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New Insights of Epithelial-Mesenchymal Transition in Cancer Metastasis

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Abstract

Epithelial-mesenchymal transition (EMT) is a key step during embryonic morphogenesis, heart development, chronic degenerative fibrosis, and cancer metastasis. Several distinct traits have been conveyed by EMT, including cell motility, invasiveness, resistance to apoptosis, and some properties of stem cells. Many signal pathways have contributed to the induction of EMT, such as transforming growth factor- β , Wnt, Hedgehog, Notch, and nuclear factor κ B. Over the last few years, increasing evidence has shown that EMT plays an essential role in tumor progression and metastasis.

Understanding the molecular mechanism of EMT has a great effect in unraveling the metastatic cascade and may lead to novel interventions for metastatic disease.

Keywords

epithelial-mesenchymal transition; metastasis; Snail; Twist; signal transduction

Although 90% of cancer deaths are caused by metastasis, the pathogenesis and mechanism underlying this event remains poorly defined. Understanding this process will provide great promise for the discovery of novel therapeutics for treating metastatic cancer. Metastasis is a 'hidden' event, which happens inside the body and is difficult to examine. It is believed to consist of four distinct steps: invasion, intravasation, extravasation, and metastatic colonization [1,2]. During invasion, tumor cells lose cell–cell adhesion, gain mobility, and leave the site of the primary tumor to invade adjacent tissues. In intravasation, tumor cells penetrate through the endothelial barrier and enter the systemic circulation. In extravasation, cells that survive the anchorage-independent growth conditions in the bloodstream attach to vessels at distant sites and leave the bloodstream. Finally, in metastatic colonization, tumor cells form macrometastases in the new host environment [1,2]. Using *in vivo* video microscopy and quantitative approaches, the first step, the acquisition of invasive ability and motility, is found to be the rate-limiting step in the metastatic cascade [1,3]. Beyond this step, survival of tumor cells in the circulation, their arrest in a distant organ, and their initial extravasation are relatively efficient processes. These findings clearly indicate that understanding the initial step of metastasis is critical to the future development of novel strategies to prevent cancer metastasis. Epithelial-mesenchymal transition (EMT), a process vital for morphogenesis during embryonic development, is attracting increasing attention as an important mechanism for the initial step of metastasis. Here we highlight the significance of EMT in cancer development and our emerging understanding of its regulation in tumor metastasis. We present some of the

current mechanisms parallel between their known roles in EMT induction during development and how these processes can be hijacked by tumor cells to enhance metastasis.

EMT is a critical cellular process

EMT, a process vital for morphogenesis during embryonic development, was first recognized as a feature of embryogenesis in the early 1980s [4,5]. During gastrulation in *Drosophila* flies and mammals, cells migrate from an epithelial-like structure to spatially reorganize one of the three main embryonic layers, the mesoderm [4,5]. In this EMT process, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility [4, 6]. EMT is essential for many morphogenetic events, such as gastrulation and organogenesis in embryonic development, tissue remodeling, fibrosis and wound healing, and heart development [7,10]. The migratory nature of these cells has prompted comparisons with metastatic cells and attracts increasing attention as an important mechanism for the initial step of metastasis, since genes implicated in EMT during embryogenesis have been shown to control metastasis [5,6]. Some pathologists were initially skeptical of this theory because they could not conclusively determine that EMT was apparent in human tumor specimens [11]. However, a growing body of evidence strongly suggests that EMT is a critical early event for the invasion and metastasis of many carcinomas [12,13]. A hallmark of EMT is the loss of *E-cadherin* expression, an important caretaker of the epithelial phenotype [4,14]. E-cadherin is a cell-cell adhesion molecule that participates in homotypic, calcium-dependent interactions to form epithelial adherent junctions [15,16]. Loss of E-cadherin expression is consistently observed at sites of EMT during development and cancer, and the E-cadherin expression level is often inversely correlated with the tumor grade and stage [15,16]. Numerous studies have shown that virtually all cases of invasive lobular carcinoma, which accounts for 8% of all breast cancers, have loss of E-cadherin expression as a result of E-cadherin gene mutation and promoter hypermethylation [17,18]. However, patients with invasive ductal carcinoma (IDC), which accounts for 80% of all breast cancers, retain E-cadherin expression. Thus, dominant transcriptional repression is mainly responsible for the transient loss of E-cadherin expression during the metastatic progression of IDC [19,23].

Several transcription factors have been implicated in the transcriptional repression of E-cadherin, including zinc finger proteins of the Snail/Slug family, Twist [14,24–27], δ EF1/ZEB1, SIP1, and the basic helix-loop-helix factor E12/E47 [28–34]. These repressors can also act as molecular triggers of the EMT program by repressing a subset of common gene that encode cadherins, claudins, cytokines, integrins, mucins, plakophilin, occluding, and zonula occludens (ZO) proteins to promote EMT [10]. Strikingly, all of these transcriptional repressors are best known for their roles in early embryogenesis. The first discovered and most important of these repressors is Snail, a DNA-binding factor that was identified in *Drosophila* as a suppressor of the transcription of *shotgun* (an E-cadherin homolog) in the control of embryogenesis [35,36]. Snail has a central role in morphogenesis, as it is essential for the formation of the mesoderm and neural crest, which requires large-scale cell movements in organisms ranging from flies to mammals. Absence of Snail is lethal because of severe defects at the gastrula stage during development [37]. Expression of Snail represses expression of E-cadherin and induces EMT in MDCK (Madin-Darby Canine Kidney) and breast cancer cells [38–40], indicating that Snail plays a fundamental role in EMT and breast cancer metastasis by suppressing expression of E-cadherin. Microarray analyses of primary human breast cancers suggest that a high level of Snail expression is correlated with a poor clinical outcome in women with early-stage breast cancer [41,42]. In fact, overexpression of Snail was recently found in both epithelial and endothelial cells of invasive breast cancer but was undetectable in normal breast [43,44]. Some studies indicated that Snail was implicated in the initial migratory phenotype of primary tumors and considered as an early marker of EMT. In contrast, Slug, ZEB1, ZEB2/SIP1, and Twist could be responsible for the maintenance of migratory cell

behavior, malignancy and other tumorigenic properties. However, this model awaits more detailed analysis owing to specific and independent roles of the different factors.

Microenvironment signals, developmental Pathways, and EMT

EMT is a dynamic process and is triggered by stimuli that emanate from microenvironments, including extracellular matrix (such as collagen and hyaluronic acid) and many secreted soluble factors, such as Wnt, transforming growth factor- β (TGF- β), Hedgehog, epidermal growth factor (EGF), hepatocyte growth factor (HGF), and cytokines [45]. The major task is to delineate the signaling pathways mediated by these microenvironmental stimuli in initiating and controlling EMT and cancer metastasis. Among many of these signaling pathways, Wnt, TGF- β , Hedgehog, Notch, and nuclear factor κ B (NF κ B) signaling pathways are found to be critical for EMT induction. These signaling pathways orchestrate a concerted and elaborate gene program and protein network needed for the establishment of mesenchymal phenotypes after disassembly of the main elements of epithelial architecture, such as cell-cell junctions and cell polarity. As many of these normal developmental pathways are also involved in EMT, morphogenesis, and motility during development, it is not surprising that tumor cells usurp these pathways for their own purposes.

The Wnt/ β -catenin pathway has a particularly tight link with EMT [46]. On one hand, β -catenin is an essential component of adherent junctions, where it provides the link between E-cadherin and α -catenin and modulates cell-cell adhesion and cell migration. On the other hand, β -catenin also functions as a transcription cofactor with T cell factor (TCF). In unstimulated cells, the level of free cytoplasmic β -catenin is kept low through a destruction complex, which consists of axin, adenomatous polyposis coli (APC), GSK-3 β , and casein kinase (CKI). GSK-3 β phosphorylates β -catenin and triggers its ubiquitination and degradation by β -Trop. In the presence of Wnt ligands, Wnts bind to frizzled and LRP5/6 receptor complexes to inactivate GSK-3 β in the destruction complex. This, in turn, results in the stabilization and nuclear accumulation of β -catenin and leads to the transcription of Wnt target genes, such as c-myc, cyclin D, and survivin [47]. Nuclear translocation of β -catenin can activate the expression of Slug and thus induces EMT. Expression of β -catenin in oocyte induces a premature EMT in the epiblast, concomitant with Snail transcription. Interestingly, Snail is a highly unstable protein and is dually regulated by protein stability and cellular location. We showed that GSK-3 β binds and phosphorylates Snail at two consensus motifs to dually regulate the function of this protein: phosphorylation at the first motif regulates its ubiquitination mediated by β -Trop, whereas phosphorylation at the second motif controls its subcellular localization [40]. Thus, Wnt can suppress the activity of GSK-3 β and stabilizes the protein level of Snail to induce EMT and cancer metastasis [48,49]. Whether the synergistic activation of Snail and β -catenin by Wnt signaling pathway is required for EMT induction and metastasis of tumor cells remains to be defined.

TGF- β is a potent inducer of EMT. It not only contributes to EMT during embryonic development but also induces EMT during tumor progression *in vivo* [50]. Overexpression of Smad2 and Smad3 results in increased EMT in a mammary epithelial model [51]. Knockout of Smad3 blocks TGF- β -induced EMT in primary tubular epithelial cells, and the reduction of Smad2 and Smad3 function is associated with the decreased metastatic potential of breast cancer cell lines in a xenograft model [52]. TGF- β can also downregulate various epithelial proteins, including E-cadherin, tight junction protein ZO-1, and several specific keratins, and also upregulates certain mesenchymal proteins such as fibronectin, fibroblast-specific protein 1, α -smooth muscle actin, and vimentin. In addition, TGF- β cooperates with numerous kinases such as RAS, MAPK, p38MAPK to promote EMT [50,53]. Furthermore, TGF- β cross-talks with other signal pathways and coordinates the regulation of EMT. Recent reports suggest that

functional interactions between TGF- β with Notch, Wnt, and NF- κ B contribute significantly to the induction of EMT [50].

The Hh signaling pathway was first identified in a large screen for *Drosophila* genes required for patterning of the early embryo [54,55]. Analysis of the Hh mutant, named after its prominent phenotype (epidermal spikes in larval segments that normally are devoid of these extensions) led to the cloning of the Hh gene. The Hh ligands, Sonic (Shh), Desert (Dhh), and Indian (Ihh) in vertebrates and Hh in *Drosophila*, are secreted proteins that undergo several posttranslational modifications to gain full activity. Key effectors of Hh signaling include zinc-finger proteins of the Gli1-3 transcription factors. Hh signaling can initiate cell growth, cell division, lineage specification, and axon guidance and can also function as a survival factor. In light of this range of biologic functions, it is not surprising that mutations in components of the Hh pathway are associated with both embryonic developmental defects and tumor progression. Indeed, mutations in Patched (PTC) and/or Smoothed (SMO) trigger inappropriate activation of the Hh pathway and have been identified in basal cell carcinoma, rhabdomyosarcoma, medulloblastoma, and other tumor types [54,55]. In mouse epidermal cells or in rat kidney epithelial cells immortalized with adenovirus E1A, Gli1 rapidly induces transcription of Snail and promotes EMT [56,57]. Targeted expression of Gli1 in the epithelial cells of mammary gland of mice induces the expression of Snail and thus results in the disruption of the mammary epithelial network and alveologenesis during pregnancy [58]. Conversely, blockade of Hedgehog signaling by inhibitor cyclopamine suppresses pancreatic cancer invasion and metastasis through inhibiting EMT in the pancreatic cancer cells [59].

Notch is an evolutionarily conserved signaling pathway that regulates cell fate specification, self-renewal, and differentiation in embryonic and postnatal tissues. Four Notch (Notch 1–4) and five ligands (Jagged1, 2 and Delta-like1, 3, 4) have been identified. Notch signaling is normally activated followed by ligand-receptor binding between two neighboring cells, Notch undergoes intramembrane cleavage by γ -secretase and its intracellular domain (NICD) is released and translocates to the nucleus to activate gene transcription by associating with Mastermind-like 1 (MAM) and histone acetyltransferase p300/CBP. Alteration of Notch signaling has been associated with various types of cancer in which Notch may act as an oncogene or as a tumor suppressor. The observation that Notch pathway is required for EMT was first made during cardiac valve and cushion formation at heart development [60]. This implies that Notch acting through a similar mechanism, may also be involved in the EMT induction during tumor progression and converts polarized epithelial cells into motile, invasive cells [61]. Indeed, overexpression of Jagged1 and Notch1 induces the expression of Slug and correlates with poor prognosis in various human cancers [62]. Slug is essential for Notch-mediated EMT by repressing E-cadherin expression, which results in β -catenin activation and resistance to anoikis. Inhibition of Notch signaling in xenografted Slug-positive/E-cadherin-negative breast tumors promotes apoptosis and inhibits tumor growth and metastasis [62]. In addition, Notch signaling deploys two distinct mechanisms that act in synergy to control the expression of Snail [63]. First, Notch directly upregulates Snail expression by recruitment of the Notch intracellular domain to the Snail promoter, and second, Notch potentiates hypoxia-inducible factor 1 α (HIF-1 α) recruitment to the lysyl oxidase (LOX) promoter and elevates the hypoxia-induced upregulation of LOX, which stabilizes the Snail protein. Thus, Notch signaling is required to convert the hypoxic stimulus into EMT, increased motility, and invasiveness of tumor cells.

NF- κ B is another key modulator for EMT. Recently, NF- κ B was identified as a central mediator of EMT in a model of breast cancer progression [6,64]. In this model, the NF- κ B signaling pathway was essential for distinct aspects of EMT (apoptosis protection, EMT induction and maintenance) as well as being required for metastasis. This suggests that both Ras- and TGF- β -dependent effects on EMT, including activation of many EMT-specific genes, are mediated,

at least in part, via NF- κ B activity [6]. Interestingly, the E-cadherin repressors Twist and Snail have been suggested as possible downstream targets of NF- κ B [6,65,66].

Cell polarity and EMT

During EMT, epithelial cells lose cell-cell junctions and polarity, leading to a more migratory, fibroblast-like “mesenchymal” cell phenotype. Many studies have emphasized the major role of signaling pathways leading to the transcriptional repression of the E-cadherin in adherens junction by Snail, Slug, SIP1, and Twist. Little is known about how EMT disrupts the formation of tight junction and cell polarity. Polarity is largely regulated by a conserved set of proteins known as partition-defective (PAR) proteins, which are required for organizing the basal-apical polarity of epithelial cells and for the establishment and maintenance of apical junction. The PAR3/PAR6/aPKC complex localizes selectively at the apical junction and the apical plasma membrane; whereas Par1, resides at the basolateral membranes of epithelia. Mutual antagonistic interactions between these two complexes results in the formation of cellular and functional asymmetry within the cell. In addition to the Par complex, the lateral resided CRUMBS/PALS1/PATJ complex and the tight junction associated SCRIBBLE/DLG/LGL complex, are also required for the formation of cell polarity. During the initial stage of epithelial cell contact, spot-like adherens junctions first appear at the tips of protrusions that contain E-cadherin, nectins, junctional adhesion molecule (JAM), and protein ZO-1. E-cadherin mediates initial intercellular adhesion, which is substantially strengthened after its connection to the actin cytoskeleton through α - and β -catenin. These connections mature into adherent junctions and promote the formation of tight junctions, which further anchors to the Par complex to establish cell polarity. Recent work has shown that TGF- β can induce phosphorylation of Par6, which in turn stimulates binding of Par6 to E3 ligase Smurf1. The Par6-Smurf1 complex then mediates the localized ubiquitination of RhoA to dissolve tight junctions during EMT [67]. TGF- β can also downregulate the Par3 expression to destroy the cell polarity [68]. Whiteman *et al* also showed that Snail disrupted the apical polarity complex by inhibiting the expression of Crumb3 [69]. In addition, ZEB1 suppresses the expression of Lgl2, Crumbs3, HUGL2 and PATJ to disrupt cell polarity [70,71]. Thus, it becomes obvious that the disruption of tight junctions and cell polarity represents a new trait of EMT.

EMT in cell survival and tumor recurrence

During EMT, epithelial cells detach from the extracellular matrix (ECM), which triggers the apoptotic process. The ability to survive in the absence of normal matrix components represents an important property for cells undergoing EMT. Several known apoptotic and anti-apoptotic proteins are involved in EMT. Overexpression of Bcl-2 and Bcl-XL increases the metastasis capacity of epithelial cells without affecting primary-tumor formation [72,73]. Moreover, integrin-mediated signaling is also attributable to the inhibition of cell death. For example, focal-adhesion kinase (FAK), a crucial activator of the tyrosine-kinase pathway, is associated with the intracellular tails of integrin and its activation is sufficient for epithelial cell survival [74]. In mouse embryo, FoxD3 requires the concomitant expression of SOX9 and Slug to induce EMT. Sox9 can inhibit cell death and specifies the neural-crest cell lineages [75].

Snail and Slug act as inhibitors of apoptosis through several mechanisms. Slug negatively regulates the expression of the pro-apoptotic p53 and Puma [76–78], while Snail represses *Cyclin D2* transcription and increases the p21^{Cip1/Waf1} level and concomitantly activates the MAPK and PI3K survival pathway to confer resistance to the lethal effects of serum depletion or TNF α administration [79–81]. Similarly, Twist, recently involved in breast cancer metastasis through regulation of EMT, functions as an oncogene in many human cancers. Twist also negatively regulates apoptosis during both embryogenesis and tumor progression [82]. All of these transcription factors exert the role in cell survival, differentiation and metastasis. Thus,

increased expression of these transcription factors during EMT is sufficient to overcome cell death provoked by proapoptotic signals. They provide a selective advantage for the invasive cells to migrate through hostile territories. This anti-apoptotic function is essential for the migratory cells to reach their final destinations during embryogenesis and is also important for malignant cells to disseminate and form metastases.

Using a mammary-specific, inducible HER2/Neu transgenic mouse model, Moody *et al* demonstrated that EMT occurred in tumor recurrence and Snail was upregulated spontaneously [42]. Snail is sufficient to induce EMT in HER2/Neu-induced primary tumor cells and to promote rapid tumor recurrence *in vivo* following downregulation of the HER2/Neu pathway. Consistent with this, breast cancer relapses in Wnt1 transgenic mice lacking either Ink4a/Arf or p53, and this relapse is accompanied with EMT with robust Snail expression and undetectable E-cadherin [83]. Moreover, ZEB1 is an important transcription factor that regulates EMT. It also maintains the proliferation of a subset of progenitor cells in gestation. The proliferative defects occur in the ZEB1 mutant mice and lead to premature replicative senescence in cultured MEFs. This cellular senescence is triggered by two cell cycle inhibitors, p15^{Ink4b} and p21^{Cip1/Waf1} [84]. Together, EMT may foster oncogene-independent survival of a crucial subset of tumor cells to promote tumor progression.

EMT and cancer stem cell

In addition to the gain of anti-apoptotic ability for cells undergoing EMT, Weinberg's group recently demonstrated that EMT also generates properties of stem cells, such as self-renewal [85,86]. Ectopic expression of Snail or Twist yields great increases in their ability to form mammosphere, which represents the presence of epithelial stem cells. Similarly, EMT generates more mammary epithelial stem-like cells from more differentiated populations of normal mammary epithelium. Surprisingly, the stem-like CD44^{high}/CD24^{low} cells exhibit strong reduction of E-cadherin, significant increased expression of fibronectin and vimentin, and robust levels of FOXC2, Snail, Twist and Slug. Consistent with this finding in mammary epithelial cells, the differentiation of human embryonic stem (ES) cells is also associated with all the characteristic EMT events, including repression of E-cadherin, increasing expression of vimentin, upregulation of Snail and Slug, high activity of gelatinase, and enhanced cell motility [87]. EMT seems to be the definitive step in human ES differentiation. Thus, EMT enables cancer cells not only to disseminate from a primary tumor but also to form the macroscopic metastases with self-renewal capability.

EMT and microRNA

As EMT plays a central role in embryogenesis, fibrosis, wound healing, and cancer metastasis, it is not surprising that a bewildering number of regulators associate with this fundamental process. Recently, microRNA has appeared as a powerful master regulator of EMT. MicroRNAs are small 20–22-nucleotide long noncoding RNAs that modulate gene expression at the post-transcriptional level [88,89]. MicroRNAs have been implicated in regulating diverse cellular pathways, such as cell differentiation, proliferation and programmed cell death and are commonly dysregulated in human cancer. Recent findings suggest that microRNAs also contribute to EMT. For example, Twist induces microRNA-10b transcription, which inhibits the translation of HOXD10 and results in elevated expression of RhoC, and thus facilitates cancer cell metastasis. Significantly, the level of miR-10b expression in primary breast carcinomas correlates with clinical progression [90]. In addition, several reports demonstrated that the microRNA-200 family were markedly downregulated in cells that had undergone EMT in response to TGF- β [91–94]. Because microRNA-200 directly targets the mRNA of ZEB1 and SIP1, expression of miR-200 induces upregulation of E-cadherin in cancer cell lines and suppresses their motility. Consistent with their role in regulating EMT, loss of microRNA-200

is commonly found in invasive breast cancer cell lines with mesenchymal phenotype and in regions of metaplastic breast cancer specimens lacking E-cadherin. In addition, an EMT specific microRNA miR-21 is found in TGF- β -induced EMT in human keratinocytes, a model of epithelial cell plasticity for epidermal injury and skin carcinogenesis [95]. MiR-21 is abundantly expressed and associated with carcinogenesis. It targeted two tumor suppressors, tropomyosin 1 (TPM1) and programmed cell death-4 (*PDCD4*), to modulate the cell proliferation, microfilament organization, and anchorage-independent growth [96,97]. Interestingly, microRNA can target distinct functions in different signaling pathways and thus contributes to several key events associated with tumor progression. Therefore, targeting microRNA can be a good therapeutic approach for cancer prevention and treatment with the effect of “one stone hitting multiple birds”.

Future perspectives

During the past few years, EMT has emerged as one of the hottest medical science topics. The role of EMT in tumor progression and metastasis provides an intriguing mechanism to explain the initial step of metastasis. However, several areas are required for further investigation to comprehensively understand the role of EMT in physiological and pathological processes. First, most traditional EMT markers are found in scenarios other than EMT. New markers of EMT are required to better distinguish EMT. Second, EMT is a kinetic conversion that varies considerably from hours to weeks. Other cellular events might embed in the EMT program. It is difficult and challenging to obtain informative results on gene expression and to discriminate between general and cell/stage-specific molecular players that are responsible for EMT. Third, additional studies are required to understand the molecular mechanisms controlling EMT. The crosstalk between different signal pathways and molecules is a crucial issue to elucidate the complicated regulation of EMT, such as the communications between cadherin and integrin, Snail and β -catenin, TGF- β PDGF. Finally, better models are particularly required to study EMT *in vivo* and powerful imaging is also needed to unveil the behavior of migratory cells in real time. New discoveries will elucidate the complex strategies of EMT and hold great promise for yielding novel therapeutic approaches for treating cancer.

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