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Review

Metastasis: cell-autonomous mechanisms versus contributions by the tumor microenvironment

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Abstract. The fatality of cancer predominantly results from the dissemination of primary tumor cells to distant sites and the subsequent formation of metastases. During tumor progression, some of the primary tumor cells as well as the tumor microenvironment undergo characteristic molecular changes, which are essential for the metastatic dissemination of tumor cells. In this review, we will discuss recent insights into pro-metastatic events occurring in tumor cells themselves and in the tumor stroma. Tumor cell-intrinsic alterations include the loss of cell polarity and alterations in cell-cell and cell-matrix

adhesion as well as deregulated receptor kinase signaling, which together support detachment, migration and invasion of tumor cells. On the other hand, the tumor stroma, including endothelial cells, fibroblasts and cells of the immune system, is engaged in an active molecular crosstalk within the tumor microenvironment. Subsequent activation of blood vessel and lymph vessel angiogenesis together with inflammatory and immune-suppressive responses further promotes cancer cell migration and invasion, as well as initiation of the metastatic process.

Key words: Angiogenesis; lymphangiogenesis; metastasis; tumorigenesis; tumor stroma.

Introduction

Carcinomas are the most frequent type of human malignancies, and the vast majority of cancer deaths are caused by the formation of metastases rather than by the primary tumor itself. Since existing metastases are difficult to target by conventional cancer therapies, a curative regimen requires the detection of the disease before the primary tumor has spread. Yet, despite evolving clinical diagnostic tools, a large proportion of tumors have already metastasized by the time of diagnosis.

The selective process of metastasis requires that cancer cells successfully complete several sequential, rate-limiting steps. They must detach from the primary tumor, invade the host stroma, intravasate into lymphatic or blood vessels, spread to the capillary bed of distant organs, ex-

travasate and proliferate in the receptive organ parenchyma [1]. The succeeding metastatic cells have undergone changes in their proliferative, survival, migratory and invasive abilities and can be seen as winners of a ‘metastasis decathlon’ [2]. It is now well established that tumor cell-autonomous changes are not sufficient to allow tumor progression and metastasis to occur. By analogy with the architecture of organs, tumors are not only composed of a ‘parenchyma’ formed by the neoplastic cells, but also of a supportive ‘stroma’, consisting of specific extra-cellular matrix (ECM) components, fibroblasts, adipocytes, vascular cells, smooth muscle cells and cells of the haematopoietic system [3]. During tumor progression and metastasis, an active crosstalk occurs between tumor cells and their stroma, mainly mediated by direct cell-cell contact or paracrine cytokine and growth factor signaling, reminiscent of the communication between epithelial and mesenchymal cells during embryonic development.

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Such signaling may activate the tumor microenvironment at the primary and secondary tumor sites, thereby undergoing morphological changes (desmoplasia) and allowing or even supporting tumor outgrowth, invasion and metastasis [4]. Hence, tumor cell-stroma interaction is an important new focus of research in the treatment of metastatic disease.

Many excellent reviews cover particular aspects of molecular pathways known to be involved in the metastatic process. This review more broadly summarizes recent progress in the elucidation of various pro-metastatic molecular events and pathways occurring during tumor progression. In particular, we emphasize the distinction between tumor cell and tumor stroma contribution, and we discuss the role of tumor cell polarity, adhesion and deregulated receptor kinase signaling, as well as endothelial cells, fibroblasts and immune cells in invasion and metastatic dissemination of tumor cells.

Tumor cell-intrinsic changes

Changes in cell-cell and cell-matrix adhesion

Loss of E-cadherin function and epithelial-mesenchymal transition

E-cadherin is a central player in the makeup of cell polarity and epithelial organization. With its extracellular domains, this cadherin mediates calcium-dependent homophilic cell-cell contact in adhesion junctions, while linking adhesion junctions to the actin cytoskeleton via a cytoplasmic cell adhesion complex consisting of α -, β -, γ - and p120 catenins [5]. Thereby cell-cell adhesion can affect localization and function of cytoskeletal regulators and influence actin-guided cell motility [6]. In most cancers of epithelial origin, E-cadherin-mediated cell-cell adhesion is lost concomitantly with tumor progression and is correlated with advanced tumor grades and poor patient survival [7]. Consistent with this observation, forced downregulation of E-cadherin function in a mouse model of carcinogenesis promotes tumor progression, invasion and metastasis [8]. These and many more results from a variety of *in vivo* and *in vitro* experimental systems demonstrate that E-cadherin is an important molecule in tumor progression [5].

How does the loss of E-cadherin promote metastasis? In order to leave the coherent epithelial cell assembly of the primary tumor, malignant tumor cells have to acquire a fibroblastoid, migratory and invasive phenotype. A similar process physiologically occurs during embryonic development, tissue remodeling, wound healing and inflammation and is termed epithelial-to-mesenchymal transition (EMT) [9]. A number of *in vitro* studies (see below) collectively support the idea that morphologic and molecular changes of tumor cells required for the first

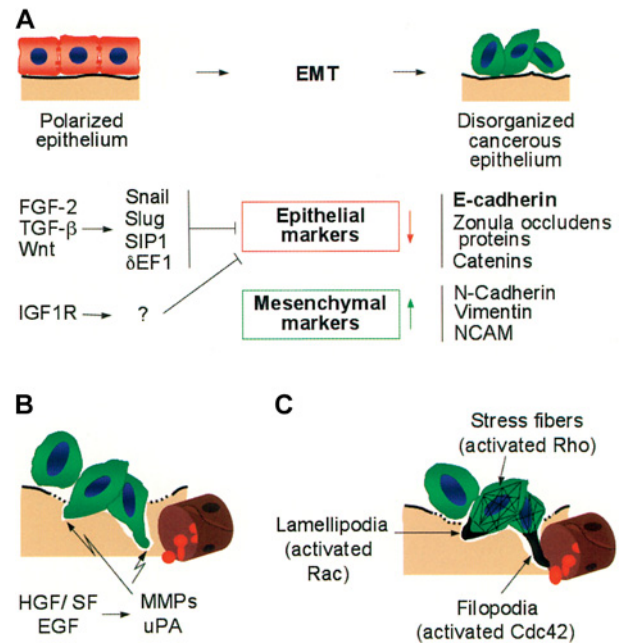


Figure 1. Mechanisms of tumor invasion and metastatic dissemination. During scattering and epithelial-to-mesenchymal transition (EMT), cells undergo major changes in morphology and lose cell-cell contacts. (A) EMT of tumor cells. Loss of E-cadherin leads to disruption of adhesion junctions and is essential for EMT. TGF- β , FGF-2 and Wnt induce or stabilize Snail1, Snail2, δ EF1 and SIP1, all direct or indirect transcriptional repressors of E-cadherin. Concomitant with the loss of E-cadherin function, the expression of mesenchymal marker proteins is induced. (B) Degradation of basement membrane (BM) and extra-cellular matrix (ECM). HGF/SF and EGF activate fibroblasts and endothelial cells to secrete matrix metalloproteases and urokinase-type plasminogen activator. The BM and ECM are thereby degraded, allowing tumor cells to transigrate into the subepithelial ECM and to finally intravasate into blood or lymphatic vessels. (C) Activation of the tumor cell actin cytoskeleton, migration and invasion of metastatic cells into blood vessels. Upon loss of cell polarity, activated Rac, Cdc42 and RhoA are no longer sequestered at adhesion junctions and exert pro-migratory activities: Rac induces lamellipodia and membrane ruffles at the leading edge of the cell, Cdc42 bundles actin into filopodia, Rho assembles actin into stress fibers. These cytoskeletal changes result in increased cell motility.

steps of metastasis mimic physiological EMT. In fact, full EMT of *in vitro* cultured tumor cells is thought to resemble, at least in part, the metastatic process in cancer patients. Nevertheless, this hypothesis is still under debate and evidence for carcinoma EMT *in vivo* has yet to be found [10]. We support the view that EMT-like events occur in tumors and that studying these events may contribute to our understanding of metastasis formation.

Oncogenic events in tumor cells as well as growth factors secreted by tumor and stromal cells, including transforming growth factor- β (TGF- β) and fibroblast growth factor 2 (FGF-2), induce EMT (fig. 1). During EMT, cells progressively redistribute or downregulate their apical and basolateral epithelial-specific proteins, such as tight and

adherens junction proteins (including E-cadherin), and re-express mesenchymal molecules, such as vimentin and N-cadherin. These changes lead to the abrogation of cell-cell contacts and the gain of cell motility necessary for invasion (reviewed in [11]). EMT is in contrast to the process of cell scattering, where different epithelial markers are reversibly downregulated and expression of mesenchymal proteins is not induced. Scattering can be mediated by a number of growth factors, including hepatocyte growth factor/scatter factor (HGF/SF), TGF- β , FGF-2, TGF- α and epidermal growth factor (EGF) via the PI3K or the Raf/MAPK signaling pathways [9, 12]. Loss of E-cadherin promotes, whereas maintenance of its expression inhibits, EMT and metastasis [5, 8]. E-cadherin is therefore considered a metastasis suppressor gene. Downregulation of E-cadherin mostly occurs at the transcriptional level. The E-cadherin promoter is frequently repressed by specific transcriptional repressors, including Snail1 (previously Snail), Snail2 (previously Slug), SIP1, δ EF1, Twist and E12/E47, and by subsequent promoter hypermethylation [13, 14]. Consistent with this observation, some of these repressors have been found expressed specifically at the invasive front of human invasive hepatocellular and breast carcinoma [15, 16]. The expression of these repressors seems to be highly regulated by pathways, including canonical Wnt signaling, TGF- β (see below), FGF, EGF, Stat3 and nuclear factor κ -B (NF κ -B) signaling [17–19]). Notably, Snail1 is a highly unstable protein. It is rapidly phosphorylated by glycogen synthase kinase 3 β (GSK3 β) and subsequently ubiquitinated and degraded by the proteasome pathway [20]. As a result of transcriptional repression, the E-cadherin promoter is frequently found hypermethylated in a large subset of cancer cases [21]. E-cadherin can also be downregulated at the protein level. Receptor tyrosine kinases (RTKs), such as EGFR, c-Met, IGF1R, FGFR and the non-receptor tyrosine kinase c-Src can induce phosphorylation of E-cadherin and catenins, resulting in their ubiquitination by the E3 ligase Hakai and subsequent endocytosis and degradation [22–25]. Finally, secreted proteases such as matrix metalloprotease (MMP)-9, for example induced by TGF- β and HGF/SF, can cleave E-cadherin and disrupt cadherin-mediated cell-cell contacts [26].

Loss of E-cadherin during EMT disrupts adhesion junctions between neighboring cells and thereby supports detachment of malignant cells from the epithelial cell layer. Yet, migration and invasion of tumor cells is also promoted by the loss of interaction of E-cadherin with the cytoskeleton, subsequent changes in the activities of Rho family GTPases, most prominently Rac1, Cdc42 and RhoA, and the concomitant reorganization of the actin cytoskeleton (fig. 1). In epithelial cells, E-cadherin-mediated assembly of adherens junctions recruits and activates Rac1 and Cdc42. Thereby, E-cadherin-bound α -

and p120-catenin directly interact with Rho family-specific guanine nucleotide exchange factors (GEFs) and with Rho family members [27, 28]. Moreover, activated Rac1 and Cdc42 promote and consolidate E-cadherin-mediated adhesion by sequestering their downstream effector IQGAP1, which in its free form inhibits the interaction of β -catenin with α -catenin/E-cadherin [29]. After an early activation phase, where RhoA is required for E-cadherin clustering, RhoA gets gradually downregulated with a concomitant repression of cell migration [30]. In contrast, forced expression of RhoA (and also RhoC) in tumor cells induces focal adhesions and stress fibers, promoting invasion and metastasis [31, 32]. Upon loss of E-cadherin function, RhoA, Rac1 and Cdc42 are released from adhesion junctions and promote cell migration and invasion. Consistent with the dual role of these molecules, the Rac1-specific GEF Tiam1 localizes to adhesion junctions and promotes cell adhesion on substrates impeding cell migration (fibronectin, laminin), whereas it is found in lamellipodia when cells are cultured on cell motility-supporting collagen type I [33]. However, the mechanisms, by which ECM and thus integrin-mediated signaling affect the activity of RhoA, Rac1 and Cdc42, and with it E-cadherin function, remain to be elucidated. Recent studies have shown that mesenchymal cadherins, in particular N-cadherin, enhance tumor cell motility and migration (reviewed in [5]), thus exhibiting an opposite effect compared with E-cadherin. N-cadherin-induced tumor cell invasion can even overcome E-cadherin-mediated cell-cell adhesion. A novel concept based on the above observations is that a ‘cadherin switch’ from epithelial to mesenchymal cadherins exists which supports the transition from a benign to an invasive, malignant tumor phenotype. Hence, in addition to the loss of E-cadherin, the gain of N-cadherin may critically contribute to tumor invasion and metastatic dissemination. In fact, N-cadherin has been shown to induce tumor cell migration, most likely by stimulating FGF receptor-mediated signal transduction [34, 35]. Hence, changes in the expression of cadherins not only modulate cell-cell adhesion but also induce pro-metastatic signaling pathways.

Integrins and tumor progression

In order to detach and migrate, tumor cells depend on changes in both cell-cell and cell-matrix interactions. It is tacitly assumed that strong cell-matrix adhesions need to be dissolved, whereas transient and weak adhesions are a prerequisite for migration. This complex situation has made it difficult to experimentally determine the functional contribution of cell-matrix adhesion to tumor progression. With integrins being the prototype mediators of cell-matrix adhesion, they have been studied in great detail, also in tumor progression [36–38]. It is beyond the scope of this review to discuss the detailed mechanisms by which integrins affect tumor cell adhesion and migra-

tion. Instead, we focus on the functional contribution of integrins to tumor metastasis. Moreover, the following sections present several mechanistic links of integrins to signaling pathways.

Integrins are heterodimeric cell surface receptors consisting of two type-I transmembrane subunits, α and β . They provide the essential link between the actin cytoskeleton and the ECM during cell migration. Different α and β subunits form distinct integrin subtypes, which link ECM ligands, such as fibronectin, vitronectin, laminin and collagen, to the intracellular actin cytoskeleton [38]. Importantly, binding to these ECM components activates integrins, which in turn induce intracellular signaling cascades modulating cell proliferation, survival, polarity, motility and differentiation [36, 38, 39]. Thereby integrins mediate anchorage dependence, since they allow these processes only in appropriately adhering cells. However, during tumor progression, mutations in oncogenes and tumor-suppressor genes can enable non-adherent neoplastic cells to survive and proliferate in the absence of proper cell-matrix adhesion [40]. Cancer cells that profit from integrin signals are being selected due to their survival and proliferation advantage. Many cancer types, including melanoma, glioblastoma, prostate, breast and ovarian cancer, exhibit increased expression of $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_1$ integrins during tumor progression [41–43]. Tumor cell migration and metastasis is supported by integrin-mediated focal adhesion and actinomyosin-dependent contractility, as demonstrated for instance for integrins $\alpha_6\beta_4$, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ [44–46]. Furthermore, activated integrins can recruit proteases, such as MMP-9, towards the site of attachment, and the subsequent ECM degradation supports migration and invasion of cells into the surrounding tissue [38, 43]. Unfortunately, various subsets of integrins seem to mediate different functions during progression of different cancer types, and more experimental work will be required to unravel their actual functional contribution.

Endothelial cell integrins have been studied in great detail during physiological and pathological angiogenesis and have been shown to play an important role in tumor angiogenesis. Distinct subtypes, including $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, support vascular endothelial growth factor (VEGF)-mediated angiogenesis in tumor xenotransplantation experiments [47]. Endothelial $\alpha_3\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are critically involved in both VEGF- and FGF-mediated angiogenesis. Peptide antagonists against these integrins are able to interfere with tumor angiogenesis in a number of experimental model systems, and initial clinical trials with some of these compounds are under way [37]. Interestingly, experiments with mice lacking $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have demonstrated increased rather than reduced tumor growth, raising the possibility of a dual function of integrins at least in the angiogenic process [48]. Thus, changes in integrin-mediated matrix

adhesion on tumor cells, endothelial cells and certainly other cells of the tumor stroma can influence tumor cell migration, invasion and angiogenesis, all rate-limiting processes in tumor metastasis.

Immunoglobulin family adhesion molecules: NCAM and L1

Among many other cell adhesion molecules (CAMs), two have been specifically implicated in promoting tumor cell invasion and metastatic spread, NCAM (CD56) and L1 (CD171, L1CAM). Both structurally belong to the immunoglobulin (Ig) superfamily and are critical for central nervous system (CNS) development. They not only mediate static neuron-neuron adhesion but also are particularly involved in neurite outgrowth, axon guidance and neural cell migration. In addition to providing mechanical cell-cell and cell-ECM adhesion, they also activate signaling receptors and induce intracellular signaling cascades [5, 49].

NCAM has been shown to play an important role in the progression to tumor malignancy. Both the shift of the adult NCAM 120-kDa isoform to the embryonic 140- and 180-kDa isoforms and a general downregulation of NCAM expression are associated with poor prognosis in a few cancer types [50, 51]. In a transgenic mouse tumor model of pancreatic β cell carcinogenesis (Rip1Tag2), loss of NCAM leads to the formation of lymph node metastasis mediated by the increased expression of the lymphangiogenic factors VEGF-C and D and with it up-regulated peri-tumoral lymphangiogenesis [52, 53]. Similar to its function in neurons, NCAM also modulates cell-matrix adhesion of tumor cells by binding to FGFR and inducing a variety of signaling pathways that lead to β_1 integrin-mediated cell-matrix adhesion and neurite outgrowth [34].

While L1 exerts functions similar to NCAM in neurons, L1 is specifically expressed at the invasive front of gliomas, melanomas, lung, prostate, renal, ovarian and endometrial carcinoma, and its expression appears to correlate with metastasis [54–59]. Similar to NCAM, L1 has been shown to enhance migration and invasion of tumor cells *in vitro*. L1 promotes cell-matrix adhesion and migration of tumor cells on ECM proteins, such as fibronectin and laminin, by associating with integrins through an integrin recognition motif. Namely, integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_3\beta_1$ have been found to associate with L1 [49, 60–62]. In these studies, integrin-mediated signaling events induced by associated L1 seem to be responsible for promoted cell migration. Interestingly, L1-integrin interaction may not only occur in cis at the cell surface but also by ectodomain shedding of L1 and subsequent autocrine/paracrine binding of soluble L1 to $\alpha_v\beta_5$ integrin. Independent of integrin binding and activation, L1 has also been shown to promote growth factor-induced mitogen-activated protein kinase (MAPK) signaling and to subse-

quently stimulate extracellular regulated kinase (Erk)1/2-mediated transcriptional changes in genes associated with cell motility and invasion and matrix remodeling [49]. Such targets include the genes for cathepsin-L and -B, osteopontin (OPN), RhoC and CD44, all proteins implicated in tumor invasion and metastasis (CD44 and RhoC are discussed below) [49, 63]. Finally, L1 was recently identified as a direct target gene of activated Wnt signaling, together with the metalloprotease ADAM10. Co-induction of ADAM10 and L1 leads to proteolytic shedding of the L1 extracellular domain, which in turn induces tumor cell motility and enhanced tumorigenesis [64].

Together, these selected examples indicate that detachment and migration of tumor cells critically depends on altered cell-cell and cell-matrix adhesion. Yet, such alterations appear not to only affect cell adhesion but also a variety of signal transduction pathways, mediated by tyrosine kinase receptors or by integrins, which all seem to lead to increased tumor cell invasiveness and metastatic dissemination.

Changes in signaling pathways during EMT and scattering

Malignant transformation with deregulation of cell growth is frequently induced by somatic mutations or overexpression of receptor tyrosine kinases [65]. Importantly, deregulated signaling of some receptor tyrosine kinases not only exerts transformation of tumor cells but also promotes their invasion and metastatic dissemination. The following sections address some of the newly emerging aspects of how receptor tyrosine kinases contribute to tumor metastasis (fig. 1).

c-Met and hepatocyte growth factor/scatter factor (HGF/SF)

Overexpression of and activating mutations in c-Met have been reported in many human tumors, and xenotransplant and transgenic mouse models demonstrate a profound pro-metastatic activity of HGF/SF and c-Met [66–68]. c-Met, encoded by the *MET* proto-oncogene, is a dimeric transmembrane tyrosine kinase receptor for HGF/SF. Intracellular transducers of c-Met activity include Ras/MAPK, PI3K, phospholipase γ (PLC- γ), Src-related tyrosine kinases and growth-factor-receptor-bound protein 2 (Grb2)-associated binder 1 (Gab-1). Internalization is a typical mechanism by which signaling of receptors such as c-Met is terminated, and poly-ubiquitylation promoted by c-Cbl is critical for c-Met downregulation by targeting it to the proteasome [69, 70].

Although the precise mechanisms underlying c-Met-mediated tumor cell migration and invasion are not completely understood, several critical events have been recently elucidated. Upon ligand binding, c-Met induces invasive growth, both during physiological tissue morph-

ogenesis and tumor progression. HGF/SF-mediated effects appear to depend on the differentiation state of the c-Met-expressing cells. Whereas HGF/SF induces morphogenesis of E-cadherin-positive polarized epithelial cells, it promotes scattering and metastasis of cells that have lost their epithelial characteristics [69]. The metastatic potential of c-Met is mediated via a signal transducer docking site, which can bind and activate multiple Src homology region 2 (SH2)-containing intracellular effectors. Interestingly, a distinct mutation of this domain (H1351N) increases the transforming but in parallel abolishes the metastatic properties of c-Met [71]. c-Met-induced dissociation of adherens junctions, scattering and metastasis appear to be primarily mediated via the PI3K pathway in a MAPK-dependent, yet protein kinase B (PKB/Akt)- or Rac-independent manner [72, 73]. Through activation of the MAPK pathway, c-Met signaling induces Ets1 transcription factors, which control the expression of genes involved in ECM remodeling and cell scattering [74]. In fact, Ets1 expression is known to correlate with metastasis incidence in a number of cancer types [75, 76].

Recent results indicate that the pro-metastatic activity of c-Met also critically depends on its ability to associate with other cell surface proteins, notably $\alpha_6\beta_4$ integrin, CD44 and Plexin B1. c-Met is constitutively associated with integrin $\alpha_6\beta_4$, which in turn is phosphorylated by activated c-Met. Subsequently, phosphorylated integrin $\alpha_6\beta_4$ recruits the signaling adaptor Shc and PI3K and potentiates HGF/SF-induced activation of the Ras and PI3K pathways [77]. c-Met also associates with and is functionally modulated by CD44, a hyaluronan receptor involved in cell-cell/cell-matrix interactions, cell migration and metastasis [78]. Upon HGF/SF stimulation, c-Met associates with a variant CD44 isoform (CD44v6) and recruits ezrin, radixin and moesin proteins (ERMs) to the complex [79]. ERMs link adhesion molecules to filamentous F-actin and participate in membrane and cytoskeletal remodeling during cell migration. Both CD44 and the ERM proteins are known to promote metastasis by various mechanisms. For instance, ezrin can stabilize mammalian target of rapamycin (mTOR) downstream signaling molecules, enhancing metastasis [80]. c-Met-mediated tumor cell invasion is CD44-dependent and may also require ERMs, since signal transduction by activated c-Met via the MAPK pathways depends on the ERM binding domain of CD44 [79, 81, 82]. Furthermore, HGF/SF specifically induces the expression of OPN and its association with the hyaluronan-CD44-ERM complex at the leading edge of metastatic cells [83, 84]. OPN is a protein of the ECM with a wide variety of cell surface binding proteins and has been shown to promote tumor cell invasiveness [85]. The functional cooperation of c-Met, CD44 and OPN thus may be critical for metastasis [83].

Plexins, which share structural homology with c-Met, are widely expressed receptors for Semaphorins (Sema). The Sema family consists of secreted and membrane-bound members that act as guidance signals for neurons [86, 87]. Recently, Plexin B1 has been shown to be overexpressed in several tumor cell lines and to be physically associated with c-Met [87]. Notably, binding of Sema 4D to Plexin B1 transactivates the tyrosine kinase activity of c-Met independent of the presence of HGF/SF [88]. Interestingly, the identical signaling pathway in endothelial cells leads to angiogenesis [89].

In addition to inducing a motile tumor cell phenotype, c-Met promotes invasion of neoplastic cells by upregulating proteases such as plasminogen activator (uPA) and MMPs via MAPK and PKC, respectively. These proteases are critical in degrading the ECM and allowing migration of tumor cells through the basement membrane and the surrounding stroma [90].

Based on the multitude of activities and interactions during tumor progression, c-Met represents an important mediator of cell migration and metastasis and, hence, c-Met-mediated signaling pathways are in the spotlight as potential targets for the development of anti-metastatic therapy [91].

Wnt signaling and tumor progression

In many cancer types, Wnt signaling is activated by mutations in a number of effector genes, including the genes encoding for adenomatous polyposis coli (APC), axin-1 and 2, and β -catenin, which predispose to cancer [92–94]. Besides their critical role in assembling the E-cadherin-mediated cell adhesion complex, β -catenin and γ -catenin also have important functions in the canonical Wnt-signaling pathway. Non-sequestered, free β - and γ -catenin are rapidly phosphorylated by GSK-3 β in the APC/axin/GSK-3 β complex and subsequently degraded by the ubiquitin-proteasome pathway. If the tumor suppressor APC is non-functional, as in many colon-cancer cells, or if GSK-3 β activity is blocked by the activated Wnt-signaling pathway, β -catenin accumulates at high levels in the cytoplasm. Subsequently, it translocates to the nucleus, where it binds to members of the Tcf/Lef-1 family of transcription factors and modulates the expression of Tcf/Lef-1-target genes, including c-Myc, cyclin D1, fibronectin, MMP-7, Id2, CD44, axin-2, Tcf-1 and others, all genes implicated in cell proliferation, transformation and tumor progression.

The dual function of β -catenin has motivated a multitude of experiments to assess whether the loss of E-cadherin function would subsequently lead to the activation of the Wnt-signaling pathway. In a number of cellular systems, it has been demonstrated that sequestration of β -catenin by E-cadherin can compete with the β -catenin/TCF-mediated transcriptional activity of the canonical Wnt-signaling pathway. The fact that E-cadherin does not com-

pletely deplete the cytoplasmic β -catenin suggests that β -catenin exists in different functional pools [95, 96]. Interestingly, in breast and prostate carcinoma cell lines, E-cadherin suppresses tumor cell invasion by binding β -catenin without repressing β -catenin/TCF transcriptional activity, indicating that a novel, as yet unknown, additional function of β -catenin may be required for cellular invasiveness [97, 98].

Furthermore, activated Wnt signaling inhibits E-cadherin-mediated cell adhesion by inducing expression of Snail1, a transcriptional E-cadherin repressor. Snail1 in turn synergizes with the Wnt/ β -catenin pathway by inducing Tcf expression, and β -catenin/Tcf can in turn repress E-cadherin transcription in co-operation with Snail1 [99, 100].

Signals elicited by ErbB receptors

Many metastatic human carcinomas are characterized by the overexpression or constitutive activation of ErbB tyrosine kinase receptor (EGFR) family members involving activating mutations of the receptor kinases or an autocrine loop with EGF family ligands. In addition to stimulating cell differentiation and proliferation, EGF promotes tumor cell motility, invasion and metastasis by affecting a large number of protein functions and signaling pathways, including Wnt signaling, Snail1 expression, focal adhesion kinase (FAK) activity and expression of MMPs [101].

Interestingly, ErbB receptors and Wnt signaling cooperate during tumorigenesis, which may be critical for metastasis formation [102]. In tumors of mouse mammary tumor virus (MMTV)-Wnt-1 transgenic mice, two members of the ErbB family of transmembrane receptor tyrosine kinases, ErbB1 (EGF receptor) and ErbB2 (Her-2/c-Neu), interact with and phosphorylate β -catenin. Interestingly, the β -catenin/ErbB interaction correlates with the incidence of pulmonary metastases, indicating a functional role of these interactions in metastasis formation [102]. Tyrosine phosphorylation of β -catenin by ErbB1 and by other receptor tyrosine kinases is known to destabilize its binding to E-cadherin. As a result, adhesion junctions disassemble, the liberated β -catenin accumulates in the cytoplasm, transfers to the nucleus and, in combination with Tcf/Lef1 transcription factors, modulates gene expression. Such activation of the Wnt pathway may result in increased tumor cell migration, invasion and metastasis (reviewed in [103]).

Notably, Snail1 and caveolin-1 seem to play antagonistic roles during EMT induced by EGF. Caveolins are structural proteins of caveolae, the invaginations of the plasma membrane that function as regulators of signal transduction. Transient EGF signaling induces caveolin-1-dependent endocytosis of E-cadherin. Long-term EGF treatment downregulates caveolin-1 and induces Snail1. Concomitantly, β -catenin/Tcf/Lef-1 transcriptional ac-

tivity is enhanced [19]. Interestingly, both EGF-mediated upregulation of Snail1 transcription as well as increased transactivation by β -catenin are dependent on caveolin-1 downregulation. EGF-induced downregulation of caveolin-1 may therefore play a central role in invasion and metastasis.

Insulin-like growth factor 1 receptor (IGF1R)

Insulin-like growth factors (IGFs) and their cognate signaling receptor IGF1R are involved in cellular and organismal growth, embryonic development and numerous pathological states, including cancer and metastasis [104]. IGF1R overexpression in the primary tumor correlates with the increased incidence of metastases in patients with gastric cancer, gastrinoma, gastrointestinal stromal tumors, thyroid cancer and other malignancies [105–108]. Forced expression of IGF1R in a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag2) results in accelerated tumor progression, with increased tumor malignancy and distant tumor metastasis to various organs [109]. Conversely, a small chemical inhibitor of IGF1R tyrosine kinase activity (NVP-AEW541) is able to repress tumor cell survival and tumor growth of a number of different tumor types in culture or in xenograft transplantation experiments, yet its effects on tumor progression and metastasis have not been explicitly assessed [110, 111]. The mechanisms of IGF1R-mediated invasive and metastatic abilities of tumor cells have been recently studied. IGF1R signaling has been famous for mediating anti-apoptotic and mitogenic effects via PI3K/PKB and Ras/MAPK pathways. Yet, IGF1R also modulates cell-substrate adhesion, migration, invasion and cell-cell interaction by signaling via FAK [112, 113]. IGF1R has also been shown to modulate the expression and function of different junctional proteins, including cadherins, catenins, ZO-1 and the small GTPases RhoA, Rac1 and Cdc42 [114]. Notably, upon IGF-II binding, activated IGF1R associates with E-cadherin and β -catenin, thereby inducing reversible scattering [115]. Through internalization of the entire complex, E-cadherin is sequestered from the plasma membrane and targeted to late endosomes and lysosomes. As a consequence, the released β -catenin translocates to the nucleus and induces Tcf/Lef1-mediated transcription of target genes (see also the previous sections). Conversely, overexpression of E-cadherin antagonizes the effects of IGF-II. Such mechanisms are currently being investigated to resolve the actual contribution of IGF1R to tumor metastasis.

TGF- β signaling

TGF- β is a member of the TGF- β superfamily of ligands and binds to a heterodimeric receptor consisting of type I and II transmembrane receptor serine-threonine kinases. TGF- β -mediated activation of the receptors induces a canonical signaling pathway via Smad proteins, which re-

sults in nuclear translocation of receptor-Smads (Smad2/3) together with common-Smad (Smad4) and in the modulation of expression of various target genes [116, 117]. Depending on the cell type, alternative signaling pathways, including MAPK, PI3K and PKC, can also be activated [118–120].

Depending on the differentiation and transformation status of a cell, TGF- β exerts two directly opposing functions; it acts as an anti-proliferative and pro-apoptotic growth factor on differentiated cells, but induces proliferation and EMT of undifferentiated or transformed cells. Hence, at early stages of tumor development, TGF- β 's cytostatic action helps to suppress tumor growth. At later tumor stages, however, transformed cells develop resistance to the growth-inhibitory effect of TGF- β and respond to TGF- β by undergoing EMT (reviewed in [121]). Moreover, TGF- β exerts immunosuppressive functions that further promote tumor progression.

Resistance of tumor cells to TGF- β -mediated growth restraint and induction of EMT is mostly due to functional changes downstream of the TGF- β signaling pathway [9]. At the transcriptional level, TGF- β cooperates with various signaling pathways stimulated by EGF, interferon γ (IFN γ) and tumor necrosis factor (TNF)- α . Activated MAPK signaling, for instance, can inactivate Smads, induce inhibitory Smads or induce an autocrine TGF- β production (for detailed reviews see [117, 122]). In immortalized (non-transformed) mammary epithelial EpH4 cells, oncogenic Ras and TGF- β R signaling are required for the induction and maintenance of TGF- β -induced EMT [123]. In Ha-Ras-transformed EpH4 cells (EpRas), upregulation of the PI3K pathway prevents TGF- β -induced apoptosis and promotes scattering. Full EMT, however, depends on the activation of the MAPK pathway. Similarly, an activated PI3K pathway correlates with tumor formation of these cells, whereas a hyperactive MAPK pathway is required for both tumorigenesis and metastasis [9, 12]). Moreover, TGF- β -induced EMT seems to depend on β_1 integrin-mediated signals and RhoA-induced actin cytoskeleton rearrangements [124]. Such cooperative activities of TGF- β together with its dual role as tumor promoter and tumor suppressor obscure a direct therapeutic exploitation of this pathway and stimulate further studies into the mechanistic details of these activities.

Genes mediating metastasis

An ongoing interesting debate in the field of cancer metastasis concerns the question whether the metastatic potential of a tumor cell is primed with the first initial genetic events of transformation or whether tumor cells by clonal selection progress to tumor malignancy. It seems that genetic alterations acquired early during tumorigenesis (including gain of oncogenes and loss of tumor-sup-

pressor genes) are sufficient for certain tumor cells to spread [125, 126]. For instance, gene microarray analysis has identified specific expression profiles in metastatic versus non-metastatic primary human carcinomas [127–130]. Yet, the pro-metastatic efficacy of those genes remains to be shown. On the other hand, circulating tumor cells are found to carry various genetic alterations, suggesting that additional genetic events are required for tumor cell dissemination [131]. Moreover, many genes are expressed and seem to be functional only during the metastatic process. Even more impressive, transgenic expression of MMP3 is sufficient to induce full-blown carcinogenesis, indicating that ‘invasive or metastatic’ processes can even affect the transformation status of a cell [132].

More refined approaches of gene expression analysis combined with functional experiments have now provided novel insights into such events. For example, work published this year has not only identified genes that may specifically mediate lung metastasis of breast cancer cells but also confirmed their pro-metastatic functionality *in vitro* and *in vivo* [133]. The genes were tested by overexpression in poorly metastatic cells or by RNA interference (RNAi) knock-down in highly metastatic cells. These were observed to specifically metastasize to the lung and not the bone when orthotopically injected into the mammary fat pad of immunodeficient mice. Nine genes have been found to be overexpressed in cells mediating lung metastaticity in *in vivo* experiments, encoding the pan-ErbB receptor ligand epiregulin, the chemokine CXCL1, the interleukin decoy receptor IL13R α 2, the proteases MMP1 and 2, cyclooxygenase-2 (COX2), the transcriptional inhibitor of cell differentiation-1 (ID1) and the cell adhesion molecules VCAM1 and SPARC [133]. Recently, the chemokine receptor CXCR4 was shown to be highly expressed in breast cancer cells, primary breast tumors and metastases. Its ligand CXCL12/stromal cell-derived factor (SDF)1 α , on the other hand, is predominantly found in organs known to be the main targets of breast cancer metastasis, namely lymph nodes, lung, bone marrow and liver. Notably, treatment with neutralizing antibodies against CXCR4 reduced the capability of a metastatic breast cancer cell line to metastasize to lymph nodes and the lung [134].

Several other genes with various functions have been identified in different gene expression profiling experiments on non-metastatic versus metastatic tumor cell lines. Some of these are already confirmed as being required for metastasis *in vivo*. For instance, the transcriptional repressor Twist is overexpressed in human invasive lobular breast cancer and has been shown to abrogate E-cadherin-mediated cell-cell adhesion as well as to induce EMT *in vitro*. RNAi knock-down of Twist in a highly metastatic breast cancer cell line reduces the number of circulating tumor cells in the blood vasculature and dramatically decreases the inci-

dence of lung metastases when tumor cells are injected orthotopically in the mammary fat pad [135].

The neurotrophic tyrosine kinase receptor TrkB has been identified as a potent metastasis-inducing gene in an elegant expression cloning experiment [136]. TrkB specifically suppresses caspase-induced anoikis of non-malignant epithelial cells. Anoikis is a physiological mechanism eliminating cells that have lost an adequate interaction with the ECM, a process that is apparently repressed in metastasizing cells. Forced expression of TrkB in normal epithelial cells confers resistance to anoikis and allows their metastatic growth in the lung and heart upon tail vein injection into nude mice. Cells expressing both TrkB and its ligand BDNF even cause metastasis throughout the body. In contrast, control-transfected epithelial cells colonize these organs but undergo apoptosis after a short period of time [136].

Rho-family GTPases function in cytoskeletal reorganization, cell migration, stress fiber formation and focal adhesion [137]. It is not surprising that enhanced expression of one of the Rho GTPase family members, RhoC, correlates with the progression of various tumor types to a metastatic phenotype [138, 139]. In gene expression profiling experiments with melanoma variants of low or high metastatic potential, RhoC has been identified as a candidate involved in the spread of malignant cells. In fact, melanoma cells expressing high levels of RhoC show a markedly increased metastatic potential compared with control cells [32]. Cells lacking RhoC display reduced motility and invasiveness *in vitro*. Moreover, when crossed to RhoC-deficient mice, transgenic mice developing metastatic breast cancer (MMTV-PymT) exhibit a significant decrease in metastasis together with increased apoptosis of disseminating cells [140].

Protease-activated receptors (PARs) constitute a family of G-protein-coupled receptors and are involved in various physiological processes. They are frequently expressed in the context of metastasis and are currently subject to intense research. PARs seem to be causally involved in the metastatic process of many human cancers, and a clear pro-migratory and pro-metastatic effect has been demonstrated for PAR1 *in vitro* and *in vivo* [141]. Activating cleavage of PAR1 by various proteases induces increased migration and invasion of cells, notably by the engagement of $\alpha_v\beta_3$ integrins and changes in cell adhesion [142]. In a xenograft mouse model, expression of PAR1 is both required and sufficient to promote growth and invasion of breast carcinoma cells. In this model, fibroblast-derived MMP1 cleaves and activates PAR1, thereby inducing Ca²⁺-dependent signaling and promoting tumor cell migration and invasion [141]. Proteases known to activate PAR1 and 2 include thrombin and plasmin, which may account for one mechanism by which the coagulation system, frequently activated during tumorigenesis, promotes cancer cell motility and metastasis [143, 144].

Some of the above-mentioned genes may not only be useful predictive screening markers for cancer patients in order to adapt a more or less aggressive therapy, but may also serve as potential targets for anti-metastatic measures. Fortunately, the number of genes and factors involved in cancer metastasis is growing daily, and, after adequate functional characterization, a number of useful novel therapeutic targets can be expected.

Metastasis-promoting activities of the tumor microenvironment

Mounting evidence from various experimental systems has demonstrated in the past years that the tumor microenvironment contributes in a pivotal way not only to tumor initiation but also to tumor progression. Normal stroma modulates cell homeostasis in order to exert tissue-specific physiological functions and to prevent inappropriate growth, apoptosis and differentiation. In contrast, aberrant stroma supports de-differentiation and transformation of cells. Tumor formation thus depends on both tumor cell-intrinsic genetic events and changes in the tumor microenvironment [40, 145].

In the following chapters, we call attention to cells of the tumor stroma and discuss some selected examples of the molecular mechanisms by which the tumor microenvironment promotes tumor cell invasiveness and spread to distant organs (fig. 2).

Tumor angiogenesis

The entry of tumor cells into blood vessels (intravasation) initiates dissemination of cancer cells from the primary tumor to distant organs via the blood vasculature (hematogenous metastasis). This first step not only depends on tumor-cell-intrinsic invasive properties but obviously also on the presence of a tumoral vascular network, which itself constitutes part of the tumor stroma. Stimulated sprouting of new tumor blood vessels from a pre-existing vascular network (angiogenesis) represents a risk factor for the development of distant metastases in at least two ways. First, an increasing number of tumor blood vessels augment the contact area between tumor cells and their potential escape routes, thereby increasing the probability of intravasation. Second, tumor blood vessels display a distinct morphology in comparison to the physiological vascular bed, facilitating entry of cells: they are larger, tortuous and leaky due to a fragmented basement membrane and an incomplete pericyte lining [146]. Furthermore, hematogenous spread of cancer cells can also occur as a result of lymphogenous metastasis. Since the lymph is drained into the venous system and lymph nodes display afferent and efferent blood vessels, tumor cells circulating in the lymphatic vasculature can successively enter the systemic circulation.

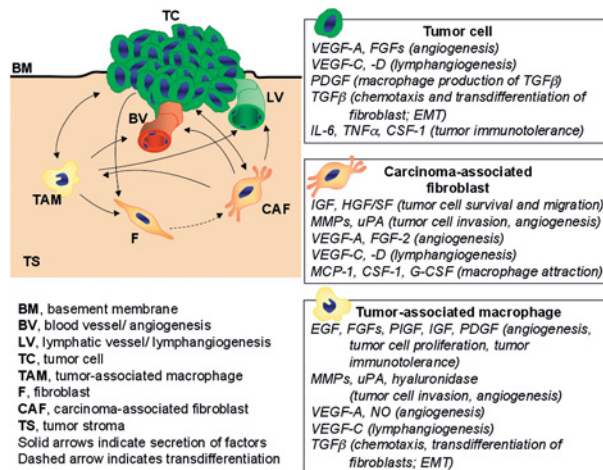


Figure 2. Contribution by the tumor microenvironment. Stromal and tumor cells cross-talk via soluble growth factors, cytokines and chemokines as schematically exemplified in this figure for tumor-associated macrophages (TAMs) and carcinoma-associated fibroblasts (CAFs). Stromal cells and tumor cells thus mutually influence their behavior in a way that promotes metastasis. For instance, tumor cell-secreted PDGF stimulates macrophages to produce TGF-β, which induces the transdifferentiation of fibroblasts into CAFs. CAFs in turn secrete macrophage chemoattractants, which promote the infiltration of macrophages into the tumor. Both TAMs and CAFs secrete growth factors, cytokines and proteases, which promote tumor cell survival, migration and invasion into the surrounding tissue. Moreover, a variety of tumor and stromal cell-secreted factors induce the formation of blood and lymphatic vessels and also modulate the immune system to tolerate the transformed cells.

Angiogenesis is orchestrated by a fine-tuned balance between secreted pro- and anti-angiogenic factors [147]. The contribution of the tumor stroma to the regulation of angiogenesis has also complicated the simplistic view that angiogenesis is mainly induced by transforming signaling pathways and by tumor hypoxia, resulting in the tumor cells' expression of angiogenic factors, such as VEGF-A and PIGF. Rather, several cell types of the tumor stroma appear to contribute in a significant way: (i) endothelial cells by secreting angiogenic factors, such as angiopoietin-2, which affect the activation status of the endothelium and its differentiation into mature vessels, (ii) infiltrating macrophages and mast cells by secreting additional angiogenic factors, including VEGF-A, FGF-2, TGF-β and IL-8, and MMPs that activate latent forms of these growth factors, (iii) cancer-associated fibroblasts (CAFs) by secreting additional growth factors, cytokines and chemokines and by modulating the ECM and (iv) additional cells of the innate and adaptive immune system or of tissue homeostasis (see below) [148, 149].

During tumor outgrowth, tumor cells and cells of the tumor stroma are stimulated for example by tumor hypoxia or lack of nutrition to produce angiogenic factors, such as VEGF-A and PIGF. These factors, together with various inflammatory chemokines and cytokines and other stimuli secreted by both neoplastic and tumor stroma

cells, tilt the balance between angiogenesis inhibitors and inducers towards the stimulation of angiogenesis (angiogenic switch). Thereby, tumor-cell and tumor-stroma-induced angiogenesis not only promotes primary (and secondary) tumor growth, but also the hematogenous dissemination of tumor cells and the outgrowth of metastasis.

Most, though not all, clinical studies demonstrate a direct correlation of high primary tumor blood microvessel densities (MVDs) with increased incidence of metastases [150, 151]. Tumor MVD is a significant and independent prognostic indicator for relapse-free and overall survival of cancer patients [152]. Similarly, elevated tumor or serum levels of the pro-angiogenic factors VEGF-A, interleukin (IL) and FGF-2 as well as a low ratio between the angiogenesis inhibitor thrombospondin-2 and VEGF-A are associated with an increased incidence of metastasis in cancer patients [153–155]. Xenograft animal models confirm the correlation of tumoral overexpression of angiogenic growth factors, increased MVD of primary and secondary tumors and metastasis formation [156–158]. Consistent with these results, transgenic mice developing prostate adenocarcinoma (TRAMP) exhibit impaired metastasis when crossed into a FGF-2 knockout background [159]. Similarly, in xenotransplant and transgenic mouse models, increased tumor and/or serum levels of angiogenesis inhibitors, such as IL-10, tissue inhibitor-1 of metalloproteinases (TIMP-1), thrombospondin-1 or endostatin, reduce the incidence of metastasis [158, 160–162]. Yet, upregulated tumor angiogenesis may not necessarily lead to metastasis formation. For example, forced expression of VEGF-A in a transgenic mouse model (Rip1 Tag2) accelerates tumor angiogenesis and growth of primary tumors but does not result in increased metastasis formation [163]. Although tumorigenic transformation, mediated for example by Ras or ErbB2, can upregulate the expression of angiogenic growth factors and repress the production of anti-angiogenic molecules, the direct contribution of the angiogenic process to tumor metastasis is difficult to demonstrate [164]. Inhibition of angiogenesis during tumor progression by specific angiogenesis inhibitors also affects primary tumor growth and thus indirectly the metastatic dissemination of tumor cells. One should also be aware of the observation that intra-tumoral hypoxia and concomitant upregulation of the expression of angiogenic factors may occur only intermittently, leading to variations in the expression in tumors and in serum levels at different time points of measurement. Moreover, methods and markers for the determination of MVD have not been standardized, hindering a direct comparison of independent studies [165]. In any case, interference with angiogenesis will not only lead to a repression of tumor growth but may also reduce tumor metastasis.

Tumor-induced lymphangiogenesis

Some tumor types, including melanoma and cancers of the breast, lung and gastrointestinal tract, preferably spread via lymphatics [166]. Sentinel lymph node biopsy of patients with these tumors is routinely used for refinement of prognostic and therapeutic measures. Since the lymphatic vasculature is specialized for entry and transport of immune cells, lymphogenous spread is more efficient than that via blood vessels. Lymphatic capillaries are larger, lack a basal lamina and display an endothelial cell arrangement facilitating intravasation of cells. Moreover, the composition of lymph is similar to interstitial fluid and flow velocities are low, thus allowing better cell viability as compared with the serum toxicity and shear stress encountered in the bloodstream [167]. Histological analyses of human primary tumor samples demonstrate a consistent correlation between the presence of enlarged peri-tumoral lymphatics and the incidence of regional lymph node metastases [168, 169]. Moreover, animal models clearly demonstrate a significant promotion of lymphogenic metastasis by tumor-induced lymphangiogenesis (reviewed in [170]). VEGF-C and D are the two most-investigated secreted lymphangiogenic factors. During secretion from the producing cell, these glycoproteins are proteolytically processed by plasmin and other (unknown) proteases. Unprocessed forms can only bind to VEGF receptor-3 (VEGFR-3), which is usually found on both normal and tumor lymphatic endothelial cells. Processing of VEGF-C and D increases the binding affinity for VEGFR-3 and allows binding to VEGFR-2, which is specifically expressed on blood vessel endothelial cells. Therefore, expression and the processing status of VEGF-C and D in a given tissue appear to determine the lymphangiogenic and angiogenic activities of these growth factors [171]. Expression of VEGF-C in tumor tissue serves as a reliable marker for ongoing tumor lymphangiogenesis and increased risk of regional lymph node metastasis in many cancer types [172, 173]. Data for VEGF-D are less consistent suggesting that its ability to promote metastatic spread via lymphatics depends on the investigated cancer type and/or grade [174–176].

The question remains whether VEGF-C and D may alter adhesive properties of both tumor and endothelial cells or modify functional features of lymphatic vessels, thus enhancing tumor cell adhesion to and intravasation into lymphatics. In addition, it is conceivable that VEGF-C and D activate lymphatic endothelial cells to secrete chemotactic factors for tumor cells. Although it is known that both tumor and stromal cells can express VEGF-C and D [53, 177], it is not clear to what extent and upon which signals cells of the tumor stroma contribute to tumor-associated lymphangiogenesis [178]. Moreover, the genetic background of a given patient may modulate expression levels and processing of VEGF family members

and thereby determine the biological behavior of a tumor [179, 180].

Carcinoma-associated fibroblasts

Besides endothelial cells of nearby vessels, neoplastic cells can also activate resident fibroblasts, which then cooperate in tumor progression (fig. 2). Activated fibroblasts or myofibroblasts (also called carcinoma-associated fibroblasts, CAFs) are often encountered at the invasive front of many different cancer types. Myofibroblasts share features of both smooth muscle cells and fibroblasts and are important promoters of growth and differentiation during embryogenesis, wound healing and other tissue-remodeling processes [181]. It is predominantly fibroblasts that transdifferentiate into myofibroblasts, but also vascular smooth muscle cells, pericytes, hematopoietic bone marrow-derived precursor cells and even cancer cells themselves are discussed as putative progenitors. Tumor cell-secreted platelet-derived growth factor (PDGF) stimulates fibroblast proliferation and the release of TGF- β from macrophages, which itself is chemotactic for fibroblasts at lower concentrations and induces their transdifferentiation into myofibroblasts at high concentrations [181]. Furthermore, tumor cells often express TGF- β themselves [121]. Myofibroblasts appear shortly before the invasive stage of tumors and promote degradation of basement membranes and ECM by secreting serine proteases, MMPs and urokinase plasminogen activator. Moreover, they express IGFs and HGF/SF, thus promoting cell-survival and migration, as well as the pro-angiogenic factors FGF-2 and VEGF and the pro-inflammatory cytokines IL-1, 6, 8 and TNF- α . By doing so, myofibroblasts not only stimulate their own migration into the tumor, but also promote survival, proliferation and invasion of adjacent cancer cells as well as angiogenesis, collectively enhancing metastasis [182–184].

Fibroblasts of the tumor stroma participate in the decision whether neighboring tumor cells proliferate and invade into surrounding tissue and metastasize to distant sites [185]. For example, epithelial cells and fibroblasts in tissues that line portals of entry to the body secrete high levels of IFN β , whereas fibroblasts of internal organs do not. It has been demonstrated in xenotransplant and *in vitro* experiments that IFN β secreted by skin fibroblasts downregulates expression of FGF-2 and secreted type IV collagenase in transplanted colon and renal carcinoma cells, thereby inhibiting their angiogenic and invasive capabilities in the skin. In contrast, when transplanted orthotopically, tumor cells express high levels of FGF-2 and secreted type IV collagenase, resulting in the development of highly vascularized and metastatic tumors [186, 187]. Moreover, transgenic mice in which TGF- β receptor type II is selectively inactivated in fi-

broblasts develop prostate and gastric cancer even in the absence of mutations in the respective epithelial cells [188]. Notably, the mutated fibroblasts secrete high levels of HGF/SF, thereby conferring a paracrine tumorigenic signal on the epithelial cells.

Contribution of the immune system to tumor metastasis

The increased incidence of cancer in immunosuppressed patients and intense research in tumor immunology over the past years provide evidence that the immune system can to some degree recognize and eliminate tumor cells [190, 191]. Nevertheless, an increasing body of evidence indicates that immune cells represent a double-edged sword during tumorigenesis. Although many tumors are potentially immunogenic, they evidently develop mechanisms to escape immunosurveillance by various mechanisms and can even ‘educate’ immune cells to support tumor cell survival and proliferation. Many recent reports have addressed these functions of the immune system and suggest new strategies how to therapeutically boost a cancer patient’s immune response against the cancer. However, only limited insights have been gained into the role of immune cells during late-stage tumor progression and metastasis. Apparently, tumor-induced immunotolerance not only allows primary tumor outgrowth but also metastasis. Such ‘immunological’ involvement of the immune system in preventing carcinogenesis has been recently summarized [192]. Here, we restrict ourselves to the question of whether ‘tumor-educated’ immune cells can actively contribute to tumor cell migration, invasion and metastatic dissemination.

The innate immune system

The innate immune system recognizes and eliminates many exogenous infectious agents as well as altered host cells independent of preexisting specific (adaptive) immunity. Macrophages, dendritic cells (DCs) and natural killer cells (NKs) are cellular components of the innate immune system. Whereas macrophages have been repeatedly shown to promote tumor growth and metastasis by various mechanisms, NKs are usually associated with suppression of tumor metastasis, and DCs exert both anti-tumor and tumor immunosuppressive activities.

Tumor-associated macrophages

Macrophages are critical players in inflammation, wound healing, tissue repair and remodeling. Due to their antigen-presenting activity, they play a central role in cell-based immunity and can execute cells via cytotoxic mechanisms [193, 194]. Tumor cell damage and hypoxia are general attractants for circulating monocytes, and the presence of leukocytes in hypoxic areas of tumors is a well-known phenomenon [195–197]. In addition, neo-

plastic cells, tumoral fibroblasts and smooth muscle cells produce distinct monocyte-attracting factors, including monocyte chemoattractant protein-1 (MCP-1), colony-stimulating factor-1 (CSF-1), granulocyte-colony stimulating factor (G-CSF), VEGF-A, PlGF and VEGF-C [198–200]. Upon invasion into the tumor tissue, monocytes differentiate into macrophages and are specifically activated or de-activated, depending on the cytokine milieu encountered.

Consistent with their antigen-presenting and cytotoxic role in inflammation, high numbers of macrophages within or in the periphery of tumors (tumor-associated macrophages, TAMs) can repress tumor growth and progression [201, 202]. Surprisingly however, most clinical and experimental data correlate high TAM counts with reduced disease-free and overall survival. This discrepancy has been explained by a tumor type-specific paracrine cytokine crosstalk between tumor cells and TAMs: tumors secreting granulocyte-monocyte colony stimulating factor (GM-CSF), IFN γ and IL-12 promote the antigen-presenting and cytotoxic activities of macrophages (reviewed in [198, 203, 204]). In contrast, most tumor cells secrete VEGF-A, TGF- β , IL-6, CSF-1 and low-dose TNF α , all factors that inhibit the TAMs' antigen-presenting activities and rather support immunosuppression, angiogenesis, tumor cell proliferation and invasion [205–208]. In turn, these TAMs promote tumor cell proliferation by secreting a variety of tumor promoting factors, including nitric oxide (NO), EGF, FGF-8b, HGF/SF, IGF-I, TGF- β , VEGF-C and PDGF. Alternatively, activated macrophages can stimulate tumor cells to secrete these factors in an autocrine fashion [209–214]. Furthermore, TAMs themselves potently stimulate tumor angiogenesis and lymphangiogenesis by secreting growth factors, cytokines and chemokines [215–217]. Interestingly, in a cornea model of inflammation-induced lymphangiogenesis, macrophages transdifferentiate and directly incorporate into the endothelial layer of nascent lymphatic vessels [217]. By releasing MMPs, plasminogen activators, hyaluronidase and other enzymes involved in the breakdown of the ECM, TAMs liberate matrix-bound angiogenic molecules, including VEGF-A and IL-1 β , thus further enhancing angiogenesis and tumor cell migration and invasion (fig. 2) [203, 218, 219]. Notably, TAMs have also been found to actively associate with tumor cells via adhesion molecules, such as ICAM-1 and sialoadhesin, and to convey them into efferent blood vessels [220]. Since macrophage activity is associated with the release of reactive oxygen and other mutagenic compounds, they might also indirectly induce additional genetic pro-tumorigenic and pro-metastatic alterations [3, 4, 221].

NKs and DCs

NKs are a derivative of the CD8 $^+$ T-lymphocyte lineage, but lack the rearrangement of T-cell receptor genes. NKT

cells represent a subtype sharing characteristics with NK and T cells. It is well-established that NKs and NKTs display important anti-tumor activity by inducing death of tumor cells through direct contact or by the secretion of pro-apoptotic cytokines [222, 223]. In parallel, NKs stimulate the maturation of DCs, which are first-line antigen-presenting cells, and thereby promote specific anti-tumor immunity mediated by cytotoxic CD8 $^+$ T lymphocytes (CTLs) and B lymphocytes [224]. In cancer patients, high peripheral blood NK activity significantly correlates with longer metastasis-free survival [225]. Experiments using perforin-deficient mice clearly demonstrate that NK-mediated cytotoxicity is one of the essential anti-metastatic mechanisms performed by the innate immune system [226–228]. Further details on NK-mediated tumor surveillance and anti-metastatic activity have been recently summarized [229]. To date, here is no evidence indicating a potential pro-metastatic role of NKs.

DCs are classical antigen-presenting cells, which can initiate strong CTL-mediated anti-tumor immune responses. They are generally divided into myeloid and plasmacytoid DCs with differences in cell-surface receptors, anatomic localization and function [192]. Recently, a third type of DCs, vascular DCs (VDCs), was identified, which simultaneously expresses endothelial and DC markers. Strikingly, VDCs can assemble into functional blood vessels [230]. Depending on the cytokine milieu encountered in tumors, DCs can either mediate anti-tumor activity or support tumor growth and metastasis. In fact, increasing evidence from analyses of human ovarian, breast, prostate and renal cell carcinoma reveals the presence of tumor-promoting rather than tumor-suppressing dendritic cells, and altered dendritic cell function and differentiation is likely to be one of the most fundamental mechanisms by which tumors escape immune responses [231–235]. Pro-tumorigenic dendritic cells can favor metastasis by inducing tumor immunotolerance and by promoting tumor angiogenesis. Whereas mature myeloid DCs act as classical antigen-presenting cells, immature or partially differentiated myeloid DCs can tolerize T cells to self-antigens by inducing either suppressive T cells or T-cell unresponsiveness. This mechanism of immunomodulation is particularly important to counteract autoimmune reactions, but can be abused by tumors as well [236, 237]. Tumor cells, TAMs and tumoral immunosuppressive T cells can secrete high amounts of VEGF-A, IL-6, macrophage colony-stimulating factor (M-CSF), COX2, IL-10, TGF- β and gangliosides, which suppress maturation of DCs [192]. Most myeloid DCs found in human ovarian, breast, prostate and renal cell carcinoma are therefore immature and may induce tumor immunotolerance [231–234]. Tumor-secreted IL-10 and VEGF also induce expression of B7-H1 on myeloid dendritic cells, a ligand for PD-1 receptor expressed on suppressor T cells [238]. A study

published this year demonstrates that the metastatic ability of melanoma and colon cancer cells expressing B7-H1 is abolished in PD-1-deficient mice and that tumors also grow more slowly [239]. These and other experiments indirectly show that tumor cell escape from immunosurveillance – in this case mediated by modulation of myeloid DC and suppressive T cell function – is essential for tumor growth and metastasis.

The involvement of dendritic cells in tumor angiogenesis has recently drawn major attention. It is thought that mature myeloid DCs suppress tumor angiogenesis and thereby impede both tumor growth and metastasis by secreting or stimulating the production of anti-angiogenic factors, such as IL-12, IFN γ and IL-10 [240]. Conversely, the tumor-induced suppression of myeloid DC maturation described above therefore enhances tumor angiogenesis. Moreover, tumors contain significant numbers of plasmacytoid and vascular DCs secreting pro-angiogenic molecules, such as TNF α and IL-8 [230, 240, 241]. Based on this rather complex situation, the involvement of DCs in tumor angiogenesis and metastasis and their therapeutic exploitation certainly deserves future investigation.

The adaptive immune system and metastasis

After a primary contact with an antigen, the adaptive immune system confers longterm antigen-specific immunological memory. Its basic mediators are T and B lymphocytes. CD8⁺ cytotoxic T cells (CTLs) together with NKs are the fundamental effectors of immune response-mediated tumor cell elimination via the release of cytokines that activate death receptors on the tumor cell surface. CD4⁺ T helper cells are central to the development of immune responses by activating antigen-specific effector cells and recruiting cells of the innate immune system [242, 243]. As mentioned earlier, suppressor T cells play an important role in inducing tumor immunotolerance, which might not only allow tumor growth but also metastasis [237, 244]. Nevertheless, stimulation of anti-tumor T cell response has been shown to be a promising strategy to counteract tumor growth and metastasis in mouse tumor models and in human cancer [245–247].

B lymphocytes are antigen-presenting cells which can differentiate into antibody-producing plasma cells. Thereby they can stimulate both cytotoxic and humoral anti-tumor immune responses. On the other hand, there is mounting evidence that B cells can also promote tumor cell invasiveness and metastasis. In one study, tumor cells producing antigenic Secreted/shed Tumor Glycoproteins (STGPs) were transplanted into immune-competent mice or mice deficient in T cells [248]. In both mouse lines, increased tumor cell invasion correlated with an increase in serum anti-STGP IgG levels and tumoral infiltration of cells of the innate and adaptive immune system. Experimental infiltration of specific anti-

STGP-IgG-secreting plasmacytoma cells further promoted tumor invasion, angiogenesis and metastasis. Apparently, the humoral anti-STGP response stimulated the tumor infiltrating stromal cells to release proinflammatory cytokines and VEGF, thereby promoting invasion and metastasis.

Recent work with a transgenic mouse model of multi-stage skin carcinogenesis (K14-HPV16) demonstrated a similar surprising pro-tumorigenic role of B but not T lymphocytes in the development of inflammation-associated de novo epithelial cancer. Upon crossing K14-HPV16 mice with T and B lymphocyte-deficient RAG^{-/-} mice resulted in reduced tumor progression [249]. Conversely, adoptive transfer of B lymphocytes or serum from K14-HPV16 mice restored the infiltration of innate immune cells into premalignant tissue and the progression to malignancy. These studies indicate that B cells and even antibodies produced by B cells contribute to tumor progression and metastasis. Of course, these surprising results raise an important caveat for the application of vaccination-based cancer therapies that aim to stimulate B cell responses in patients with pre-malignant disease.

Conclusions

Apparently, cancer needs to be treated at an early tumor stage or grade in order to prevent the formation of metastases. Nevertheless, even when cancer may have already spread throughout the body, targeting the growth of primary and secondary tumors as well as further tumor cell spread may significantly improve quality of life and overall survival of patients. Although ‘curing cancer’ may be an unrealistic notion in advanced-stage tumors, a significant fraction of patients may nevertheless profit from a cocktail of drugs inhibiting tumor progression at different levels. Most of the experimental results presented here highlight many pathways and mechanisms that may be appropriate targets for the development of such therapeutic interventions. Prevention of further metastatic dissemination, in addition to the reduction of tumor load, is certainly a prerequisite to convert a rapidly fatal disease into a chronic illness.

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