REVIEW

Epithelial–mesenchymal plasticity in carcinoma metastasis

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Tumor metastasis is a multistep process by which tumor cells disseminate from their primary site and form secondary tumors at a distant site. Metastasis occurs through a series of steps: local invasion, intravasation, transport, extravasation, and colonization. A developmental program termed epithelial–mesenchymal transition (EMT) has been shown to play a critical role in promoting metastasis in epithelium-derived carcinoma. Recent experimental and clinical studies have improved our knowledge of this dynamic program and implicated EMT and its reverse program, mesenchymal–epithelial transition (MET), in the metastatic process. Here, we review the functional requirement of EMT and/or MET during the individual steps of tumor metastasis and discuss the potential of targeting this program when treating metastatic diseases.

Epithelial and mesenchymal cell types have long been recognized by their unique cell morphology and organization in tissues. Epithelial cells form polarized sheets or layers of cells that are connected laterally via several types of cellular junctions, including adherens junctions, desmosomes, and tight junctions. In addition, epithelial cells anchor themselves to the underlying basement membrane via hemidesmosomes to maintain apical–basal polarity. Both desmosomes and hemidesmosomes further connect with the epithelial-specific cytokeratin intermediate filaments. In contrast, mesenchymal cells embed themselves inside the extracellular matrix (ECM) and rarely establish tight contact with neighboring cells. During specific embryonic morphogenesis processes such as mesoderm formation and neural crest development, epithelial cells can exhibit enormous plasticity and transit into a mesenchymal state by activating the epithelial–mesenchymal transition (EMT) program. After EMT, these cells lose their epithelial junctions and switch to producing vimentin filaments. The functional hallmark of the EMT program is to allow stationary epithelial cells to gain the ability to migrate and invade during developmental morphogenesis (Boyer and Thiery 1993; Hay 1995).

Although epithelial cells convert into the mesenchymal state during developmental EMT, entering the EMT program is not necessarily an irreversible commitment, as evident during kidney tubule formation. These epithelial cells can activate a transitory EMT program and then undergo a reverse process called mesenchymal–epithelial transition (MET) to continue their differentiation paths (Thiery et al. 2009; Lim and Thiery 2012). In many instances, the identification of an epithelial versus a mesenchymal state can be relatively fluid, and a partial EMT/MET frequently occurs to fulfill unique developmental tasks. These dynamic EMT/MET events highlight the enormous flexibility of presumably differentiated cells during morphogenesis.

In the past decade, an increasing number of studies have provided strong evidence for the reinitiation of the EMT developmental program in carcinoma progression and metastasis. This EMT program in tumor metastasis possesses many morphological and molecular features similar to those of the developmental EMT program. Importantly, due to the heterogeneity and constantly evolving microenvironment in human tumors, the EMT program in metastasis adapts to these conditions to allow tumor cells to successfully metastasize.

While EMT has been accepted as a critical program during embryogenesis, its role in carcinoma metastasis has been under debate (Tarin et al. 2005; Thomson et al. 2005; Garber 2008; Ledford 2011; Chui 2013). Many cell culture and mouse tumor model studies have clearly demonstrated the importance of EMT in tumor progression. However, the EMT process in human cancer, if present, remains difficult to identify, since carcinoma cells undergoing EMT share many similar morphological and molecular features with surrounding stromal fibroblasts. Furthermore, although primary tumors and circulating tumor cells (CTCs) present EMT features, distant metastases are generally epithelial in morphology, suggesting that,

if functional, the EMT program is likely to be dynamically regulated during metastasis. In 2002, Jean Paul Thiery (2002), proposed the reversible EMT metastasis model in which primary epithelial tumor cells activate EMT to invade and disseminate throughout the body, while, upon arriving at distant sites, disseminated tumor cells (DTCs) undergo a reversion process, or MET, to form epithelial metastases. This intriguing hypothesis has brought EMT research to the forefront of carcinoma metastasis study.

The molecular program of EMT

Given that a number of recent reviews have focused on the molecular pathways regulating EMT/MET (Thiery et al. 2009; De Craene and Berx 2013; Zheng and Kang 2013), this section aims to provide an overview of the cellular and molecular definition for EMT/MET and establish a foundation for detailed discussions of these pathways in the context of tumor metastasis. The complex morphological and cellular changes during EMT require the cooperation of a large number of molecular signaling pathways and regulators. Based on their functionalities during EMT, we categorize them into three groups: the effector molecules that execute the EMT program (EMT effectors), the transcription factors that orchestrate the EMT program (EMT core regulators), and the extracellular cues that activate the EMT program (EMT inducers).

EMT effectors

Many of the hallmark EMT effector molecules are subcellular structure proteins that demarcate the epithelial or mesenchymal identity of a cell. During EMT, key molecular components of these structures are subjected to various levels of regulation. For example, the genes encoding various epithelial junction proteins, such as E-cadherin, α-catenin, and γ-catenin, are down-regulated at the mRNA and protein levels. Among them, E-cadherin is regarded as a gatekeeper of the epithelial state in various epithelial cell types. During EMT, E-cadherin gene transcriptional repression (Batlle et al. 2000; Cano et al. 2000; Hajra et al. 2002), promoter methylation (Graff et al. 1995; Kanai et al. 1997; Saito et al. 1998), and protein phosphorylation and degradation (Zhou et al. 2004; Bachelder et al. 2005; Lester et al. 2007) have all been observed in response to various inducing signals. Furthermore, intermediate filaments are shown to switch from cytokeratin to vimentin during EMT. Increased vimentin levels are also a consistent marker during various EMT events, while cytokeratin subtype switches tend to be variable and tissue type-specific.

Some additional key EMT effector molecules are proteins that promote cell migration and invasion during EMT. Fibronectin, an extracellular protein required for mesenchymal cell migration, is frequently induced upon activation of EMT. To promote cellular invasion through the ECM during EMT, a PDGF/PDGF receptor (PDGFR) autocrine loop is activated to promote invadopodia-mediated

ECM degradation (Eckert et al. 2011). A number of nonepithelial cadherins, such as N-cadherin, and cell surface proteins, such as CD44 (Kuo et al. 2009) and integrin β6 (Bates et al. 2005), are induced and thought to be critical for proper cell migration. All of these proteins have been used to define the occurrence of EMT in tumors.

EMT core regulators

Execution of the EMT program involves the transcriptional alteration of many genes regulating cell adhesion, mesenchymal differentiation, cell migration, and invasion. In general, three core groups of transcriptional regulators have been consistently shown to be critical during various EMT events, thus being regarded as the core EMT regulators.

The first group is the transcription factors of the Snail zinc finger family, including Snail1 and Snail2, both of which are capable of directly binding to the E-boxes of the *E-cadherin* promoter to repress its transcription (Batlle et al. 2000; Cano et al. 2000; Hajra et al. 2002). The second group is the distantly related zinc finger E-box-binding homeobox family proteins Zeb1 and Zeb2, which are also able to suppress *E-cadherin* transcription (Comijn et al. 2001; Eger et al. 2005) via a double-negative feedback loop controlling Zeb1/Zeb2 and miRNA-200 family expression (Christoffersen et al. 2007; Bracken et al. 2008; Burk et al. 2008; Gregory et al. 2008; Korpal and Kang 2008; Korpal et al. 2008; Park et al. 2008; Kim et al. 2011b). Both the Snail and Zeb families of transcription factors have also been shown to repress the expression of other cellular junction proteins, such as claudins and ZO-1 (Ohkubo and Ozawa 2004; Vandewalle et al. 2005). The third group is the basic helix–loop–helix (bHLH) family of transcription factors, including Twist1 (Yang et al. 2004), Twist2 (Fang et al. 2011), and E12/E47 (Perez-Moreno et al. 2001), all of which can induce EMT alone or cooperatively. For example, Twist1 can not only repress *E-cadherin* through induction of Snail transcription factors (Li et al. 1995; Yang et al. 2004; Casas et al. 2011) but also activate programs associated with tumor invasion (Eckert et al. 2011), thus coordinating two major aspects of the EMT program.

EMT inducers

During tumor progression, EMT induction in tumor cells has not been associated with genetic alterations of the EMT core transcription factors, perhaps due to their essential roles in embryonic morphogenesis. Instead, carcinoma cells are thought to undergo EMT in response to a combination of extracellular signals in the tumor microenvironment. Many EMT-inducing signals tend to be cell type- or tissue type-specific and probably require the cooperation between multiple pathways. All major developmental signaling pathways, including TGF-β, Wnt, Notch, and growth factor receptor signaling cascades, have been implicated in some aspect of the EMT program. Most notably, the TGF-β pathway appears to be a primary inducer of EMT (Katsuno et al. 2013). For example, TGF-β and BMPs have been shown to induce the EMT core transcription factors Snail1/2, Zeb1/2, and

Twist1 (Thiery et al. 2009; Eckert et al. 2011). Interestingly, the ability of TGF-β/Smad signaling to induce EMT depends on the cooperation of several other pathways, including activation of the Ras kinase cascade via activated receptor tyrosine kinases (RTKs) or Ras mutations (Grunert et al. 2003) and cooperation from the Wnt/β-catenin/LEF-1 signaling pathway (Nawshad et al. 2005). One of the major sources of TGF-β in tumors is the stromal fibroblast cells in the tumor microenvironment (Hanahan and Weinberg 2011).

In addition to growth factor signaling, inflammatory cytokines and hypoxia in the tumor microenvironment have also been shown to promote EMT. The inflammatory cytokine TNFα can stabilize Snail1 via NF-κB activation (Wu et al. 2009) and induce Twist1 expression via IKK-β and NF-κB p65 activation (Li et al. 2012). Cytokines in the tumor microenvironment can also activate Stat3 via JAK kinases to induce Twist1 expression (Lo et al. 2007; Cheng et al. 2008). Hypoxic responses mediated by HIF-1 were also shown to induce the expression of Twist1 and Snail1 to promote EMT (Yang et al. 2008; Mak et al. 2010). Together, these studies indicate that extracellular cues from the tumor microenvironment play a critical role in activating EMT.

In summary, the EMT program involves a large number of cellular and molecular alterations. Since EMT-inducing signals are diverse and often context-dependent, EMT effectors and core transcription regulators are most widely used as molecular markers of EMT in human cancers. Further analysis of how individual EMT-inducing signals impinge on the EMT core regulators and effectors will provide a more comprehensive inventory of key players in EMT.

EMT/MET in tumor metastasis

The metastatic process is thought to consist of several steps. The initial escape from the primary site requires the epithelial tumor cells to become motile and degrade the underlying basement membrane and ECM; breakdown of these barriers initiates invasion into the nearby tissue parenchyma (step I: invasion). The next step of metastasis is termed intravasation, during which tumor cells invade across the endothelial lamina, penetrate into the blood or lymphatic vessels, and thereby enter the systemic circulation (step II: intravasation). Once in the circulation, only a small number of the disseminated neoplastic cells appear to be capable of surviving various insults within the circulation (step III: systemic transport). Eventually, some of the surviving cells may arrest in the vascular lumen and extravasate through the capillary endothelium into the parenchyma of distant organs (step IV: extravasation). In the new stromal environment, an even smaller subset of tumor cells establish micrometastases with the potential to proliferate into fully malignant, secondary tumors that are clinically detectable and eventually life-threatening (step V: colonization) (Thiery 2002; Fidler 2003; Kalluri and Weinberg 2009).

To clearly define the role of EMT in metastasis, we discuss both experimental and clinical evidence of EMT and its reversion program, MET, during the appropriate individual steps of tumor metastasis (Fig. 1).
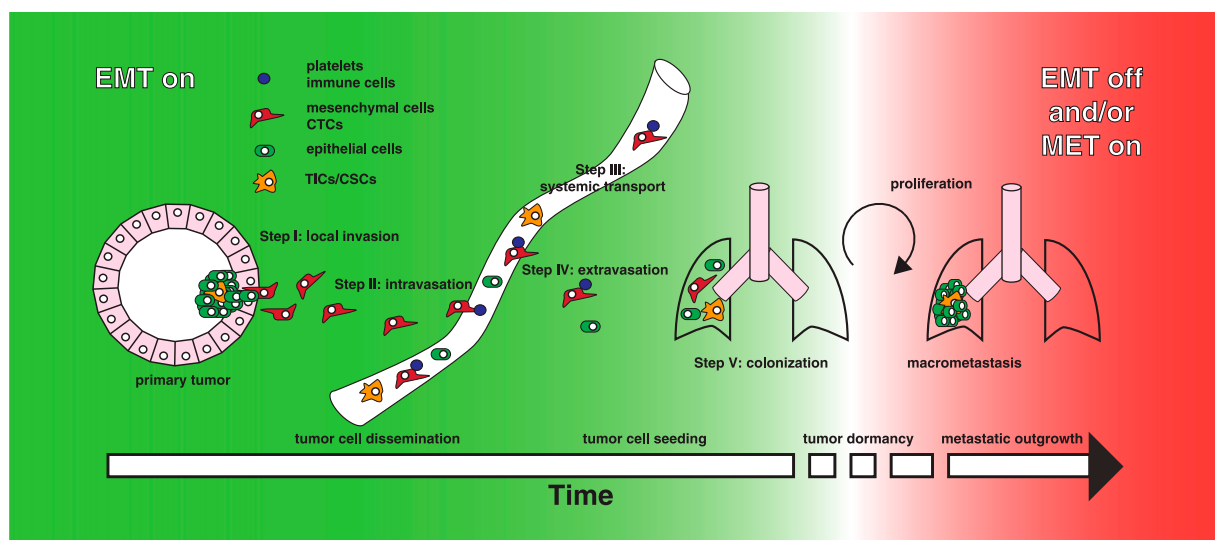

Figure 1. Model for reversible EMT over time. Epithelial cells undergo genetic transformation to become carcinoma in situ. Microenvironmental and genetic factors can promote the malignant conversion of these cells to activate the EMT program. During these early stages of tumor development, tumor cells that have undergone EMT can invade the local matrix (step I) and intravasate into the vasculature (step II). These epithelial–mesenchymal-transitioned cells are then transported in the circulation and survive via various prosurvival mechanisms (step III). At the distant tissue site, maintenance of the EMT program is required to help tumor cells extravasate into the parenchyma (step IV) to establish micrometastases. This initial seeding of tumor cells at distant sites can occur rapidly, after which cells may remain "dormant" for a long period of time. Subsequent colonization in distant organs requires the reversion of EMT and/or activation of the MET program to establish secondary tumors (step V).

Step I: EMT in malignant conversion and local invasion

Malignant conversion

Tumor initiation is often characterized by both genetic changes intrinsic to the tumor cells and alterations in the local microenvironment that promote tumor progression. Activation of the EMT program is classically thought to be a late stage event in malignant cancer to promote metastasis. However, several studies have implicated a possible role of EMT core transcription factors in the initial malignant transformation. Expression of Twist1 mRNA was detected in atypical ductal hyperplasia, a very early stage of primary breast tumor development in the MMTV-Neu mouse tumor model (Husemann et al. 2008). Twist1 was also shown to override oncogene-induced senescence and apoptosis by binding to p53 and promoting its degradation (Valsesia-Wittmann et al. 2004; Ansieau et al. 2008; Lee and Bar-Sagi 2010; Piccinin et al. 2012). By inhibiting p53, Twist1 was able to cooperate with oncogenes such as Her2 and H-ras to promote malignant transformation (Valsesia-Wittmann et al. 2004; Ansieau et al. 2008; Morel et al. 2012; Piccinin et al. 2012), suggesting a potential role of EMT genes in tumor initiation.

Activation of EMT is considered essential to allow carcinoma cells to lose cell–cell junctions and dissociate from each other for single-cell migration and invasion. For example, TGF-β pathway activation in EpH4-Ras mouse mammary carcinoma cells resulted in the loss of E-cadherin-mediated adherens junctions and gain of mesenchymal markers in cell culture and mouse tumor xenografts (Oft et al. 1996, 1998; Janda et al. 2002). Intercrossing the mouse pancreatic β-cell tumor (RIP-Tag2) model with transgenic mice that maintain E-cadherin expression in β-cell tumors arrested tumor development at the early adenoma stage, whereas expression of a dominant-negative form of E-cadherin induced early invasion and metastasis (Perl et al. 1998). Furthermore, mice carrying a genetic deletion of the *E-cadherin* gene on a mammary-specific p53-null background developed invasive lobular carcinomas, a subtype of breast cancer that presents individual migrating tumor cells (Derksen et al. 2006). Together, these studies strongly support a role of EMT in promoting single-cell invasion in primary tumors.

Degradation of the ECM

Carcinoma invasion requires tumor cells to gain the ability to degrade the underlying basement membrane and ECM. The EMT program is involved in this process through up-regulation of various matrix degradation enzymes by the EMT core regulators. Snail1 expression in MDCK epithelial cells and MCF-7 breast carcinoma cells increased MT1-MMP, MT2-MMP, and MMP9 expression (Olmeda et al. 2007a; Ota et al. 2009) and facilitated the breakdown of the basement membrane (Hotary et al. 2006; Ota et al. 2009). Conversely, Snail1 inhibition in epidermal and breast carcinoma cells decreased MMP9 expression and tumor growth and metastasis (Olmeda et al. 2007a,b). Consistent with these results, Snail2 was found to play an essential role in regulating tumor metastasis through induction of MT4-MMP and MMP2 (Shih et al. 2005; Huang et al. 2009). Interestingly, proteases have also been implicated in activating EMT by disrupting cell–cell junctions. Radisky et al. (2005) showed that MMP3 induces expression of Rac1, which increases reactive oxygen species (ROS) production and Snail1 expression to promote EMT (Orlichenko and Radisky 2008). Together, these results suggest a possible interplay between the loss of cellular junctions and the induction of proteases during EMT to promote tumor invasion.

More recently, there is emerging evidence that EMT transcription factors can also induce the formation of specialized subcellular structures called invadopodia to invade local ECMs (Eckert et al. 2011; Murphy and Courtneidge 2011). Invadopodia are actin-based protrusions that recruit various proteases such as membrane-tethered proteases (MT-MMPs), ADAMs, and MMPs, etc. to cell–matrix contact points to degrade ECM (Murphy and Courtneidge 2011). The EMT core transcription factor Twist1 was found to promote invadopodia formation through induction of PDGFRα expression and Src activation (Eckert et al. 2011). TGF-β was also shown to induce invadopodia formation in EpH4 and MCF10A mammary epithelial cells through up-regulation of Twist1 (Eckert et al. 2011) and the focal adhesion protein Hic-5 (Pignatelli et al. 2012) to promote matrix degradation and invasion. Furthermore, Zeppo1, a novel metastasis promoter that can repress E-cadherin expression, was found to induce EpH4.9 cells to form invadopodia-like structures in three dimensions (Slorach et al. 2011). Together, these studies suggest that the EMT program not only allows carcinoma cells to dissociate from each other but also provides them the ability to degrade ECM for single-cell invasion to initiate the metastatic cascade (Fig. 2).

Clinical evidence of EMT in primary tumor invasion

In the past, identification of carcinoma cells undergoing EMT in human tumor tissues has largely relied on histological analysis of E-cadherin expression. A partial loss of E-cadherin is associated with carcinoma progression and poor prognosis in various human tumor types, consistent with the role of E-cadherin as a caretaker of the epithelial state in carcinomas (Hirohashi 1998; Vincent-Salomon and Thiery 2003). In a few carcinoma subtypes, E-cadherin is lost at an early stage of the disease, so the tumor types present a permanent EMT phenotype. For example, a portion of lobular breast carcinomas (Berx et al. 1995, 1998) and diffuse gastric cancers (Becker et al. 1994; Oda et al. 1994) contain *E-cadherin* gene nonsense or frameshift mutations, while transcriptional suppression or post-translational modification of E-cadherin also contributes to the lack of E-cadherin expression in these tumors (Droufakou et al. 2001). Furthermore, in many late stage human carcinomas, E-cadherin expression appears to be heterogeneous, with E-cadherin-negative tumor cells interspersed within foci of E-cadherin-positive areas in the tumor (Bukholm et al. 1998, 2000), suggesting that some carcinoma cells might have undergone EMT.
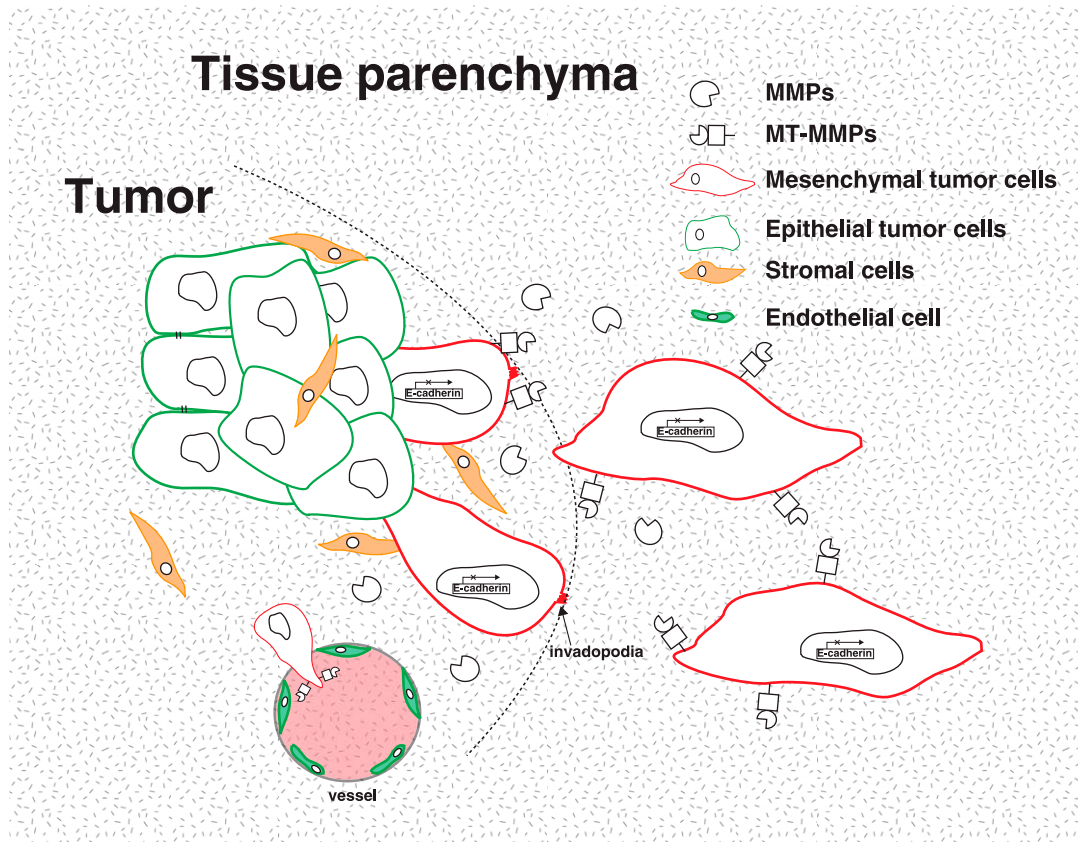
Figure 2. EMT in local invasion and intravasation. Activation of the EMT program is mostly characterized by the loss of E-cadherin expression. In order to invade through local basement membrane (surrounding the tumor or the tumor vasculature), these mesenchymal tumor cells up-regulate several secreted (MMPs) and membrane-tethered (MT-MMPs) proteases to break down ECM components. In addition, EMT factors can up-regulate specialized cellular structures such as invadopodia to promote local invasion. Expression of proteases can further induce EMT by breaking down cell–cell junctions, resulting in a positive feedback loop during malignant transformation of these cells.

Recent progress in gene expression profiling has shed more light on the prevalence of EMT in human cancer. Most notably, microarray analysis classified a claudin-low subtype of breast ductal carcinoma with low expression of E-cadherin and found that this subtype was enriched with an EMT gene signature including EMT core transcription factors Snail1, Twist1/2, and Zeb2 (Prat et al. 2010). In addition, immunohistochemical analysis of 28 molecular markers in 479 invasive breast carcinomas revealed clustering of EMT markers in tumors with a basal-like phenotype (Sarrio et al. 2008). Further analysis of these invasive basal/triple-negative subgroups found tumor cells that are double-positive for keratin and vimentin at the invasive front (Sorlie et al. 2001; Livasy et al. 2006; Rakha et al. 2006; Bonnomet et al. 2012). In colorectal carcinoma, ZEB1- and Snail1-positive cells with a mesenchymal morphology at the invasive front showed strong nuclear β-catenin signals (due to an APC mutation), suggesting that colon carcinoma cells, but not stromal cells, have undergone EMT (Brabletz et al. 2001, 2005; Spaderna et al. 2006). Recently, Celesti et al. (2013) performed Twist1 immunostaining in combination with fluorescent in situ hybridization (FISH)

analysis of chromosome translocation unique to human colorectal tumor cells and found that 17 out of 20 human colon tumors contained Twist1^{+} tumor cells with a mesenchymal phenotype. Together, these studies provide convincing clinical evidence of the occurrence of EMT (or at least partial EMT) in primary human carcinomas.

Step II: EMT in tumor cell intravasation

Following local invasion, tumor cells need to undergo an intravasation process to enter into the vasculature (lymphatic or blood vessels) for systemic dissemination. The precise mechanism of how tumor cells cross the endothelial barrier is largely unknown. Because of their size, tumors cells may require additional machinery to intravasate, unlike the smaller leukocytes that rely on diapedesis to migrate between endothelial cell (EC) junctions (Miles et al. 2008). The EMT program is thought to modulate the migratory and invasive properties of carcinoma cells to promote entry into the vasculature.

Technological advances in transendothelial assays, chick chorioallantoic membrane (CAM) assays, and intravital live imaging have facilitated the investigation of

EMT during the intravasation process. Using a transendothelial migration assay, Drake et al. (2009) found that Zeb1 expression in PC-3 human prostate cancer cells was required for enhanced migration through the EC barrier and increased metastatic colonization. Using a modified CAM assay that allows visualization of chorionic epithelium-derived vascular basement membrane, Ota et al. (2009) found that MCF-7 breast cancer cells expressing Snail1 could transmigrate through the underlying chorionic basement membrane and intravasate into the host vasculature. More interestingly, they showed that over-expression of Snail1 promoted intravasation through activation of membrane-bound MT1-MMP and MT2-MMP but not secreted MMPs, suggesting that direct contact between tumor cells and the endothelium is likely to be required for intravasation (Ota et al. 2009). Although further advances in in vivo imaging techniques are needed to carefully investigate the mechanisms regulating intravasation, these studies support a role of EMT in promoting carcinoma cells to breach through endothelium during intravasation (Fig. 2).

Step III: EMT in systemic transport

Upon entering systemic circulation, surviving tumor cells must possess the proper machinery to survive anoikis and then attach onto the blood vessel wall to prepare for extravasation from the circulation. Many recent studies in human cancer patients and mouse tumor models have identified the presence of EMT molecular markers in CTCs using various methods, including immunostaining, in situ hybridization, RNA sequencing, real-time quantitative PCR, and expression analysis (Yu et al. 2011). In general, most studies detected expression of EMT markers in CTCs; however, the functional significance of EMT in CTCs still awaits further evaluation.

Experimental evidence for EMT in CTCs

Currently, only a limited number of reports using mouse tumor models directly examine the involvement of EMT in producing CTCs. This may be due in part to the challenges in capturing CTCs that have fully undergone EMT, given that current isolation methods rely primarily on using epithelial markers, such as EpCam, to capture CTCs from the blood or bone marrow (Yu et al. 2011). To investigate tumor cell dissemination in vivo, Husemann et al. (2008) used the MMTV-Her2 mouse mammary tumor model and found increased Twist1 expression in hyperplastic lesions during early primary tumor development. Concomitantly, these mice presented increased DTCs in the bone marrow at this stage, suggesting that EMT may be partially responsible for the induction of CTCs/DTCs (Husemann et al. 2008). Consistent with these findings, in a K-Ras-driven mouse pancreatic tumor model, Rhim et al. (2012) detected circulating pancreatic tumor cells at the premalignant stage of tumor progression. The majority of these CTCs presented a mesenchymal phenotype and expressed Zeb2, indicating activation of the EMT program in these cells (Rhim et al.

2012). To demonstrate that activation of EMT directly promotes the production of CTCs, Tsai et al. (2012) examined the number of CTCs in a squamous cell carcinoma mouse tumor model in response to Twist1 induction. Indeed, Twist1 induction dramatically increased the number of CTCs compared with control mice, and these CTCs presented an EMT phenotype with loss of E-cadherin and expression of vimentin (Tsai et al. 2012). In the MDA-MB-468 breast tumor xenograft model, expression of Snail1 and Snail2 also increased the presence of vimentin-positive CTCs (Bonnomet et al. 2012). In these experimental studies, an increase in CTCs was associated with an increase in metastasis incidence, suggesting that EMT-induced CTCs directly contribute to effective metastasis formation.

Recent insightful studies have also revealed a potential mechanism for maintaining the mesenchymal state of CTCs. Labelle et al. (2011) found that CTCs were preferentially associated with platelet cells, which are a major source of TGF-β production in the blood. Importantly, depletion of platelets or inhibition of TGF-β secretion solely in platelets drastically reduced distant metastases (Labelle et al. 2011). Consistent with these experimental data, mesenchymal CTCs isolated from breast cancer patients were found clustered with platelet cells, and gene expression profiling of these CTCs found an enrichment of the TGF-β pathway (Yu et al. 2013). Together, these studies suggest that successful metastasis may depend on the maintenance of a mesenchymal state in CTCs.

One potential hypothesis for why CTCs need to maintain the EMT program is that EMT can prevent single tumor cells from detachment-induced anoikis while in circulation. For example, CTC survival may be aided by microtubule-based membrane protrusions called microtentacles that are thought to allow CTC aggregation and/or cell attachment to the blood wall. These structures have previously been shown to form in detached breast tumor circulating cells (Matrone et al. 2010a,b; Whipple et al. 2010). Expression of Twist1 or Snail1 in human mammary epithelial cells promotes microtentacle formation in detached cells, suggesting that EMT could aid CTC survival via microtenacle-based attachment of CTCs to leukocytes, platelets, and endothelium (Fig. 3; Matrone et al. 2010a).

Clinical evidence for EMT in CTCs

Many recent clinical studies have highlighted the use of CTCs as a prognostic marker for cancer progression and an indicator of therapeutic response. Studies in patients with metastatic breast and colorectal cancer showed that CTCs serve as an independent predictor of progression-free survival and overall survival (Christoffersen et al. 2007; Cohen et al. 2008). Interestingly, many clinical studies also detected the presence of EMT molecular markers in the CTCs. For example, CTCs from breast cancer patients showed reduced expression of epithelial markers and/or increased mesenchymal markers (Aktas et al. 2009; Kallergi et al. 2011; Raimondi et al. 2011;
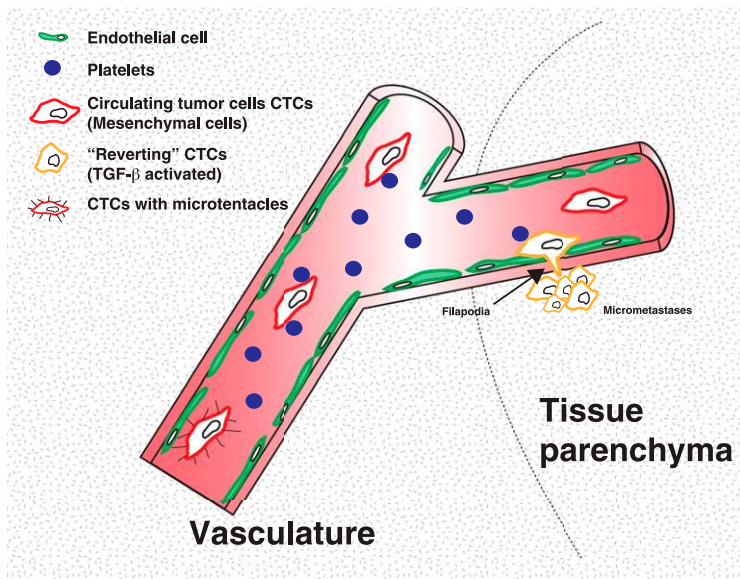
Figure 3. EMT in systemic transport and extravasation. Experimental and clinical samples have revealed an EMT signature in CTCs. This signature provides a possible biomarker to monitor tumor progression and/or therapeutic response. Experimental evidence suggests that platelets play a critical role in maintaining EMT activation in CTCs by providing the TGF-β signal. Furthermore, studies suggest that activation of EMT promotes microtentacle formation that allows tumor cell attachment to the endothelium and promotes cell survival. In order to extravasate at distant sites, tumor cells maintain an EMT phenotype and express cellular protrusions that allow extravasation, which is mediated by β1 integrin signaling.

Mego et al. 2012). Expression of Twist1 in DTCs isolated from bone marrows of breast cancer patients was correlated with early distant relapse (Watson et al. 2007). Using a similar approach, CTCs isolated from hepatocellular carcinoma (HCC) patients with metastasis expressed almost 20 times more Snail1 transcripts when compared with patients with no metastasis, demonstrating a potential functional role of Snail1 in HCC metastasis (Min et al. 2009). A recent study by Yu et al. (2013) quantified the proportions of CTCs with epithelial or mesenchymal phenotypes and found an association of mesenchymal CTCs with disease progression. One caveat of many studies involving CTCs is that, as previously discussed, the current CTC isolation methods rely primarily on using epithelial markers to capture CTCs. Thus, CTCs in the mesenchymal state may be missed during isolation (Paterlini-Brechot and Benali 2007; Pantel and Alix-Panabieres 2010; Kang and Pantel 2013). New technical advances are required to explore the potential utility of CTCs in both monitoring therapeutic responses and predicting cancer patient survival.

Step IV: EMT in tumor cell extravasation

Many classic studies of EMT regulators have used experimental metastasis assays, such as tail vein injection and intracardiac injection, to investigate tumor cell extravasation in the organ of interest. Studies have suggested that extravasation is a relatively rapid process (i.e., tumor cells extravasate 1–2 d following tail vein injection) (Cameron et al. 2000; Mendoza et al. 2010). However, tail vein assays involve injecting high numbers of tumor cells directly into the circulation. These injected tumor cells arrive at the lung microvasculature in such a large quantity that this process often results in intravessel growth rather than the sequential step of tumor growth in the tissue parenchyma following tumor cell extravasation. Thus, experimental models that more closely

mimic physiological dissemination are needed to analyze the extravasation step.

Recently, new experimental systems have been developed to investigate how EMT is involved in the extravasation step. Stoletov et al. (2010) established an extravasation assay in zebrafish, which are optically transparent and allow real-time imaging of cell movement. Using this elegant system, they found that Twist1 expression in breast tumor cells promoted tumor cell extravasation through a β1 integrin-independent mechanism. Furthermore, they showed that Twist1-expressing cells formed large dynamic membrane protrusions during extravasation (Stoletov et al. 2010). Interestingly, Shibue et al. (2012) found that upon arriving at the distant tissue parenchyma, tumor cells present filopodium-like protrusions (FLPs) that contain integrin β1 to interact with the ECM. Not only was FLP formation found to be essential for successful metastasis, but the ability of various breast tumor cells to generate FLPs was correlated with their mesenchymal states, and their formation can be induced by the expression of Twist1 and Snail1 (Shibue et al. 2012). Together, these data indicate that the EMT program may promote extravasation and the initial lodging of tumor cells in distant organs (Fig. 3).

Step V: Reversion of EMT in metastatic colonization

As discussed, primary carcinomas and CTCs show strong cellular and molecular signatures of EMT; in contrast, resulting macrometastases are largely epithelial, which suggests that the involvement of EMT during metastasis is likely to be dynamic. Indeed, Chao et al. (2010) showed that tail vein injection of mesenchymal MDA-MB-231 cells into the secondary organ environment resulted in re-expression of E-cadherin through the passive loss of methylation in the *E-cadherin* promoter. Using vimentin as the mesenchymal marker, Bonnomet et al. (2012) found that primary MDA-MB-468 tumor xenografts and

the resulting lung metastases showed a heterogeneous expression pattern of vimentin, while CTCs expressed high levels of vimentin, Snail1, and Snail2. This suggests that vimentin-positive CTCs might have undergone MET to form vimentin-negative macrometastasis. Two recent studies have provided concrete experimental data to support such epithelial–mesenchymal plasticity during tumor metastasis. Using an inducible Twist1 mouse bearing skin tumors, it has been demonstrated that activation of EMT promotes the early steps of metastasis, including local invasion, intravasation, and extravasation. However, the loss of an EMT-inducing signal at the distant site was essential for cell proliferation and macrometastasis formation (Tsai et al. 2012). Consistent with these studies, Ocana et al. (2012) showed that downregulation of a novel EMT inducer, Prrx1, in BT549 human breast cancer cells was required for lung metastasis colonization upon tail vein injection. Specifically, Prrx1 cooperated with Twist1 to promote a more invasive phenotype, while loss of Prrx1 was required to revert EMT (Ocana et al. 2012). Together, these studies strongly argue that reversion of EMT is essential for metastasis colonization.

Why do tumor cells need to revert to an epithelial state to grow into macrometastases? Studies in cell culture showed that induction of EMT by Snail1 and Zeb2 directly represses cell division by inhibiting Cyclin D activity (Vega et al. 2004; Mejlvang et al. 2007). In an in vivo skin tumor model, activation of Twist1 was found to be associated with reduced tumor cell proliferation (Tsai et al. 2012). Since colonization demands tumor cells to restart proliferation upon extravasation into a foreign microenvironment, reversion of EMT may be required to provide such growth advantage. While these studies suggest that the induction of proliferation likely plays a key role in the reversion of EMT during colonization, it remains unanswered which signaling pathways couple EMT with cell proliferation. Other studies suggest that EMT regulators might provide additional assistance for metastatic colonization. The miR-200 family members are negative regulators of the EMT inducer Zeb1 and vice versa (Christoffersen et al. 2007; Bracken et al. 2008; Burk et al. 2008; Gregory et al. 2008; Korpal and Kang 2008; Korpal et al. 2008; Park et al. 2008; Kim et al. 2011b). Interestingly, re-expression of miR-200 family members was shown to enhance colonization possibly by repressing Sec23a-mediated secretion of metastasis-suppressive proteins, including Igfbp4 and Tinagl1 (Fig. 4; Korpal et al. 2011).

Another unanswered question is how the EMT reversion process occurs in distant organs. In other words: Is the absence of an EMT-inducing signal sufficient for EMT reversion? Are additional MET-inducing signals required to actively promote MET? Recent studies indicate that both scenarios are possible in promoting EMT reversion. In a skin tumor model, the absence of a Twist1 signal (or the withdrawal of Twist1-activating signal) in DTCs resulted in macrometastasis formation (Tsai et al. 2012). However, this study does not exclude the possibility that additional MET-inducing signals may also contribute to
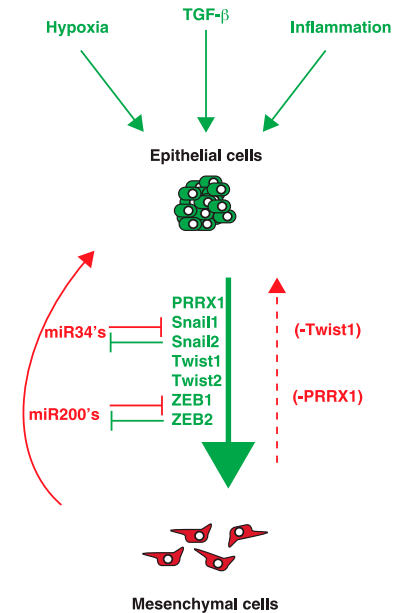

Figure 4. Mechanisms of EMT reversion. Colonization at distant sites requires the reversion of EMT to promote tumor cell proliferation. The interplay between EMT activators and inhibitors (i.e., MET activators) plays a critical role in metastatic outgrowth. The loss of EMT activators such as Twist1 or Prrx1 appears to be required to promote EMT reversion. However, signals from the microenvironment in distant sites may also shift the balance from EMT activators to EMT inhibitors or MET activators. It is unknown when or how these factors are regulated during tumor progression, which may impact treatment of metastatic disease.

colonization. Gao et al. (2012) showed that versican expression by myeloid cells in the lung metastatic niche promoted lung colonization by inducing MET, thus supporting the notion that signal inputs from the metastatic niche could regulate epithelial–mesenchymal plasticity in distant sites. Although it is currently unknown how microenvironmental signals regulate MET, EMT core transcription factors are considered primary targets for such regulation. For example, a number of EMT core transcription factors are negatively regulated by miRNAs, including miR-200 family members that regulate Zeb2 as well as miR-34 family members that regulate Snail1 and Zeb1. It is possible that microenvironmental signals could impinge on these miRNAs to turn off EMT at distant organs (Fig. 4; Kim et al. 2011a; Siemens et al. 2011). Given that micrometastasis outgrowth is a key rate-limiting step in metastasis, more studies on the molecular regulators of MET could shed light on therapeutic approaches to inhibit tumor colonization.

Adding to the complexity of metastatic colonization is the clinical observation that DTCs can remain "dormant" for many years before regrowth. These cells are thought to reside in the secondary tissue parenchyma as a micrometastatic lesion or in the bone marrow and mobilize to secondary sites prior to regrowth (Hedley and Chambers 2009). Due to the lack of specific molecular markers to detect and isolate dormant micrometa-

stases from epithelial organs in cancer patients and mouse tumor models, our current understanding of tumor dormancy is largely based on micrometastases isolated from the bone marrow. In one study, micrometastases from the bone marrow of breast cancer patients were isolated, and Twist1 expression was identified as a marker for early distant metastasis relapse (Watson et al. 2007). This result is consistent with a role of EMT in promoting tumor cell dissemination, as discussed earlier. More importantly, together with the notion that reversion of EMT is required for macrometastasis colonization, they suggest that the inability to revert EMT in DTCs might contribute to metastasis dormancy. Further studies using relevant experimental tumor models are needed to better evaluate the involvement of EMT in tumor cell dormancy.

While large numbers of studies have demonstrated a cell-autonomous role of EMT in promoting tumor cell dissemination, an alternative "cooperative" model proposes a supporting role for tumor cells that have undergone EMT to aid metastasis. By mixing uniquely labeled epithelial tumor cells and mesenchymal tumor cells to generate primary tumors, Tsuji et al. (2008) reported that epithelial cells require the cooperation of mesenchymal cells to lead the way for intravasation and CTC generation. However, only the epithelial tumor cells, but not the mesenchymal tumor cells, could generate macrometastases (Tsuji et al. 2008). In addition, the collaboration between tumor-initiating cells (TICs) and cells having undergone EMT accelerated metastatic colonization by the TICs (Celia-Terrassa et al. 2012). Both studies suggest that epithelial and mesenchymal tumor cells may cooperate to successfully form metastatic tumors (Tsuji et al. 2009). However, given that the mesenchymal tumor cells used in these studies have undergone a permanent EMT and cannot colonize in distant organs, it is plausible that the epithelial tumor cells still need to undergo a dynamic EMT/MET process to succeed in generating macrometastases.

Emerging frontiers of EMT

EMT and cancer stem cells (CSCs)

The role of CSCs or TICs in tumor progression has been at the forefront of cancer research in recent years (Nguyen et al. 2012). CSCs are generally characterized by their ability to initiate tumors in serial dilution transplantation assays. They express multiple cell surface markers, including $CD44^{high}$, $CD24^{low}$, and $CD133^{high}$, depending on the tumor type. Interestingly, a number of studies have demonstrated that EMT activation can generate CSCs or disseminated cells that have tumor-initiating properties. Studies by the Weinberg group (Mani et al. 2008) and the Puisieux group (Morel et al. 2008) showed that activation of EMT by TGF-β, Snail1, Twist1, and Zeb1 in normal human mammary epithelial cells can promote a CSC-like phenotype with tumor-initiating properties. In mouse mammary epithelial cells, Snail2 was found to be a critical player in regulating normal mammary stem cells (Guo et al. 2012). Twist1 was also found to be capable of

suppressing CD24 expression, providing a direct connection between an EMT transcription factor and CSC generation (Vesuna et al. 2009). In breast cancer cells, activation of urokinase-type plasminogen activator receptor (uPAR) was found to reversibly activate EMT, and activation of this pathway was capable of generating CSC-like properties (Lester et al. 2007; Jo et al. 2009, 2010). More interestingly, recent studies also found that basal breast cancer non-CSCs are populations that can generate CSCs de novo, and the CSC plasticity is controlled by the chromatin state of the Zeb1 promoter (Chaffer et al. 2011), further highlighting the critical role of EMT regulators in regulating CSC plasticity during tumor progression.

Consistent with these experimental results, numerous studies have provided correlative evidence relating EMT to the emergence of a CSC phenotype in human cancers. In breast cancer patients, disseminated breast cancer cells from pleural effusions, which likely have undergone EMT, are enriched for a $CD44^{high}$, $CD24^{low}$ CSC-like population (Al-Hajj et al. 2003). In addition, stem cells isolated from normal breast tissue or breast cancers express a number of canonical EMT markers. Of clinical significance, studies have shown that the tumor immune response resulted in EMT-associated emergence of $CD44^{high}$–$CD24^{low}$ CSCs (Santisteban et al. 2009).

Despite the strong evidence of a pro-CSC-forming role by EMT core regulators, there are experimental data that implicate EMT as having a negative impact on TICs. Celia-Terrassa et al. (2012) showed that cancer cells with a pronounced epithelial phenotype were enriched with highly metastatic TICs, whereas the mesenchymal-like cells lacked TICs. Furthermore, forced expression of Snail1 in the phenotypically epithelial cells suppressed their self-renewal and metastatic capabilities, suggesting that EMT activation may in fact suppress the tumor-initiating properties of CSCs (Celia-Terrassa et al. 2012). These contradictory results could be due to the difference in additional genetic/epigenetic alterations in the individual cell types studied. A recent study found that removing an EMT regulator, Prrx1, was required for the tumor-initiating ability of breast cancer cells expressing Twist1 (Ocana et al. 2012). Future studies to clearly define the difference in signaling pathways regulating EMT versus TICs will provide much-needed information on how the EMT program and TIC regulation are intervened.

EMT and drug resistance

One major obstacle in cancer therapy is that cancer patients can develop resistance to treatment over time. An increasing number of reports suggests a potential role of EMT in conferring drug resistance. Studies using colorectal cell lines have shown that expression of EMT inducers Snail1 or TGF-β in SMAD4-null cells increased resistance to chemotherapy (Hoshino et al. 2009; Papageorgis et al. 2011). Conversely, colorectal cell lines that were rendered oxaliplatin-resistant showed phenotypic and gene expression changes consistent with EMT (Yang et al. 2006; Kawamoto et al. 2012). Interestingly,

tumor specimens taken from patients who had received 1 wk of preoperative chemotherapy prior to surgery resection displayed a more mesenchymal gene signature compared with prechemotherapy biopsy samples. Furthermore, recurrent tumors frequently exhibited an EMT gene signature (e.g., decreased E-cadherin and increased vimentin) (Kawamoto et al. 2012). Shintani et al. (2011) found that, in non-small-cell lung carcinoma (NSCLC) patients, tumor biopsy prior to chemotherapy treatment showed epithelial markers but that this phenotype shifted toward mesenchymal markers following treatment. Consequently, the disease-free survival rate was lower in patients whose tumors presented an EMT phenotype compared with EMT-negative tumors (Shintani et al. 2011).

Besides the evidence of drug resistance to chemotherapy, recent studies have also suggested a role of EMT in drug resistance toward targeted therapies. Using several human NSCLC lines, Thomson et al. (2005) demonstrated that cell lines expressing epithelial markers were more sensitive to epidermal growth factor receptor (EGFR) inhibition, whereas cells lines presenting mesenchymal markers were more resistant to treatment. In gefitinib-resistant PC9/AB2 lung cancer cells, Notch1 was found to promote EMT. Knockdown of Notch1 reverted the EMT phenotype and rendered these cells sensitive to gefitinib (Xie et al. 2012), implicating a strong correlation between EMT activation and drug resistance.

While it remains unknown whether and how the EMT program directly impinges on drug resistance, several possible mechanisms might be in play. First, since activation of EMT reduces cell proliferation (Vega et al. 2004; Mejlvang et al. 2007), slowing the cell cycle machinery will increase resistance to chemotherapy, which generally targets highly proliferative cells. Second, EMT core transcription factors, including TGF-β, Snail1, Snail2, and Twist1, have been shown to confer resistance to cell death via various pathways, most notably by antagonizing p53-mediated apoptosis (Inoue et al. 2002; Kajita et al. 2004; Vega et al. 2004; Gal et al. 2008), thus providing a potential survival advantage. Third, as discussed above, the EMT program can promote CSC properties. CSCs were found to be inherently more resistant to conventional cancer chemotherapies than rapidly proliferating progenitor cells and differentiated tumor cells. These CSCs were also found to be responsible for tumor reoccurrence and capable of establishing metastases (Phillips et al. 2006; Li et al. 2008). Indeed, conventional chemotherapy is shown to enrich CSCs in breast cancer patients. For example, mammospheres isolated from chemotherapy-treated breast tumors showed similar mammosphere-initiating capacity after eight passages in culture, whereas cells from untreated tumors vanished within three passages, suggesting again an increase in CSCs after chemotherapy (Yu et al. 2007). Li et al. (2008) further demonstrated that chemotherapy increased the number of CD44^{high}–CD24^{low} cell population and that these cells have stem-like features. Together, these studies strongly indicate that activation of EMT contributes to cancer therapy resistance.

Future directions in therapeutic targeting of EMT/MET

Metastatic diseases are responsible for >90% of carcinoma-related deaths. Given the strong evidence supporting a critical role of EMT in tumor metastasis, targeting this process is thought to be a promising approach to treat invasive cancer. However, current treatment modalities remain limited in their efficacy in targeting cells undergoing EMT. This may be due in part to potential drug resistance in this population of cells (as discussed above) and the lack of appropriate targets in the core EMT program. Because EMT core transcription factors remain technically challenging to target, targeting the activation or the functional consequence of EMT is perhaps the more effective approach. Several groups have performed high-content drug screens to identify potential inhibitors of EMT in response to various EMT-inducing signals. Interestingly, the majority of the compounds obtained appear to be involved in inhibiting the specific EMT-inducing signals used in the screen. For example, rapamycin and 17-AGG were identified as inhibitors of TGFβ-induced EMT by modifying the TGFβ pathway (Reka et al. 2011), while inhibitors of ALK5, MEK, and SRC could interfere with EMT in response to EGF, HGF, and IGF-1 (Reka et al. 2011; Chua et al. 2012). Salinomycin was also identified as inducing selective killing of E-cadherin-null breast epithelial cells compared with E-cadherin-positive cells (Gupta et al. 2009), although its molecular action toward EMT is unknown.

The plasticity of EMT in metastasis provides another level of complexity regarding the appropriate time window to target EMT in patients. Several studies suggest that targeting the TGFβ pathway to inhibit EMT or blocking tumor cell invasion through inhibiting PDGR signaling may be appropriate as metastasis prevention strategies in early stage carcinomas. Once tumor cells have disseminated from the primary tumor, inhibiting EMT could be counterproductive, since reversion of EMT appears to be beneficial for disseminated carcinoma cells to regain proliferation and colonize distant organs (Tsai et al. 2012). Instead, the EMT features in CTCs and disseminated dormant tumor cells present several unique opportunities for therapeutic intervention. First, since DTCs present molecular markers of EMT, unique EMT surface markers expressed in these cells could be ideal targets for T-cell-based immunotherapy. Second, since an increasing number of studies suggest a role of EMT in promoting chemoresistance, combining chemotherapies with EMT inhibitors holds promise to overcome chemoresistance in dormant tumor cells, thus providing a unique therapeutic approach to eradicate dormant tumor cells. Last, preventing the reversion of the EMT program in dormant micrometastases would be a novel approach to prevent resurrection of dormant tumor cells. In the near future, improving our understanding of the molecular regulation of the dynamic EMT/MET programs during tumor metastasis will help to provide much-needed effective treatment to eradicate metastatic diseases.

Acknowledgments

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Epithelial−mesenchymal plasticity in carcinoma metastasis

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