Pivotal Role of Pervasive Neoplastic and Stromal Cells Reprogramming in Circulating Tumor Cells Dissemination and Metastatic Colonization

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Abstract Reciprocal interactions between neoplastic cells and their microenvironment are crucial events in carcinogenesis and tumor progression. Pervasive stromal reprogramming and remodeling that transform a normal to a tumorigenic microenvironment modify numerous stromal cells functions, status redox, oxidative stress, pH, ECM stiffness and energy metabolism. These environmental factors allow selection of more aggressive cancer cells that develop important adaptive strategies. Subpopulations of cancer cells acquire new properties associating plasticity, stem-like phenotype, unfolded protein response, metabolic reprogramming and autophagy, production of exosomes, survival to anoikis, invasion, immunosuppression and therapeutic resistance. Moreover, by inducing vascular transdifferentiation of cancer cells and recruiting endothelial cells and pericytes, the tumorigenic microenvironment induces development of tumor-associated vessels that allow invasive cells to gain access to the tumor vessels and to intravasate. Circulating cancer cells can survive in the blood stream by interacting with the intravascular microenvironment, extravasate through the microvasculature and interact with the metastatic microenvironment of target organs. In this review, we will focus on many recent paradigms involved in the field of tumor progression.

Keywords Microenvironment · Stromal reprogramming · ECM remodeling · CTCs · EMT · Invasion · Intravasation · MET · Dormancy · Metastasis · Tumor self-seeding

Abbreviations

COX2	Cyclooxygenase 2
PDGF	Release platelet-derived growth factor
PDGFR	Release platelet-derived growth factor receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
HIF-1	Hypoxia-inducible factor 1
HGF	Hepatocyte growth factor
VEGF	Vascular endothelial growth factor
FSP-1	Fibroblast-specific protein 1
SDF-1	Stromal cell-derived factor 1
EGFR	Epidermal growth factor receptor
RANKL	Receptor activator of nuclear factor kappa-B ligand
TGF-β	Transforming growth factor <i>beta</i>
FGF	Fibroblast growth factors
IFNs	Interferons
TNF1α	Tumor necrosis factor 1 Alpha
LOX	Lysyl oxidase
MCT1	Monocarboxylate transporter 1
MCT4	Monocarboxylate transporter 4
Shh	Sonic Hedgehog
LC3	Microtubule-associated protein 1 light chain 3

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Introduction

Carcinomas are highly heterogeneous tissues represented by neoplastic epithelial cells and tumor microenvironment composed of non-carcinomatous cells and modified extracellular matrix (ECM) [1]. Complex interactions between cancer cells

and microenvironment result in cell-cell and cell-matrix interactions, allowing tumor cells to obtain advantage during cancer progression by acquiring numerous adaptative properties [2]. Cancer cells survival seems to be dependent on their ability to control and to shape the surrounding microenvironment. Tumor progression is now considered as a process of epithelial and stromal cells which interact together and influence each other in reciprocal manner.

Reciprocal Stromal and Epithelial Reprogramming at the Site of Primary Tumor

Tumorigenic Microenvironment

Cancer is classically considered as a genetic disease characterized by alterations of oncogenes and suppressor genes implicated in cell proliferation, apoptosis, local invasion, angiogenesis and dissemination. Tumorigenic microenvironment (TM) is a complex network composed of ECM, immune/inflammatory cells and non immune stromal cells that drives cancer cells fate from invasion to intravasation and metastasis. Following cancer initiation, autonomous proliferating tumor cells with genetic and epigenetic changes cannot survive in isolation [3]. Cancer cells need cellular, biochemical and biophysical stimuli originating from a more adapted microenvironment by recruiting and educating various types of normal cells into their neighborhood [4]. Modulation of this complex stromal cellular niche from an initial hypoxic mesenchymal tissue to a TM plays fundamental roles in the successful survival and expansion of malignant cells. Mechanisms involved in heterotypic cooperations between cancer cells and stromal cells comprise cell-to-cell interactions and secretion of exosomes, ECM components, growth factors (GFs), signaling molecules and inflammatory mediators [5]. Stromal remodeling and acquisition of a more aggressive phenotype by cancer cells are provided by a pervasive stromal and epithelial cellular reprogramming [6]. Development of a TM is driven by genetic instability of cancer cells associated with extrinsic and intrinsic stress stimuli. Extrinsic factors include hypoxia, aberrant microcirculation, acidosis, glucose deprivation, oxidative stress and pH changes. Intrinsic stressors associate imbalanced cell growth, increased mutation rate, errors in glycoprotein and lipid biosynthesis and decreased amino acid supplies [7]. Microenvironmental variations in local distribution and intensity are pivotal components of intra-tumoral heterogeneity. By using cell-cell paracrine loops or diffusible molecules, reciprocal interactions between cancer cells and tumor, intravascular and distant organ microenvironments are crucial in selecting cancer cells

subpopulations with more aggressive properties. Conversely, cancer cells may interact with and modify stromal cellular functions by using a specific program resulting in cancer-associated alternating splicing events [8].

Pervasive Stromal Cellular Reprogramming

Recruitment, activation, reprogramming and persistence of stromal cells and bone-marrow derived cells in the extracellular space are the consequences of a reciprocal relationship between TM and cancer cells. Stromal cells can be grouped into two classes comprising non immune/inflammatory and immune/inflammatory cells. Opposite tumor front, stromal inflammatory interface represents a dynamic space characterized by reversible stromal and epithelial events. Stromal cells reprogramming comprises fibroblasts, endothelial cells and pericytes transitions to myofibroblasts and macrophages M1 conversion to macrophages M2. Epithelial cancer cells reprogramming comprises epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET). Within this dynamic space, exchange of numerous molecular informations is associated with these transitions and includes GFs, cytokines, chemokines, enzymes, matrix proteins and metabolic intermediates.

Non Immune/Inflammatory Cells

Cancer associated fibroblasts (CAFs) represent a heterogeneous population of three main cell types: fibroblasts, myofibroblasts and cancer associated adipocytes (CAAs). These three subtypes represent the majority of stromal cells [9]. CAFs may be also derived from pericytes, cancer stem cells (CSCs), bone-marrow derived fibrocytes (BMDFs), local and bone-marrow derived mesenchymal stem cells (BMSCs), smooth muscle cells, endothelial cells, normal epithelial and cancer cells [10]. According to their different origins, CAFs variably express smooth muscle actin (SMA), tenascin-C, PDGFR and mesenchymal biomarkers (vimentin, fibronectin and FSP-1).

Fibroblasts are pivotal in recruitment, functionality and interactions of the different stromal cells and induce biochemical and biophysical modifications of ECM. Interestingly, pathways that activate CAFs and EMT are similar by engaging COX2, NF- κ B and HIF-1.

Myofibroblasts are characterized by expression of SMA and are particularly implicated in cytokines and GFs production and ECM fibrosis [11]. Fibroblasts to myofibroblasts reprogramming is driven by ED-A splice variant of fibronectin, TGF- β , ECM stiffness, GFs and metalloproteases (MMPs).

Cancer associated adipocytes (CAAs) have been recently identified as a new stromal sub-population in TM [12]. In addition to their metabolic functions, adipocytes possess important secretory properties that may enhance tumor

aggressiveness. They produce adipokines (leptin, adiponectin and apelin), angiogenic factors (VEGF), cytokines (TNF α , IL-1 β , IL-6), chemokines (MCP-1) and GFs (HGF) [13]. Mature adipocytes reprogramming to CAAs depends on Wnt/ β -catenin pathway reactivation in response to Wnt3a secreted by cancer cells. Once reprogrammed, CAAs are found in close proximity with invasive epithelial cells and initiate protumoral heterotypic paracrine and endocrine interactions. Compared to mature adipocytes, CAAs are characterized by loss of adipocyte differentiation, a smaller size and FSP-1 but not SMA expression. They exhibit increased secretion of IL-6, MCP1, VEGF, fibronectin, collagen I/VI and MMP-11/Stromelysin-3 [14]. The latter promotes adipocyte dedifferentiation and cleavage of collagen VI to endotrophin, a signaling molecule involved in inflammation, angiogenesis and stiffness. Interestingly, endotrophin inhibition results in tumors sensitization to cisplatin. Recently, it has been observed in breast and prostate carcinomas that CAAs could serve as energy sources for cancer cells via β -oxidation of fatty acids and can promote resistance to treatments.

All subtypes of CAFs are activated in response to TGF- β produced by adjacent cancer cells [15]. In turn, CAFs play key roles in all stages of tumor progression by issuing numerous oncogenic cytokines (IL-1, IL-6, IL-8), chemokines (CXC chemokines CXCL1 to CXCL12/SDF1, CC chemokines CCL2 and CCL20), chemotactic GFs (VEGF, PDGF, EGF, FGF, HGF), ECM proteins (fibronectin, collagen I and III, EDA-fibronectin, tenascin C, SPARC), enzymes (MMP-1, -2, -7, -9, -13, -14; LOX family oxidases LOX and LOX-like proteins 1–4) and COX2 [16]. PDGF/PDGF α axis is of paramount importance as master regulator of respectively normal and cancer cells trafficking [17] and of tumor microenvironment development [11]. CAFs are partly responsible for cancer-associated inflammation and neoangiogenesis. They are instrumental in ECM remodeling that alters its composition via excess of EDA-fibronectin, tenascin C and SPARC. These protumoral matrix proteins make ECM more permissive for cancer cells invasion and play a complementary role to that of MMPs secreted by cancer cells. CAFs are also implicated in ECM stiffness and fibrosis via deposition of collagen fibers type I. Increased rigidity augments release of active TGF- β from ECM-associated LTBP-1 via interaction of integrins α v β 3, α v β 5, α v β 6 and α v β 8 expressed by myofibroblasts [18]. Finally, CAFs play a central role in collective migration by serving as leader cells to cancer cells and by promoting development of invasion paths via collagen fibers arranged perpendicularly to tumor cells. This reorganization is based on activity of MMPs, integrins and their connections with actin cytoskeleton that generate contractile forces.

Angiogenic vascular cells Blood vessels are composed of perivascular cells termed as pericytes, endothelial cells which form the inner lining of the vessels wall and vascular smooth

muscle cells. Tumor blood vessels are often inefficient in their function, leading to high interstitial fluid pressure, hypoxia, and acidosis in the TM, rendering tumors refractory to delivery of chemotherapeutic agents and immune effector cells [19].

Pericytes have gained new attention as functional and critical contributors to tumor angiogenesis. Recent data suggest that pericytes serve as important gatekeepers against cancer progression and metastasis [20]. Clinical studies demonstrated that low numbers of vessel-associated pericytes correlated with a drop in overall survival of patients with breast cancer. Using genetic mouse models or pharmacological inhibitors, pericyte depletion was associated with increased hypoxia, EMT, and Met pathway receptor activation [21].

Endothelial cells are organized in a thin single layer and are closely linked by specialized cell junctions. They are interconnected with pericytes and a specialized basement membrane [22]. Endothelial cells contribute to exchange (nutrients and O₂), repair and tissue growth (proteins, circulating cells). These physiological functions are diverted when tumor growth is more than 2 mm³ with development of a cancer-associated neovasculature. Endothelial cells function not only as static structural cells of perfusing vessels but also as active stromal regulators implicated in proliferation, invasion, secretion of inflammatory and growth mediators and metastatic spread [23].

Immune/Inflammatory Cells

TM contains higher densities of immune/inflammatory cells than normal tissues. They include lymphoid cells (CD8+ cytotoxic TLs/CTLs, CD45+ memory TLs, Th1, Th2, Tregs, Th17, TFH, NK/T cells, B cells and pDCs) and myeloid cells (TAMs, MDSCs, cDCs, neutrophils, mast cells and plaquets). Invasive margins of carcinomas comprise tertiary lymphoid structures (TLS) that coordinate specific Th1/CD8 and B cell responses [24]. These immune cells infiltrate malignant tissue via a network of inflammatory chemotactic cytokines and chemokines produced by cancer cells [25].

Lymphocytes play a critical role in tumor progression and metastasis. Whereas cytotoxic CD8+ and CD4+ Th1 T cells function as major anti-tumor immune effector cells, CD4+ CD25+ FoxP3+ Tregs play an important role in promoting tumor growth [26]. Tregs inhibit activation of both CD4+ and CD8+ T cells, which may serve to suppress anticancer cell immunity [27]. Th17 could antagonize and counter the tumor-suppressive IFN- γ producing CD4+ Th1 cells [28].

Dendritic cells (DCs) are a heterogeneous population of innate immune cells of bone marrow origin derived from myeloid precursors (conventional DCs, cDCs) and lymphoid

precursors (plasmacytoid DCs, pDCs) [29]. DCs are divided into three differentiation stages: precursor, immature, and mature DCs [30]. They are believed to be among the first cells migrating to the tumor site [31] through VEGF, HGF, β -defensin, CXCL12 and CXCL8 release by cancer and stroma cells. Immature DCs act as sentinels and after interaction with antigens, migrate in secondary lymphoid organs and become antigen-presenting cells (APCs) [32]. PDCs are located in blood and around high endothelial venules in T cell areas of lymphoid organs and have morphologic features similar to plasma cells [33, 34]. They regulate inflammation and constitute an important link between innate and adaptive immunity [35, 36].

Myeloid-derived suppressor cells (MDSCs) are immune cells with two distinct monocytic and granulocytic subsets [37]. They coordinate tumor progression, angiogenesis through releasing MMP-9 and VEGF and promote immune evasion by suppressing anti-tumor CTLs and NK cells. They can differentiate into TAMs and may be osteoclast progenitors [38].

Tumor-associated macrophages (TAMs) represent as much as 50 % of the tumour mass. These highly flexible multifunctional cells are characterized by plasticity and secretion of numerous immune/inflammatory modulatory cytokines. Most commonly activated macrophages are classified as M1 or M2 cells [39]. M1 are involved in response of Th1 cells to pathogens and are characterized by high capacity to present antigen, production of proinflammatory cytokines (IL-1, IL-12, TNF α , IFN- γ), generation of reactive oxygen species (ROS) and nitric oxide (NO) with ability to kill pathogens and cells. By contrast, M2 express an immunosuppressive phenotype initializing Th2 type responses through production of IL-10 and inhibition of CTLs. Moreover, M2 promote angiogenesis, ECM remodeling and repair. In the TM, TAMs have generally a M2 skewed phenotype. Recruitment of monocytes to the tumor is mainly driven by CCL2, a chemokine produced principally by CAFs, while macrophages differentiation and growth are regulated by several GFs, including CSF-1 and GM-CSF [40].

Immune/inflammatory cells present important functional, temporal and topographic plasticity. Immune cells promoting tumor growth associate Th2, MDSCs, M2, B lymphocytes and mastocytes, whereas immune cells inhibiting immune cells include CTLs, Th1, NK cells, DCs and M1. Tregs, Th17 and neutrophils have both promoting and inhibiting properties [41]. During tumor progression, pre-invasive microenvironment with anti-tumor properties comprises predominantly Th1 and M1 subpopulations with production of IL-12, IFN γ and inducible NOS. Transition to invasive carcinomas is marked by establishment of a pro-tumoral microenvironment with a shift from Th1 to Th2 and from M1 to M2, decrease of IFN γ and increase of IL-1, IL-6, indoleamine 2, 3-dioxygenase and VEGF. Topographically, immune cells are

not randomly located within tumors [42]. Th1 and cytotoxic memory T cells are found at the invasive margins and in the core of tumors. NK cells are dispersed within the stroma and at the invasive margins. Immature DCs are located in the tumor core whereas mature DCs infiltrate T cell zones where they are in close contact with CD4 + and CD8 + T cells. Follicular DCs, TAMs and T FH are in contact with B lymphocytes within B cell zones. B cells are located in TLS and at the invasive margins [43]. High densities of Th1/cytotoxic CD8+ T cells, memory CD45+ T cells, NK cells and B lymphocytes are correlated with good prognosis. Conversely, high levels of TAMs and Tregs are associated with poor prognosis in most malignant tumors. Th2 and Th17 lymphocytes densities are associated with variable prognosis.

Biophysical and Biochemical Stromal Remodeling

TM acquires new characteristics associating ECM stiffness, hypoxia, acidity, pro-oxidant and pro-inflammatory properties.

ECM stiffness controls motility strategies of cancer cells [44]. Normal ECM is a biopolymer fiber network that generates a mechanically stable support for epithelial cells. ECM is composed of proteins, proteoglycans and glycosaminoglycans. Mechanistic properties of ECM are dependent on collagen content (collagen I or IV), fiber thickness and extent of intrafibrillar cross-links, porousness and mesh size [45]. During tumor progression, ECM composition and structure change continuously. Increased matrix stiffness plays a role in regulating focal adhesion maturation, cell spreading, actin stress fiber formation and cell mobility. Interestingly, mechanical forces generated by myofibroblasts and pericytes remodel ECM stiffness and vascular niche respectively. This may contribute to cancer cell invasion via invadopodia in a fibronectin and cofilin dependent manner. In TM, decreased stiffness of ECM with large mesh sizes allows cancer cells to migrate in an amoeboid fashion [46]. Cancer cells using amoeboid-like invasion filter through gaps within ECM and move at relatively high velocities, thus resisting to drugs targeting proteases or integrins [47]. Alternately, an increased stiffness favors a mesenchymal motility with up-regulation of integrin-mediated contacts and focal adhesions and enhances the rate of migration by degrading fibers matrix with MMPs, serine proteases and cathepsins [48].

Insufficient blood supply leads to hypoxia and ischemia, which activate the dimeric hypoxia inducible transcription factors (HIFs). HIFs stabilization activates numerous genes modifying stromal and cancer cells phenotypes [49]. CAFs acquire profibrogenetic and proangiogenic properties. Cancer cells activate glycolytic metabolism, reverse acid-outside pH gradient, promote cell migration, induce survival pathways and acquire stemness properties [50]. Furthermore, HIF-1 and Met activation induces EMT and stimulates neoangiogenesis.

Recently, HIFs have been also associated with metastasis cascade through transcriptional activation of genes implicated in circulating tumor cells (CTCs) extravasation and metastatic niche development [51].

A reversed pH gradient with an increased intracellular pHi (>7.4) and a decreased extracellular pHe (<7.1) with acidification of the stroma is observed in invasive carcinomas. This biochemical response is the consequence of inefficient blood perfusion and tumour cells metabolic adaptation to hypoxia by enhancing glycolysis and increasing acid production [52]. In cancer cells, maintenance of intracellular alkalization is regulated by HIF-1 through up-regulation of plasma membrane ion pumps and carbonic anhydrases family (CA-IX and CA-XII) [53]. CAFs also contribute to TM acidification by using a CA-IX dependent mechanism [54]. Dysregulation of pH confers new aggressive properties to cancer cells: GFs-independent proliferation, resistance to apoptosis and anoikis, invasion, ECM remodeling, intravasation and metastatic spread through pH sensors activities [55]. High pHi is pivotal in enhancing cancer cell invasion by regulating dynamics of integrin-ECM attachments [56]. Low pHe allows redistribution of lysosomes to stromal cells periphery and secretion of Cathepsin B and MMPs by fibroblasts which promote EMT and invasion [57]. Acidic tumor microenvironment is also involved in regulation of inflammation, tumor immunity and angiogenesis [58].

ROS are produced by cancer and stromal cells and induce a pro-oxidant microenvironment through diffusion of ROS compound H₂O₂ [59, 60]. In TM, ROS modulate several factors and signaling pathways including HIF, CXCL12, PDGFR- β , TGF- β and caveolin, which drive onset of inflammation and promote fibroblasts to myofibroblasts transition [61]. In CAFs, ROS levels increase ECM remodeling, modulate tumor cells metabolism, activate invasion, neoangiogenesis and metastasis [62]. In cancer cells, high levels of ROS result from increased metabolism, cytokines and GFs secretion by stromal cells, mitochondrial DNA mutations, hypoxia and reduction of antioxidant defenses [63]. They are implicated in cancer cells proliferation, metabolic reprogramming, genetic instability, plasticity, stemness, escape from immune system and therapeutic resistance [64].

Cancer-associated inflammation (CAI) is a new hallmark of cancer implicated in genome instability, tumor cell growth, angiogenesis and metastatic spread. Many activated immune cells accumulate within TM and induce dysregulation of local homeostasis. Maintain of CAI is provided by a chemokine inducer initiated by simultaneous implication of NF- κ B and STAT3 and located in fibroblasts, macrophages, endothelial cells, normal epithelial and cancer cells [65]. This chemokine inducer is activated by molecules secreted by immune cells that act as NF- κ B stimulators (IL-17, TNF- α , norepinephrine) and STAT3 stimulators (IL-6, INF- γ). This activation

ultimately results in expression of genes coding for chemokines that amplify inflammation and tumor-related genes implicated in tumor progression.

Cancer Cells Reprogramming With Acquisition of Adaptive Strategies

Biochemical, biophysical and cellular components of TM act through numerous adaptive mechanisms employed by epithelial cancer cells to adjust their phenotype to unfavorable environmental conditions.

Epithelial mesenchymal plasticity (EMP) is a multistep morphogenetic and biological program situated centrally in mechanisms that govern tumor progression, metastatic spread and response to therapies. It seems to be a highly heterogeneous and variable dedifferentiation process with acquisition of mesenchymal traits and stem-like properties [66]. These events are highly contextual and driven by genetic/epigenetic mechanisms and microenvironmental factors. The most widely known plasticity processes are EMT and MET, which are fundamental reversible mechanisms [67]. EMT is characteristic of a bistability state with epithelial state genetically and environmentally determined during embryogenesis while mesenchymal state is almost determined by TM pathological signals [68]. Since seminal data published by Elizabeth Hay, evidence is accumulating that the same molecular mechanisms are implicated in cancer progression. In normal cells, EMT is a latent and inactivated epigenetic program while during cancer progression EMT is reactivated and allows cells to convert from an epithelial to a mesenchymal state with profound changes in cellular phenotype and molecular properties. Static epithelial cancer cells simultaneously shed their epithelial characteristics and gain mesenchymal traits and stem-like properties. Most importantly, acquisition of tumor-initiating and stem-like traits is crucial for successful completion of invasion-metastasis cascade characterized by dissemination of cancer cells and colonization of target organs via MET [69]. In fact, cancer cells may reside in an intermediate state in which they coexpress epithelial and mesenchymal traits (partial EMT). Invasive carcinomas are frequently composed of different subpopulations with epithelial, metastable and mesenchymal phenotypes. Cancer cells with metastable phenotype are implicated in local invasion and those with a fully and stable mesenchymal phenotype allow intravasation and anoikis resistance during dissemination and extravasation [70]. Moreover, recent phenotypic studies suggest that CSCs, cells undergoing EMT and metastatic cells are fundamentally the same in tumors. EMT markers have been found to be predictors of clinical outcome. CSCs and EMT biomarkers are found in CTCs, indicative of local and distant recurrence. Autocrine and paracrine signaling networks converge from nearby microenvironment to activate intracellular signaling factors that drive initiation, execution and maintenance of

EMT. External drivers are mainly represented by CAFs and TAMs. Intracellular pathways comprise TGF- β , Wnt, Notch and Shh [71]. EMT core regulators are transcription factors (TFs) included in three subgroups: (i) SNAIL1 and SNAIL2, (ii) ZEB1 and ZEB2 and (iii) Twist1, Twist2 and E12/E47. EMT effectors are subcellular structural proteins that define epithelial and mesenchymal identities of cells [72]. During EMT, TFs repress epithelial traits (E-cadherin, desmoplakin and claudins) and induce expression of a mesenchymal and migrating phenotype (vimentin, N-cadherin, CD44, β 6-integrin, fibronectin and MMPs). EMT irreversibility involves important epigenetic mechanisms implicating covalent modifications of DNA and histone proteins and dysregulations of non coding RNAs (ncRNAs). Crucial links have been recently identified between classical EMT-TFs that bind DNA and numerous co-activators and co-repressors implicated in epigenetic regulation of EMT [73]. While several EMT-TFs are implicated in a transient transcriptional repression of CDH1, a stable epigenetic silencing of E-cadherin is coordinated by histone-modifying enzymes belonging to the two polycomb repressive complexes (PRCs) PRC1 and PRC2 and the histone demethylase Lysine-specific demethylase1A (LSD1). In colon carcinomas, SNAIL1 is associated with CDH1 promoter and enables subsequent recruitment of the two chromatin-modifying enzymes EZH2 and SUZ12 belonging to the PRC2 complex to catalyze trimethylation of H3K27 in nearby nucleosomes and to silence CDH1 gene transcription. Interaction between SNAIL1 and LSD1 allows silencing of several important epithelial genes [74]. MiRNAs are implicated in regulation of EMT through binding to other regions of targeted mRNAs [75]. A repertoire of miRNAs maintains epithelial phenotypes by post-transcriptionally inhibiting mRNAs that encode EMT-TFs. Classic EMT-related TFs are transcriptional regulators of miRNAs. During EMT, members of the epithelial miR200 and miR205 families, which are physiologically implicated in suppression of TGF- β , NF- κ B, cell migration and resistance to anoikis, are frequently repressed by ZEB TFs [76]. Twist1 and ZEB1 respectively activate transcription of two pro-metastatic miRNAs, miR10b and miR21. Recently, long non-coding RNAs (lncRNAs) have been identified as a major class of ncRNAs longer than 200pb involved in epigenetic regulation of gene expression, maintenance of pluripotency and metastatic spread. Recent data have demonstrated that numerous lncRNAs are dysregulated in carcinomas. lncRNA *MALAT1* promotes EMT by activating Wnt signaling pathway, upregulating EMT-TFs ZEB1, ZEB2 and SNAIL2 and downregulating E-cadherin [77].

The concept of CSCs postulating that tumors are clonal and arise from a rare sub-population of dysregulated or mutated stem cells is increasingly accepted [78]. CSCs present ability to self-renew through asymmetric division, cause constant expansion of existing tumors and initiate new heterogeneous

tumors through metastatic cascade [79]. Their longevity in the stem cell niche could explain their malignant transformation via accumulating genetic and epigenetic alterations [80]. In fact, the stemness state of CSCs seems to be dynamic and flexible rather than a fixed state. The TM could play crucial roles via hypoxia in maintenance of CSCs properties, quiescence, activation of CSCs associated pathways and cell motility [81]. Origin of CSCs from normal embryonic or adult stem cells, more mature progenitors or differentiated epithelial cancer cells is still unresolved [82]. CSCs and EMP paradigms share striking parallels because EMT gives rise to cells with stem-like properties and CSCs exhibit an EMT gene expression profile. Physiologic stem cell niche normally orchestrates balance between self-renewal, cell proliferation and differentiation. Conversely, dysregulated CSC niche allows CSCs to initiate a complex epigenetic reprogramming during tumor progression with generation of heterogeneous subpopulations of tumorigenic CSCs and more differentiated non-tumorigenic cells [83]. Recent data have revealed that their epigenetic landscape is evocating of an embryonic rather than an adult stem origin with expression of Dnmt1 and HDAC1 rather than MLL family of histone methyltransferases. They exhibit dynamic epigenetic profiles enriched in euchromatin with low levels of DNA methylation, absence of bivalent mark H3K4me3/H3K27me3, high levels of activation mark H3K4me3 and repressive histone mark H3K27me3 at tumor suppressor genes [84]. CSCs regulation is driven by key regulatory and morphogenetic pathways (Wnt, Notch, BMP, Shh) through direct cell-cell contact (endothelial cells via Notch pathway in glioblastoma) or secreted signaling components (HGF) provided by stromal cells of the CSC niche [85]. Numerous biomarkers have been identified and render identity of CSCs a matter of debate [86]. Expression of these biomarkers is regulated by DNA methylation and histones modifications controlled by chromatin modifying enzymes, miRNAs and lncRNAs. CSCs typically resist to cancer therapies by having upregulated abundance of DNA repair enzymes, lower levels of ROS and dormant or slow-cycling properties [87].

Unfolded protein response (UPR) is a cellular stress response related to accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (ER). ER is a cytosolic membranous network implicated in synthesis, post-translational modifications and folding of proteins, lipid biogenesis and vesicular trafficking. Both extrinsic and intrinsic stressors implicated in development of a tumor microenvironment can induce ER stress, accumulation of misfolded proteins and activation of the UPR to restore ER proteostasis [88]. UPR has three aims: restoring normal function of cells by halting protein translation, degrading misfolded proteins and activating signaling pathways that lead to increasing production of molecular chaperones involved in protein

folding. After sequestration of unfolded proteins, ER stress initiates UPR through three proximal sensors (ATF6, IRE1 and PERK) to transmit stress signal to cytosol and nucleus [89]. Induction of UPR regulates epithelial cancer cells fate by using a paradoxical apoptotic or survival switch (death, transient dormancy or re-growth), depending of duration and severity of ER stress. UPR activation in endothelial cells promotes angiogenesis and metastatic spread [90].

Metabolic reprogramming occurs both in cancer and stromal cells [91]. In cancer cells, high levels of GFs or constitutively activated GF-receptors allow cells to double their biomass and then divide by increasing their nutrient uptake (glucose, glutamine) and adopting an anabolic metabolism resulting in amino acid, lipid and protein synthesis [92]. Compared to quiescent cells, oxidative phosphorylation-dependent production of ATP is rather a secondary event and metabolic changes are in fact a direct adaptive reaction to stimulating growth pathways via glycolysis and macromolecular production in mitochondria [93]. It has been recently observed that aggressive cancer cells use oxidative stress to extract nutrients from surrounding microenvironment in a symbiotic relationship. Oxidative stress in CAFs leads to induction of an autophagic program, via activation of HIF-1 α and NF κ B. During mitophagy, caveolin-1 destruction is performed by lysosomal degradation, leading to production of recycled nutrients to feed tumor cells. CAFs are forced to undergo aerobic glycolysis, and produce energy-rich nutrients, such as lactate and ketones, to feed cancer cells. To accomplish this metabolic coupling, CAFs export L-lactate and ketone bodies via MCT4 transporters. In turn, cancer cells import and recycle these fuels via MCT1 transporter to produce ATP. This stromal catabolism, via autophagy and mitophagy, fuels the anabolic growth of tumor cells, and allow tumor cells, which migrate with CAFs, to seed elsewhere by inducing oxidative stress. Conversely, CAFs can also obtain energy from cancer cells by using same mechanisms [94]. Connections between metabolism reprogramming and metastasis have been identified by revealing that the metastasis-suppressor KISS1 reverses Warburg effect [95]. Moreover, recent studies have developed the hypothesis that altered metabolism in tumor cells is not a passive reaction to damaged mitochondria, but rather an active oncogene-induced metabolic reprogramming [96]. Activation of PI3K/Akt/mTORC1, Myc and Kras mutations and loss of p53 function, reprogram cancer cells metabolism by increasing glucose and glutamine uptake, promoting mitochondrial biogenesis and regulating transcription and translation [97–100].

Autophagy is an adaptive catabolic pathway that protects cancer cells in response to microenvironmental stressors [101]. When tumor cells are exposed to hypoxia, nutrient deprivation, ECM detachment and ER stress, autophagy allows them to survive by degradation of organelles and proteins to provide alternative source of energy and metabolites

[102]. ER stress stimulates autophagy through PERK-eIF2 α and IRE1-JNK1 pathways and Ca²⁺ release [103]. Hypoxia activates autophagy through AMPK to inhibit mTOR and via inhibition of Bcl2 and activation of beclin1 and LC3 [104]. Conversely, if extrinsic or intrinsic stressors persist, excessive autophagy may lead to cell death by cellular self-degradation.

Extravesicular vesicles (EV) can be produced by cancer cells and stromal cells [105]. Stromal stressors like acidosis and hypoxia act by modulating EVs molecular content, secretion, trafficking and activity. EVs are implicated in tumour progression mechanisms, including ECM degradation, angiogenesis, escape to immune surveillance, metastatic spread and resistance to anti-cancer therapies [106]. Two subtypes of EVs have been identified: (i) exosomes which are intracellularly generated vesicles of size ranging from 30 to 100 nm and (ii) shed microvesicles, which comprise a heterogeneous population of vesicles larger than exosomes (>100 nm) generated by direct budding off from plasma membrane. EVs components associate transmembrane proteins, lipids, DNA, mRNA and ncRNAs [107]. They interact with various cells by using membrane receptors, membrane fusion or endocytosis and represent a protective signal for neighbor cells. EVs can enter the circulation and support formation of the pre-metastatic niche [108]. Exosomes components transmit oncogenic signals to cancer cells, normal epithelial cells and stromal cells. Oncogenic mutant EGFRvIII expressed by subpopulations of glioblastoma cells is associated with aggressiveness and transferred via exosomes to cancer cells lacking this mutant receptor. In colon carcinomas, exosomes from neoplastic cells expressing mutant K-ras are transmitted to cells expressing wild-type protein, leading to enhanced aggressiveness. Prostate cancer cells exosomes induce MET of mesenchymal stem cells (MSCs) [109]. Similarly, breast and ovarian cancer cells exosomes allow MSCs to acquire myofibroblasts properties [110]. Exosomes also participate to TM development by promoting neoangiogenesis via VEGF and by inducing a prometastatic inflammatory response via immune cells TLRs activation. They may suppress immune responses by promoting MDSCs differentiation, Tregs expansion and CTLs apoptosis. Interestingly, components exported in exosomes are also implicated in signaling pathways modulation. TGF- β receptors released by cancer cells exosomes induce activation of TGF- β signaling pathway in stromal and immune cells, promoting myofibroblastic reprogramming and immune suppression. Release of Wnt proteins, CD9 and CD82 tetraspanins and β -catenin by CAFs modulate Wnt signaling pathway in cancer cells and promote growth, survival and invasion [111]. Conversely, release of PTEN inhibits Notch-1 survival pathway and activates apoptosis in cancer cells.

Migration of cancer cells is an initial and crucial event in tumor progression and metastasis. Different types of cell migration are observed, including single cell migration (SCM) and collective cell migration (CCM) [112]. To move

and invade ECM in 2D or 3D, individual cancer cells need actin cytoskeleton remodeling (lamellipodia and filopodia) or edification of actin-based structures (invadopodia) respectively. Underlying process in both types of migration is rearrangement of cytoskeleton coupling with cell surface receptors that engage with surrounding microenvironment [113]. Locomotion in 2D requires a shift from a non-polarized into a polarized state to acquire a motile phenotype. This major rearrangement of cytoskeleton includes actin, tubulin, myosin and their binding proteins [114]. By using SCM, cancer cells invade locally ECM through two interconvertible motility modes. Mesenchymal mode is driven by Rac activation and defined by elongated cell morphology and strong interactions with ECM. Amoeboid mode is driven by Rho/ROCK activation and actomyosin contractility and characterized by round cell morphology and weak interactions with ECM [115]. Switch from one mode to the other, which characterized plasticity in cell invasive motility, is controlled by the balance between Rho/ROCK inhibition by Rac through its effector WAVE2 and Rac inhibition by Rho/ROCK through the GTPase ARHGAP22 [116]. While lamellipodia and filopodia are involved in 2D forward movement, invadopodia allow cancer cells locomotion in 3D by coupling actin polymerization with metalloproteases-induced ECM degradation. These actin-rich protrusions are required for penetration through the basement membrane, ECM invasion and intravasation [117]. Invadopodia are enriched in actin regulators, such as cortactin, cofilin, and fascin. Invadopodia formation and SCM in 3D result from three molecular steps: polarization via EGFR/Src/Cdc42 signaling with N-WASP activation, FAKs depletion with integrins-induced redistribution of Src in invadopodia and dissolution with tyrosine phosphorylation of cortactin [118]. However, because lamellipodia, filopodia and invadopodia are similar in composition, these structures interconvert and could form an “invadosome” [119]. Conversely, CCM is predominantly implicated in metastatic spread and endocytic pathways are pivotal in coordinating activation of Rac and actin dynamics [120]. Recent studies identified Rab5 and Rab11 as key players of collective migration by acting through control of Moesin in spatial regulation of Rac activity and polarization [121]. Moesin plays a critical role in organizing epithelial properties of clusters by unifying cortex of different cells, upregulating Rac activity in front cells while inhibiting Rac in other cells and generating invasion force in one direction [122]. Moreover, it has been recently demonstrated that Rb1 inhibits CCM and intravasation of SCM and clusters. This mechanism depends on CD44, whose expression is activated by Rb1 suppression. Low levels of Rb1 and overexpression of CD44 are observed in aggressive triple negative breast carcinomas and are associated with partial EMT, CCM, lymphovascular invasion and metastatic progression [123].

De novo angiogenesis and vasculogenesis are crucial adaptive events for tumor growth beyond 1–2 mm³ [124] and

involve several cellular events including proliferation, migration, vascular transdifferentiation and ECM degradation [125]. Angiogenesis results from a balance between activating and inhibitory factors. During tumor growth, an imbalance between pro- and anti-angiogenic signals, the angiogenic switch, promotes proliferation of endothelial cells, migration and rearrangement in neovessels [37]. These vessels are dilated, irregular, permeable (loss of V-cadherin) and disorganized. They are devoid of pericytes and smooth muscle cells. Neoangiogenesis occurs through several forms from preexisting endothelial cells or progenitors. Sprouting angiogenesis is developed from existing vessels. Intussusception is the result of lumen invagination and splitting of vessels. Vessel co-option is characterized by enfolding of vessels by cancer cells. Lymphangiogenesis is the development of lymphatic vessels from pre-existing ones [126]. Neovasculogenesis is an angiogenesis-independent morphomolecular entity termed vasculogenic mimicry (VM). Morphologically, these intratumoral channels are lined externally by tumor cells and lack an inner lining of endothelial cells [127, 128]. Erythrocytes and plasma are able to flow in these vessel-like structures [129]. VMs are connected with host vessels for blood supply. They have been observed in numerous epithelial and non-epithelial malignant tumors [130–138]. VMs allow tumor cells to express locally a vascular phenotype [139] with endothelial properties [140, 141]. CSCs may be involved in this transdifferentiation process via EMT [142–144].

To intravasate tumor cells need to invade through local tissue to move to neovessels. These vessels have weak cell–cell junctions through which tumor cells can enter vasculature [145]. Macrophages can attract cancer cells toward blood vessels by secreting EGF. They can also induce retraction of endothelial junctions and TEM by secreting TNF α [146]. Tumor cells can then enter the circulation by transmigrating either paracellularly through endothelial cell junctions or transcellularly through endothelial cells body [147]. MMP-1 seems to be required for paracellular intravasation while ADAM12 leads to disruption of endothelial junctions [148]. Tumor cells also use Notch receptors to bind to Notch ligands on endothelial cells and thereby transmigrate through endothelial junctions. TGF- β and VEGF reduce the local endothelial barrier function and increase the number of cancer cells entering into blood circulation. During transcellular intravasation, Ca²⁺–calmodulin complex located in endothelial cells activates myosin light chain kinase at the site of cancer cells attachment, which leads to light chain myosin phosphorylation and actomyosin contraction. Finally, this rapid cytoskeletal and membrane remodeling creates a transitory pore-like structure for cancer cells to cross endothelial cells [149].

Reciprocal Interactions Between Cancer Cells and Stromal Cells

Cancer cells may induce ECM remodeling, neoangiogenesis, metabolic reprogramming of CAFs and immune tolerance.

ECM is degraded and disrupted by tumor cells. This remodeling contributes to cancer cells proliferation by releasing mitogenic factors and to cells dissemination by dissociating their interactions with ECM [150]. Moreover, they increase ECM rigidity by expressing high levels of LOX, an enzyme involved in crosslinking of collagen fibrils and by stimulating EMT, angiogenesis and inflammation [151].

Neoangiogenesis is induced by cancer cells by secreting VEGF, FGF, CXCL8 and PlGF or by taking over the role of thrombocytes, attracting immune cells and transforming stellate cells to highly proliferating CAFs [152]. VEGF secreted by tumor cells leads to development of new irregular and disorganized vessels, facilitating their dissemination. Chemotactic molecules produced by cancer cells induce infiltration of inflammatory cells which secrete factors promoting tumor growth, angiogenesis and immune tolerance [153, 154].

A hypoxic microenvironment is created by tumor cells. Lactate and ROS accumulation stimulates protumoral activities of CAFs. Once activated, these stromal cells have crucial functions in reprogramming cancer cells toward a respiratory metabolism, promoting EMT and CSCs properties, inducing motility and interacting with CTCs [155].

Immunosuppression is developed by cancer cells to escape immune system via reduction of MHC1 molecules and by stimulating lymphangiogenesis that allows local infiltration of immunosuppressive cells. They also promote development of an immune tolerance by producing PDGF and Shh that promote fibroblasts reprogramming to immunosuppressive CAFs [156].

Stromal and immune/inflammatory cells may also increase proliferation and survival of cancer cells, evade growth suppressors, stimulate metabolic activity and angiogenesis, induce immune tolerance and promote invasion and metastasis.

Tumor proliferation is stimulated by endothelial cells via production of GFs and neoangiogenesis development. Immune/inflammatory cells promote tumor growth by inhibiting oncogene-induced senescence and induce production of GFs and proteolytic enzymes that release mitogenic agents normally sequestered in ECM [37].

Neoangiogenesis is induced by CAFs via production of chemokines (CXCL8, CXCL12), MMP-1, -3, -7, -9, -13, -14, VEGF and COX-2 [157, 158]. MDSCs secrete proangiogenic chemokines by stimulating ERK1/2 signaling pathway [159].

Fibroblasts reprogramming to CAFs is associated with loss of their oncosuppressive functions and acquisition

of procarcinogen properties (secretion of proteases and paracrine GFs) that alter epithelial architecture. Immune/inflammatory cells secrete proteolytic enzymes that cleave oncosuppressive adhesion complexes regulating epithelial homeostasis [160].

Tumor survival and reduced apoptosis are promoted by an angiogenic switch. CAFs secrete paracrine survival factors IGF-1 and IGF-2. Myeloid cells promote infiltration of precursors and vascular growth by secreting CXCL12, VEGF and MMPs. TAMs and CAAs provide survival signals to cancer cells and promote resistance to cell death induced by loss of cellular interactions [161]. During progression of breast carcinomas, juxtacrine interaction between $\alpha 4$ integrin-expressing TAMs and VCAM-1-expressing cancer cells activates PI3K/Akt and prevents apoptosis of circulating tumor cells [162].

ROS-induced activation of glycolysis by CAFs provides lactate and pyruvate which are sources of metabolites for tumor growth. CAAs produce fatty acids reused by cancer cells to generate ATP by generating mitochondrial oxidation. Hypoxia and HIF-1 induced vascular insufficiency stimulates aerobic glycolysis enabling cancer cells to proliferate [163].

Immunosuppression is induced by pericytes, immune cells and cytokines. Immunosuppressive cells Tregs, Th17, DCregs, MDSCs, M2, neutrophils and mast cells inhibit proliferation and infiltration of T cells [164]. Immunosuppressive molecules include cytokines IL-1 β , IL-4, IL-6, IL-10, TGF- β and PGE2. Lack of permissive HEVs also favors immunosuppression by preventing tumor infiltration by CTLs. CAFs suppress antitumor cytotoxic activity of CTLs and NK/T cells by secreting TGF- β .

Neoangiogenesis is orchestrated by CAFs via production of proangiogenic proteins VEGF, FGF2, IL-8/CXCL8 and PDGF, ECM proteins, enzymes and chemoattractants for proangiogenic macrophages, and by neutrophils and other myeloid cells [165].

Invasion is stimulated by hypoxia and HIF-1 overexpression via VEGF synthesis in tumor cells, loss of tight junctions in endothelial cells and reduced number of pericytes [166]. TAMs promote invasion by inducing paracrine loops with tumor cells and by secreting proteases which reshape ECM and synthesize fragments with proinvasive properties. Cleavage of laminin-5 $\gamma 2$ chain mimics EGFR ligand which is a potent inducer of cell migration. They stimulate invasion via secretion of RANKL which reduces Maspin activity, an inhibitor of serine proteases. They can also combine with cancer cells and create hybrid cells which have acquired migratory properties. CAFs promote invasion by secreting HGF, promoting TGF- β -induced EMT and producing ECM proteins and enzymes [167].

Interactions Between CTCs and Their Intravascular/Circulatory Microenvironment

CTCs

The concept of intravascular dissemination of cancer cells is widely accepted as a central paradigm. The literature on metastases is one of the most abundant, starting in the middle of 19th century and termed in french “diathèse cancéreuse” by Sanson in 1834. Presence of isolated tumor cells in blood was firstly described by Ashworth in the last century (1869) [168]. Working on this phenomenon, Ryall introduced the term cancer infection in 1908 [169]. In 1960s and 1970s many reports on cytological demonstration of tumor cells in peripheral blood were published [170–173]. Actually, according to the path followed by tumor cells to generate metastases, the terms circulating tumor cells (CTCs) and circulating tumor microemboli (CTMs) are defined as tumor cells originating from either primary or secondary metastatic sites and circulating freely in the peripheral blood and lymphatic vessels [174]. They have been detected during progression of most epithelial tumors, including breast, prostate, ovary, lung, colorectal and kidney carcinomas. CTCs may constitute seeds for subsequent growth of other distant metastasis, with ability of self-seeding back to primary tumor and thus increasing their aggressiveness [175]. CTCs can settle in other organs such as bone marrow, a point at which they are termed DTCs and serve as a reservoir of tumor cells responsible for future recurrences [176, 177]. CTCs represent a heterogeneous population of tumor cells with the potential of forming metastases. They have been approximated to average 1 CTC for every 1 billion peripheral blood cells, emphasizing the challenges in their isolation and analysis [178]. From experimental models, it has been estimated that around 1 million cells per 1 g of tumor tissue can be introduced daily into the bloodstream. In animal models, among tumor cells directly introduced into systemic circulation, only 1 CTC in 40 was able to establish micrometastases, and approximately 1 in 100 micrometastases give rise to macrometastasis. Metastasis inefficiency is mainly the effect of anoikis and explains low survival rates of CTCs in vessels after leaving the tumor mass. CTCs are collected with the goal of understanding their role in the metastatic cascade and for their utility in disease monitoring as well as assessing prognosis and therapeutic response [179]. CTCs can provide a repeatable, non-invasive approach which provides a real-time insight into a patient's tumor history. However, the interpatient and inpatient variations suggest a greater underlying complexity in CTCs, including heterogeneous subpopulations and progressive genomic aberrations typical in later-stage or metastatic disease. If EMT induces mesenchymal features and directs epithelial cells to a more stem-like state, it means that invasive capacities reside in CSCs [83]. Several reports give more evidence that among

primary tumor cells and CTCs, there are subpopulations of cancer cells with CSC-like characteristics more suitable to colonize distant organs. In accordance, majority of breast DTCs in bone marrow show a CSC phenotype based on $CD44^{high}CD24^{-/low}$ expression [180]. Consistently, $CD44^{+}CD24^{-/low}$ breast CSCs display an invasiveness gene signature which correlated with poor overall and decreased metastasis-free survival [181]. Moreover, most CTCs express epithelial cell markers such as epithelial cell adhesion molecule (EpCam) and cytokeratins (CKs) [182], meaning that EMT is not a crucial requirement for invasion. Although CTCs do not necessarily need to be in a mesenchymal state, a recent study indicates that passing through an EMT significantly increases metastatic potential of DTCs [183]. Hermann and colleagues found that human pancreatic cancer tissue contains highly tumorigenic CD133+ CSCs, compared to CD133- pancreatic cancer cells [184, 185]. Direct and indirect methods have been developed to detect, isolate, and characterize CTCs in different tumors. Traditionally, CTCs can be recognized by microscopic examination using cytopathologic criteria of malignancy, and modified May-Grünwald-Giemsa stain, already used in routine conventional cytology [186]. Classical criteria associate nucleo-cytoplasmic ratio, anisonucleosis, nuclear membrane irregularity, nucleus size, nuclear hyperchromasia and presence of three dimensional sheets. CTCs are defined by the presence of at least four of the following criteria: [1] anisonucleosis (ratio >0.5), [2] nuclei larger than three calibrated pore sizes of the membrane (8 μ m) (i.e. >24 μ m), [3] irregular nuclei, [4] high nucleo-cytoplasmic ratio, and [5] presence of three-dimensional sheets. Cells are defined as circulating uncertain malignant cells when they have fewer than two criteria. Circulating benign cells are characterized by absence of criteria [187]. Efficacy of other methods is attributed to use of epithelial, mesenchymal and/or stem cells antibody cocktails designed for CTCs enrichment and feasibility of performing postcapture molecular cytogenetic analysis. However, CTCs genomic analysis is still in its early days, mainly due to a lack of technologies capable of isolating sufficient number of cells and lack of suitable material with which to compare results due to CTCs heterogeneity [188, 189].

Intravascular/Circulatory Microenvironment

CTCs have to transit within a stressful intravascular microenvironment. Cancer cells are subjected to hemodynamic forces, immunological stress and collisions with host cells, such as blood cells and endothelial cells lining vessel walls. All of these stresses could affect cell survival and ability to establish metastatic foci. Only CTCs that overcome or even exploit effects of fluid shear and immunosurveillance will adhere to vascular endothelium of distant organs, exit circulation and successfully enter foreign tissues.

Trajectory of CTCs is influenced by physical and mechanical parameters: pattern of blood flow, diameter of blood vessels and complex interplay between shear flow and intercellular adhesion that leads to arrest in larger vessels [190]. Shear flow influences translational and rotational motion of CTCs and hence determines orientation and time constant associated with receptor ligand interactions that lead to adhesion. It may also induce deformation of CTCs and margination towards vessel walls (Fig. 1).

Three subtypes of intravascular leucocytes belonging to the circulatory microenvironment interact with CTCs:

Neutrophils are the predominant circulating granulocytes in humans [191]. Clinical and experimental studies suggest that elevated numbers of circulating neutrophils are an independent marker of adverse prognosis in cancer [192–194]. Lipopolysaccharide-induced systemic inflammation results in increased CTCs adhesion within hepatic sinusoids. Conversely, neutrophils depletion is associated with a decrease

in overall gross hepatic metastasis formation and this phenomenon is reversed by neutrophils reinfusion. In lung carcinomas, neutrophils have been shown to stabilize adhesion of CTCs to pulmonary microvasculature and to facilitate gross pulmonary metastasis formation [195, 196]. Three main mechanisms by which neutrophils may act to promote development of metastasis have been proposed: (i) contact-dependent mechanism, whereby neutrophils act as bridges, tethering CTCs to end-organ endothelium [197]; This interaction is mediated by interaction between β_2 integrins on neutrophils and ICAM-1 on CTCs [198–201], (ii) contact-independent mechanism, where neutrophils secrete soluble factors activating endothelium and enhancing CTCs adhesion in distant sites [202–204], and (iii) development of neutrophil extracellular traps (NETs), composed of extruded DNA decorated with antimicrobial proteins [205]. NETs are formed in response to infectious stimuli and serve as a host defense against pathogens [206].

Fig. 1 Reciprocal interactions between stromal and cancer cells at primary site. Induction of a TM is driven by genetic instability of cancer cells, extrinsic stress stimuli; (hypoxia, aberrant microcirculation, acidosis, glucose deprivation, oxidative stress, pH changes) and intrinsic stressors (imbalanced cell growth, increased mutation rate, errors in

glycoprotein and lipid biosynthesis and decreased amino acid supplies). Cancer cells develop adaptive strategies to overcome specific microenvironmental growth constraints. Stromal cells reprogramming, ECM remodeling and acquisition of an aggressive phenotype by cancer cells are crucial events in promoting intravasation, dissemination and metastasis

In the context of severe infection, recent data have demonstrated that NETs are able to trap CTCs, thus promoting early adhesion of tumor cells to distant organ sites [207, 208]. These findings implicate NETs in metastasis progression and identify NETs as potential therapeutic targets [209].

Host circulating platelets are frequently aggregated with CTCs. Tumor cell-induced platelet aggregation (TCIPA) contributes significantly to hematogenous metastasis. Recent data have demonstrated that metastatic MCF-7 human breast cancer cells induced dose-dependent aggregation of washed platelets. In this study, four major platelet activation pathways, glycoprotein GP-Ib-IX, GPIIb/IIIa, thromboxane-A2 and adenosine diphosphate were activated during TCIPA [210]. Antiplatelet drugs may represent a promising strategy to prevent cancer metastasis. Egan and colleagues have recently described the “platelet cloak phenomenon” where platelets directly adhere to cancer cells [211]. Gene expression profiling of cancer cells exposed to platelets revealed an EMT-like phenotype in tumor cells, with decreased expression of E-cadherin and increased expression of serpine-1, Snail, Slug and vimentin, providing clear evidence that platelets can induce a more invasive mesenchymal-like phenotype in CTCs [212].

MDSCs facilitate tumor growth and metastasis. Systemic immune microenvironment could have direct interactions with CTCs to exert an immunosuppressive effect while they are in transit, thus allowing CTCs to escape immunosurveillance. Immune cells might also form cluster interactions with MDSCs to prevent immune attack and destruction by systemic shear stress. Furthermore, CTCs/immune cell clusters extravasation from circulation could induce CTCs migration across blood vessel barriers [213].

Extravasation

Extravasation refers to a process involving CTCs that exit circulation and enter into distant organs. Physical occlusion and cell adhesion are two mechanisms of CTCs arrest. Physical occlusion by mechanical trapping occurs when CTCs enter vessels whose diameters are less than CTCs. As CTCs of epithelial origin are typically $>10\ \mu\text{m}$ in size, physical occlusion occurs in capillaries or small vessels of $<10\ \mu\text{m}$. In contrast, CTCs with a diameter less than diameters of blood vessel wall will follow a trajectory determined by local flow pattern and collisions with host cells and vessel walls. The second type of arrest is the consequence of CTCs extravasation out of circulation and homing to endothelial surface receptors using integrins, which help to mediate cell-ECM and cell-cell adhesions. Integrins on CTCs bind to fibrinogen present in ECM [147] and target basement membrane receptors, allowing CTCs to pass through gap junctions in the endothelium [214]. In lung metastasis, $\alpha3\beta1$ integrins expressed on CTCs bind to laminin-5 located on the vascular

basement membrane. CTCs that have already formed microthrombi will be encapsulated in platelets, thus restricting presentation of receptors and slightly altering CTCs properties. Platelets strongly bind to the surface of injured blood vessels which have exposed basement membranes [215]. In this way platelets could act as homing signals for CTCs by finding gaps in the epithelium. After arrest, the next step is characterized by transendothelial migration of CTCs, which happen in a variety of ways. Rapidly dividing mass of cells can mechanically burst vessel through application of pressure more than walls can handle [216]. Cancer cells break through endothelial cell junctions by promoting VEGF-induced signaling pathways. Endothelial cells respond to VEGF by activating Src kinases, resulting in cell junction disruption. This disruption is pivotal for TEM, which allows CTCs to squeeze through and enter target tissues [217]. Once these cell junctions are weakened, CTCs induce endothelial retraction through structural rearrangements of cytoskeleton. CTCs then push between endothelial cells in a process called intercalation. Among important factors implicated in TEM, Rho family of GTPases regulates cytoskeleton and turnover of cell-cell and cell-ECM adhesion, resulting in control of actin turnover and polymerization for intercalation. Adenine nucleotides released by tumor cell-activated platelets induce opening of endothelial barrier, which allow transendothelial migration of CTCs [218].

Interactions Between Extravasated CTCs and Their Foreign Microenvironment at the Metastatic Site

Pre-Metastatic Niche

The term pre-metastatic niche has been coined to describe microenvironmental conditions required for CTCs survival and outgrowth at the metastatic site [219]. At the primary site, cancer cells orchestrate development of pre-metastatic niches that promote mobilization and recruitment of bone marrow-derived dendritic cells (BMDCs) to future metastatic sites. In foreign tissues, CTCs encounter either an endogenous environment or a pre-metastatic niche induced by the primary tumor. Processes implicated in induction of pre-metastatic niches associate hypoxia, ECM and vasculature remodeling, exosomes production and expression of signaling molecules [220]. Their development is also dependent of recruitment of BMDCs, endothelial cells, inflammatory cells and fibroblasts [221]. Deposition of fibronectin produced by CAFs has been implicated in pre-metastatic niche initiation [222]. LOX and LOX-like proteins are additionally actively involved in pre-metastatic niche development due to their ability to cross-link collagen and elastin [223]. Recent study indicates that

angiopoietin 2, MMP-3 and -10 combine synergistically to increase vascular permeability in lung and disrupt vascular integrity [224]. Mobilization of hematopoietic progenitor cells that express VEGFR-1 happens early during formation of pre-metastatic niche. These cells express $\alpha 4\beta 1$ integrin (VLA-4), a surface receptor for fibronectin, which mediates their recruitment to fibronectin-rich microenvironments [225]. They contribute to ECM remodeling through secretion of MMP-9 [226] and to the niche vasculature development through recruitment of VEGFR2⁺ circulating endothelial progenitor cells and CD11b⁺Mac1⁺ myeloid cells. LOX activity at these sites cross-links collagen IV, providing a substrate to which the CD11b⁺ cells adhere and remodel. Production of MMP-2 by the CD11b⁺ cells cleaves collagen IV, releasing chemoattractive collagen IV peptides. Collectively, these events attract further CD11b⁺ cells and BMDCs and recruit metastatic cells. Inflammatory cells that drive primary tumor growth are also important components of the pre-metastatic niche, including TAMs, Tregs, neutrophils and MDSCs [227]. S100A8 and S100A9 of the S100 proteins family are implicated in MDSCs recruitment and development of an inflammatory microenvironment at the primary site and pre-metastatic niches [219]. Moreover, TGF- β family is not only a master regulator of EMT and metastasis, but may play a crucial role in the regulation of metastatic niche as well [228].

Homing and Dormancy

Homing of cancer cells to distant organs are complex multi-step processes associating survival in the circulation, initial arrest, extravasation, seeding, establishment of a supportive niche and growth into micrometastasis and macrometastasis [229, 230]. According to the widely accepted “seed and soil” hypothesis suggested by Steven Paget (1889), metastatic cancer cells (seeds) home to at a distant site and form metastases if it provides a favorable microenvironment (soil) [231, 232]. Tumor cells initially proliferate in blood vessels and then invade or extravasate as single cells and then infiltrate foreign tissues [233, 234]. Once extravasated, cancer cells may experience a period of dormancy while they adapt to their secondary microenvironment. Numerous mechanisms, including cross-talk between tumor cells and their microenvironment, angiogenesis, immunosurveillance, ECM molecules, GFs and cytokines have been proposed to explain how cancer cells survive and remain in dormancy [235, 236]. During dormancy, cancer cells may undergo very slow proliferation, a balanced turnover due to equal rates of cell deaths and proliferation or cellular quiescence [237]. Exit from dormancy and development of metastases may result from accumulation of tumor mass that eventually exceeds detection limit, onset of successful angiogenesis, evasion from immunosurveillance and interactions with stromal cells [238].

Micrometastasis and Macrometastasis

Probability for both CTCs and DTCs to become clinically relevant metastases is low because most CTCs fail to seed productively in a distant site and many DTCs remain dormant for several years [239]. Dormant cells may progress to micrometastatic deposits where their size is kept in check because of a balance in proliferation, apoptosis and phagocytosis by the host-tissue immune system. Dormancy of DTCs may also be linked with their EMT state, as previous studies showed reduced proliferation or quiescence of cells undergoing EMT [240]. Recent data have shown that DTCs require MET to resume proliferation and colonize foreign tissues [241, 242]. The new microenvironment may express factors such as versican, which drives miR-200 expression by tumour cells to repress E-cadherin repressor genes and hence permit MET and E-cadherin re-expression [243, 244]. Thus, genes like cyclin D1 and D2 that are directly repressed by SNAIL and ZEB2 may be reactivated, restoring proliferation and tumour growth. To develop into a macrometastasis, cancer cells must recruit an adequate blood supply which is necessary for growth beyond 1 to 2 mm. Factors secreted from activated platelets can induce angiogenesis and stimulate growth at the metastatic site. It has also come to light that reduced immune surveillance is a novel mechanism through which primary tumors create favorable niches in secondary organs [245].

Tumor Self-Seeding

CTCs can colonize their tumors of origin, in a process that Kim and colleagues called “tumor self-seeding” [246]. This study suggested that development of a more aggressive metastatic progeny requires ability of primary tumors to attract their own CTCs back. Moreover, molecular profiling of isolated CTCs showed that these selected cells have transcriptional signatures resembling those of metastatic subpopulations, thus suggesting that re-seeding might prime CTCs to acquire these signatures [247]. However, self-seeding and presence of a tumor mass may not be a *sine qua non* requisite because metastasis develops in patients after undergoing surgery (Fig. 2).

Biomarkers and Targeted Therapies

Although several molecular pathways are common to oxidative stress, hypoxia, EMT, metabolic switch and resistance to therapies, a combined strategy seems preferable, considering the too large number of cancer cells adaptive mechanisms. Thus, immunomonitoring and genetic/epigenetic characterization of CTCs seem of paramount importance in understanding such a complex morpho-molecular network. Actually, the most promising pharmacological approach could associate

Fig. 2 Reciprocal interactions between cancer cells and intravascular and metastatic microenvironments. Establishment of metastasis at secondary sites requires several steps: (1) survival of CTCs in the intravascular microenvironment via interactions with circulating MDSCs, neutrophils

and platelets, (2) initial arrest of CTCs by physical occlusion, (3) extravasation by TEM, (4) MET, establishment of a supportive premetastatic and metastatic niche, dormancy, (5) development of micro and macrometastasis and (6) self-seeding

conventional therapies combined with immune checkpoint inhibitors and targeted inhibitors of epigenetic reprogramming of plasticity, hypoxia and de novo angiogenesis [248]. Finally, xenograft models may provide a consistent and producible platform for investigating mechanism of interactions between tumor cells and their different subtypes of microenvironment.

Conclusion

Recent data have revealed new unexpected levels of microenvironmental and epigenetic/genetic tumor heterogeneity induced by complex interactions between stromal cells and cancer cells. In addition to clonal selection and reversible transcriptional reprogramming, intratumoral cooperation at the primary site between subpopulations of cancer cells with high and low aggressiveness, may explain preservation of heterogeneity in metastases. Thus, a better understanding of molecular and cellular mechanisms implicated in reciprocal cooperation between cancer cells and their primary, intravascular and metastatic microenvironments is mandatory to identify pertinent biomarkers and to develop new targeted therapies.

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