Epithelial-Mesenchymal Transition and Cell Cooperativity in Metastasis

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Abstract
The role of epithelial-mesenchymal transition (EMT) in metastasis remains controversial. EMT has been postulated as an absolute requirement for tumor invasion and metastasis. Three different models including incomplete EMT, mesenchymal-epithelial transition (MET), and collective migration have been proposed for the role of EMT in cancer invasion and metastasis. However, skepticism remains about whether EMT truly occurs during cancer progression, and if it does, whether it plays an indispensable role in metastasis. Our recent findings suggest that EMT cells are responsible for degrading the surrounding matrix to enable invasion and intravasation of both EMT and non-EMT cells. Only non-EMT cells that have entered the bloodstream are able to re-establish colonies in the secondary sites. Here, we discuss an alternative model for the role of EMT in cancer metastasis in which EMT and non-EMT cells cooperate to complete the entire process of spontaneous metastasis. [Cancer Res 2009;69(18):7135–9]

Epithelial-Mesenchymal Transition and Cancer Metastasis

Epithelial-mesenchymal transition (EMT) was first recognized as a central differentiation process in early embryogenic morphogenesis (1). It is a coordinated molecular and cellular change defined as a reduction in cell-cell adhesion, apical-basolateral polarity, and epithelial markers, as well as an acquisition of motility, spindle-cell shape, and mesenchymal markers. The definition of EMT in embryo development, which includes an ordered series of transcriptional events and a switch in cell fate, has been more liberally referred to as a recognizable change in cellular phenotype characterized as loss of cell junctions and gain of migratory behaviors (2).

This more inclusive EMT process has been proposed and supported by numerous publications to be a potent mechanism that enhances the detachment of cancer cells from primary tumors. However, it is still controversial whether transformation of a noninvasive tumor into a metastatic tumor truly represents an EMT, and if it is, how important it is in the process of cancer metastasis (3, 4). The main argument for the lack of a role of EMT in cancer is that metastases seem histopathologically similar to the primary tumors from which they are derived. To resolve this apparent contradiction, a mesenchymal-epithelial transition (MET) process in the metastatic sites has been postulated as part of the process of metastatic tumor formation (5). MET is an attractive hypothesis that can explain the histopathological similarity between primary and metastatic tumors. In support of the MET hypothesis, dynamic expression of E-cadherin (CDH1) in cancer progression has been documented. However, direct experimental data supporting MET in cancer metastasis are still lacking. For example, Graff and colleagues showed that the DNA methylation status of the CDH1 promoter varies at different stages of the metastatic process (6). In primary breast cancers, the tumors that undergo transient hypermethylation and repression of CDH1 are more invasive and metastatic. Subsequently, demethylation of the CDH1 promoter occurs and E-cadherin is re-expressed during metastases (6). Unfortunately, the clonal identities of these metastatic cancer cells have not been determined. There is still a lack of convincing experimental data to support the notion that MET is an integral part of metastasis. Dumont and colleagues have also reported that de novo methylation during cancer progression has a deterministic rather than stochastic pattern (7). As such, the model of sequential EMT and MET has yet to be further tested. There is also strong evidence that tumor invasion can occur in the absence of EMT. For example, Wicki and colleagues have shown that podoplanin promotes breast cancer cell invasion through downregulation of the activities of small Rho family GTPases, which results in remodeling of the actin cytoskeleton but does not alter the expression and localization of EMT markers (8).

On the other hand, tumors are made up of a highly diverse population of cells that exhibit a wide range of phenotypes from stem cell-like cells to well-differentiated cells (3). There are several lines of evidence suggesting that many invasive and metastatic carcinomas have not undergone a complete transition to a mesenchymal phenotype or even lack signs of EMT, and that invasive carcinomas do not invade adjacent connective tissue as individual mesenchymal-like cells. These carcinoma cells invade as multicellular aggregates or clusters (9). Accordingly, a cell cooperativity theory has been proposed and experimentally proven to play an important role in cancer metastasis (10). In view of the contention that cancer EMT is a result of reactivation of a normal embryonic development program, the cell cooperativity theory also has developmental roots, because cooperation also occurs during normal development of embryos (11).

Similarly, a “class action” theory has been proposed in which successive waves of homogeneous and heterogeneous circulating cancer cells form a premetastatic niche that promotes colonization of metastases (12). This premetastatic niche hypothesis is in agreement with the observation that cancers can remain dormant for years or even decades before overt progression and metastasis.
Ectopic expression of p12 in HCPC-1 cells induced morphological change from polygonal to fibroblastoid structure, accompanied by a complete loss of E-cadherin and desmoplakin expression and the gain of vimentin and N-cadherin expression. Twist 2, an E-cadherin repressor, was upregulated in p12-transfected cells. Although the mechanism by which Twist 2 suppresses CDH1 expression is not clear, its function in p12-induced EMT was confirmed by morphological reversion of EMT cells after treatment with Twist 2 siRNA (19).

Twist proteins are essential for proper gastrulation, mesoderm formation, and neural crest migration during development, which represents a typical EMT event. Twist 2 expression is detected in cranial mesenchymal cells around the nose and pharyngeal arches and tongue of mouse embryos, and is progressively restricted to the superficial regions in tongue and jaws during embryonic development (20), indicating its involvement in maintenance of mesenchymal lineage. Twist 2 is overexpressed in a large variety of human primary tumors and cancer cell lines. Ectopic expression of Twist 2 induces EMT of Madin-Darby canine kidney cells (MDCK) cells by repressing expression of E-cadherin, α-catenin, occludin, and claudin-7, and promoting expression of vimentin and N-cadherin (21). Twist 2 knockout mice have severe growth retardation with atrophic dermis, thymus, liver, and fat tissues (22). p12 knockout mice died during gestation, but two mice that survived exhibited craniofacial defects, with a short snout and a round forehead compared with wild-type animals (23). These results indicate that p12 and Twist 2 are linked to EMT and to the maintenance of mesenchymal lineage during embryogenesis. Therefore, p12-induced, Twist 2-mediated EMT is not merely an isolated experimental phenomenon but has physiological and pathological relevance.

Cooperativity between EMT and Non-EMT Cells

Besides the overt morphological and molecular changes, overexpression of p12 also results in behavioral changes of HPCP-1 cells. The p12 transfectants have clearly enhanced motility both in vitro and in vivo, and have acquired the ability to invade into the surrounding tissues from the primary tumors grown at subcutaneous regions. Both non-EMT (vector transfectants) and EMT (p12 transfectants) cells were able to establish ectopic tumors with a 100% tumor take rate and a similar growth curve when they were inoculated subcutaneously into athymic mice. However, only the EMT cells showed invasive fronts, penetrated into the surrounding muscle tissues, and intravasated into the blood vessels. EMT cells were detected inside the capillaries around the primary tumor site and in the blood stream. However, no non-EMT cells were detected inside the blood vessels or in circulation, indicating that they either failed to invade into the surrounding tissues or failed to intravasate. It is thus clear that EMT of HCPC-1 cells enhances their invasive behavior, consistent with numerous previous reports of the property of EMT cells. However, no metastasis was detected in either group of animals, even when the primary tumors had grown to 20% of body weight, at which time the animals had to be sacrificed.

**EMT Regulators**

A variety of extracellular signals have been shown to trigger transition of epithelial cells into mesenchymal or mesenchymal-like cells during embryogenesis and in tumorigenesis. Transforming growth factor-β (TGF-β), epidermal growth factor (EGF) family members, fibroblast growth factors (FGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) have all been shown to induce EMT in an autocrine or paracrine manner (14). TGF-β was the first EMT inducer described in normal mammary epithelial cells by signaling through its receptor serine-threonine kinase complex. It remains to be the main and the best-characterized inducer of EMT phenotype in a variety of biological and pathophysiological conditions. TGF-β has an important tumor suppressor function at the early stage of tumorigenesis by inducing apoptosis and cell cycle arrest. However, it acts as a positive modulator of tumor progression in the late phase of tumorigenesis. This tumor promotional function of TGF-β, which is consistent with its EMT-induction activity, plays an important role in tumor progression including invasion and metastasis. TGF-β-mediated signaling during EMT involves both gene expression-dependent and -independent pathways. The type II receptor of TGF-β, upon activation by TGF-β binding, interacts with occludin, a component of the tight junction, and phosphorylates Par6 protein. This direct protein-protein interaction and the subsequent phosphorylation of Par6 protein recruits Smurf1, thereby leading to ubiquitin-dependent protein interaction and the subsequent phosphorylation of Par6 protein. This direct protein interaction and the subsequent phosphorylation of Par6 protein recruits Smurf1, thereby leading to ubiquitin-dependent protein interaction and the subsequent phosphorylation of Par6 protein. Twist 2, an E-cadherin repressor, was upregulated in p12-transfected cells. Although the mechanism by which Twist 2 suppresses CDH1 expression is not clear, its function in p12-induced EMT was confirmed by morphological reversion of EMT cells after treatment with Twist 2 siRNA (19).

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**EMT of HCPC-1 Cells Induced by p12CDK2-AP1**

*p12<sup>CDK2<sub>AP1</sub></sup>* was first identified and isolated from hamster normal oral keratinocytes as a putative tumor suppressor gene by subtractive hybridization (17). p12 expression is induced by TGF-β (18) and serves as its down-stream effector to mediate the growth inhibitory activity of TGF-β by interacting with DNA polymerase α/primase and cyclin-dependent kinase 2 (CDK2). In our recent study, we showed that p12 also mediates TGF-β-induced EMT of hamster cheek pouch carcinoma-1 (HCPC-1) cells (19). Moreover, overexpression of p12 directly induces EMT of HCPC-1 cells (19), at least according to the more inclusive definition of EMT in cancer biology (2). Ectopic expression of p12 in HCPC-1 cells induced morphological change from polygonal to fibroblastoid structure, accompanied by a complete loss of E-cadherin and desmoplakin expression and the gain of vimentin and N-cadherin expression. Twist 2, an E-cadherin repressor, was upregulated in p12-transfected cells. Although the mechanism by which Twist 2 suppresses CDH1 expression is not clear, its function in p12-induced EMT was confirmed by morphological reversion of EMT cells after treatment with Twist 2 siRNA (19).

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Metastasis of carcinoma is a complex process including detachment of tumor cells from primary tumors, invasion through the basement membranes and the local mesenchymal tissues, intravasation into blood or lymphatic vessels, survival in the circulation and from immune clearance, lodging and extravasation in distant organs, and proliferation at the secondary sites. It is possible to model various stage of the metastatic cascade by varying the site of inoculation of cancer cells. The

**Figure 1.** A novel model for the role of EMT in cancer progression and metastasis. A, EMT invade into the blood stream but do not establish lung metastasis. B, Non-EMT cells do not invade but establish lung metastasis once it gets into the blood stream. C, cooperation between non-EMT and EMT cells complete the entire metastatic process. D, model of cell cooperativity. Cancer cells with an EMT phenotype invade into the surrounding tissues, enable non-EMT cells to migrate and intravasate so that both cell types enter the circulation. However, only non-EMT cells are able to regrow in the distant organs and establish metastasis.
growth and development of metastases from the orthotopic or subcutaneous site requires tumor cells to complete the entire metastatic process. Intravenous injection can bypass the initial stages of metastasis and measures the ability of the cells to survive in the blood circulation, extravasation, and formation of metastatic deposits at a secondary site. Surprisingly, EMT HCCP-1 cells failed to establish lung metastases even when they were directly inoculated into the blood stream by tail vein injection. However, overt lung metastases formed from non-EMT cells when they were injected via tail vein. These results indicate that p12-induced EMT is accompanied with a decreased ability to establish metastatic tumors in the lung although they have enhanced migratory and local invasive phenotypes. This is somewhat in contrast to the mainstream theory that EMT cells have increased ability of metastasis.

Re-expression of E-cadherin by transfection of a cytomegalovirus (CMV) promoter-controlled CDH1 cDNA changed the morphology of EMT cells from the fibroblastoid structure back to polygonal ones. Therefore, MET did occur from the viewpoint of cell morphology. However, E-cadherin re-expressed MET cells also failed to establish lung metastasis when they were directly injected into the tail vein of athymic mice. It is notable that the ability to establish and to grow xenograft tumors in the subcutaneous region was not different among the non-EMT, EMT, and MET cells.

More surprisingly, lung metastases formed when a mixture of non-EMT and EMT cells were co-inoculated subcutaneously. In this experiment, non-EMT and EMT cells were labeled with DsRed and green fluorescent protein (GFP), respectively, so that the origin of the metastatic cells could be determined by fluorescence and immunohistochemistry (IHC). In the primary tumors, non-EMT and EMT cells coexisted with an enrichment of non-EMT cells in the center and EMT cells on the periphery. The edges of the primary tumors had invasive appearances. They invaded the muscle and fat tissues, in a very similar way to the tumors derived from EMT cells alone. Importantly, both EMT and non-EMT cells were detected in the blood stream, indicating that intravasation of both cell types occurred under this circumstance. Because inoculation of non-EMT cells alone failed to intravasate, this result showed a cooperation between non-EMT and EMT cells in the process of local invasion and intravasation, adding another line of evidence to the cell cooperativity theory in cancer metastasis (10).

The metastatic tumors in the lung were entirely composed of non-EMT cells. No EMT cells were detected in the lung tissue either by IHC for GFP or by PCR for its DNA. Because both cell types were detected in the blood stream with a half-life of the likely reason for the failure of EMT cells to establish metastasis may be related to their inability to adhere to the lung vasculature or to extravasate, rather than a difference in escaping immune clearance. These results show that at least in HCCP-1 cells, p12-induced EMT is not sufficient for metastasis, and cooperation of non-EMT cells is necessary.

These findings are summarized in Fig. 1. Cancer cells with an EMT phenotype can invade into adjacent connective tissues, and intravasate; but they are unable to form metastatic nodules in the lung even when they are directly injected into the blood circulation (Fig. 1A). Cancer cells without an EMT phenotype cannot invade into adjacent connective tissue, but they have the ability to form metastatic nodules in the lung when they are intravenously injected (Fig. 1B). More importantly, cancer cells with a mixed EMT and non-EMT phenotype can complete the entire process of spontaneous metastasis (Fig. 1C). On the basis of these findings, an additional model can be proposed for the role of EMT in cancer metastasis (Fig. 1D). EMT and non-EMT cells cooperate to complete the spontaneous metastasis process. EMT cells, with enhanced migratory and invasive phenotype, are responsible for degrading the surrounding matrix and penetrating the local tissues and blood or lymphatic vessels, thereby leading the way to intravasation. Non-EMT cells migrated either together with EMT cells, or immediately following them to enter the blood or lymphatic streams. Both cell types survive in circulation, but EMT cells fail to lodge to the vessel wall at the secondary site, probably because of reduced adhesive properties. Circulating non-EMT cells, with an unaltered adhesive phenotype, will be able to attach to the vessel wall, extravasate, and reestablish colonies in the secondary sites.

Although the significance and relevance of this model in cancer therapy are not clear at present, these findings support the cell cooperativity as well as the premetastasis niche theories, and suggest that a linear conversion of cancer cells through the successive EMT and MET processes for establishing metastasis may not be entirely correct.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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