Chapter 6
Microenvironmental Control of Metastatic Progression

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Abstract As solid tumors progress, the surrounding microenvironment is altered dramatically. This microenvironment contains stromal and immune cells, some resident and some newly recruited, that are often activated due to factors released by the tumor cells themselves. These activated cells then release soluble factors that feed forward on the tumour cells in a symbiotic manner. Activated cells also alter the deposition and processing of extracellular matrix molecules in the microenvironment which further affects both the genotype and the phenotype of tumor cells. More specifically, these microenvironmental alterations can have profound effects on the genome and epigenome of tumor cells as well as their signal transduction pathways, both biochemical and mechanical. All of these effects contribute to the invasion and progression of the metastatic tumor organ.

Keywords Microenvironment · Tumor progression · Stroma · Extracellular matrix

Abbreviations

CAFs	Cancer-associated fibroblasts
CIMP	CpG island hypermethylation phenotype
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
HIF1α	Hypoxia inducible factor 1alpha
MET	Mesenchymal to epithelial transition
MSI	Microsatellite instability
TAMs	Tumor-associated macrophages
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

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Introduction

Given that the overwhelming majority of cancer victims succumb to the formation and expansion of metastatic lesions, an overarching goal of modern cancer research is to determine how changes in defined cohorts of oncogenes and tumor suppressors facilitate the emergence of the malignant phenotype within individual tumor cells and their progeny. As a result, identifying actionable targets that are the products of such dysregulated genes has become the cornerstone of rational cancer therapeutics. However, even when they are directed against bona fide targets, such therapies are often only partially effective and they are invariably susceptible to acquired resistance as the tumor evolves towards full blown malignancy. The recognition that many non-tumor cell autonomous events contribute to this progressive evolution, together with data generated by genome-wide profiling both at the transcriptomic and epigenomic levels, are starting to give us a systems-based picture of why this is the case. What is becoming increasingly clear from such studies is that the tumor microenvironment is a major multifactorial contributor to this progressive evolution [23].

In early *in situ* lesions that have been initiated by mutagenic insult, the microenvironment still closely resembles the normal tissue that the lesion arises in. In many cases, notably the breast [48], the prostate [56, 60], and the thyroid gland [25], this near 'normal' tissue microenvironment actually acts to suppress the further expansion of microcarcinomatous lesions. However, as the tumor progresses, the surrounding tissue microenvironment is replaced by an ever-changing milieu that is itself abnormal. Importantly, this abnormal microenvironment co-evolves with the tumor cells as part of the malignant tumor 'organ'. Rather than being suppressive, the microenvironment of the malignant tumor organ instead functions to promote the invasion and metastatic spread of the expanding lesion [5].

In addition to cellular differences, the microenvironment of the malignant tumor organ also differs in its extracellular components compared to the near normal microenvironment that surrounds early tumorigenic lesions. For example, factors released into the extracellular milieu by the tumor cells themselves can act to 'activate' nearby stromal cells in the surrounding microenvironment. The responding stromal cells can consist of those that already reside within the tissue affected or they can be recruited from other sites, most notably the bone marrow. The latter cells often have an ability to expand due to their progenitor characteristics which can further expand the activated stromal cell pool. This expansion and activation has been best documented in the case of cancer-associated fibroblasts (CAF's; [24]), although many other stromal cell types can also be 'activated' by factors in the tumor microenvironment.

CAF's are capable of modifying the acellular architecture of the tumor microenvironment by altering its insoluble extracellular matrix (ECM). This is achieved through changes in the production and deposition of matrix molecules as well as the alteration of the structure and interaction of those matrix molecules already present in the ECM. Examples include CAF-mediated changes in the deposition of

collagens, their extracellular processing by metalloproteases and their cross-linking by lysyl oxidases [11, 31]. CAF's also release soluble factors into the tumor microenvironment that act in a feed-forward way to further stimulate the growth of, or alter the phenotype of, tumor cells and to further recruit and activate more stromal cells. Tumor cell proliferation and survival can be facilitated by the CAF-mediated release of factors such as IGF-1, EGF, HGF and IL6, while a major tumor cell phenotype modifier that can be released by CAFs is transforming growth factor beta (TGF-β) (see epithelial-mesenchymal transformation section, below; [64]). Stromal cell recruitment and their feed forward activation can be initiated by the CAF-mediated release of cytokines such as CCL5 and SDF-1 [32, 35]. Importantly, the cytokines and growth factors that are released into the primary tumor microenvironment can act as short range paracrine factors as described above or they can also travel to distant sites and sow the microenvironmental soil to facilitate the expansion and local invasion of secondary metastatic lesions [76].

Acting in concert, soluble factors released by both the tumor cells and the activated stromal cells in the microenvironment can act over considerable distances to recruit immune cells to the lesion site. This immune infiltration, which has many of the hallmarks of a chronic inflammatory state, consists of varying numbers and ratios of lymphoid and myeloid cells, the precise nature of which depends on the tumor site involved and the malignant state of the lesion [59]. These infiltrates produce their own growth factors and cytokines that then further influence nearby and tumor and stromal cells in the manner described above. They also secrete proteolytic enzymes that can remodel the ECM [42]. Such remodeling can have profound effects on the tissue microenvironment as it can release and/or activate soluble factors that are normally sequestered within the insoluble portion of the ECM.

Soluble factors and ECM fragments released into the tumor microenvironment by proteolytic degradation can also act in a paracrine manner on nearby vascular endothelial cells and their surrounding pericyte stem-like population to initiate an angiogenic response. A critical factor that helps drive this angiogenic response is vascular endothelial growth factor (VEGF); [61], although VEGF-independent factors come to the fore as the malignant tumor organ evolves which has been confounding to targeted anti-angiogenic therapy development [63]. Regardless, the resulting formation of new blood vessels leads to an increase in blood supply that is critical to the malignant tumor organ's survival. Concurrently, the angiogenic process itself further alters the landscape of the tumor microenvironment because the resulting newly formed blood vessels are often tortuous, porous and 'leaky' which increases the hydrostatic pressure within the tissue. In addition, tumor cells are subjected to widely varying oxygen tensions depending how far they are from these new vessels [7, 19]. In areas of low oxygen, the resulting hypoxia induces metabolic alterations and a suite of gene expression changes in both the tumor and the stromal cells that lead to further microenvironmental changes in soluble factor production and ECM production and modulation that can affect a number of tumor characteristics including genome stability (see below). It is becoming increasingly clear that a wide variety of responses to metabolic alterations within the malignant tumor microenvironment are also mediated by the activated

stromal cells. For example, in response to the release of reactive oxygen species by tumor cell, nearby CAFs upregulate the expression of glycolytic enzymes which leads to their increased production and secretion of pyruvate and lactic acid that are then secreted and utilized by the surrounding tumor cells as a critical alternative energy source for their continued rapid proliferation [55].

While the cellular and acellular aspects of the microenvironment broadly influence tumor and stromal cell phenotypes, they also impinge upon specific processes that are critical for malignant progression. These include alterations to genomic stability, the epigenome, non-coding RNAs, immunomodulation, mesenchymal transformation and mechanotransduction. We provide specific examples below and expand on individual processes in the chapters that follow.

Hypoxia-Induced Genomic Instability

In normal tissues, the microenvironmental oxygen tension can be as high as 10%. In contrast, the oxygen tension within rapidly expanding locally advanced solid tumors is often less than 1% due in large part to their high metabolic rate [71]. The resulting hypoxia leads to the increased production of factors by both tumor and stromal cells that are transcriptional regulators, the prototype of which is hypoxia inducible factor 1alpha (HIF1α). These regulators both stimulate and suppress the expression of a wide variety of genes whose products modify the microenvironment to facilitate metastatic progression. These include the afore-mentioned angiogenic factor VEGF as well as osteopontin, an ECM protein that facilitates tumor cell invasion. The latter factor facilitates the movement of tumor cell cohorts even farther away from blood vessels which further exacerbates the hypoxic state locally within the lesion [43].

Hypoxia also acts to suppress the expression of a number of homologous recombination (HR) genes involved in repairing the DNA double strand breaks caused by ionizing radiation and radiomimetic drugs [44]. In some contexts this suppression is HIF1α-dependent [35] while in others it is not [4]. Interestingly, in some cases these two means of hypoxia-induced suppression can act on the same HR gene. This is the case, for example, with the suppression of BRCA1. Hypoxia also suppresses the expression of genes involved in DNA mismatch repair. This leads to an increase in spontaneous mutations that are associated with microsatellite instability (MSI) during experimental colorectal carcinoma progression [17, 62]. Furthermore, in human colorectal tumors HIF1α expression, which is used as an indication of hypoxia, and MSI are associated are associated with poor outcome/progression [21].

There is some evidence that hypoxia can also affect chromosome segregation during mitosis. Mechanistically, it appears that this occurs due to an alteration of the centrosome that leads to a defect in mitotic spindle formation [46]. Taken together, these and many other observations [43] indicate that hypoxia-mediated defects in DNA repair and chromosome segregation accelerate the genomic instability that

is already intrinsic to the growing tumor organ, thereby facilitating the continued evolution of malignant progression [9].

Epigenetic Dysregulation

The most widely studied epigenomic change that is correlated with tumorigenesis is the CpG island hypermethylation phenotype (CIMP). Essentially, this is a broad measure of suppressive promoter methylation that has been observed in bladder, breast, endometrial, gastric, colorectal, hepatocellular, lung, ovarian, pancreatic, renal cell and prostate carcinomas as well as leukemias, melanomas and gliomas. Drivers of this phenotype, including mutations in the isocitrade dehydrogenase-1 gene that result in the accumulation of the hypermethylating oncometabolite 2HG [70], are now being identified. However, while it is clear that the CIMP phenotype contributes to tumor formation, its role in tumor progression is less clear. For example, in colorectal cancer CIMP functionally contributes to the initial tumor formation but not tumor progression. Instead, a subsequent trend towards hypomethylation becomes more prominent as lesions progress from adenomas to invasive cancers [67]. In addition to contributing to a general increase in genomic instability, this hypomethylation has been shown to specifically trigger the production and release of soluble growth factors and modulators including insulin-like growth factor-2 (IGF2) and IGF2 binding protein-3 into the tumor microenvironment [29, 41]. These factors act in an autocrine fashion to increase tumor cell proliferation and invasion and they act in a paracrine fashion to activate stromal cells which, as was described above, have feed forward effects on tumour cells that contribute to malignant progression.

LINE-1 hypomethylation within the long interspersed nucleotide element-1 (LINE-1) often occurs in metastatic prostate [74] and metastatic endocrine pancreatic [10] carcinomas. While this epigenetic mark is often used as a general indicator of hypomethylation, it is also known to be functionally significant in tumor progression in a number of ways. Specifically, it can lead to the activation of adjacent genes as well as an increase in chromosomal instability [18, 65, 72] as well as genomic instability [1, 50].

Immunomodulation

Cytokines such as interleukin-4 and -13, produced by malignant and stromal cells within the tumor microenvironment in a manner that mimics the end stages of wound healing, cause an immune suppression that is tumor promoting. This is initiated, in large part, by the cytokine-mediated recruitment of monocytes to the lesion. These new recruits then differentiate into alternatively activated tumor-associated macrophages (TAMs) that skew towards an ‘M2’ phenotype that is immunosuppressive

[47, 59]. More specifically, alternatively activated TAMs do not exhibit the cytotoxicity of typical macrophages [54]. Instead, they release paracrine-acting factors such as the chemokine CCL22 [12] and they generate nitrogen species, particularly under hypoxic microenvironmental conditions [14], that suppress the infiltration and proliferation of T-lymphocytes into the tumor microenvironment. Immunosuppressive TAM's also secrete VEGF-A [40] which augments the hypoxia-induced increase in angiogenesis within the tumor microenvironment described above. Alternatively activated TAM's are an attractive anti-metastatic therapeutic target given their profound ability to facilitate tumor progression by contributing to an escape from immune surveillance while simultaneously promoting angiogenesis. Experimentally, TAMs can be targeted by blocking the cytokine CSF-1 [13, 45], which is required for the proliferation and differentiation all macrophage populations. Unfortunately, this approach is a very broad one and is likely to have long term side effects in patients. A more specific approach would be the reprogramming of TAM's into more conventional 'antigen-presenting' immune response-promoting macrophages that are known to have anti-tumor effect. Experimentally, this has been achieved using histidine-rich glycoprotein [57].

Interestingly, some cytotoxic drugs (eg. paclitaxel) can suppress the M2 TAM phenotype and skew it towards a pro-inflammatory M1 phenotype that is then antitumoral [34]. Thus, a goal of the field has been to identify mediators that drive this proinflammatory M1 TAM skewing in a predictable manner. One such mediator is the microRNA miR-511–3p [66]. Thus, non-coding RNA's are capable of modulating effectors in the microenvironment that play a critical role in tumor progression.

Immunomodulatory changes during tumor progression are discussed in more detail by Gregor Reid in Chap. 8 of this volume.

Mesenchymal Transformation

A major driver of tumor cell invasion is the epithelial-to-mesenchymal transformation (EMT; [69]). During the EMT process there is a breakdown in apical-basal polarity followed by the loss of adhesive junctions between stationary epithelial cells. The resulting individual cells acquire an anterior-posterior polarity and they become motile and mesenchymal [22]. Therefore, classical markers of this transformation are the loss of the epithelial cell-cell adhesion molecule E-Cadherin and the upregulation the mesenchymal intermediate filament protein vimentin. These changes, particularly at expanding tumor fronts, are often used as an indicator of invasive progression and the onset of the metastatic process [30].

During normal development, EMTs contribute to organogenesis and the formation of the body plan in a manner that is tightly regulated, both spatially and temporally [26]. For example, this occurs during gastrulation where a precisely controlled EMT leads to the production of invasive mesenchymal cells that move into the embryo and later re-aggregate to form the mesodermal condensations during

primary germ layer formation [39]. While microenvironmental organizing centers (eg. the primitive knot, Spemann's organizer) that regulate the position and timing of developmental EMT's have been identified, the precise nature of the instructive paracrine factors they release have still not been well characterized. In contrast, the core transcriptional machinery that acts to initiate the gene expression changes that initiate an EMT has been determined. This includes the Snail, Zeb and Twist transcription factors which act on the E-cadherin promoter to inhibit the gene's expression as well as stimulate the expression of secreted factors that further stimulate an EMT [52]. An example of the latter is platelet-derived growth factor (PDGF) which itself stimulates the localized production and activation of metalloproteases that degrade the ECM in the microenvironment to facilitate the migration and invasion of mesenchymal cells produced by the EMT [16].

During malignant tumor progression the precise spatial and temporal control of EMT is disrupted, most often because of an inappropriate accumulation and/or activation of EMT-inducing factors within the tumor microenvironment. One such factor is TGF-ß which, when it acts on epithelial-derived tumor cells (but not normal epithelial cells) stimulates the activity of the core EMT transcription factor complex [33]. TGF-ß is often produced and secreted by cancer-associated fibroblasts in the microenvironment [8]. Interestingly, TGF-ß that is produced by platelets and released into the microenvironment due to the leakiness of new vessels formed by angiogenesis can also contribute to transient tumor cell EMT at sites of thrombosis [36]. This has important implications for the movement of tumor cells from the stroma into the vasculature by a process known as intravasation. Additionally, it may also help explain why circulating tumor cells themselves can be mesenchymal [75]. The latter point is not trivial in terms of survival in the circulation as mesenchymally-transformed cells tend to be resistant to the suspension-mediated apoptosis that normally occurs when epithelial cells are detached from the ECM [27].

Other factors found in the tumor microenvironment that can act to stimulate the core EMT transcriptional machinery including the afore-mentioned PDGF produced by CAF's as well as WNT or WNT-like factors produced by recruited mesenchymal stem cells and interleukin-6 (IL-6) produced by TAMs [49]. Importantly, the removal of such factors can shift the tumor cell phenotype from the mesenchymal back to the epithelial in a process known as mesenchymal-epithelial transformation (MET). This often occurs during the later stages of metastatic progression where an MET is proposed to contribute to the colonization of distant sites after tumor cells have left the vasculature by extravasation. Such transient shifts between epithelial and mesenchymal phenotypes can also be regulated by the oxygen tension in the microenvironment given that hypoxia upregulates the core EMT transcriptional complex via the actions of HIF-1α [73]. Thus, the mesenchymal phenotype is often plastic, unless mutations within the E-cadherin gene and/or stable, epigenetically-driven changes in E-cadherin expression occur. As such, there are varying tumor microenvironment-driven metastable and stable states of mesenchymal transformation within tumor lesions that have important implications for therapeutic treatment strategies bent on reversing the process [68].

Mechanotransduction

Once they have acquired the ability to become invasive, either by varying degrees of mesenchymal transformation or by other means that can include either collective or single-celled amoeboid migration [20], tumor cells move through the tumor microenvironment by interacting with the ECM, the components of which are highly modified due to changes in component deposition, molecular cross-linking, and proteolytic processing within that microenvironment [58]. Ultimately, the molecular composition of the ECM greatly contributes to changes in motile phenotype of the invading tumor cells based on, for example, the soluble factors it sequesters and the specific nature of the cell surface integrins that it engages [28]. However, it is becoming increasingly clear that the mechanical properties of the ECM also play an important role in regulating the phenotype of the invading tumor cells. In this case, physical changes in the ECM can dramatically alter mechanical signals within tumor cells that influence proliferation, survival and the invasive phenotype itself [15]. Experimentally, this can be achieved by artificially crosslinking ECM components, particularly collagens, to increase the stiffness of the matrix which increases intracellular tension and integrin-mediated biochemical signaling within the tumor cell [38]. Thus, mechanical cues in the ECM are translated intracellularly by the cytoskeleton and signaling moieties that are modulated by tension applied through integrin-containing adhesion complexes. Such collagen cross-linking can be achieved by the actions lysyl-oxidase which is released into the tissue microenvironment by CAFs. In yet another example of a feed forward mechanism, lysyl oxidase-dependent collagen crosslinking will further activate CAF's themselves in an integrin signaling-dependent manner [3] and this effect can be so strong that it can facilitate tumor invasion and metastatic progression even when TGF-ß is removed [53].

Importantly, collagen crosslinking-dependent increases in radiologically observable mammographic 'density' is a major risk factor for breast carcinoma formation and progression [6]. The latter effect may be facilitated by the fact that ECM stiffness-mediated mechanotransduction augments the ability of soluble factors sequestered within the tumor microenvironment to efficiently induce an EMT [37].

Mechanotransduction events that contribute to tumor progression are discussed in more detail by Celeste Nelson's group in Chap. 7 of this volume.

Summary

It is now clear that the microenvironment that a tumor cell finds itself within can greatly affect its phenotype regardless its genotype. These microenvironmental effects are mediated by surrounding stromal cells, soluble factors, and the extracellular matrix all of which act together, with tumor cells, to form the tumor organ. Therefore, given the molecular and cellular complexity within the tumor organ, it is very difficult to predict the response of any one component of the organ to

a particular therapeutic treatment when that component is viewed in isolation. While this complexity can be extremely problematic when viewed from a reductionist point of view, particularly when it contributes to the failure of agents targeted against specific tumor cell-intrinsic oncogenes or tumor suppressors, it also provides myriad new therapeutic opportunities to halt the emergence of those microenvironment-dependent tumour progression phenotypes that contribute to the overwhelming majority of cancer deaths due to metastasis.

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