

## Mechanism of the Mesenchymal–Epithelial Transition and Its Relationship with Metastatic Tumor Formation

Dianbo Yao, Chaoliu Dai, and Songlin Peng

### Abstract

Cancer metastasis consists of a sequential series of events, and the epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are recognized as critical events for metastasis of carcinomas. A current area of focus is the histopathological similarity between primary and metastatic tumors, and MET at sites of metastases has been postulated to be part of the process of metastatic tumor formation. Here, we summarize accumulating evidence from experimental studies that directly supports the role of MET in cancer metastasis, and we analyze the main mechanisms that regulate MET or reverse EMT in carcinomas. Given the critical role of MET in metastatic tumor formation, the potential to effectively target the MET process at sites of metastasis offers new hope for inhibiting metastatic tumor formation. *Mol Cancer Res*; 9(12); 1608–20. ©2011 AACR.

### Introduction

Cancer metastasis accounts for the majority of cancer deaths (1). Carcinomas derived from epithelial cells represent the most prevalent malignancies (~90%) in humans (2). It is well recognized that metastasis consists of distinct steps in which tumor cells (i) detach and migrate away from the primary tumor site, (ii) invade neighboring tissue and penetrate through basement membrane, (iii) enter the blood or lymphatic vessels, (iv) survive the condition of anoikis while they are detached from the tumor mass and in circulation, (v) exit the blood or lymphatic vessels at a distant organ, (vi) form micrometastatic nodule, (vii) adapt and reprogram the surrounding stroma, and form macrometastases (3). Investigators seeking to understand the cellular and molecular bases of tumor metastasis inevitably are challenged by the fact that metastasis is a complex, multistep biological process that most likely is controlled by distinct genes and signaling pathways during each step. Elucidating these mechanisms would aid in the intervention of metastasis or recurrence; however, although great efforts have been made, the mechanisms remain largely elusive.

Changes in cell phenotype between the epithelial and mesenchymal states, defined as the epithelial–mesenchymal

transition (EMT) and mesenchymal–epithelial transition (MET), are central to the complex remodeling of embryo and organ architecture during gastrulation and organogenesis, and they are recognized as critical events for metastasis of many carcinomas (4). Epithelial cells acquire fibroblast-like properties and exhibit reduced cell–cell adhesion and increased motility via EMT, which facilitates the escape of tumor cells from primary tumors (5, 6). EMT represents a fundamentally important process that is conducive to tumor dissemination, prompting investigators to explore the mechanism of EMT and methods to inhibit or even reverse this process and thereby inhibit tumor metastasis (7). Studies have shown that many metastatic lesions and their primary tumor counterparts share a similar epithelial nature. It is surprising that some metastases of a number of carcinomas, including prostatic cancer (8, 9), breast carcinoma (10, 11), colorectal cancer (12, 13), ovarian cancer (14), pulmonary cancer (15, 16), and hepatic carcinoma (17), seem even less dedifferentiated than their corresponding primary tumors. These findings are inconsistent with the theory of EMT. To resolve this apparent contradiction, a MET process in the metastatic sites was postulated to be part of the process of metastatic tumor formation (18, 19). Progression of solid tumors involves spatial and temporal occurrences of EMT, whereby tumor cells acquire a more invasive and metastatic phenotype (4). Subsequently, the disseminated mesenchymal tumor cells undergo the reverse transition, MET, at the site of metastases, as metastases recapitulate the pathology of their corresponding primary tumors. EMT is thought to be critical for the initial transformation from benign to invasive carcinoma, whereas MET (the reverse of EMT) is critical for the later stages of metastasis. Just as a critical EMT event is the down-regulation or silencing of E-cadherin, the reexpression of E-cadherin is proposed to be the important hallmark of MET (20).

**Authors' Affiliation:** Department of Hepatobiliary and Splenic Surgery, Shengjing Hospital of China Medical University, Shenyang, China

**Corresponding Author:** Chaoliu Dai, Department of Hepatobiliary and Splenic Surgery, Shengjing Hospital of China Medical University, No. 36, San Hao St., Heping District, Shenyang 110004, Liaoning Province, China. Phone: 86-24-96615-31511; Fax: 86-24-96615-31511; E-mail: daicl@sj-hospital.org

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## Association of MET with Metastatic Tumor Formation

In support of the MET hypothesis, as well as pathological morphometric examinations and molecular findings, fundamental experimental data supporting the role of MET in cancer metastasis are gradually increasing. By using the bladder carcinoma TSU-Pr1 (T24) progression series of cell lines selected *in vivo* via systemic seeding, Chaffer and colleagues (21) showed the importance of the epithelial phenotype in the formation of secondary tumors, as epithelial characteristics were dramatically associated with increased bone and soft-tissue colonization after intracardiac or intratibial injection. Furthermore, Oltean and colleagues (22, 23) observed an unexpected MET among lung micro-metastases in the organ parenchyma and immediately adjacent to blood vessels by visualizing the fibroblast growth factor receptor-2 (FGFR2) exon IIIc in a prostate cancer model. In addition, non-EMT hamster cheek pouch carcinoma-1 cells, but not EMT cells, were found to be able to form overt lung metastases after being inoculated into the bloodstream by tail-vein injection (24), suggesting that EMT is not conducive to the formation of metastases and that EMT cells *in vivo* might possibly be converted into the non-EMT cells via MET at the site of metastases. Lastly, when 4 isogenic mouse breast cancer cell lines (67NR, 168FARN, 4TO7, and 4T1) with different abilities to metastasize were implanted into mammary fat pads to model the steps of metastasis, only 4T1, which acquired epithelial properties (e.g., high expression of E-cadherin and cytokeratin-18), formed macroscopic lung and liver metastases, indicating again the importance of the epithelial properties (25). As such, increasing experimental data point to a critical role of the epithelial phenotype in metastatic tumor formation. Intriguingly, when the mesenchymal-like breast cancer cells (MDA-MB-231), in which E-cadherin expression is transcriptionally repressed by methylation of the E-cadherin promoter, were injected into the mammary fat pads of mice, E-cadherin-positive metastatic foci were detected (11), presenting a more direct demonstration that these E-cadherin-expressing metastases may arise from E-cadherin-negative cells. Because the EMT of carcinomas is critical for the initial escape by enabling individual cell migration and invasion, these experimental findings suggest that cancer cells should further undergo a MET in the secondary organ environment following the EMT that allows for escape. However, we still cannot absolutely exclude the possibility that *in vivo* the metastases might arise from the unusual escape of E-cadherin-positive cells in the primary mass, with the help of EMT cells (24).

## Mechanism of MET

Previous research into the mechanisms of metastasis focused mainly on the kinds of factors that contribute to initiation of metastasis, and relatively few studies have examined the formation of secondary tumors. The importance of MET in cancer metastasis has been gradually recognized, and studies on the mechanism of MET are

increasing. A number of pathways are related to the changes in cell phenotype. In the following sections, we discuss both the mechanism of MET and the mechanism that reverses EMT, because in the near future we might find that it could also promote the formation of metastases by reverting tumor cells to the epithelial phenotype.

## Cytokines and their receptors, intercellular signaling elements, and regulatory proteins

Multiple complex signaling systems are required for the induction of EMT and are also closely related with MET (Table 1). The *FGFR2* gene, which is located at human chromosome 10q26, encodes for FGFR2b and FGFR2c isoforms due to alternative splicing and mutually exclusive use of exon IIIb or exon IIIc. FGFR2b primarily binds FGF10 and FGF7 and is the isoform of choice in epithelial cells, whereas FGFR2c binds FGF2 and is mainly expressed in cells of mesenchymal origin. FGF/FGFR2 signaling governs the EMT that is required for organogenesis in mouse embryos. In addition, a class switch from FGFR2b to FGFR2c occurs during the progression process of prostate cancer and bladder cancer, and this switch is accompanied by EMT with increased potential for invasion and metastasis (26). The proliferation and tumorigenicity of prostate or bladder cancer cells with decreased FGFR2b expression were shown to be significantly suppressed by transfection of the FGFR2b expression construct (27, 28), suggesting that EMT might be

**Table 1. Cytokines Involved in Induction of MET**

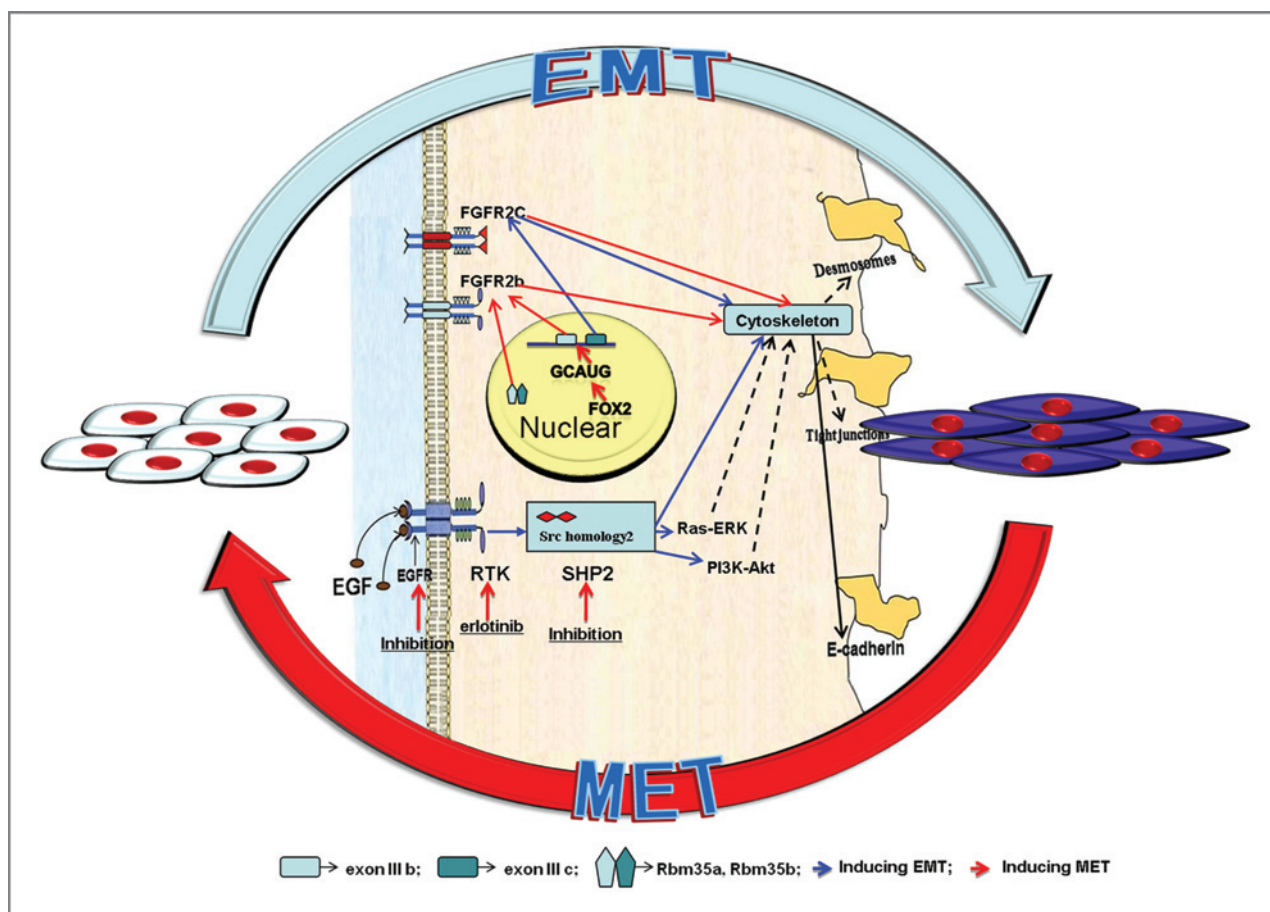
MET-associated cytokines <sup>a</sup>		Inducers or related elements	Reference
FGF	FGFR2b	Transfection of FGFR2b expression construct	(27, 28)
		Exon IIIb	(22, 23)
		GCAUG element	(29)
		Fox-2	(30)
		Rbm35a, Rbm35b	(31)
	FGFR2c	Non	(21)
EGF	EGFR/ErbB1; HER2/ErbB2	EGFR kinase inhibitor	(36, 78)
		Inhibition of SHP2	(38)
		Erlotinib	(39)
BMP2	Alk2	BMP2 siRNA	(42)
BMP7		Recombinant human BMP7	(45–48)
Wnt	WntR	$\beta$ -catenin siRNA	(51)
		FZD7 siRNA	(52, 53)
		FZD4 siRNA	(54)
		WIF1	(55)
Akt		PIA	(57)

<sup>a</sup>Cytokines and their corresponding receptors.

reversed. By imaging the alternative splicing decisions, Oltean and colleagues (22, 23) revealed that expression of FGFR2b induced MET in a model of Dunning prostate tumors. As for the regulation of FGFR2 isoforms' alternative splicing, a highly conserved GCAUG element was shown to be required for efficient exon IIIb activation (29). Afterward, Fox protein family members, especially Fox-2, were shown to regulate the FGFR2 exon choice, and this regulation was absolutely dependent on the GCAUG elements present in the FGFR2 pre-mRNA (30). Fox-2 induced the FGFR2c to FGFR2b switch, accompanied by molecular and morphological changes consistent with MET (30). Recently, Warzecha and colleagues (31) identified 2 paralogous epithelial cell type-specific RNA binding proteins, Rbm35a and Rbm35b, which also are essential regulators of FGFR2 splicing. Ectopic expression of either protein in cells that express FGFR2c caused a switch in endogenous FGFR2 splicing to the epithelial isoform. Of note, there are now several reports of FGFR2c expression in normal epithelia and tumor cells (32, 33). FGFR2c was greatly upregulated across

the TSU-Pr1 series acquired by Chaffer and colleagues (21) and was found to play a key role in determining the epithelial phenotype in these cell lines. Furthermore, targeted abrogation of FGFR2c in B2 cells reversed the MET. Together, these findings suggest that FGFR2 plays a critical role in the MET of tumor cells (Fig. 1).

Epidermal growth factor receptor (EGFR or ErbB1) and human epidermal growth factor receptor-2 (HER2 or ErbB2) are members of the receptor tyrosine kinase family and play a key role in normal development. They are always overexpressed in malignant tumors and are thought to contribute to tumor progression (34, 35). Yates and colleagues (36, 37) found that *in vitro* inhibition of autocrine EGFR signaling increased E-cadherin expression and cell-cell heterotypic adhesion and that E-cadherin upregulation was prevented by expressing a downregulation-resistant EGFR variant. E-cadherin and catenins, but not activated EGFR, were also found in human prostate cancer metastases to the liver, supporting the notion that the inverse relationship between E-cadherin expression and EGFR also exists in *de novo* human tumors (36). In addition, the EGFR tyrosine



**Figure 1.** Roles of the FGFR and EGFR pathways in the process of MET. The expression of FGFR2b can induce MET, and some elements, including exon IIIb, GCAUG element, Fox-2, Rbm35a, and Rbm35b, have been found to regulate the expression of FGFR2b and induce a change in cell phenotype. Studies have shown that FGFR2c can also induce MET in some kinds of tumor cells. In addition, it was found that MET can be induced by inhibition of the FGF signaling pathway, such as by inhibition of EGFR tyrosine kinase or SHP2.

kinase inhibitor erlotinib inhibited cell motility and invasiveness and reverted the mesenchymal phenotype of inflammatory breast cancer cells to the epithelial phenotype in a three-dimensional culture as upregulation of E-cadherin and downregulation of vimentin were induced and  $\beta$ -catenin was recovered in the cell membrane (38). In addition, Src homology phosphotyrosine phosphatase 2 (SHP2), which possesses 2 in tandem arranged Src homology 2 domains (a common target of receptor tyrosine kinases) in the N-terminal region (39), seem to play an important role in the EGF system. Inhibition of SHP2 suppressed EGF-induced activation of the Ras extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways in breast cancer cell lines, induced epithelial cell morphology, and led to reversion to a normal breast epithelial phenotype (40). Furthermore, E-cadherin was upregulated, and fibronectin and vimentin were downregulated (40). These data suggest that ERK also plays an important role in the process of MET (Fig. 1).

Bone morphogenetic proteins (BMP) are part of the TGF- $\beta$  superfamily and compose a large, evolutionarily conserved family of secreted signaling molecules that are required for numerous developmental processes (41). Activation of the BMP receptor complex is found to initiate intracellular signaling through phosphorylation of Smad proteins (42). The mRNA and proteins of BMP2 were found to be overexpressed in some cases of prostatic cancer, breast carcinoma, and lung cancer. Recently, in highly metastatic mesenchymal colon carcinoma cells (CT26), it was found that blockade of BMP2 signaling by BMP2 siRNA could reduce motility and invasiveness and cause a MET, possibly via activation of Akt (43). BMP7, another member of the BMP family, was shown to counteract TGF- $\beta$ 1-induced EMT in developmental stages (44), and this counteraction was also observed in renal tubular epithelial cells (44, 45). In cell lines of breast carcinoma and prostate cancer, BMP7 was also found to be inversely related to the metastatic potential and positively related to the expression ratio of E-cadherin and vimentin, suggesting that BMP7 may be associated with MET (46, 47). In esophageal adenocarcinoma (TE7) cells, TGF- $\beta$ 1-induced EMT was also reversed by 100 ng/mL of BMP7 (48). Furthermore, upon exogenous addition of increasing concentrations of BMP7 to human melanoma cells, the morphology of the cells changed to that of epithelial-like cells (49). MET was induced by BMP7 in melanoma cells, possibly by upregulation of the specific receptor BMP receptor (Alk2). Expression of Slug, smooth muscle actin (SMA), Twist, and Snail was also decreased in the process of MET induced by BMP7, and BMP receptor (Alk2) siRNA transduction was shown to restore Twist protein expression via blocking of Smad 1, 5, and 8 signaling (49). These findings suggest that BMP7 can induce MET, possibly by upregulation of Alk2; phosphorylation of Smad 1, 5, and 8; and inhibition of Slug, SMA, Twist, and Snail.

The Wnt signaling pathway has a particularly tight link to EMT (50). The best-studied Wnt signaling pathway is the Wnt/ $\beta$ -catenin pathway, which is comprised of secreted

Wnt ligands and cell-surface receptors called Frizzled and lipoprotein receptor-related protein 5/6 (LRP5/6; refs. 51 and 52).  $\beta$ -catenin is the Wnt-pathway effector. It serves as an essential component of adherent junctions; provides the link between E-cadherin and  $\alpha$ -catenin; modulates cell-cell adhesion and cell migration; and functions as a transcription cofactor with T-cell factor (TCF).  $\beta$ -catenin is the main oncoprotein in colorectal cancer and in most cases is overexpressed due to mutations in the adenomatous polyposis coli (APC) tumor suppressor (53, 54). Intriguingly, nuclear  $\beta$ -catenin was found in dedifferentiated mesenchyme-like tumor cells at the invasive front, but as in central areas of the primary tumors,  $\beta$ -catenin was localized to the membrane and cytoplasm in polarized epithelial tumor cells in the metastases (13). This expression pattern was accompanied by changes in E-cadherin expression, suggesting that  $\beta$ -catenin plays an important role in the metastasis of colorectal cancer, participating in EMT at the invasive front and in MET in metastases (13, 55). In support of these findings, it was observed that silencing  $\beta$ -catenin in hypoxic MHCC97 and Hep3B cells also reversed EMT (56). Using a variant of the human cell line LIM1863 (LIM1863-Mph), Vincan and colleagues (57, 58) established a unique model of colorectal cancer morphogenesis and showed that FZD7 plays a pivotal role in phenotype transitions, suggesting that Wnt signaling participates in orchestrating colorectal cancer morphogenesis. Similarly, silencing of FZD4 was shown to induce the phenotypic transition and activate  $\beta$ 1-integrin and E-cadherin expression (59). Wnt inhibitory factor 1 (WIF1), a Wnt inhibitor that exists *in vivo*, can also modulate Wnt signaling by binding to Wnt ligands (60), and expression of WIF1 was found to be downregulated in numerous cancers (61, 62). Ectopic expression of WIF1 in PC3 cells resulted in a dramatic increase in the protein levels of E-cadherin and cytokeratin-8 and a decrease in N-cadherin, vimentin, and fibronectin, suggesting that WIF1 expression caused a reversal of EMT (61). The mRNA expression and the protein levels of Slug and Twist were also decreased by WIF1 expression (61). It is conceivable that the WIF1-induced reversal of EMT is associated with inhibition of Wnt signaling, again highlighting the important role of Wnt signaling in the MET.

The Akt/PKB family of kinases is a downstream effector of PI3K and is frequently activated in human cancers. As noted above, the activity of Akt in breast carcinoma was shown to be repressed during MET, suggesting that repression of Akt activity may be related to MET (40). It was also shown that MET induced by BMP2 in mesenchymal colon carcinoma cells (CT26; ref. 43) or by progesterone (P4) in basal phenotype breast cancer (63) was related to the activity of Akt signaling. Intriguingly, inhibition of Akt activity by PIA (Akt inhibitor, phosphatidylinositol ether lipid analogs) decreased NF- $\kappa$ B signaling and led to downregulation of Snail and Twist expression (64). PIA treatment induced the expression of E-cadherin and  $\beta$ -catenin; downregulated vimentin; restored the epithelial morphology of a polygonal shape; and reduced tumor cell migration in KB and KOSCC-25B cells (64). These findings suggest that Akt

is an important intercellular signaling element during the changes in cell phenotype, and its activity is usually repressed during MET.

E-cadherin, an important transmembrane protein that is localized to the adherens junctions and basolateral plasma membrane, represents the best-characterized molecular marker expressed in epithelial cells. Cadherin-mediated adhesion is a critical element in determining and maintaining epithelial phenotype. In addition to this, it was found that E-cadherin alone can induce MET. As early as 1988, expression of E-cadherin was found to induce MET in pleiomorphic mouse sarcoma S180 cells (65). Reexpression of E-cadherin also induced MET in pancreatic tumor cell line MIA PaCa-2 cells and resulted in upregulation of  $\alpha$ - and  $\beta$ -catenin mRNAs and protein concentrations (66). Recently, it was found that ectopic expression of full-length E-cadherin in MDA-MB-231 cells resulted in a morphological and functional reversion of the epithelial phenotype, and even just the cytosolic domain of E-cadherin yielded a partial phenotype (11). These data suggest that reexpression of E-cadherin is not only an important hallmark of MET (20) but also may be an important inducer of MET.

Growth factors, whether derived from tumor cells or the surrounding parenchyma, play a key role in determining the balance of epithelial and mesenchymal traits of tumor cells. Extensive cross-talk between the signaling pathways is activated by growth factors, and further research is needed to explore the complex mechanisms involved. It is important to consider that the initiation of signal transduction cascades may lead to disparate outcomes in different cell types. EMT and MET programs can be induced by a variety of contextual signals that cancer cells may experience in diverse tissue sites throughout the body. This issue also requires further experimentation.

### Transcriptional factors

Several transcription factors, including zinc finger proteins of the Snail and Twist families (e.g., *DEF1/ZEB1/TCF8* and *SIP1/ZEB2/ZFH1B*) and the basic helix-loop-helix factor *E12/E47*, which have been shown to be associated with the repression of E-cadherin (22), are also depressed during MET. As described above, downregulation of ZEB1, Slug, Snail, SMA, or Twist is usually induced during MET (49, 61, 64). It was also found that progesterone (P4) can regulate the expression of Snail and other EMT-relevant proteins in the human breast cancer cell line MB468 and induce cell morphological reversion from mesenchymal to epithelial phenotypes via membrane progesterone receptor  $\alpha$  (mPR $\alpha$ ; ref. 63). Downregulation of Notch signaling by siRNA also led to partial reversal of the EMT phenotype and decreased expression of vimentin, ZEB1, Slug, Snail, and NF- $\kappa$ B (67). Furthermore, Snail and Twist2 were also significantly downregulated during MET-induced hyperbaric oxygen treatment in a 7,12 dimethylbenz(a)anthracene (DMBA)-induced mammary rat adenocarcinoma model (68). All of these findings suggest that these transcription factors are closely associated with MET.

Several other pieces of evidence support the notion that these transcription factors themselves can induce MET. ZEB1 and ZEB2 are able to initiate EMT by binding to E-boxes within the E-cadherin promoter and repressing its transcription, and silencing of ZEB1 was shown to increase the expression of E-cadherin (69, 70). Downregulation of ZEB2 mRNA via ZEB2 siRNA in 4TO7 cells increased E-cadherin mRNA, and the cells that had stably silenced ZEB2 expression also adopted an epithelial-like morphology (25). These data reveal an important role of ZEB2 in MET. Supporting this notion, it was found that decreasing ZEB1 and ZEB2 expression in mouse mammary gland cells with shRNA was also sufficient to upregulate expression of epithelial proteins, such as E-cadherin, and to reestablish epithelial features (71). Furthermore, inhibition of 2 other EMT regulators, Snail and Twist, led to upregulation of E-cadherin and MET (72, 73), and knockdown of Twist or Snail in hepatocellular carcinoma cell lines, such as Mahlavu cells, reversed EMT (74).

Given the correlation between the downregulation of such transcription factors and E-cadherin expression, it is important to study their roles in diverse cancer cells. Targeting these transcriptional factors may be a more direct and effective way to control the MET.

### MicroRNAs

MicroRNAs (miRNA) are small, noncoding RNAs that modulate gene expression posttranscriptionally and play essential roles in many physiological and pathological processes, including tumor development. The expression of several miRNAs was changed during EMT or MET, and the change in expression of several miRNAs may even induce EMT or MET. The breast carcinoma cell lines that express E-cadherin and retain the features of well-differentiated epithelial cells were found to express the miR-200 family and miR-205, whereas cells that are invasive and generally mesenchymal in phenotype expressed low or undetectable levels of the miR-200 family and miR-205 (75). The miR-200 family was shown to inhibit the initiating step of metastasis and EMT by maintaining the epithelial phenotype through direct targeting of the transcriptional repressors of E-cadherin (75), suggesting that the miR-200 family is greatly associated with the epithelial phenotype. Furthermore, the ectopic expression of miR-200c in lung and breast cancer cells (A549 and MDA-MB-231) was shown to reduce levels of ZEB1, restore E-cadherin expression, and alter cell morphology (76). In a study of 4 isogenic mouse breast cancer cell lines (67NR, 168FARN, 4TO7, and 4T1), the 4T1 cells (the only ones that could form macroscopic metastases when implanted into mammary fat pads) also had elevated expression of miR-200 family miRNAs and high expression of E-cadherin and cytokeratin-18, and of interest, overexpression of miR-200 in 4TO7 cells enabled them to metastasize to lung and liver (25). These results show that the miR-200 family can induce MET and contribute to the formation of metastases. In addition, miR200 miRNAs were found to directly target the 3'-untranslated regions of the mRNA and

inhibit the expression of WAVE3, an actin cytoskeleton remodeling and metastasis promoter protein, resulting in a significant reduction in the invasive phenotype of cancer cells and inducing MET of the cells (77). Expression of miR-200 and miR-30 in mesenchymal anaplastic thyroid carcinoma-derived cells also reduced their invasive potential and induced MET by regulating the expression of MET marker proteins (78).

MiRNA has been found to target distinct functions in different signaling pathways, thereby contributing to several key events associated with tumor progression. Recent research suggests that miRNAs may also be important regulatory factors of the MET. Therefore, targeting miRNA may be a good method to regulate changes in the cell phenotype and a good therapeutic approach for cancer treatment.

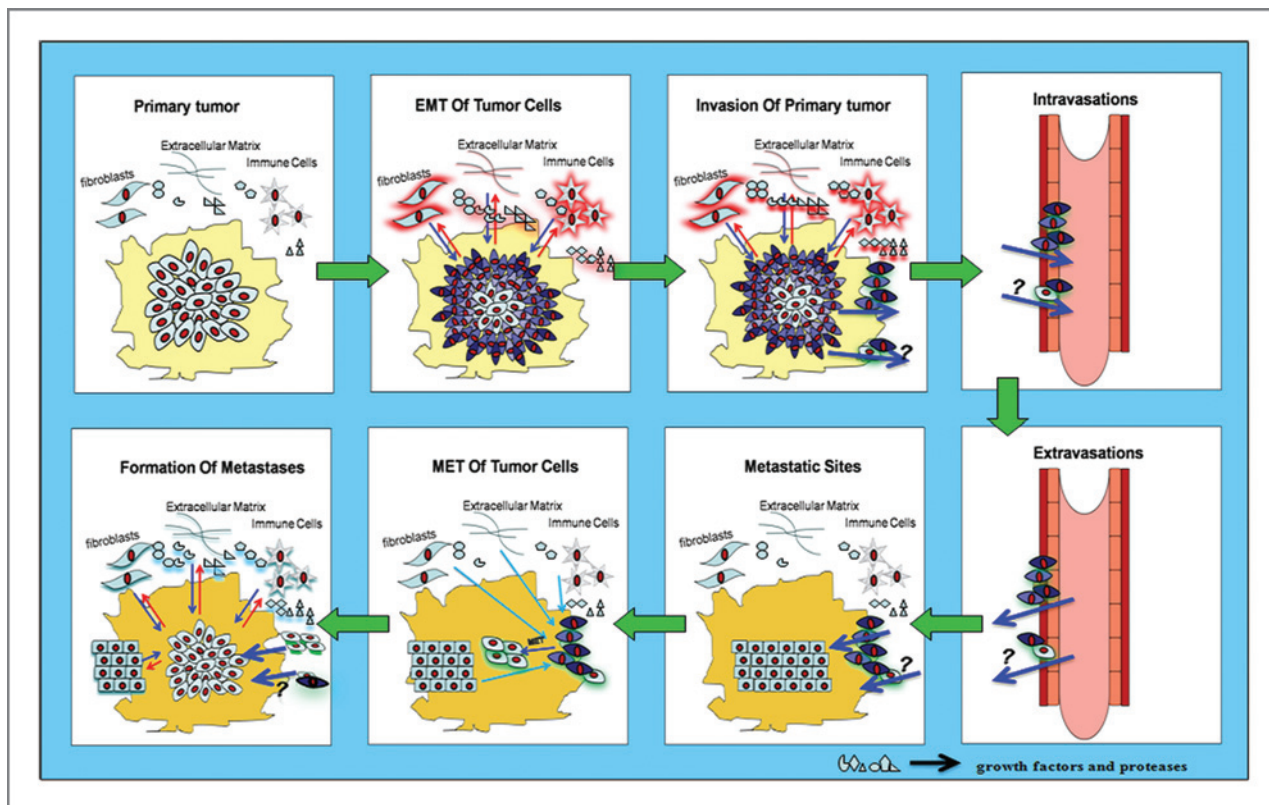
**Mechanisms of MET in Promoting the Formation of Metastatic Tumors**

Although the role of MET in metastatic tumor formation is gradually being proved, the exact mechanisms of this process, such as where and how MET takes place and how it facilitates the formation of metastases, remain largely elusive.

**Microenvironment and changes in cell phenotype**

As indicated above, the factors that induce EMT or MET in carcinomas are often components of heterotypical signaling pathways that originate in the tumor-associated stroma from cells creating the tumor microenvironment, and the changes in gene expression observed during EMT or MET are reversible (7, 79). Studies have shown that cancer cells can activate local stromal cells, such as fibroblasts, smooth muscle cells, and adipocytes, and recruit endothelial and mesenchymal progenitors and inflammatory cells. In turn, this stromal activation could further favor cancer cell proliferation and invasion via the secretion of additional growth factors and proteases and promotion of EMT (80–82). EMT occurs all along the tumor-host interface of carcinomas, supporting the notion that the environment triggers EMT at the tumor-host interface (13). Cancer cells may also undergo MET because of influences originating in their microenvironment (Fig. 2). This was shown by the upregulation of E-cadherin expression and the acquisition of differentiated epithelial cell features when prostate cancer cells were cocultured with normal hepatocytes (36).

Another plausible mechanism has been proposed to explain the changes in the cell phenotype: numerous signals in primary tumors actively promote the induction and continued expression of an EMT, whereas tumor cells that



**Figure 2.** Mechanisms of cell phenotype changes in the process of metastasis. EMT and MET play important roles in the process of cancer metastasis. During transition of the cell phenotype, complex communications occur among the host fibroblasts, extracellular matrix, immune cells, and cancer cells. Interactions with host elements may influence the progression of cancer at all stages.

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leave the primary site may revert to the epithelial state due to the absence of EMT-inducing signals (83). Thus, in the absence of the EMT-inducing signals received from the activated stroma that are present in primary tumors, metastatic cancer cells may simply fall back to an epithelial state when entering into sites of dissemination (84, 85). However, some studies found that the induction of an EMT seemed to be able to create a heritable state, even long after the EMT-inducing stimulus was removed (86). Furthermore, increasing evidence suggests that the EMT process is greatly associated with resistance to chemotherapy, radiotherapy, and even targeted therapy (see the "Clinical importance of EMT and MET" section). This suggests that the induction of EMT may contribute to not only the invasion and dissemination of tumor cells in the primary sites but also help the tumor cells survive in the circulation or sites of dissemination. Therefore, EMT may also occur even when the cancer cells leave the primary tumors, and MET should not be induced only by reason of the absence of EMT-inducing signals.

Recently, Aokage and colleagues (16) immunostained 13 molecular markers of EMT and MET, and then scored the immunostaining intensity of cancer cells floating in lymphatic vessels, migrating into the connective tissue surrounding vessels, and growing in lung parenchyma. They showed that when tumor cells invaded and grew in lung parenchyma in the early phase of metastatic tumor formation, the tumor cells that had extravasated and invaded the connective tissue surrounding vessels from within the lymphatic vessel underwent an EMT and later underwent a reverse transition (i.e., MET). The authors concluded that the MET of tumor cells took place after the tumor cells arrived at the lung parenchyma and considered that these results would be applicable to hematogenous metastasis. Therefore, the microenvironment in which the metastatic tumor would be formed was suggested to contribute to the MET. Moreover, when cultured in a hepatic microenvironment, MDAMB-231 was found to exhibit a reversion to an epithelial phenotype, in terms of both morphology and E-cadherin reexpression. Some initial studies found that neither conditioned media nor a hepatocyte-derived matrix could trigger E-cadherin reexpression in this breast carcinoma line, indicating that the E-cadherin reexpression may not be driven by the extracellular matrices but by contact with the hepatocytes (11). In addition, Yates and colleagues (36) found that when prostate cancer cells (DU-145) were cocultured with hepatocytes, E-cadherin expression was elicited by the hepatocytes at peripheral sites of contact. These findings suggest that it may be the normal parenchymal cells, with which the tumor cells would be in contact in the microenvironment, that contribute to MET; however, some other, as yet unidentified factors may also be involved.

#### Construction of metastases

Presently, it is unclear how MET facilitates the formation of metastases. Nascent efforts are being made to uncover these related mechanisms. Some findings suggest a possible

mechanism whereby MET helps the tumor cells to construct connections with the resident normal cells. These close connections are suggested to exist between tumor cells and the resident normal cells. In one study (87), many breast cancer metastases to the liver seemed to recreate hepatocyte cords with carcinoma cells. Moreover, in an *ex vivo* model of carcinoma metastasis to the liver, ultrastructural evidence for close connections was also found (88). In addition, cadherin-mediated adhesion is not only a critical element of homologous cells but it also occurs between heterotypic cells. As the important marker of MET, E-cadherin is currently suggested to play a very important role in promoting the formation of metastases. Histopathological analyses of a number of tumors suggested close associations between metastatic carcinoma cells and the neighboring parenchyma cells, supporting the possibility of carcinoma-parenchyma binding via E-cadherin (9, 10, 14). It was also found that when prostate carcinoma cells were induced to reexpress E-cadherin, both DU-145 and PC3 cells were able to form heterotypic adhesions with rat hepatocytes (36). These data suggest that carcinoma cells may reexpress E-cadherin in response to the ectopic organ microenvironment to establish connections with the resident, nonneoplastic epithelial cells.

#### Survival advantage of cancer cells with MET

In addition to their role in constructing connections, the formation of cell heterotypic E-cadherin adhesions in the metastatic target organ may result in dormancy and enable the tumor cells to survive at a lower metabolic load at the micrometastasis stage (20). E-cadherin may also serve as an upstream regulator that triggers downstream kinases activation and helps the disseminated mesenchymal tumor cells survive at the site of metastases and then form metastases. It was found that E-cadherin binding could activate intracellular proliferation and survival signals by activating the survival-associated mitogen activated protein kinase (MAPK) and Akt/PKB cascades via the classical Raf-MEK-MAPK pathway and PI3K, respectively (89). Moreover, it was found that E-cadherin cell-cell adhesion suppressed anoikis and increased the resistance of cells to cytotoxic agents by activating ErbB4, which also led to induction of the PI3K-Akt pathway (90). The exact mechanisms of this process and more related signals require further investigation.

#### EMT, MET, and Cancer Stem Cells

Cancer stem cells (CSC) constitute a small minority of neoplastic cells within a tumor and are defined operationally by their ability to seed new tumors (91). For this reason, they have also been termed tumor-initiating cells (92). The existence of CSCs, or tumor-initiating cells, was first reported by Lapidot and colleagues (93). Since then, CSCs have been identified in numerous solid tumors, including breast, colon, endometrial, pancreas, prostate, ovary, and brain tumors (94–102). High tumorigenicity was shown in CSCs. For example, in the work of Al-Hajj and colleagues

(99), 100 tumor cells exhibiting the CD44<sup>high</sup>/CD24<sup>low</sup> cell surface marker profile were sufficient to initiate tumors in mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors. Similar results were also observed in other studies (98).

Recently, the EMT program, which is typically associated with motility and invasiveness (4), was suggested to be greatly linked with CSCs (103–105). In what appears to be the first demonstration that EMT leads to the generation of breast cancer cells with stem cell–like characteristics, Mani and colleagues (103) showed that the induction of EMT in differentiated HMLE cells by either overexpression of Snail or Twist or exposure to TGF- $\beta$ 1 caused the cells to acquire the CD44<sup>high</sup>/CD24<sup>low</sup> stem cell profile. In support of this finding, another independent group showed that in mammary epithelial cells, treatment with TGF $\beta$  increased the number of stem cells, as defined by their cell surface antigenic profiles, ability to form mammospheres in culture, and ductal outgrowths in xenotransplant assays (106). Furthermore, it was shown that in ovarian cancer, transfection with 2 EMT inducers, Snail and Snail2, led to derepression of stemness genes, including *Nanog* and *KLF4*, and 4- to 5-fold increases in the size of a CD44<sup>high</sup>/CD117<sup>high</sup> CSC population (107), giving us another example that the induction of EMT in more-differentiated cancer cells can generate CSC-like cells.

The development of metastasis may also involve the dissemination of CSCs, including cells at the tumor margins that have undergone EMT, known as migrating CSCs (108). These CSCs have undergone EMT for dissemination and retain stem cell functionality for formation of metastases (12). Studies have shown that CSCs are enriched in cancer cells disseminated in the circulation or sites of metastases. For example, breast cancer cells disseminated in the circulation and bone marrow were found to be enriched for the CD44<sup>high</sup>/CD24<sup>low</sup> antigen phenotype (109–111). Furthermore, it was found that chemokine receptors can express on CSCs and that CSCs can migrate along a gradient of the CXCR4 ligand CXCL12 (also known as SDF-1), originating from hematopoietic niches and many other tissue sites, thereby facilitating metastasis of CSCs to particular sites (112–114).

Mounting evidence indicates that CSCs are also involved in colonization and metastases formation (115–117). In addition to contributing to the generation of CSCs, EMT may give differentiated tumor cells the ability to self-renew, thus allowing the successful establishment of secondary tumors composed of cancer cells with heterogeneity at distant sites (103, 118, 119). Not only can CSCs expand in number by symmetric divisions, but they can also undergo self-renewal by asymmetric cell division, contributing to the heterogeneity of cancer cells. The cellular processes enabled by EMT during cancer metastasis are possibly analogous to the processes that adult stem cells use when participating in tissue reconstruction (120). When the migrating CSCs generated by EMT arrive at distant tissues, they can form secondary tumors that even exhibit an epithelial phenotype via MET. The reverse of EMT,

MET, observed during embryonic development, is also suggested to be operational in the formation of secondary metastatic nodules.

### Clinical Importance of EMT and MET

EMT is believed to be a major mechanism by which cancer cells become migratory and invasive, enabling the dissemination of cancer cells (4). In the EMT process, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility (4). The expression of proteins that are characteristic of mesenchymal cells and the loss of epithelial markers correlates with tumor progression (121), and invasion of adenocarcinomas is accompanied by the release of single cells through the EMT process (4). Many researchers have observed some loss of epithelial characteristics paired with a gain of mesenchymal markers in the invasive front of various cancers (13), pointing to a possible contribution of EMT to the acquisition of an invasive phenotype leading to metastasis.

Recent studies have shown that EMT can lead to the generation of cancer cells with stem cell–like characteristics, including escape from immune surveillance, increased resistance to apoptosis, and diminished senescence, as well as an increased ability to self-renew and initiate new tumors, and these characteristics further lead to therapy resistance of cancer cells (122). For example, EMT induced by Twist or Snail was found to be related to chemoresistance of ovarian carcinoma cells (123) and the lung carcinoma cell line A549 (124). Gemcitabine-resistant pancreatic tumor cells also exhibited phenotypic changes associated with EMT and acquired stem cell–like characteristics (125). EMT induced by EGFR signaling was also linked to tamoxifen resistance in MCF-7 cells (126, 127). Of interest, in pancreatic cells, EMT was also shown to contribute to drug resistance, and reversal of EMT by silencing Zeb-1 restored drug sensitivity (69). In support of the relationship between therapy resistance and EMT, it was found that endometrial carcinoma cells with resistance to radiotherapy exhibit a mesenchymal phenotype, including decreased expression of E-cadherin (128). Upregulation of Snail and Slug in ovarian cancer cells is also associated with acquisition of radioresistance and chemoresistance of ovarian cancer cells (107). Furthermore, the EMT process has been proposed to be associated with resistance to targeted therapy (127, 129, 130), potentially bypassing the dependence on this pathway by activation of its downstream targets (131). Moreover, the resistance to apoptosis that is integral to cells generated by an EMT should be critical for the ability of carcinoma cells to survive the passage from primary tumors to sites of dissemination (132). These selective advantages may enable the dissemination of cancer cells and their long-term survival at distant sites and may even make cancer cells resistant to conventional therapies. It is conceivable that due to the presence of therapeutically resistant CSCs, possibly as a result of the EMT process, many patients experience relapse, and tumors become refractory to further treatments.



These findings provide convincing support for the role of MET in sites of dissemination. Classical chemotherapy and endocrine therapy generally target more-differentiated epithelial cells and may cause a substantial proportional increase in tumor cells with stem/progenitor phenotypes (117). Therefore, metastases that are histopathologically similar to the primary tumors should be formed via MET of disseminated MCS cells.

## Conclusions

Metastasis is a fatal step in the progression of cancer, with death from metastases accounting for approximately 90% of all human cancer mortalities (133). Most cancer patients die of metastases rather than from their primary tumors. Therefore, it is critical to study the molecular mechanisms of metastasis and elucidate therapeutic targets to prevent the spread of cancer.

To explain the similarity between metastases and their corresponding primary tumors, a MET process in the metastatic sites has been postulated to be part of the process of metastatic tumor formation (18, 19). However, investigators have proposed other explanations, such as the collective migration theory (24). According to this theory, during the progression of invasive and metastatic carcinoma, epithelial cancer cells can invade the surrounding tissue and metastasize, via functional cooperation between mesenchymal and more-differentiated epithelial cancer cells, and then participate in the formation of metastases that are histopathologically similar to the primary tumors. Although this kind of cooperation is conceivable and cannot be excluded easily, this hypothesis does not sufficiently explain therapy resistance, tumor cell dormancy, or disease recurrence. This observation suggests that the processes of EMT and MET provide an important and more reasonable explanation, although more supporting data are still needed.

At present, the process of metastasis is still poorly understood. This unfortunate lack of conceptual understanding is partially due to the difficulties inherent in direct observation of this phenomenon. Experimental metastasis models have helped to reveal numerous mechanisms involved in metastasis, but they entail certain limitations. For example, in some experimental metastasis models, cancer cells of different phenotypes were inoculated into the arterial circulation through the left heart ventricle of female nude mice to cause selective development of metastases and reveal the role of MET inducers in the formation of metastases. However, owing to the selective disadvantages of MET cells as reviewed above, cancer cells with an epithelial phenotype may not survive

before they reach the sites of metastases formation and then form metastases, and the results may not exactly reveal the roles of phenotype transitions in metastases formation. This situation may explain why some investigators obtained results that seemed to contradict the conclusion that MET promotes metastasis (46, 47, 134). Certainly, the exact reasons for these results remain to be revealed in the future, perhaps by improving the experimental metastasis models. In addition, thanks to recent advances in intravital videomicroscopy techniques, studies are shedding light on numerous critical steps in tumor metastasis (135–142). Such techniques represent a powerful tool for studying fluorescently labeled proteins within individual tumor cells in animal models. Clearly, techniques to facilitate real-time observations *in vivo* will greatly enhance our understanding of metastasis and answer many nagging questions about the role of EMT and MET in this process.

Studies suggest that formation of micrometastases and the process in which micrometastases progress to macrometastases are the main rate-limiting steps in the process of metastasis (139). The mechanisms of metastatic tumor formation are complex, and much remains to be learned. Although MET has been revealed to play an important role in metastatic tumor formation, there must be other mechanisms that participate in the process because some phenomena are difficult to explain only by phenotype transitions. These include formation of metastases of some rare carcinomas (e. g., diffuse type gastric cancer, lobular breast cancer, and endometrial cancer) in which E-cadherin expression seems to be irreversibly lost due to mutations in the E-cadherin gene. Although normal E-cadherin expression was revealed in some metastatic foci of lobular carcinoma, the E-cadherin expression was still lost in major metastases (10). Nevertheless, given the reversibility of EMT in the vast majority of carcinomas and the importance of these tumor-associated phenotypes in metastasis (122), targeting such cellular plasticity and its effects on cancer cells is still likely to be an attractive, albeit challenging, approach to improve clinical management of cancer patients, especially for patients with a high risk of metastasis and recurrence.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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