the Scandinavian Sarcoma Group (SSG) has pursued a pathway of biomarker-dependent risk stratification in two clinical studies of adjuvant chemotherapy (SSG XIII and SSG XX; see www.ssg-org.net). In order to meticulously select patient to undergo toxic treatment, prognostic tumor characteristics such as size, growth pattern, pleomorphic appearance, vascular invasion, mitotic count, and tumor necrosis have been systematically recorded since 1998 [30, 31]. An important feature of these panels is that they are developed carefully, stepwise, and over many years. This lengthy development is frustrating for the patients and for the physicians treating advanced cancer. However, attempts to shorten this development phase have so far shown limited success [21].

Biomarkers in Cancer Research

Biomarkers are biological features that can be used as diagnostic indicators and predictors for disease trajectory and therapy response [32]. Typical biomarkers used in cancer research are genetic alterations or proteins expressed by the tumor itself or in peripheral blood circulation. An emerging field of research is the investigation of biomarkers in the peripheral blood, including cell-free nucleic acids (eventually enveloped in cellular-derived microvesicles). Such liquid biopsies also include circulating tumor cells and endothelial cells derived from the tumor locations [33]. Designated biomarkers that assess the effect of a treatment by substituting clinical endpoints are called surrogate endpoints.

Survival rates are used as a measure of success in cancer therapy development. Overall survival is the final endpoint, with event-free survival as the most successful

surrogate endpoint. In cancer with superior survival, overall survival may be a largely irrelevant endpoint and may not provide information about possible detrimental adverse events due to high dose or particular toxic susceptibility of the patients. This is exemplified in chronic-phase chronic myeloid leukemia (CML), where 5-year survival (after diagnosis) is more than 90% after the introduction of targeted kinase inhibitor (TKI) therapy in contrast to 50% 5-year survival before the imatinib era. In CML, quantitative PCR of the pathognomonic fused gene product *BCR-ABL1* is measured in the peripheral blood as a surrogate endpoint and used to accurately determine response and tumor load under therapy. PCR determination allows monitoring of persistent deep or complete molecular response in TKI-treated CML and could facilitate stopping treatment of patients with potentially toxic TKI therapy.

In GIST, the detection of a driving mutation in the c-*KIT* or *PDGFRA* proto-oncogene, which could be effectively targeted with imatinib, has improved the overall survival dramatically since introduced in 2001 [34]. However, wild-type GIST or tumors that harbor *PDGFRA* substitution mutation D842V do not respond to the same extent to imatinib [35]. These cases may demonstrate genetic alterations in downstream intracellular pathways such as *BRAF* and *RAS* or defects in the succinate dehydrogenase (SDH) complex and should be treated accordingly. Extended panels including potential prognostic and predictive genetic markers are therefore crucial in research trials. GIST may respond to sequential treatment with different TKIs, with each drug potentially adding months to the overall survival time. The clinical relevant endpoint to evaluate efficacy of either one of the drugs is progression-free survival. Detection of molecular markers predicting resistance to a drug has become increasingly important to prevent overtreating cancer patients with toxic compounds. Likewise, overcoming acquired resistance against initially effective treatment remains a challenge. A new strategy to prevent drug-related induction of resilient cancer cells is pre predefined alternating treatment targeting different molecular aberrations within the kinase receptors. This is investigated in the ongoing phase III ALT GIST trial, in which first-line imatinib monotherapy in advanced GIST is compared with imatinib alternating with regorafenib (Clinical Trials.gov Identifier: NCT02365441). The primary outcome measure will be progression-free survival with overall survival as a secondary outcome.

Proteins involving the tumor microenvironment may serve as other biomarkers. Osteosarcoma is a rare mesenchymal bone tumor arising in young adults, where tumor development and growth prerequisite degradation of the tumour-hostile bonye environment. It has recently been demonstrated that human osteosarcoma cells harbor strong expression of an endocytic collagen receptor (uPARAP/Endo 180), enabling tumor cells to directly mediate bone degradation [36]. Blocking uPARAP/Endo 180 in a murine model using a monoclonal antibody significantly reduced bone destruction, indicating a promising target for improved neoadjuvant therapy.

In some metastatic cancers, there has been limited improvement in survival until the emergence of immunological checkpoint inhibitors [2, 37]. In these cases, a rapid estimation of therapy response by surrogate endpoints may be needed for stratification of responders versus nonresponders.

Similar molecular features of different cancers experience a wide range of clinical responses to targeted therapy. Therefore, it is increasingly clear that the tumor cells are highly dependent on their contextual setting [10, 38–40]. Among the most complex environments of a tumor may be the involvement of the gut microbiota, identified as an important co-player in the development of colon cancer [41]. In allogeneic hematopoietic stem cell transplantation, the gut microbiota regulate graft-versus-host reactions of the immune system [42], indicating a role in immunological graft-versus-tumor mechanisms. Along the same line, gut microbiota modulate responses to immunological checkpoint inhibitors in cancer patients and therefore may provide an explanation for some responders and non-responders [43]. Novel biomarkers will increasingly focus on tumor context for therapy selection.

Design of Clinical Trials in Cancer Research

Traditionally, cancer clinical trials are divided into phase I, II, III, and IV (Fig. 22.1). The focus of phase I trials is usually on safety, and the primary objective is to find a maximum tolerated dose for use in subsequent trials. In phase II trials, the primary objective is usually to quickly determine whether the new treatment regime has sufficient efficacy. The use of biomarkers (surrogate endpoints) is

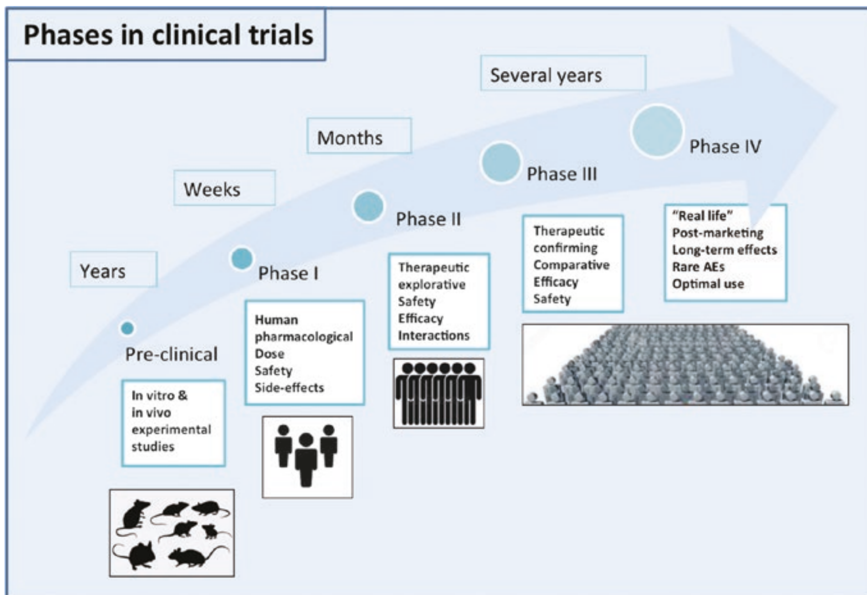

Fig. 22.1 Advancement through different phases in clinical trials

important for making early treatment decisions. Phase III trials are designed for establishing definitive clinical benefit, and survival is often used as a primary endpoint. Despite the division of trials into different phases, clinical trials should not be seen as distinct entities. Due to the failure rate of approximately 20% in phase III for approval of new cancer therapies, trial design and the strict trial phase design have been challenged [44]. There are now examples of phase Ib studies with expansion cohorts of patients numbering more than 1000 patients and where the US Food and Drug Administration (FDA) has used these data for a fast track approval. It is important to design trials in the context of an extended scientific process that starts first with human trials and, if the process is not discontinued due to lack of sustained efficacy or safety problems, ends with a new treatment or medicine on the market. At the same time, a new therapeutic molecule may represent only a modest incremental improvement in one group of patients, while others may have an outstanding response. This is observed in the use of kinase inhibitors in sarcoma versus CML [45–47] and the use of immunological checkpoint inhibitors in non-small cell lung carcinoma versus Hodgkin's disease [48, 49]. Use of various trial designs will be needed to develop the full potential of a therapeutic molecule.

Clinical trial design for oncology studies can broadly be categorized into rule-based and model-based designs. Rule-based designs are often used in early-phase (I/II) cancer trials. A small number of patients start with an initial dose, and the occurrence of unacceptable dose-limiting toxicity (DLT) determines, based on pre-specified rules, the dose for the next group of patients. Commonly used designs are the single- and two-stage up-and-down designs and optimal/flexible multiple-stage designs; see Chow and Liu [50]. The use of rule-based designs in early trials is appealing since they allow minimized exposure of patients to doses with unacceptable DLT. These designs can, however, be suboptimal from a statistical standpoint resulting in low efficiency. Model-based designs are usually more efficient. They employ statistical dose-response models to guide the dose-finding process and for estimating the effect (safety or efficacy) at different doses of the new investigational drug.

Clinical Trials for Targeted Therapy

The altered focus from cytotoxic agents to targeted therapy and predictive biomarkers has called for a shift from the classical paradigm in phase I studies where the primary focus is on safety (MTD) and phase II studies with primary focus on efficacy (see Mandrekar et al.) [51]. Within the new targeted therapy paradigm, the focus is on identifying molecularly defined subgroups of patients who will benefit from the targeted therapy and on understanding the drug activity for these patients. Novel designs have been developed, in which predictive biomarkers play a central role. Among these are enrichment, adaptive, umbrella, and basket designs. Brief descriptions of these designs are given below.

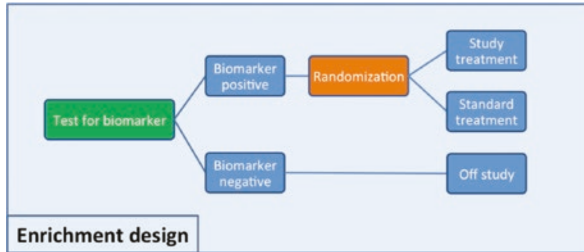

Fig. 22.2 Enrichment design, i.e. only biomarker positive patients are recruited into the clinical trial to receive experimental treatment

Enrichment Designs

Enrichment designs involve diagnostic tests that are used to identify which patients are positive for a biomarker and thereby eligible for the trial (Fig. 22.2). The eligible patients are then randomized to either a test group or a control group. Obviously, enrichment designs are most appropriate when patients who are biomarker negative are unlikely to benefit from the new treatment. Non-eligible patients are spared from adverse events, and overall safety of the trial is increased. Moreover, because the focus is on a targeted subgroup of patients, enrichment designs have high efficiency meaning smaller patient groups, faster trials, and hence overall cost reduction [52]. Sometimes the biomarker-negative patients can be included in a later separate study for the same treatment, but this is only meaningful if the treatment has proven to be successful for the targeted patients and if there is a reason to believe that biomarker-negative patients could benefit as well. Before enrichment designs are employed, it is important that the mechanism of the predictive biomarker is well understood. Enrichment designs are hence most appropriate in late phases of cancer drug development.

Umbrella Designs

Umbrella designs consist of two or more cohorts or sub-studies, e.g., two enrichment designs connected through an initial screening procedure. Patients are screened for predictive biomarkers and allocated into different cohorts (Fig. 22.3). Patients in cohort 1 are positive with respect to biomarker 1, patients in cohort 2 are positive with respect to biomarker 2, etc. Patients that are negative for all of the defined biomarkers are allocated to the last cohort. Within each of the biomarker-positive cohorts, patients are randomized to a targeted agent or a control group. Umbrella designs can be very useful, in particular, when a large number of patients are screened for low-prevalence biomarkers. As with enrichment designs, it is important that there is a strong rationale for using predictive biomarkers. These studies can be exploratory and have been used in different phases of drug development. The umbrella designs usually focus on a single histology or tumor subtype.

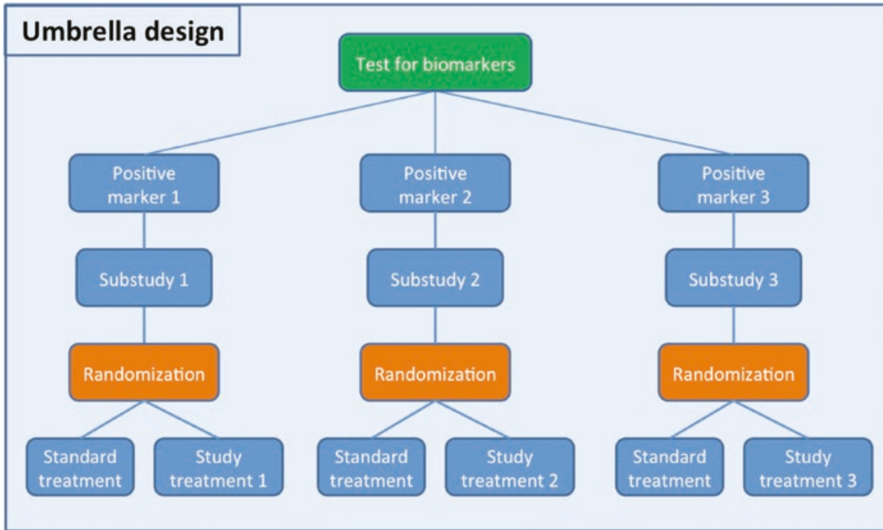

Fig. 22.3 Umbrella design, i.e. patients are screened for biomarkers and allocated into different marker-dependent substudies (different treatments) with subsequent randomization (study drug versus standard treatment)

Basket Designs

Different from umbrella designs, the basket designs are often used when several tumors or histological types are under investigation. Basket designs are of an explorative nature and can be appropriate for phase I targeted therapy studies. Patients are first screened and then allocated to cohorts depending on cancer type and molecular profile (Fig. 22.4). All patients within a cohort are then given the same targeted treatment.

Adaptive and Optimal Designs

Clinical trials are sequential in nature, and as a trial progresses, increasing amounts of data and information become available. The FDA has published guidance on adaptive design both for drugs and medical device clinical studies [53]. Adaptive designs allow a flexible approach to clinical trials enabling study teams to modify the design during the recruitment phase. Bayesian decision rules enable optimization of trial parameters based on real-time data at defined intervals to determine patient pathways in a “learn-as-you-go” principle [54]. The hope is that the FDA guidance will encourage the proper use of adaptive designs, reducing resource requirements and increasing the chance of study success and regulatory compliance. Optimal design theory can be used to find the most efficient designs. Adaptive designs and Bayesian analysis (Fig. 22.5) are employed to utilize the most

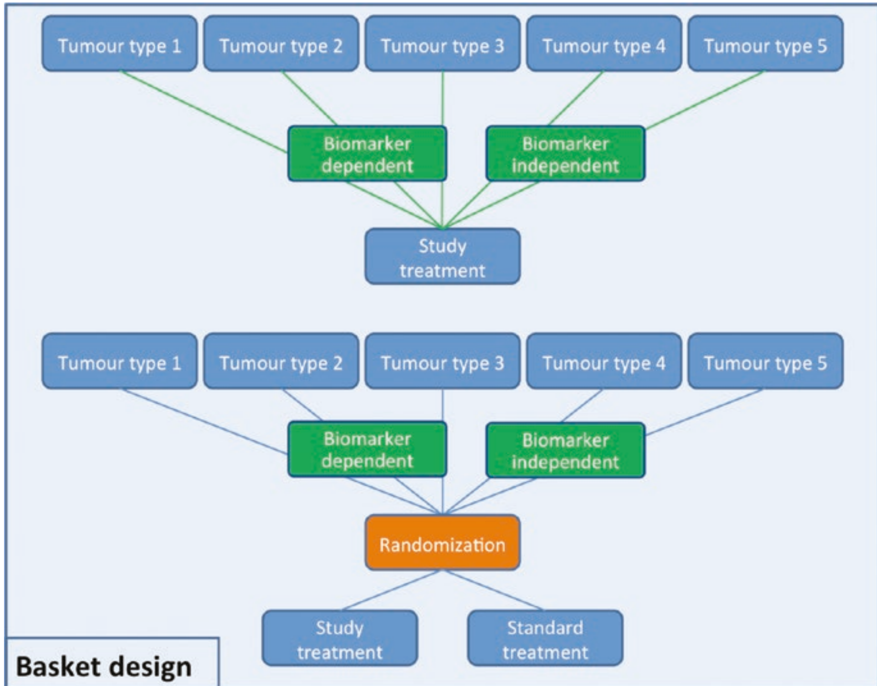

Fig. 22.4 Basket design, i.e. multiple cohorts based on different tumour types or biomarker status are given the same experimental treatment, with or without randomization

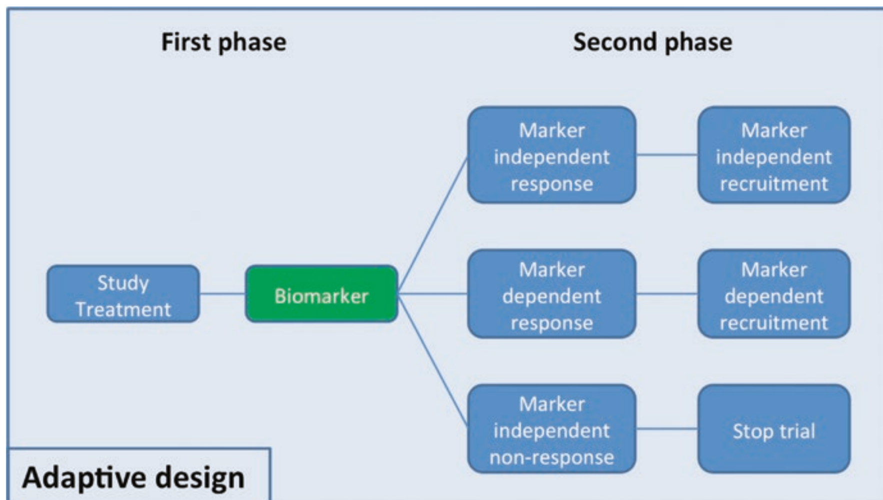

Fig. 22.5 Adaptive design, i.e. modifying the design during the recruitment phase by optimizing trial parameters based on interim analyses of treatment outcomes

up-to-date cumulative knowledge, and the use of surrogate endpoints together with early stopping rules can shorten the study period [55, 56].

Biomarker Panels in Late-Phase Trials of Hematopoietic-Derived Blood Cancer: Acute Lymphoblastic and Acute Myeloid Leukemia

The Nordic pediatric trial of acute lymphoblastic leukemia (ALL; NOPHO ALL 2008) represent the standard therapy of patients in the age group 1–45 years in Iceland, Finland, Denmark, Lithuania, Norway and Sweden. Patients are stratified into three categories: normal, intermediate, and high risk for relapse. Therapy intensity is adjusted according to risk stratification [57]. This risk stratification is guided by a panel of markers, including white blood cell counts [58] and advanced flow cytometric determination of leukemic cell remnants, so-called minimal residual disease (MRD), after treatment. The impact of the biomarker panel in this trial is significant due to the increased toxicity experienced in the high-risk arm of therapy.

The Dutch hemato-oncology network (HOVON) has, in collaboration with the Swiss oncology society (SAKK), been running larger randomized phase III trials in acute myeloid leukemia (AML) patients 18–65 years of age [59]. The later trials have incorporated an increasing number of molecular markers [60]. Not only have the presence of such markers been used but also have exclusion of high-risk markers, e.g., the length mutation or internal tandem repeat in the juxta-membranous domain of the receptor tyrosine kinase FLT3. Like for NOPHO, a panel of markers has been formed to guide therapy, using flow cytometry-based minimal residual disease assessment (MRD) or NPM1 mutation-specific quantitative PCR. MRD is used to select intermediate-risk patients between allogenic and autologous hematopoietic stem cell transplantation. For the patients, this implicates careful selection between two cellular immunotherapies with large differences in mortality. Therapy-related mortality is below 5% in autologous HSCT, while approximately 20% in allogenic HSCT [61].

The use of single-gene mutations has been used in adult and older AML to guide therapy. The molecular target FLT3 has been explored since 2000, but results of FLT3-targeted therapy have been limited or even detrimental for the experimental group [62]. The third-generation FLT3 targeting agent quizartinib (AC-220) has been shown to have effect both in FLT3-mutated patients and to a certain degree in FLT3 wild-type AML cases. A phosphoproteomics panel examining proteins in AML cells has indicated a distinction between responders and nonresponders independent of the FLT3-ITD status [63].

Based on the heterogeneity of most aggressive cancers, it is maybe a surprise that single biomarkers are able to predict therapy responses. Failure of FLT3-ITD to determine successful inhibitor therapy is likely from a biological perspective, even before we examine the quality and efficiency of the inhibitors. One of the important questions that arise is how an ideal biomarker panel could be constructed.

Biomarker Panels in Early-Phase Trials of Mesenchymal-Derived Cancer: Sarcoma

Mesenchymal stem cells or progenitor cells of connective tissues represent the origin of sarcomas, a rare heterogeneous group of diseases often with a specific morphology and a clearly defined macro- and microanatomical location [64, 65]. Many of the various subtypes of sarcoma are characterized by defined chromosome translocations, recurrent gene amplifications, and mutations.

Clinical trials have taken advantage of the defined genetic features and are testing targeted therapy based on the signaling pathways involved. Multidimensional analysis of biomarkers for future prognostic classifiers that reflect inter- and intratumor heterogeneity is in development [66], but such patient-stratifying tools have not been employed in clinical trials so far. GIST is the most common subset of sarcoma with high expression of the receptor tyrosine kinase c-*KIT*, and most patients with inoperable or recurrent GIST obtain temporary and often long-term disease control with kinase inhibitor therapy [46]. Like most oncogene-driven tumors that are sensitive to small-molecule inhibitors, relapse or disease progression due to secondary mutations of the kinase is a clinical problem in these sarcomas. Acquired imatinib resistance is typically associated with substitutions between the first and second receptor kinase domains, rarely found in untreated tumors. A likely mechanism explaining secondary clonal evolution is gatekeeper mutations interfering with binding of imatinib at the receptor site [67]. The oncogene activations in c-*KIT* are however still the driving force and the main target for therapy. In lack of secondary mutations, feedback mechanisms may be responsible for reactivation of c-*KIT*. Another explanation suggested is a morphologic shift with altered immune phenotyping insensitive to imatinib emerging as a result of the targeted therapy [68]. Novel multi-target TKIs, either alone or in combination, may be effective in imatinib-resistant GIST; again, genotype biomarkers may predict the likelihood of response. Hence, design of clinical research protocols must take into account the impact of these molecular hallmarks.

Within the field of sarcoma, there is an ongoing search for effective targeted therapy related to pathway aberrancies detected by mutational biomarker analyses. In Norway, a population-based recording of mutational status in all new incidences of sarcoma over a period of 2–3 years has recently been initiated. This Norwegian Sarcoma Consortium (NoSarC) project assesses frozen tumor tissue according to a panel of approximately 900 mutations described in various types of cancers [69]. The project aims at increasing insight into molecular mechanism in sarcoma development as well as identifying aberrations which may be further exploited as targets for new therapeutic drugs. Potential targets will be validated in preclinical studies prior to further investigations in small-scale academic studies. The accumulation of functional information emerging from systematic genome sequencing may facilitate development of second-line personalized medicine in orphan cancer types for which the rarity hampers large-scale clinical trials.

One interesting trial design is the EORTC trial, "CREATE," testing the ALK kinase inhibitor crizotinib in six defined rare diagnoses including three rare sarcoma

subtypes (ClinicalTrials.gov Identifier: NCT01524926). However, specific *ALK* and/or *MET* pathway alterations in tumor tissue are not mandatory for patient registration, thereby allowing control patients for pathway-targeted therapy. Furthermore, substantial tumor material should be available for central review and molecular diagnostics, and previous therapy is permitted. Trials have previously demonstrated timely use of biomarkers in targeted therapy of rare diagnoses, including therapeutic antibodies against IGF-1R as well as the tyrosine kinase inhibitor pazopanib in soft tissue sarcomas [70, 71].

Summary and Discussion

Biomarker panels are an increasingly integrated part of clinical trials, used for pivotal decision-making. It is likely that combinations of biomarkers that involve tumor environment and stromal features will increase prognostic precision with immunotherapy and targeted therapy. Optimal trial design theory can be used for finding the most efficient designs. Adaptive designs and Bayesian designs are used to utilize the most up-to-date cumulative knowledge, and the use of surrogate endpoints together with early stopping rules can shorten the study period. Ethical perspectives need to be taken into account when designing a cancer study: for example, will the patients benefit from taking part in the trial?

FDA guides the process of obtaining an approved biomarker program in a clinical trial [72]. This secures the quality of the biomarker assays but has also likely limited the use of biomarker assays in many US trials.

Even if genomics alone as guidance for therapy is unlikely to prove relevant for all types of cancer, the proportion of actionable mutations is likely to be so high that cancer genetics may provide significant clinical benefit [20]. Therefore, genomic profiling may become relevant to some but not all cancer types. Development of functional biomarker assays are emerging and prepared for use in clinical trials, such as single-cell immune and signaling profiling or in vitro cancer sensitivity assays for selection of optimal therapy. AML patients responding to the combination of vitamin A, theophylline, and valproic acid displayed a low signaling signature of phospho-signaling proteins [73]. Functionality through in vitro testing of therapy response has been employed in single patients and with promising responses [7, 74]. Limitations of this in vitro drug sensitivity assay include the absence of combination testing and novel therapy availability.

Studies in AML and ALL, as well as in soft tissue sarcoma including GIST, potentially illustrate a robust use of biomarker panels. A combination of various modalities, from clinical data with cancer cell numbers via karyotype, gene expression, and mutational analyses, seems to guide therapy intensity at a higher accuracy. Similarly, surrogate markers for therapy effect need to be selected based on the nature of the disease, e.g., minimal residual disease determination by quantitative PCR in the presence of reliable markers or protein expression of the malignant clone by immunophenotype. The complexity of the decision tools for the physician

seems to be increasing, and the future challenge will be to create a simplified and validated diagnostic system that provides precision medicine to the heterogeneous tumor diseases of our patients.

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Erratum to: Models of Tumor Progression in Prostate Cancer

Waqas Azeem, Yaping Hua, Karl-Henning Kalland, Xisong Ke, Jan Roger Olsen, Anne Margrete Øyan, and Yi Qu

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