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Drug Development for Metastasis Prevention

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Abstract

Metastatic disease is responsible for 90% of death from solid tumors. However, only a minority of metastasis-specific targets has been exploited therapeutically, and effective prevention and suppression of metastatic disease is still an elusive goal. In this review, we will first summarize the current state of knowledge about the molecular features of disease, with particular focus on steps and targets potentially amenable to therapeutic intervention. We will then discuss the reasons underlying the paucity of metastatic drugs in the current oncological arsenal and potential ways to overcome this therapeutic gap. We reason that the discovery of novel promising targets, an increased understanding of the molecular features of the disease, the effect of disruptive technologies and a shift in the current pre-clinical and clinical settings have the potential to create more successful drug development endeavors.

Keywords

EMT; MET; metastasis; cancer; tumorigenesis; prevention; drug development

Introduction

Metastasis is defined as the dissemination of transformed cells from their site of origin to distant sites, where these transformed cells can eventually lead to growth of secondary tumor colonies. A substantial therapeutic effort in oncology has been focused on halting cancer growth. However, more than 90% of death from solid tumors is due to metastasis, rather than to the primary tumor [1]. Several factors have led to the paucity of therapeutic options that specifically target metastasis. First, metastatic spread is a multi-step and multi-factorial process in which many of the potential molecular targets remain to be defined. Second, the majority of the metastasis-specific targets identified to date act as suppressors, thus drug development efforts are faced with the challenging task of activating suppressors, rather than inhibiting overactive effectors. Third, the development of drugs targeting metastatic disease requires preclinical models that address the metastatic process rather than the standard models that are predominately based on primary tumor growth. Fourth, the paradigm that underlies most clinical trials is currently focused on growth inhibition; trial design for metastasis prevention in many clinical settings is often challenging to due to both the

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number of patients required and increased study duration. Despite these challenges, the increasing understanding of the metastatic process and the existence of metastatic molecular targets largely shared across multiple cancer subtypes lend optimism to more successful drug development endeavors.

Steps of the metastatic cascade

The long-standing view of metastasis as the consequence of late-stage tumor shedding has been challenged in some instances by evidence supporting early dissemination and the proposal of a model of parallel progression for metastasis. In the prototypical model of cancer dissemination, based on the initial description by Leslie Foulds [2], metastasis arise at late stages of primary tumor development, following a stepwise linear progression of morphological changes. In this scenario, metastasis occurrence depends on the formation of a fully malignant tumor of significant size. Conversely, the parallel progression model postulates the early dissemination of cells from tumors at initial stages of development, possibly even before malignant conversion, and the development of some of these cells into secondary tumor masses. Both models are supported by histological, ontogenic, genetic and molecular evidence [3], thus it is possible that both mechanisms can contribute to metastasis formation.

Irrespective of the process that takes place, hematogenous metastasis can be summarized into six major steps, collectively known as the metastatic cascade: mobilization, invasion, intravasation, transit within the vasculature and arrest, extravasation and colonization (figure 1).

Mobilization

Cell-cell adhesion is a key factor in maintaining a compact primary tumor mass. Structures such as adherens junctions (including desmosomes), gap junctions and tight junctions all contribute to epithelial tissue cohesiveness. In the primary tumor, alterations of these cohesion structures lead to increased potential for cell detachment and dissemination [4]. An expanding body of evidence supports a role for Epithelial-Mesenchymal Transition (EMT) in the steps that allow transformed cells to actively detach and migrate from the primary tumor. Importantly, several studies suggest that EMT, by triggering the acquisition of stem cell properties in cancer cells, would facilitate dissemination and metastasis [5,6].

EMT, originally described as a feature of morphogenesis, is the process whereby polarized epithelial cells, closely connected and adherent to the basal lamina, assume mesenchymal properties, including lack of polarity and diminished cell-cell adhesion. Key molecular features of EMT are the down-regulation of the transmembrane adhesion protein E-Cadherin (a master regulator of EMT), claudins and ZO-1 and increase in mesenchymal markers such as N-cadherin, vimentin and fibronectin, in addition to a series of cytoscheletal reorganization events [7]. Although the relevance for EMT in the metastatic process has been long debated, recent work in colorectal carcinoma with a budding invasion phenotype has provided morphological evidence supporting this involvement [8]. One of the reasons justifying the caution surrounding the relationship between EMT and metastasis is the heterogeneity of the primary tumor population, in which cells may be at different stages of

EMT, and only a fraction of cells that have fully undergone EMT will metastasize successfully.

During embryogenesis, EMT is driven by up-regulation of several transcription factors, including: 1) the Snail zinc-finger family members SNAI1/Snail1 and SLUG/SNAI2; 2) the zinc-finger E-box-binding homeobox family proteins ZEB1 and ZEB2 (SIP1); 3) the basic helix–loop–helix (bHLH) family of transcription factors TWIST1, TWIST2 and E12/E47 [9,10]. All these transcription factors have been associated with repression of the adhesion protein E-Cadherin (CDH1), a key event in metastasis [11–19].

Multiple mechanisms act in concert to down-regulate CDH1, including direct and indirect repression of the CDH1 promoter and additional modulatory effects by miRNAs [7]. Snail, ZEB and bHLH factors, regulated at a transcriptional and at a post-translational level, have been shown to be key regulators of this process, as they bind directly to E-box consensus sequences in the E-Cadherin promoter, activity facilitated by local chromatin remodeling [11,20–23]. Complex mechanisms of regulation have emerged from the study of these factors. For example, in the case of SNAI1, its subcellular localization is modulated by Pak1-dependent phosphorylation [24,25], whereas interaction with Glycogen synthase kinase 3ß (GSK3ß) and Lysyl-oxidase-like 2 and 3 (LOXL2 and LOXL3) are determinants for Snail1 stability [26,27]. Importantly, LOX family members have been shown to play a part in the mediation of the metastatic and pre-metastatic niches [28]. Upstream, recent studies have revealed an intricate cross-talk between TGFß, RTKs-Ras, Notch, hedgehog and Wnt / ß-catenin pathways (reviewed in [29]. Further modulation is provided by the activity of the high-mobility group protein HMGA2, by prostaglandin E2 (PGE2) [30,31], and by a series of autocrine and paracrine signals [23,32]. Snail1 can also bind and repress its own promoter, providing an additional layer of auto-regulatory complexity to this network [33]. While some of the above signals appear to be common regulators for Snail, ZEB and bHLH factors, others seem to be specific. Further to E-Cadherin, the regulation of other structural proteins involved in cell-cell adhesion is altered during the execution of the EMT program. This list includes the claudin family of proteins associated with tight junction formation [4].

A plethora of other downstream effectors are targeted by Snail, ZEB and bHLH (reviewed in [23], which effectively act as triggers for the EMT program. In the context of EMT in carcinogenesis, this activity is confirmed by substantial evidence showing the overexpression of SNAI1, SNAI2, ZEB1, ZEB2 and TWIST1 in a variety of human tumors including breast, ovarian, colon, lung, gastric, hepatic and skin; in the majority of these cancer types, the overexpression of the above CDH1 repression factors is generally associated with invasive phenotype, secondary metastasis and poor prognosis [23].

Another feature that appears to be relevant for tumor progression and metastasis is the pro-survival and anti-apoptotic properties of SNAI1, SNAI2, TWIST and E47 [34–39]. These pro-survival features appear to be also relevant for resistance to anoikis, a form of programmed cell death that transformed cells need to overcome in order to survive detachment from the extracellular matrix (ECM) [40]. Thus, the role of EMT appears not be confined to conferring migratory and invasive properties to tumor cells. Several studies support a role for EMT in maintenance of stem cell features, anti-apoptotic and anti-senescence properties, in the

suppression of immune reactions and in acquired resistance against chemotherapy and radiotherapy [7].

Invasion

The basement membrane, a type of extracellular matrix (ECM), is a dense matrix of tissue which separates the epithelium from stroma and interstitial matrix. The basement membrane consists of proteins such as laminin, collagen IV and heparin sulphate proteoglycans. Broadly speaking, matrix metalloproteinases (MMPs) are the predominant factors responsible for EMC degradation, enabling tumor invasion [41].

In recent years, a complex dynamic interplay between tumor cells and ECM has emerged. While transformed cells need to degrade the ECM surrounding them in order to disrupt the basement membrane and ultimately invade neighboring tissues, the ECM orientation, organization and chemical modification has been shown to act as a facilitator, as well as a hurdle, in this process [42]. Evidence for tumor-driven ECM degradation has been found, for instance, with the E-cadherin repressors Snail1 and Slug –dependent up-regulation of MMP-2 and MMP-9 [43]. Furthermore, increased expression of the collagen receptor discoidin domain-containing receptor 2 (DDR2), observed in invasive breast carcinoma, stabilizes Snail1, which in turns promotes invasion and migration through collagen I-enriched matrices [44].

Despite being recognized as a key step in the early stages of metastatic spread, evidence suggests that EMT is not the only mechanism that enables tumor cell migration and invasion. Single amoeboid invasion, collective passive invasion, collective to amoeboid transition (CAT) and mesenchymal to amoeboid transition (MAT) have been observed in several human cancer types [45,46], depicting a scenario where cancer cell mobilization and invasion are plastic and dynamic mechanisms, likely dependent on tumor localization and interplay with the surrounding microenvironmental conditions. Another important aspect of cell motility and invasion is represented by Rho proteins and effectors like the Rho-associated protein kinases ROCKs. These are key regulators of actin cytoskeleton movement, required for cell contraction and enhanced cell motility during invasion [47]. Additionally, tumor cells have been shown to recruit macrophages through secretion of chemoattractants such as colony-stimulating factor-1 (CSF-1). Tumor-associated macrophages play a direct role in facilitating ECM remodeling, invasion and intravasation [48].

Intravasation

Although the hematogenous spread via the vascular space has been considered as the main route for cancer cell dissemination, increasing evidence supports an important role for lymphangiogenesis and the lymphatic system as an alternative vehicle for disseminating cancer cells, at least for some solid tumor types [49].

Although it is generally assumed that cancer cells transmigrate through blood and lymphatic vessels by active migratory mechanisms, evidence supports the notion that passive and coincidental intravasation is a common occurrence and contributes to the entry of tumor cells into the vasculature [50]. The poorly organized and highly permeable nature of blood

vessels stimulated by angiogenesis around the tumor might allow this passive modality of transmigration [51].

Another area of active investigation surrounds the involvement of cytokines and chemokines that promote cell intravasation. While transforming growth factor-ß (TGFß) and vascular endothelial growth factor (VEGF) released by tumor cells increase entry into vasculature and favor metastasis, tumor-associated macrophages appear to play an equally important role by secreting epidermal growth factor (EGF) and tumor necrosis factor 1α (TNF1α), which further facilitate the transmigration process [52]. Several other factors are shared between the invasion and the transmigration processes, primarily with the involvement of metalloproteinases such as metalloproteinase 1 (MMP1) and membrane-type-4 matrix metalloproteinase (MT4MMP, also known as MMP17), secreted by cancer cells and favoring disruption of cell-cell junctions in the endothelial barrier [52–54]. Intriguingly, factors expressed on the surface of tumor endothelial vasculature such as a disintegrin and metalloproteinase 12 (ADAM12) additionally contribute to disruption of endothelial junctions and favor tumor cell intravasation [55].

Transit within the vasculature and arrest

Once in the vasculature, tumor cells interact with blood and immune cells such as endothelial cells, platelets, mast cells, lymphocytes, macrophages, and progenitor cells derived from bone marrow. Amongst all these cell types, the interaction between cancer cells and platelets appears to be particularly significant. Seminal work by Trousseau in 1865 [56] led to the notion that alterations in the blood coagulation system support metastatic cancer progression [57]. Following activation by thrombin, platelets attach to injured vessels and trigger downstream coagulation processes [58]. As the vasculature represents a hostile environment for circulating cancer cells, activated platelets appear to play an essential role in survival of hematogenous metastatic cells. In the current model, following intravasation, tumor cells activate and bind platelets, which favor immune evasion and ultimately tumor cell adhesion to blood vessels and extravasation towards secondary sites. However, a complex interplay is emerging between the coagulation system and tumor cells in which the role for platelet support of tumor metastasis seems to extend to earlier stages of tumor progression such as vasculature remodeling, angiogenesis and maintenance of tumor vasculature integrity [58].

The formation of hetero-aggregates involving tumor cells, platelets and leukocytes is suggested to promote the initial adherence of metastatic cells to the endothelium, similarly to the “rolling and tethering” scenario observed during leukocyte recruitment in inflammatory processes. The interaction between the hetero-aggregate and the endothelium is enabled by a class of membrane proteins referred to as selectins [59]. Firm arrest of the hetero-aggregate to the endothelium is mediated by platelet transmembrane integrins (primarily integrin αIIbß3), CD44 and MUC1 [52,58].

Adhesion is not the only mechanism responsible for the arrest of cancer cells in the capillary beds: physical entrapment has been shown to play a part, for instance, in murine models of melanoma and colon carcinoma [60,61].

Extravasation

The role for platelets and macrophages in supporting metastatic cells could extend to promoting tumor cell extravasation by modulation of vasculature permeability, a process that physiologically helps immune cells to reach sites of inflammation. This process could be influenced, directly or indirectly, by release of factors such as platelet-derived growth factor (PDGF), TGFß, EGF, insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) [52,58].

A role for chemokines has been shown in the regulation of metastatic cell extravasation. In this context, the complementarity between chemokines and their receptor expression plays a part in the clinically observed organ-specific colonization of distant sites [62,63]. In particular, the CXC-chemokine ligand 12 (CXCL12) has been frequently implicated in attracting cancer cells expressing its receptors C-X-C chemokine receptor type 4 (CXCR4) in promoting retention of those tumor cells at specific secondary sites [62].

Chemokines produced by cancer cells have been shown to promote extravasation and to attract leukocytes, which facilitate immune evasion [52]. In addition, neutrophils, monocytes and macrophages play a direct role in metastatic cell extravasation. For instance, neutrophils have been shown to potentiate the attachment of melanoma, breast and lung cancer cells to the endothelium, whereas monocytes are recruited by breast cancer cells secreting the chemokine CCL2 (C-C motif ligand 2) to induce VEGF-dependent promotion of extravasation [52,64,65].

Mechanistic details are currently lacking regarding the steps following cancer cells crossing of the endothelial cell barrier and the invasion of the basement membrane surrounding the vasculature.

Of note, extravasation is not a mandatory step for all cancer types prior to colonization of the secondary site, as several cancer cell lines have been shown to start proliferating within the vasculature lumen before extravasation [52,66].

Colonization

Intravasation, transit through vasculature and extravasation represent extremely stressful steps for invading cancer cells. Early studies have shown that an estimated 0.01% of the millions of cells that are released every day from a fully formed tumor are able to survive and form metastatic colonies [50,52]. Mechanical shear stress and cell-mediated cytotoxicity are likely to be responsible for the majority of tumor cell death. Tumor cells that survive extravasation can find a challenging environment at the secondary sites that can often lead to further cell death. A considerable amount of evidence indicates that the tumor-host interaction is a key determinant of survival and proliferation of metastasizing cancer cells. In particular, the interaction of tumor cells with the ECM and with the host stromal cells in the secondary site, as well as effective vascularization and escape from immune surveillance, determine whether tumor cells will encounter death, go into dormancy or proliferate into macrometastases [66]. The concept of the complex crosstalk between cancer cells and host microenvironment was proposed in the early years of cancer research in the “seed and soil”

hypothesis by Stephen Paget, in which the cancer cells (the “seeds”) need a suitable recipient (the “soil”) in order to proliferate [67].

Tumor cell integrins and surface receptors such as CD44 mediate the interaction with ECM components in the secondary site. The contribution of growth factors such as TGF-ß, bone morphogenic proteins (BMPs) and VEGF, present in the ECM, is also likely to influence the survival and the progression of metastasizing cells [66]. Other factors such as the interaction with immune cells such as macrophages, efficient angiogenesis supporting the metastasis survival and the interaction with particular cell types have a major impact in determining the next steps following early colonization [68]. Primary tumors seem to further contribute to this scenario by directly or indirectly priming host sites for the favorable receipt of metastasizing cells, a concept that is referred to as the formation of “pre-metastatic niches” [66].

Once in the parenchyma, these tumor cells can grow into full overt metastases. However, an increasing body of literature supports the notion that tumor cells that have colonized a secondary site (often called disseminated tumor cells, DTCs) often enter a long-lasting state of dormancy, which might be the underlying cause of tumor reoccurrence.

Tumor dormancy and reactivation

Despite the potential relevance of this process for tumor reoccurrence after surgical resection of the primary tumor, this aspect of oncological research has been relatively understudied, partly due to lack of relevant models. Tumor dormancy was first proposed by Rupert Willis in 1934 as a state of tumor growth arrest, later redefined by Geoffrey Hadfield as ‘temporary mitotic arrest’ [69,70]. It should be noted that primary tumor dormancy and metastatic dormancy are thought to be mechanistically distinct processes: while the former is considered as a lag time necessary for the newly formed neoplastic cells to bypass apoptosis or senescence, the latter is the delay in growth due to adaptation to the new microenvironment [5].

Metastatic dormancy can be classified according to the signals responsible for antagonizing cell proliferation: cellular dormancy (due to intrinsic and extrinsic cellular signals that lead to G0-G1 arrest), angiogenic dormancy (due to limited vascularization) or immune-mediated dormancy (due to immune-mediated cytotoxicity)[71]. During dormancy, enhanced survival signals appear to be a pre-requisite, for example in the form of paracrine interactions with the tumor microenvironment that lead to enhanced Akt signaling via the Src [72] or VCAM1-Ezrin [73] axes. Additionally, endogenous stress signals via the p38 mitogen-activated protein kinases (MAPK) may contribute to the same aim [5]. Conversely, the identification of metastasis-specific suppressor genes involved in inhibition of mitogenic signals and activation of stress response signaling suggests that deregulation of signals from the tumor microenvironment is partly responsible for the maintenance of tumor dormancy itself [74]. Contextual and systemic paracrine signals limiting the capacity for self-renewal in tumor cells may also play a part in keeping tumor cells dormant, as shown for bone morphogenetic proteins (BMPs) [75,76]. Thus, it appears plausible that reactivation of tumor cells from dormancy is mediated by overcoming inhibitory signals from the microenvironment. Recent studies support the importance of stem cell signals such as the tenascin C – mediated

elevation of Wnt and Notch pathways [77,78] and Periostin-driven facilitation of the Wnt pathway [79].

Even when metastasizing cells are released from the G0-G1 arrest, these cells need to be supported by increased vascularization in order to proliferate into a growing secondary tumor mass. The steps that actively induce angiogenic dormancy, and/or allow angiogenesis reactivation, are not currently clear. However, it is likely that this process parallels the so-called angiogenic switch for primary tumors [71], where a balance between pro- and anti-angiogenic factors determines whether tumor cells will be served by vascularization sufficient to sustain their growth. Local as well as systemic signals can affect angiogenic dormancy, for instance as shown by the activity of Prosaponin, VEGF and Angiopoietin 2 in prostate, lung and breast cancer respectively [5].

Immune suppression adds a further layer of complexity to the maintenance of micrometastatic lesions in a dormant state. It has been known for years that the immune system has a role in controlling tumor growth, a property that is being successfully exploited with the recent success with immune therapies which elicit immune responses against tumor cells [80–83]. Studies confirm that tumor cells that escape immune-mediated cytolysis are kept in a dormant state by the immune system [84–86].

The involvement of MET in tumor reactivation

The postulated role for Mesenchymal-Epithelial Transition (MET), the reverse process of EMT, as a pre-requisite for metastatic colonization has received so far only limited experimental support [87,88], and it is not clear whether MET precedes or follows reactivation. The requirement for cancer cell proliferation in the colonized niche would suggest that it would be advantageous for these cells to retain the enhanced self-renewal capacity that is conferred by the stem cell-like properties associated with the activity of the EMT-inducing factors Twist, Snail and ZEB1 [7]. In fact, a recent study suggested that the above states are not mutually exclusive, as the EMT status can be uncoupled from its associated stem cell properties, for instance by suppression of the EMT inducer Prrx-1, which allows retention of stem cell properties while reverting to the epithelial phenotype [89].

The current status of antimetastatic strategies

Although the metastatic cascade offers several potential drug targets in its multiple steps, the paucity of current treatments targeting metastasis is staggering. The reasons underlying this gap in the modern anti-oncological drug arsenal are multifaceted and they will be discussed in the following section.

The current section focuses on approaches at various steps of the metastatic cascade that have shown some pre-clinical and clinical efficacy (summarized in table 1). Broadly speaking, anti-metastatic approaches can be classified into three categories: 1) approaches that inhibit early steps of metastasis, thereby preventing metastatic spread (primary prevention); 2) approaches that prevent further dissemination after initial dissemination has occurred; 3) approaches that kill dormant cells or maintain them in a dormant state (secondary prevention).

Targeting EMT

The importance of migration in early steps of tumorigenesis has been recently confirmed by a spatial model for tumor evolution, which showed that short-range movement of cells within a tumor mass significantly affects tumor growth rate and drug resistance. [90] Because of its involvement at the very early steps of cell mobilization in the metastatic cascade, EMT is considered a particularly promising target as adjuvant therapy with the objective of preventing early metastatic spread or drug resistance in patients with no pre-existing metastases [91]. The first approach could be to target intracellular signals triggering the EMT cascade. Despite being considered challenging targets, the development of inhibitors of transcription factors such as STAT3 [92–94] encourages similar efforts to target Snail, ZEB and bHLH EMT-inducing transcription factors directly. Because it can be anticipated that the success of these approaches may be thwarted by signal transduction pathway redundancy, which could lead to rapid resistance, combined therapies could represent a preferred therapeutic route. Alternative indirect approaches to target EMT-inducing factors like SNAI1 have been suggested through the use of inhibitors of the nuclear exporter CRM1/XPO1 [95]. A second approach is represented by targeting mesenchymal markers such as vimentin, N-cadherin and fibronectin, the expression of which increases after EMT induction has taken place [7]. This strategy could be useful also to target more advanced stage of the metastatic disease. Compounds such as Whitaferin-A have been shown to induce the degradation of vimentin and to inhibit cell migration, invasion and metastasis formation in *in vitro* and *in vivo* models of breast cancer [96], proving the feasibility of this approach. Another promising example of therapeutic targeting of mesenchymal markers was shown by the use of an N-cadherin antibody inhibiting prostate cancer growth and metastasis *in vitro* and *in vivo* [97]. Finally, a fourth approach could be aimed at maintaining the mesenchymal phenotype, for instance as shown by targeting of the Axl kinase with the small molecule inhibitor BGB324 [98].

A broader approach is represented by targeting factors acting as upstream modulators of the EMT program. Complex networks of paracrine signals mediated by the microenvironment and affecting EMT have been identified [32,99–102]; some of these factors have already been explored as potential therapeutic targets. The inhibition of several receptor tyrosine kinases (RTKs) for growth factors showed effects on EMT, such as the partial EMT inhibition shown for the epidermal growth factor receptor (EGFR) inhibitors gefitinib and AG1478 [103,104], while EMT status is a determinant of sensitivity to erlotinib [101,105]. Other RTK targets that have been explored include the HGF receptor / c-Met [106–108] and PDGF receptor [109]. The TGFß / SMAD axis has also been considered as a target, showing that inhibiting TGFß signaling attenuates migratory properties in several models of human cancer [110,111]. Antibodies directed against TGFß such as Fresolumimab / GC1008 are currently tested in clinical trials for oncological indications [112].

As Wnt, Notch and Hedgehog signaling cascades are implicated in regulatory networks of physiological EMT [113], it is not surprising that interference with these pathways is being explored as a therapeutic avenue for cancer treatment. For example, Wnt signaling factors WNT1, CTNNB1 or AXIN2 induce EMT in breast cancer cells, whereas other factors like WNT5A, SFRP3 and dickkopf 1 homologue (DKK1) have suppressive effects on EMT-

associated markers [114–116]. However, the possibility of targeting Wnt signaling to inhibit EMT might be partially hampered by positive feedback loops such as the one observed for WNT5A, the suppression of which leads to decreased motility in epidermal carcinoma cells [117]. Notch signaling has been shown to directly and indirectly regulate SNAI1 expression [113]. Accordingly, Notch inhibition has been successfully employed in pre-clinical studies where it was shown to reverse EMT in lung cancer [118] and inhibit metastasis associated with upregulated Snail signaling in breast and liver cancer models [119,120]. Members of the Hedgehog signaling pathway promote the transcription of EMT factors such as SNAI1 [121]. The disruption of the Hedgehog factor Shh with cyclopamine, for instance, has been shown to inhibit EMT in several models of cancer cells, including colon and pancreas [122,123]. Targeting the Wnt, Notch or Hedgehog pathways may represent a successful strategy that could address the dynamic nature of the paracrine EMT regulation in metastatic colonies. However, considering the complexity of these regulatory networks, further studies are needed to define the most effective targets able to elicit a consistent and durable decrease in EMT signaling.

Targeting motility, invasion and altered cell adhesion

Based on substantial preclinical data, matrix metalloproteinase inhibitors had been hailed amongst the most promising approaches in targeting metastasis. However, more than 50 MMP inhibitors have failed to show any efficacy in clinical trials to date [124]. Several reasons can be imputed to this failure, including lack of precise understanding of the complex biology of these proteases. For instance, it is now known that MMP inhibition can cause a switch to amoeboid motility [125]; additionally, many MMPs can have opposing effects that can ultimately have an impact on many aspects of tumor progression in an undesirable manner [124]. Despite this, new approaches for drug optimization and a deeper understanding of the biology underlying MMP structures and functions may eventually lead to a new, more effective generation of inhibitors, which may include highly specific monoclonal antibodies as catalytic domain inhibitors [126].

The urokinase-type plasminogen activator (uPA) and its receptor uPAR seem to be particularly promising targets. uPAR has pleiotropic functions that bear relevance for EMT initiation, tumor angiogenesis, invasion and metastasis [127]. Several inhibitors of the uPA system are currently being investigated in phase I and II clinical trials [127].

Targeting the motility regulators Rho proteins and their effectors, such as the Rho-associated protein kinases ROCKs, represents another promising approach. Elevated levels of ROCK types I and II have been found in several human cancers, including breast, osteosarcoma, liver and bladder, and are generally correlated with poor prognosis [47]. To this date, there are no clinical trials employing ROCK inhibitors, although pre-clinical data with the inhibitors Fasudil, Wf-536, Y-27632, and RKI-1447 show consistent reduction in tumor progression in murine models of liver, lung, breast cancer and myeloma [128–132].

Alternative approaches to perturb the ECM are currently being investigated. Strategies targeting integrins have been proposed, mainly based on peptidomimetics, peptide inhibitors and antibodies. Interference with αVβ3, αVβ5 and ß1 integrin activity has shown encouraging results in several types of cancer (for a complete review, see [133]. In particular,

the use of Cilengitide, an Arg-Gly-Glu(RGD)-containing pentapeptide, has shown efficacy in phase I and II clinical trials for the treatment of glioblastoma [134,135]. A recent failure in phase III for the same indication [136] has not stifled further studies and interest in this class of compounds [137].

LOX family proteins have been proposed as potential targets, encouraged by pre-clinical data showing reduction in hypoxia-induced metastasis upon their inhibition [138]. To date, the only approach that has reached the clinic is based on the monoclonal antibody Simtuzumab, targeting LOXL2 for the treatment of colorectal adenocarcinoma and metastatic pancreatic adenocarcinoma in two clinical trials currently being evaluated.

The cell-ECM receptors of the DDR family DDR1 and DDR2 can be also considered as potential targets in metastatic disease. DDRs can be targeted at the level of ECM-extracellular domain interaction, by blocking the conformational change necessary for activation, or at the level of DDR kinase activity. Several of these strategies have been explored (reviewed in [139]. To date, the identification of highly specific and effective DDR inhibitors for metastasis has not been successful, but research is ongoing.

Targeting anoikis resistance

The resistance to apoptosis triggered by cell detachment (anoikis) can be targeted by antagonizing survival signals. Considering the short time window of cell circulation into the bloodstream, anoikis resistance may be a more relevant target at early stages of the metastatic disease, prior to intravasation. Very few mechanistic details are currently known about this process and about its relevance in metastasis.

Studies show that the brain-derived neurotrophic factor (BDNF) receptor trkB has a key role in anoikis resistance [140] and has been successfully targeted by Tkb tyrosine kinase inhibitors like CEP701 and CEP2563 [141]. Other proapoptiotic strategies have involved the use of PI3K pathway inhibitors ZSTK474, PI103 and LY294002 in *in vitro* and xenograft models [142,143].

Targeting cell survival during the transit within the vasculature

Disrupting the protection against apoptosis provided by platelets has been considered a viable therapeutic option following several studies observing inhibition of metastasis upon genetic or antibody-mediated depletion of platelets in mouse models [58]. Administration of anticoagulants is commonly utilized in clinical practice to prevent and treat cancer-related venous thromboembolism and there has been some success in preclinical studies suggesting this approach for metastasis prevention. For example, entities such dipyridamole and RA-233 have been shown to prevent hepatic metastasis in nude mice [144], while the use of antibodies directed against tumor integrin αVβ3 disrupted tumor-platelet interaction, with anticancer and antiangiogenic effects [145]. The use of anticoagulants as a specific metastasis prevention strategy has so far been confined to animal models, with limited success and significant risk of bleeding complications [146]. A recent study suggested that the anti-coagulant properties of proteins such as Serpine2 and Slpi are able to promote intravasation and metastasis by preventing clotting at the vascular-extravascular interface [147]. This event might be important in vascular mimicry, a process whereby tumor cells form endothelial-like channels to bypass the requirement for true angiogenesis [148]. Thus, critical questions remain

as to whether anti-coagulant approaches should be considered for metastasis prevention, and whether this strategy could be safely used for extended treatments.

Targeting arrest and adhesion

Studies revealing the interaction between tumor hetero-aggregates and the endothelium have suggested that the disruption of this binding could prevent the next steps in the cascade. The inhibition of the interaction between P(latelet)-, E(ndothelium)- and L(eukocyte)-selectins has been successfully exploited in experimental models of metastasis and in mice [141]. Other approaches proposed include hyaluronic acid and its receptor CD44; for instance, suppressing the hyaluronan synthase with 4-methylumbelliferone suppressed liver metastases of melanoma cells in mice [149].

Targeting extravasation

The interaction between the chemokine SDF1/CXCL12 (CXC-chemokine ligand 12) and its receptor CXCR4 has been the focus of drug development efforts as HIV entry inhibitors [150]. Considering the key importance of this chemokine in determining metastatic organ tropism, some of those drugs have been tested in the context of metastatic disease, which demonstrated successful metastasis inhibition in mice models, for instance with the CXCR4 antagonists 4F-benzoyl-TE14011 and 4F-benzoyl-TN14003 [151,152]. Invasiveness and metastasis inhibition was shown also for the CXCR4 antagonist AMD3100 / plerixaflor in models of metastatic breast and pancreatic cancer, amongst others [153,154]. Several small molecules and biopharmaceutics acting on the CXCL12-CXCR4 axis have been patented, some of them currently being tested in the clinic for oncological indications from leukemia to a range of refractory metastatic solid tumors [155]. Importantly, recent studies have extended the therapeutic scope for CXCR4 antagonists as modulators of immune system responses that can lead to increased sensitivity to anti-PD-L1-based immunotherapy [156]. CXCR3 has also been recognized as an alternative potential target, as its antagonism by the small molecule inhibitor AMG478 led to suppression of lung metastasis of metastatic breast cancer in mice [157].

Targeting colonization

Recognizing the key role of tumor-host interactions in the early steps following extravasation for the successful establishment of macrometastasis [66], several different therapeutic approaches have been proposed and tested. First, the use of entities that alter the dynamic interaction between the tumor cell and the host microenvironment (discussed above for earlier steps: paracrine EMT signal disruptors, integrin antagonists, LOX and DDR inhibitors, CD44-hyaluronan interaction disruptors) may as well affect this later stage of the metastatic cascade. Second, interactions between the host organ parenchyma and tumor cells may be successfully modulated, especially when organ-specific cells are involved (such as cytokine-mediated interactions of osteoclasts or osteoblasts with breast and prostate cancer cells, respectively) [66,158]. Immune cells such as macrophages, CD8+T and NK cells represent other crucial interactors for tumor cells in the host niche. The surge and recent success of immunotherapies suggests that modulating the immune system to elicit a response leading to tumor cell dormancy or kill is a feasible strategy for metastatic disease.

Although a detailed account of immunotherapy approaches is outside the scope of this review, other recent reviews highlight the importance of this area [85,159–161].

Third, active remodeling by tumor cells appears to play another fundamental role for macrometastasis formation. Consistently, anti-angiogenic drugs have shown promising results in a metastatic context. The anti-angiogenic drug TNP-470, inhibiting endothelial cell migration and proliferation, was amongst the first drugs to show efficacy on metastasis as well as on primary tumor growth in *in vitro* and *in vivo* human cell lines [162]. To date, several drugs targeting angiogenesis have shown inhibitory effects on newly established metastatic colonies, including the VEGF / c-MET antagonists crizotinib, sunitinib and cabozantinib [163–165]. However, recent preclinical studies have reported contradictory results that suggest that anti-angiogenic therapy could in fact, in some cases, increase invasiveness and metastasis formation [166,167]. A number of plausible reasons could explain these results: 1) malignant cells display high tolerance to hypoxic conditions and vigorous aggressiveness in response to hypoxia caused by antiangiogenic therapies; 2) antiangiogenic agents might create pre-metastatic niches that are favorable to micrometastasis growth; 3) metastatic colonies might have derived from tumors resistant to antiangiogenic treatment and might be less reliant on neoangiogenesis in the metastatic niche [163]. Further studies will shed light on these mechanisms and establish optimal utilization of antiangiogenesis drugs in these settings.

Targeting the dormant niche

The persistence of disseminated tumor cells, the behavior of which is regulated at the level of their microenvironmental niche, opens promising therapeutic avenues for metastasis prevention. Two general strategies can be envisaged: DTCs can be kept dormant with chronic treatments; or, dormant cells can be eradicated with selective cytotoxic agents. Population-level dormancy (caused by angiogenic dormancy and immune-mediated dormancy, which contribute to steady-state levels of cell numbers in the metastatic colony) can be potentially modulated by several approaches, as described above. As far as cellular dormancy is concerned, it should be noted that evidence of the origin of metastases from dormant DTCs is still indirect and mostly circumstantial. Nevertheless, clinical evidence confirms that DTCs can be particularly resistant to chemotherapy, possibly due to multiple factors including: pre-existing mutations that confer resistance; quiescent cell cycling state; cellular architecture and polarity that shields DTCs from the effect of chemotherapeutics [168].

To date, some evidence supports the presence of specific cues that keep DTCs in a dormant state. Broadly speaking, proliferation/survival pathways and stress signaling pathways appear to be key determinants in this scenario, offering several potential targets with drugs that are already currently used in the clinic. For instance, reduced uPAR levels induced a prolonged state of cell dormancy *in vivo* via an integrin and MAPK signaling – dependent mechanism [169]. Several reports confirm the importance of the suppressing the MAPK pathway for maintaining the dormant state [168], offering several potential targets such as integrins, EGFR, matrix metalloproteinases and PI3K. More recently, targeting members of the Src pathway has shown encouraging results [170]. Another strategy could be to induce or

sustain the p38 stress pathway, known to induce a state of dormancy in a range of tumors [171,172].

The alternative approach would be to selectively kill dormant DTCs, possibly after their mobilization and/or awakening. The treatment of patients with refractory acute myeloid leukemia (AML) offers a possible paradigm for this option. Treatment-resistant leukemic stem cells have been mobilized from osteoblastic niches and perivascular niches in bone marrow with priming agents such as Granulocyte-colony stimulating factor (G-CSF) and CXCR4, CD44 and integrin antagonists. Combination treatment with cytotoxic agents have led to successful disease remission in the clinic and in preclinical models [173–175]. Whether such an approach would awaken dormant DTCs, and whether targets could be found that prevent this from happening, are still open questions. Further strategies could be explored to kill mobilized DTCs by exploiting vulnerabilities before they can induce further tumor spreading.

Finally, as suggested above, targeting the mesenchymal-epithelial transition might be a successful means to keep DTCs in a dormant state [91]. However, this therapeutic avenue remains at the moment only theoretical, until more studies elucidate the requirement and the timing for MET in DTC awakening.

Targeting metastasis suppressors

Over the past 25 years, more than 23 genes that play a part specifically in suppressing metastasis, without altering the primary tumor, have been identified in a wide range of malignancies. These metastasis suppressor genes (MSGs) affect a breadth of metastasis-related processes such as EMT, invasion and anoikis resistance [176]. The prognostic value of these genes in the management of the metastatic disease is obvious and has been investigated in many studies; the significance of some of the most promising MSGs with prognostic value can be found in [177]. Brief accounts of two MSGs with significant translational relevance are presented below as examples.

The first-identified MSG, and the most studied, is *NME1*, coding for the nucleoside diphosphate and histidine protein kinase NM23. Increasing NME1 transcription with pharmacologic doses of methoxyprogesterone acetate (MPA) led to significant reduction of soft agar colonization of metastatic breast cancer cell lines [178]. *In vivo* administration of MPA resulted in reduction of pulmonary metastases in a murine model [179]. Despite a clinical trial that was terminated in 2011 (NCT00577122) without showing clinical benefit for MPA administration, further studies are exploring alternative means to restore metastasis-suppressing levels of NM23. One possible avenue that has been proposed is to antagonize the type 1 lysophosphatidic acid receptor (LPA1), the expression of which negatively correlates with NM23 levels [180]. Functional suppression of LPA1 led to reduction of bone metastasis progression in mice [181]. Rho GDP dissociation inhibitor ß (RHOGDI2) was found as a metastasis suppressor gene in bladder cancer [182,183]. As RHOGDI2 inhibits endothelin 1 (ET1), administration of the ET1 antagonist atrasentan showed dramatic reduction of lung metastasis formation in mice injected with metastatic bladder carcinoma cells [184]. Other MSGs that have been explored in preclinical studies are reported in detail in [176]

Despite the great potential for targeting MSGs, the impact of this approach in the clinic has been very limited so far. Probably the major obstacle to overcome is the restoration of the levels of a suppressed gene product, a task that has been historically difficult to achieve. However, targeting compensatory pathways, employing novel biotherapeutical approaches or modulating epigenetic regulation of gene expression could provide significant progress and successful outcomes in this clinical space. Clinical attempts such as the restoration of wild-type p53 in lung cancer patients via gene therapy [185] raise the possibility that future investigations may eventually lead to the development of delivery systems capable of inducing MSGs.

Obstacles in the current metastatic drug development space

Several reasons underlie the paucity of effective treatments in the prevention of metastatic disease in the clinical setting. The complexity of the disease process plays a major role in this therapeutic gap. In addition, systematic obstacles are recognized as serious impediments towards more successful translational efforts.

Limitations in the current metastasis preclinical in vivo murine models

The majority of preclinical models has so far focused on short-term reduction of primary tumor size, despite a number of preclinical studies reported that many drugs have differential effects on primary tumor versus metastatic disease [186]. Furthermore, the majority of our preclinical data for metastatic studies relies heavily on murine models, although their predictive power is often diminished by significant caveats.

Experimental murine models, in which metastatic cells are injected directly into the blood flow, offer several advantages in terms of versatility and reproducibility, but their capacity to recapitulate the early steps of the metastatic cascade is questionable [141].

Spontaneous models, in which murine (syngeneic) or human tumors are implanted orthotopically in mice, can recapitulate the disease in a satisfactory way. In these models, the use of metastases from patients instead of cell lines may add value. Because at least some of the metastatic features of established cancer cell lines may be lost in culture due to genetic drift or epigenetic plasticity, the direct xenotransplantation of metastatic cells into animal models may give answers that have more clinical relevance in a metastatic setting. Patient-derived xenografts (PDXs) and patient-derived orthotopic xenografts (PDOXs) seem to be generally robust and predictive models of metastasis [187], although interspecies differences in tumor and stroma need to be taken into account. Increased access to and use of metastatic material from patients, for example by extending the reach of rapid autopsy programs [188], could lead to more accurate and predictive models.

Transgenic mice, where specific genes are modified with recombinant DNA technologies, may recapitulate the metastatic disease in a more accurate way.

Another significant limitation of each one of the above model systems is that they typically recapitulate only some stages of the metastatic disease, rendering fully comprehensive studies challenging. Finally, most of the current preclinical models fail to address the

question of whether the development and the treatment of metastases could be altered in the absence of the primary tumor, i.e. mimicking the adjuvant setting.

Limitations in the current clinical setting

Clinical trials for agents directed at metastasis prevention are difficult to design and conduct given the required study duration and number of patients required. Also, because inhibitors of metastasis are not necessarily intended to be cytotoxic or to effectively synergize with traditional chemotherapy regimens, usual study endpoints may not be met for agents designed for metastasis prevention. Thus, there has been a call for new trial designs to address these problems [189]. In this type of metastatic setting, it has been proposed that time to first metastases (primary prevention trials) or time to new metastases (secondary prevention trials) would be more relevant endpoints than tumor shrinkage [189]. Another requirement for these settings would be the implementation of successive biopsies and advanced imaging probes to accurately monitor disease progression [190].

Challenges for dormancy maintenance therapies

Amongst all the therapeutic options for metastasis prevention, therapies for chronic maintenance of the dormant niche face the most significant challenges. Similarly to the case of antiretroviral therapy for HIV, these therapies would reduce metastatic disease to minimal residual disease, without achieving full eradication. It has been suggested that the HIV paradigm would argue against a similar avenue in oncology [168].

A chronic regimen would require preceding, potentially expensive, clinical trials that should assess the maximum tolerated chronic dose over the course of several years of treatment. One potential drawback of this strategy could be that local imbalance of paracrine factors (for example due to inflammatory processes) could lead to reactivation of DTCs and overcoming of drug efficacy [168].

Finally, patient compliance and adherence to chronic therapies could pose significant challenges to determining true efficacy.

Future perspectives

More than 60 years after Leslie Foulds' initial description of the steps involved in metastasis, limited effective therapeutic options are available for patients that focus on prevention or inhibition of the metastatic process. However, the dramatic progress seen in oncological research in recent decades lends optimism to a future where anti-metastatic drugs will be part of the therapeutic arsenal against cancer. As seen above, the metastatic process is extremely inefficient, due to the intensity of the stress that metastasizing cells are exposed to, as well as to healthy tissues being refractory to invading cancer cells. Thus, ultimately, successful metastatic therapies will be the ones that exploit the steps in the cascade which offer the highest vulnerability; the aim for future metastasis research will be to find the most targetable "Achilles heel(s)" of the cascade.

The prospect of determining biomarkers for metastatic potential that can guide drug development in chemoprevention is particularly intriguing. The discovery of

polymorphisms, genetic mutations (either germline or acquired) and plastic epigenetic alterations that determine susceptibility to metastasis (as opposed to metastasis-specific mediators) will be a required achievement for therapeutic endeavors that aim at preventing metastatic spread in neoadjuvant as well as adjuvant settings. These biomarkers may be dependent on mutational or gene expression profiles as well as microenvironmental factors within each malignancy, thus requiring a deeper understanding of the biology of the complex interplay between tumors and their surrounding stroma, especially of interactions driven by plastic mechanisms. In addition to epigenetic-driven plasticity, an increasing body of evidence points at the key role of microRNAs (miRNAs) in modulating malignant transformation and metastasis. For example, the regulation of EMT by the miRNA-200 family members and their role in suppressing metastasis is perhaps the most prominent example [191,192]. The autocrine TGF-ß/ZEB/miR-200 regulatory network has been shown to regulate the plasticity of the transition between epithelial and mesenchymal states [193], but it is clear that this axis represents only a limited portion of a much more extensive network including the possible activity of modulatory elements such as the recently discovered competitive endogenous RNAs (ceRNAs) [194]. It can be anticipated that more advanced network-based approaches will be essential for unraveling the complexity of this crosstalk and guide towards the therapeutic use of these biological entities [194]. In fact, despite the considerable challenges in delivering miRNAs to the target sites, two clinical trials are currently ongoing [195]. miRNAs represent particularly attractive therapeutics, due to their specific while pleiotropic effects on multiple metastasis-related traits [192].

Exploring novel therapeutic avenues can extend to after metastatic spread has occurred. Appreciation of the impact that systemic modulation by primary tumors has on priming future metastatic niches and on influencing the growth of established metastases will likely bear consequences in choosing the best therapeutic options, either as single agents or in combination, prior to and following resection of the primary tumor. As discussed above, the maintenance of metastatic cells in a dormant state is likely to face considerable challenges. Non-genetic mechanisms are likely to play a particularly prominent role in the awakening of dormant cells, as they are arrested in their cell cycle progression [5]. Thus, further unraveling of molecular details underlying these mechanisms may open effective and sustainable therapeutic avenues that aim to convert metastatic disease into a chronic state.

Changes in the current clinical trial setting are warranted, if true metastatic drugs are to be tested in a satisfactory and effective manner. With an increasing body of evidence supporting the case for true antimetastatic therapies, a paradigm shift may be necessary. In these restructured clinical settings, the implementation of advanced imaging techniques will provide an essential tool to monitor disease progression and inform therapeutic choices. The recent advances seen in single-cell high resolution fluorescent imaging and computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) imaging recording is, in this respect, very promising [196]. Other advances in diagnosis can provide further support to these clinical efforts. In particular, the development of robust assays for the detection, collection, and analysis of circulating tumor cells (CTCs) may lead to minimally invasive and highly dynamic monitoring of genomic changes at a single-cell level. Applications of this technique can be envisioned at different stages of the clinical path,

from detection of minimal residual disease through to monitoring of metastatic development (CTCs used as biomarkers) and new target identification in response to arisen resistance [45].

Finally, it cannot be excluded that emerging technologies will be able to change the landscape of metastasis prevention and treatment. Clustered regularly interspaced short palindromic repeat (CRISPR) / Cas9 – based genome editing and immunotherapy approaches have recently entered preclinical and clinical settings, respectively, with disruptive force. The application of the CRISPR/Cas9 system in a genome-wide screen in a metastasis murine model led to the discovery of loss-of-function mutations that drive tumor growth and metastasis [197]. In vivo applications of this powerful genome editing technique might, for example, determine new avenues for restoration of metastasis suppressor genes, or for resensitization against acquired resistance. Immunotherapies, in particular immune checkpoint therapy, have already proven to be successful in treating numerous tumor types [161], thus the application of this technique for eradication of metastatic cells kept in immune-mediated dormancy, or in combination might be brought to bear on this problem.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

EMT	Epithelial-mesenchymal transition
LOX(L)	Lysyl-oxidase(-like)
MET	Mesenchymal-epithelial Transition
bHLH	Basic helix–loop–helix
CDH1	E-Cadherin
ECM	Extracellular matrix
MMP	Metalloproteinase
DDR	Discoidin domain-containing receptor
TGFß	Transforming growth factor-ß
VEGF	Vascular endothelial growth factor
DTCs	Disseminated tumor cells
uPA/uPAR	Urokinase-type plasminogen activator and receptor

MSGs Metastasis suppressor genes

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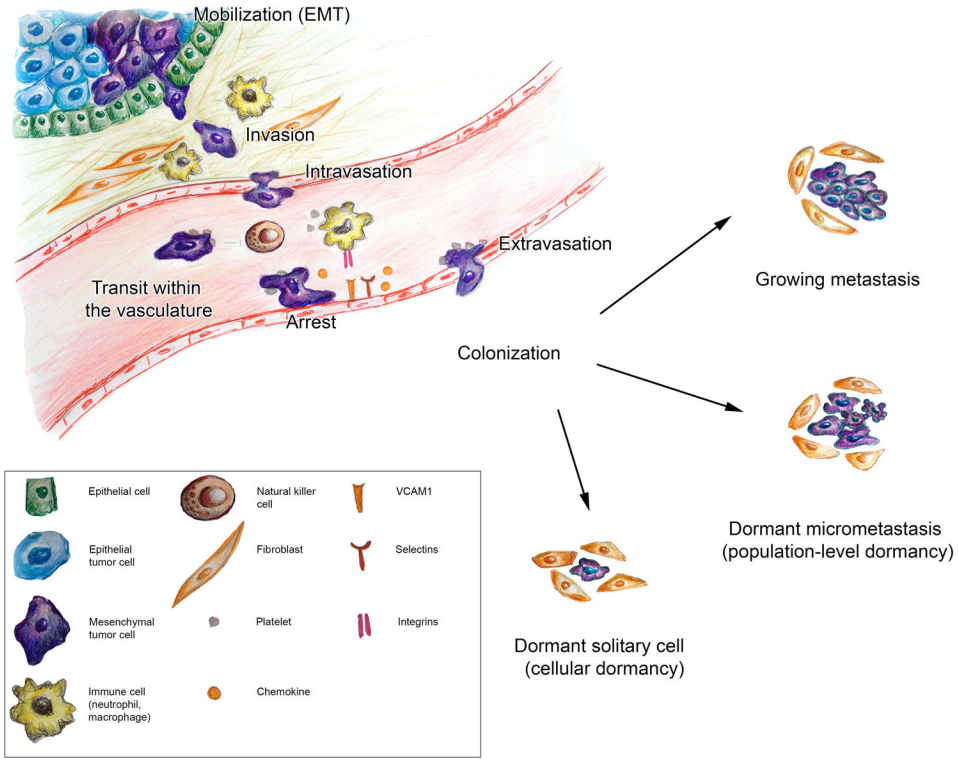

Figure 1 |. Steps of the metastatic cascade amenable to drug targeting.

For simplicity, only hematogenous metastasis is represented here. The metastatic cascade can be potentially targeted at different stages. First, drugs acting to inhibit Epithelial-Mesenchymal Transition (EMT) could prevent motility in mesenchymal tumor cells, thereby halting tumor spread at very early stages of the disease. Once mesenchymal cells have left the primary tumor site, further spread could be prevented by targeting tumor cell enhanced invasion and migration properties. Overlapping targets might be effective also for the intravasation step, whereby tumor cells cross the endothelial barrier lining the vasculature. At this level of the cascade, resistance to apoptosis triggered by cell detachment (anoikis) can also be a target. Tumor cells that transit within the vasculature interact with platelets and a variety of immune cells including macrophages, neutrophils and natural killer cells. Activated platelets appear to have a crucial role for the survival of metastasizing cells within the bloodstream, for example by protecting tumor cells from the cytolytic activity of circulating natural killer cells. Thus, disrupting tumor cell-platelet interaction has been considered as a therapeutic option, although the safety of this approach is still a matter of debate. Macrophages and neutrophils, in conjunction with activated platelets coating the migrating tumor cells, have a crucial role in facilitating extravasation from the blood vessels. The interaction between extracellular proteins such as integrins, selectins and endothelial molecules such as vascular cell adhesion molecule 1 (VCAM1) determines the successful transmigration of tumor cells across the endothelial barrier. Chemokines like SDF1/CXCL12 (CXC-chemokine ligand 12), secreted by tumor cells, aid this process and have equally been considered as promising targets. Following colonization, solitary dormant cells can be suppressed, ideally without the need for reawakening them. Further therapeutic strategies have been devised and developed to suppress a micrometastasis that is being kept dormant at

a population-level by either active immune surveillance or limited angiogenesis. VCAM1, vascular cell adhesion molecule 1; EMT, Epithelial-Mesenchymal Transition; CXCL12, CXC-chemokine ligand 12.

Table 1 |

Examples of actionable pathways and molecules for metastasis prevention.

EMT, Epithelial-Mesenchymal Transition; HGFR, hepatocyte growth factor receptor; STAT3, Signal transducer and activator of transcription 3; Shh, sonic hedgehog; MMPs, metalloproteinases; ROCKs, Rho-associated protein kinases; LOXL, Lysyl-oxidase-like; DDR, Discoidin domain-containing receptor; BDNF, Brain-derived neurotrophic factor; CXCR, C-X-C chemokine receptor; ECM, Extracellular matrix; VEGF, Vascular endothelial growth factor.

Step in the metastatic cascade	Context	Target	Examples	Refs
Mobilization (EMT)	Paracrine EMT-inducing signals	EGFR (inhibition)	AG1478, gefitinib	[102,103]
		HGFR / c-Met (inhibition)	SU11274	[106,107]
		TGFβ (inhibition)	SB431542, LY36497, Fresolumimab / GC1008	[109–111]
	Intracellular EMT inducing signals	STAT3 (inhibition)	Stattic, S31-201	[91–93]
	Mesenchymal markers	Vimentin (degradation)	Withaferin A	[95]
		N-cadherin (degradation)	Anti-N-cadherin monoclonal antibody	[96]
	Maintaining the mesenchymal state	Axl (inhibitor)	BGB324	[97]
	Notch pathway inhibition			[117]
	Wnt pathway inhibition			[112]
	Hedgehog pathway inhbition	Shh (disruption)	Cyclopamine	[121,122]
Motility, invasion, adhesion	Matrix metalloproteinase inhibition	MMPs (inhibition)		[125]
	Rho pathway / ROCK	ROCK I and II (inhibition)	Fasudil, Wf-536, Y-27632, RKI-1447	[127–131]
	Tumor-ECM interaction	Integrins (antagonism)	Cilengitide	[133]
		LOXL family (inhibition)	Simtuzumab	[137]
		DDR (inhibition)		[138]
Anoikis resistance	Anoikis restoration	BDNF receptor (inhibition)	CEP-701, CEP2563	[139]
	Pro-apoptotic strategies	PI3K pathway inhibitors	ZSTK474, PI103 and LY294002	[142,197]
Transit within the vasculature	Anti tumor-platelet interaction		Dipyridamole and RA-233	[143]
Arrest and adhesion	Selectin-mediated interaction	Selectins (antagonism)		[140]
	Hyaluronic acid – CD44 interaction	Hyaluronan synthase (inhibition)	4-methylumbelliferone	[198]
Extravasation	Chemokine – mediated chemoattraction at distant sites	CXCR4 (antagonists)	4F-benzoyl-TE14011, 4F-benzoyl-TN14003, AMD3100 / plerixaflor (Mozobil)	[150–153]
		CXCR3 (antagonism)	AMG478	[156]

Step in the metastatic cascade	Context	Target	Examples	Refs
Colonization	Paracrine EMT-inducing signals	See above		
	Tumor-ECM interaction	See above		
	Hyaluronic acid – CD44 interaction	See above		
	Matrix metalloproteinase inhibition	See above		
	Angiogenesis inhibition	Endothelial cells (inhibition of migration and proliferation)	TNP-470	[161]
		VEGF / c-MET antagonists	crizotinib, sunitinib, cabozantinib	[163,164]