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Review

EMT Transition States during Tumor Progression and Metastasis

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Epithelial–mesenchymal transition (EMT) is a process in which epithelial cells acquire mesenchymal features. In cancer, EMT is associated with tumor initiation, invasion, metastasis, and resistance to therapy. Recently, it has been demonstrated that EMT is not a binary process, but occurs through distinct cellular states. Here, we review the recent studies that demonstrate the existence of these different EMT states in cancer and the mechanisms regulating their functions. We discuss the different functional characteristics, such as proliferation, propagation, plasticity, invasion, and metastasis associated with the distinct EMT states. We summarize the role of the transcriptional and epigenetic landscapes, gene regulatory network and their surrounding niche in controlling the transition through the different EMT states.

Highlights

EMT occurs through distinct intermediate states *in vivo*.

Distinct EMT transition states can be identified using cell surface markers and single-cell RNA-sequencing.

Distinct EMT transition states present different functions, with the hybrid EMT state presenting the highest metastatic potential.

Distinct EMT transition states present different gene expression and chromatin landscape.

Distinct EMT transition states are localized in different niches that regulate cell fate transitions.

EMT Transition States

Epithelial–mesenchymal transition (EMT) is a cellular process in which cells lose their epithelial characteristics and acquire mesenchymal features. EMT has been associated with various tumor functions, including tumor initiation, malignant progression, tumor stemness, tumor cell migration, intravasation to the blood, metastasis, and resistance to therapy [1–3]. EMT has long been viewed as a binary process with two distinct cell populations, epithelial and mesenchymal [1,4], and is often defined by the loss of the epithelial marker E-cadherin and the gain of the expression of the mesenchymal marker vimentin. However, recent studies indicate that EMT occurs in a gradual manner characterized by several cellular states expressing different levels of epithelial and mesenchymal markers and exhibiting intermediate morphological, transcriptional, and epigenetic features, between epithelial and mesenchymal cells [5–10]. The intermediate states between epithelial and fully mesenchymal states have been referred to as partial, incomplete, or hybrid EMT states.

Researchers have investigated the expression of epithelial and mesenchymal markers in various cell lines, patient derived xenografts [9], and primary cancers. In some breast [6,11,12], pancreatic [12], renal [13], lung [14], colorectal [12,15], and ovarian [5,16] cancer cell lines, these two markers are coexpressed in the same cells, suggesting the existence of an EMT hybrid state. *In vitro* the hybrid phenotype is associated with increased invasion and migration [5,11,14,17], and increased cell survival in suspension [5]. Similarly, the coexpression of epithelial and mesenchymal markers has been documented in human primary cancers, such as breast [18–20], colorectal [21,22], head and neck [23], lung [24], and pancreatic [25] cancers, as well as in carcinosarcomas including: uterine [26], renal [27], lung [28], breast [12,29], esophagus [30], and skin [31,32] cancers (Table 1). Carcinosarcomas are rare tumors that contain epithelial and mesenchymal parts of clonal origin within the same tumor and represent the paradigm of spontaneous EMT observed in primary human cancers from different organs [12,26–34]. Moreover, the coexpression of epithelial and mesenchymal markers evaluated by immunostaining or enrichment of hybrid EMT RNA signature has been

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Table 1. Coexpression of Epithelial and Mesenchymal Markers in Different Cancers and Cancer Cell Lines[a]

Experimental model	Cancer type	Markers used	Method	Refs
Cell line	Ovarian carcinoma	E-cadherin, N-cadherin, Zeb1	IF	[5]
Cell line	Breast carcinoma	E-cadherin, vimentin	IF, FC	[6]
Cell line	Oral SCC	Vimentin, keratin 5, keratin 14	IF, IHC, WB	[23]
Cell line	Breast carcinoma	Vimentin, keratins	IF	[11]
Cell line	Pancreatic cancer	E-cadherin, pancytokeratin, Zeb1, vimentin	IF	[12]
Cell line	Clear cell renal carcinoma	E-cadherin, Snai1	IF	[13]
Cell line	Lung adenocarcinoma	E-cadherin, vimentin	IF	[14]
Cell line	Colorectal cancer	E-cadherin, occludin, Snai1, vimentin	WB	[15]
Xenograft primary cancer cell lines	Ovarian cancer	EpCAM, vimentin, CD44	IF, FC	[16]
Xenograft primary cancer cell lines	Ovarian cancer	E-cadherin, Tie2, CD133, CD44	IF, FC	[17]
In vivo mouse model	Skin SCC Luminal-like breast cancer Metaplastic breast cancer	EpCAM, CD106, CD51, CD61	FC	[9]
PDX	Lung and esophagus carcinomas	Pancytokeratin, vimentin	IF	[9]
Human primary tumors	Breast cancer	Cytokeratin 8/18, cytokeratin 5/6, vimentin	IHC	[18]
Human primary tumors	Breast cancer	Keratin, vimentin	IF	[19]
Human primary tumors	Breast cancer	E-cadherin, vimentin	IHC	[20]
Human primary tumors	Prostate cancer	E-cadherin, N-cadherin, vimentin, fibronectin	IHC, IF	[21]
Human primary tumors	Lung SCC and ADC	E-cadherin, cytokeratin, vimentin	IHC	[24]

[a]Table 1 summarizes the epithelial and mesenchymal markers reported to be coexpressed in different cancer cell lines, patient derived xenografts (PDX), and primary human cancers. Abbreviations: ADC, adenocarcinoma; IF, immunofluorescence; IHC, immunohistochemistry; FC, flow cytometry; WB, Western blot.

associated with poor survival and resistance to therapy in several tumor types [19,23,25,35–37]. Single-cell transcriptomics used to assess tumor heterogeneity in head and neck cancers identified partial/hybrid EMT programs, defined by incomplete activation of EMT transcription factors (TFs). Interestingly, cells exhibiting partial EMT were spatially localized at the leading edge of the tumor [38].

In this review article, we describe the increasing evidence demonstrating the existence of different EMT states and their functional role during tumorigenesis, invasion, and metastasis. We further discuss the genes associated with each EMT state, their chromatin landscape, their regulatory network, their spatial location, and the mechanisms regulating their transition and plasticity.

EMT in Mouse Cancer Models

Until recently, most studies on EMT were performed using cancer cell lines *in vitro* or by assessing pathological specimens of human cancers, precluding the assessment of the functional significance and the cellular plasticity of EMT *in vivo*. Moreover, due to the lack of expression of epithelial markers in full EMT, it is difficult to determine with high confidence whether cells expressing only mesenchymal markers correspond to tumor cells or to cancer associated fibroblasts. For these reasons, researchers have developed genetically engineered mouse models combining lineage tracing to assess EMT *in vivo* (Table 2). In *Pdx1CRE/KRasG12D/P53cKO/Rosa-YFP* or *Pdx1CRE/KRasG12D/Ink4a+/−/Rosa-YFP* mice [39], which results in oncogenic recombination and YFP expression exclusively in embryonic pancreatic epithelial cells, more than half of the tumors showed EMT features, characterized

Table 2. EMT in Mouse Cancer Models[a]

Tumor type	Mouse models	Markers used to define epithelial and mesenchymal states	Refs
Pancreatic cancer	*Pdx1CRE/KRasG12D/P53cKO/Rosa-YFP* *Pdx1CRE/KRasG12D/Ink4a+/−/Rosa-YFP*	Zeb1, Fsp1, E-cadherin	[39,47]
Pancreatic cancer	*Pdx1-cre;KrasLSL.G12D/+;Tp53LSL.R172H/+;Zeb1fl/fl*	E-cadherin, vimentin	[54]
Pancreatic cancer	*Pdx1-cre;LSL-KrasG12D;P53R172H/+;Twist1loxP/loxP* *Pdx1-cre;LSL-KrasG12D;P53R172H/+;Snai1loxP/loxP*	αSMA, Krt8, Krt19	[56]
Prostate cancer	*Probasin-CRE/Pten cKO/KRasG12D/Vim-GFP*	EpCAM, pancytokeratin, vimentin	[40]
Colorectal cancer	*VilinCREERT2/p53KO/NICD-IRES-GFP*	E-cadherin, vimentin	[41]
Breast cancer	*MMTV-PyMT, Rosa26-RFP-GFP/Fsp1-Cre*	E-cadherin, vimentin, Fsp1	[42,57]
Breast cancer	*K8-CreERT2/Pik3caH1047R/p53fl/fl/Rosa26-YFP*	Krt8, Krt14, vimentin, CD106, CD61, CD51	[9,44]
Breast cancer	*Lgr5-CreERT2/PIK3CAH1047R/Tomato,* *K8-CreERT2/PIK3CAH1047R/Tomato*	Krt8, Krt14, CD24, Sca-1	[45]
Skin SCC	*Lgr5CREER/KrasG12D/p53cKO/Rosa-YFP*	EpCAM, Krt14, vimentin, CD106, CD61, CD51	[9,46]

[a]Table 2 summarizes the mouse models describing the role of EMT during tumorigenesis *in vivo*.

by the gain of mesenchymal markers Zeb1 or Fsp1 or the loss of E-cadherin. A smaller proportion of tumor cells coexpressed epithelial and mesenchymal markers. Interestingly, EMT was observed at the early stage of tumorigenesis in areas of metaplasia associated with inflammation, and the presence of circulating pancreatic cells presenting the oncogenic recombination could be identified before the presence of macro- or micro-metastasis, supporting that EMT and blood dissemination occur early during pancreatic tumorigenesis [39].

Similarly, in a mouse model of prostate cancer using *probasin-CRE/Pten cKO/KRasG12D* together with a vimetin-GFP reporter gene, different subpopulations of prostate tumor cells could be identified: $EpCAM^+$ tumor epithelial cells, hybrid $EpCAM^+$/vimentin-GFP+ TCs, and $EpCAM^-$/vimentin-GFP+ tumor mesenchymal cells [40]. The hybrid and mesenchymal tumor cells exhibited increased invasive features, circulating tumor cells (CTCs), and tumor propagating characteristics, suggesting an important role for EMT during the early stages of metastatic dissemination [40]. *VilinCREERT2/p53KO/NICD-IRES-GFP* mice, that present p53 deletion and expression of active Notch1 receptor in the gut epithelium after tamoxifen administration had an increased rate of malignant progression to colorectal tumors expressing a moderate to poorly differentiated phenotype, which was associated with metastasis to the lymph node, liver, and peritoneum [41]. Immunohistological analysis revealed that these aggressive intestinal carcinomas presented EMT features, including an elongated shape and expression of mesenchymal markers together with the loss of E-cadherin [41]. Triple transgenic mouse model *MMTV-PyMT*, *Rosa26-RFP-GFP*, and *Fsp1-Cre* allows to follow the conversion of RFP-positive breast epithelial tumor cells to GFP-positive tumor mesenchymal cells [42]. In this model, some tumor cells marked with the mesenchymal Cre presented a spindle shape, long membrane extensions, and were located close to blood vessels, where these cells were able to migrate along the vessels much faster than individual EMT cells surrounded by epithelial tumor cells, suggesting that the microenvironment and the proximity to blood vessels play an important role in the motility of EMT tumor cells [42,43]. In the mammary gland, activation of oncogenic *Pik3ca* mutation and simultaneous deletion of *p53cKO* in the luminal lineages lead to metaplastic mammary tumors characterized by EMT [44,45].

K14CREER/KrasG12D/p53cKO/Rosa-YFP, which targets the cells of the interfollicular epidermis in the skin, leads to the development of well-differentiated squamous cell carcinoma (SCCs)

without signs of EMT. In contrast, most of the SCCs that arise from the hair follicle (HF) lineages using *Lgr5CREER/KrasG12D/p53cKO/Rosa-YFP* present EMT features. The vast majority of the tumors consist of carcinosarcoma presenting epithelial and mesenchymal features that are characterized by a fraction of the tumor cells that lost EpCAM expression. Intravenous injection of epithelial ($EpCAM^+$) and mesenchymal ($EpCAM^-$) tumor cells demonstrates the higher capacity of lung colonization of $EpCAM^-$ cells as compared to $EpCAM^+$. The molecular profiling of these tumors and their cells of origin demonstrate that HF lineages are transcriptionally and epigenetically primed to undergo EMT during tumorigenesis [46].

Altogether, these different mouse models illustrate that EMT is relatively common in poorly differentiated tumors arising from different tissues.

EMT Transition States *In Vivo*

In HF derived SCCs presenting features of carcinosarcoma, EpCAM is expressed in a bimodal pattern in YFP+ tumor cells, suggesting that EMT may occur as a binary switch. However, a screen of a large panel of cell surface markers performed in these tumors revealed that $EpCAM^-$ mesenchymal tumor cells were heterogeneous and expressed different levels of the cell surface markers CD106, CD61 and CD51 [9]. Combinatorial multicolor FACS analysis revealed that $EpCAM^-$ mesenchymal tumor cells could be separated into six distinct subpopulations. Immunostaining of (keratin 14) K14 and vimentin revealed that these different subpopulations present different degrees of EMT. Interestingly, loss of EpCAM expression coincided with a gain of vimentin expression in all tumor cells, representing the first molecular switch to the mesenchymal state. However, some $EpCAM^-$ subpopulations continued to coexpress K14 and vimentin, representing hybrid tumor cells, whereas other populations completely lost the expression of K14, representing full EMT tumor cells (Figure 1A,B). Single-cell RNA-sequencing of $EpCAM^+$ and $EpCAM^-$ tumor cells further confirmed the heterogeneity of EMT tumor mesenchymal cells and the existence of hybrid and full EMT tumor populations (Figure 1C). The existence of this population heterogeneity during EMT, where cells express different levels of CD106, CD61, and CD51, was also found in *MMTV-PyMT* luminal and in metaplastic *Pik3ca/p53cKO* mammary tumors [9] (Table 3 and Figure 2).

Transcriptional profiling of the different tumor cell populations arising in SCCs presenting EMT revealed that some markers traditionally used to define epithelial state such as *Cdh1* or *EPCAM* were lost in the early step of EMT, while others such as *Krt14*, *Krt5*, or *Krt8* were maintained in the hybrid states and were completely lost in the late stages of EMT (Figure 3) [9]. Similarly, mesenchymal markers exhibited different patterns of expression: some known EMT genes and TFs, such as *Cdh2*, *Vim*, *Snai1*, *Twist1/2*, and *Zeb1/2* were highly upregulated in early hybrid states and were maintained at the same level in the more mesenchymal populations, while the expression of *Cdh11*, *Pdgfra*, *Pdgfrb*, *Fap*, *Loxl1*, *Col24a1*, *Mmp19*, or *Prrx1* increased in late stages of EMT (Figure 3) [9].

Recently an alternative post-transcriptionally regulated program that promotes a hybrid EMT phenotype *in vivo* has been described in pancreatic tumors [47]. Transcriptional profiling of E-cadherin+ and E-cadherin− tumor cells from *Pdx1CRE/KRasG12D/P53cKO/Rosa-YFP* mice identified two types of pancreatic tumors. One subgroup of YFP+/E-cadherin− tumor cells was associated with low levels of epithelial gene expression, whereas the other subgroup was characterized by stable levels of E-cadherin and expression of other epithelial genes. These EMT tumor cells exhibited intracellular localization of E-cadherin, suggesting that a hybrid EMT phenotype can be achieved through the relocalization of epithelial proteins [47].

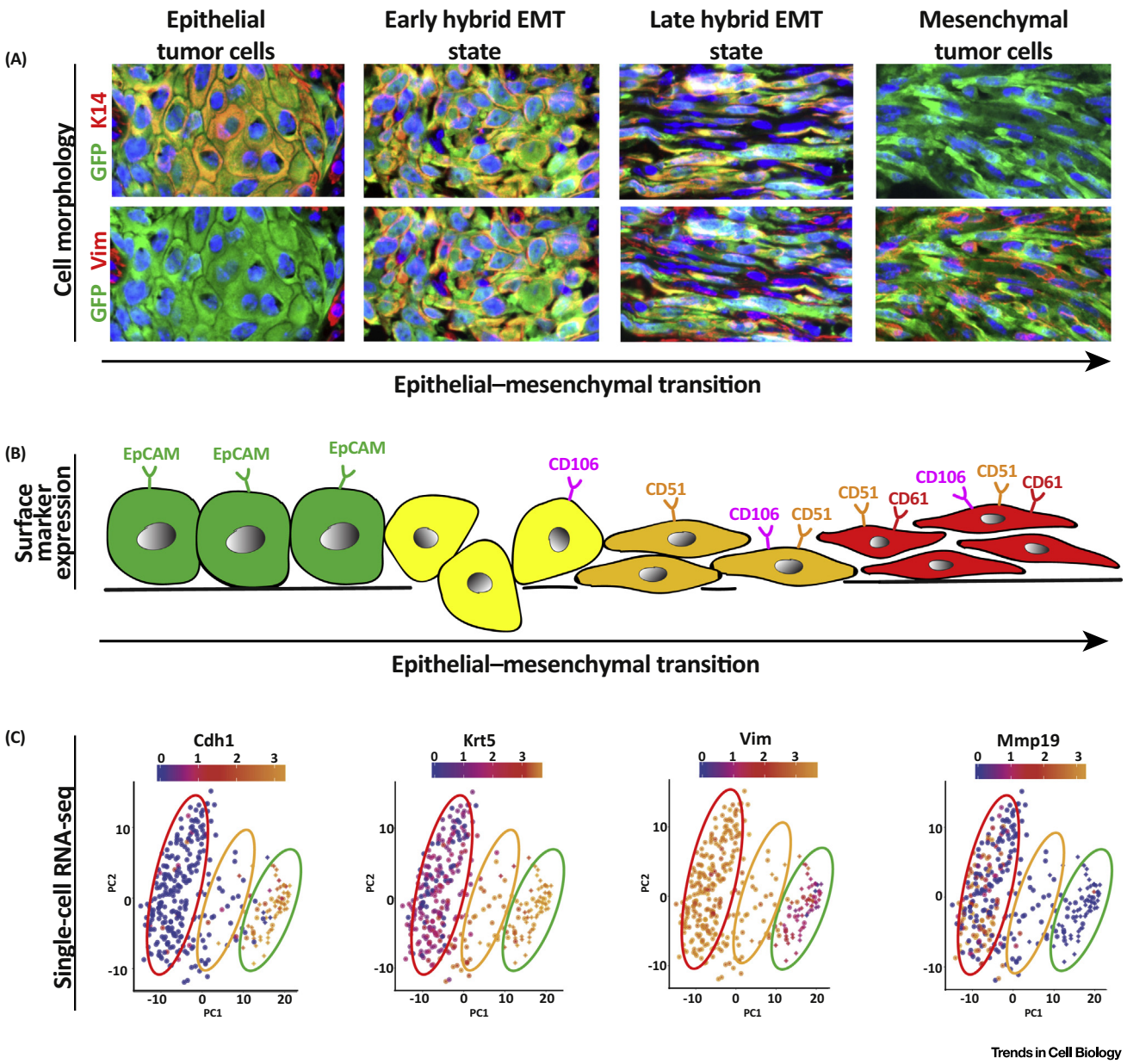

Figure 1. Definition of Tumor Transition States Occurring during Epithelial–Mesenchymal Transition (EMT). (A) Immunostaining for keratin 14 (K14) and vimentin (Vim) showing changes in their expression and in the morphology of skin tumor cells during EMT. Epithelial tumor cells have round shape and remain closely attached one to another, express K14, and are negative for Vim. Cells in early hybrid EMT state coexpress K14 and Vim, are more elongated, but still cohesive. Cells in late hybrid EMT coexpress K14 and Vim and are further elongated, acquiring fibroblast-like appearance. Mesenchymal tumor cells lost the expression of K14 while are uniformly expressing Vim, have fibroblast-like shape, and do not form cell–cell junctions [9]. (B) Expression of cell surface markers EpCAM, CD106/Vcam1, CD51/Itgav, and CD61/Itgb3. Epithelial tumor cells express EpCAM. Early hybrid EMT state is characterized by loss of EpCAM expression and triple negative (TN or CD106−CD51−CD61−) or CD106+ phenotypes. Late hybrid EMT state is characterized with expression of CD51 or CD106/51. Mesenchymal tumor cells express CD51/61 or have triple positive (TP or CD106+CD51+CD61+) phenotype. Green color denotes cells with epithelial phenotype, yellow color denotes cells with early hybrid EMT phenotype, orange color denotes cells with late hybrid EMT phenotype, and red color denotes cells with full EMT phenotype [9]. (C) Examples of principal component analysis (PCA) plots of single-cell RNA-sequencing of genes expressed in different stages of EMT. Dots represent single cell, colored scale represents the normalized expression of each gene [9]. Green circle highlights cells with epithelial phenotype, orange circle highlights cells with hybrid EMT phenotype, and red color highlights cells with full EMT phenotype.

Table 3. Characteristics of EMT Transition States[a]

EMT state	Epithelial	Early hybrid EMT	Late hybrid EMT	Full EMT
Cell shape Cell adhesion	Round-shaped, strong adhesion between cells	Round-shaped, adhesion decreased	Elongated shape, adhesion lost	Elongated shape, adhesion lost
Surface markers	EpCAM, Cdh1	TN, CD106	CD51, CD106/51	CD51/61, TP
Markers	Krt5, Krt14, Dsg2, Esrp1/2	Krt5, Krt14, Vim, Cdh2	Krt5, Krt14, Vim, Pdgfrb, Fap, Cdh2	Vim, Aspn, Cdh2, Fap, Mmp19, Lox
Transcription factors	Trp63, Klf4, Ovol1, Grhl1-3,	Trp63, Grhl1-3, Zeb1/2, Twist1/2, Snai1	Zeb1/2, Twist1/2, Snai1	Prrx1, Zeb1/2, Twist1/2, Snai1

[a]Table 3 summarizes the cell shape, the adhesion, the markers, and the transcription factors specific for each EMT transition state. Abbreviations: TN, triple negative (CD106−CD51−CD61−); TP, triple positive (CD106+CD51+CD61+).

Stemness and Plasticity of EMT Transition States

Cancer stem cells describe a population of tumor cells with increased tumorigenic potential that self-renew and differentiate into different types of tumor cells present in primary tumors. Cellular assays, including tumor transplantation, lineage tracing, and lineage ablation have been developed to assess tumor stemness [48]. EMT has been associated with tumor stemness by their increased tumor propagating potential following their transplantation into immunodeficient mice. Forced expression of TFs that promote EMT such as *Twist1* or *Snail1* in mammary epithelial cells increase their ability to give rise to secondary tumors upon transplantation [49,50].

Isolation of different tumor cell populations from primary tumors based on EpCAM or E-cadherin have shown that EMT tumor cell populations are often associated with increased tumor propagating potential [39,46]. However, tumor cells with an epithelial phenotype can also

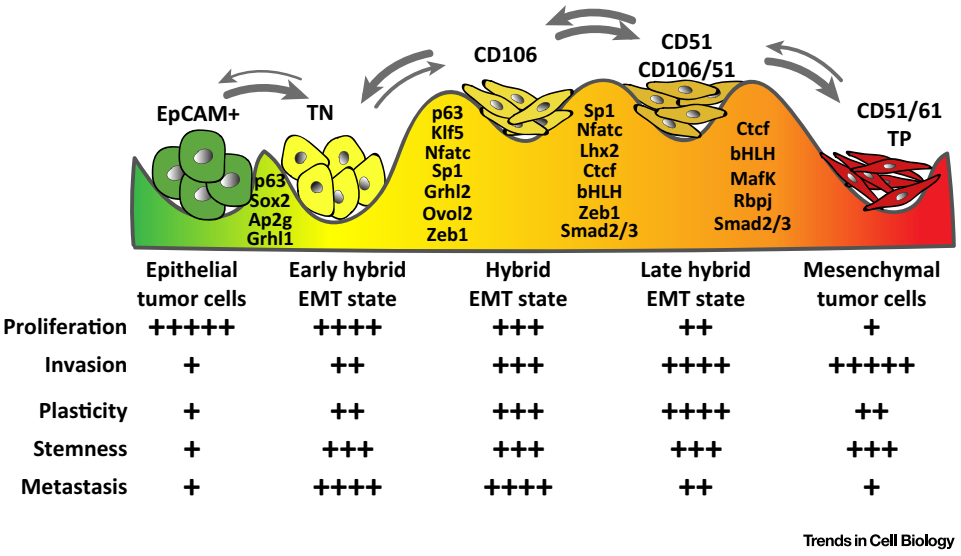

Figure 2. Epithelial–Mesenchymal Transition (EMT) Transition States Exhibit Different Functional Characteristics. Schematic representing EMT transition states and the transcription factors driving each transition. Thickness of the arrows represent the plasticity of the different EMT states. Proliferation, invasion, plasticity, stemness, and metastatic capacity of the different EMT transition states are summarized as follows: (+: low to ++++: very high).

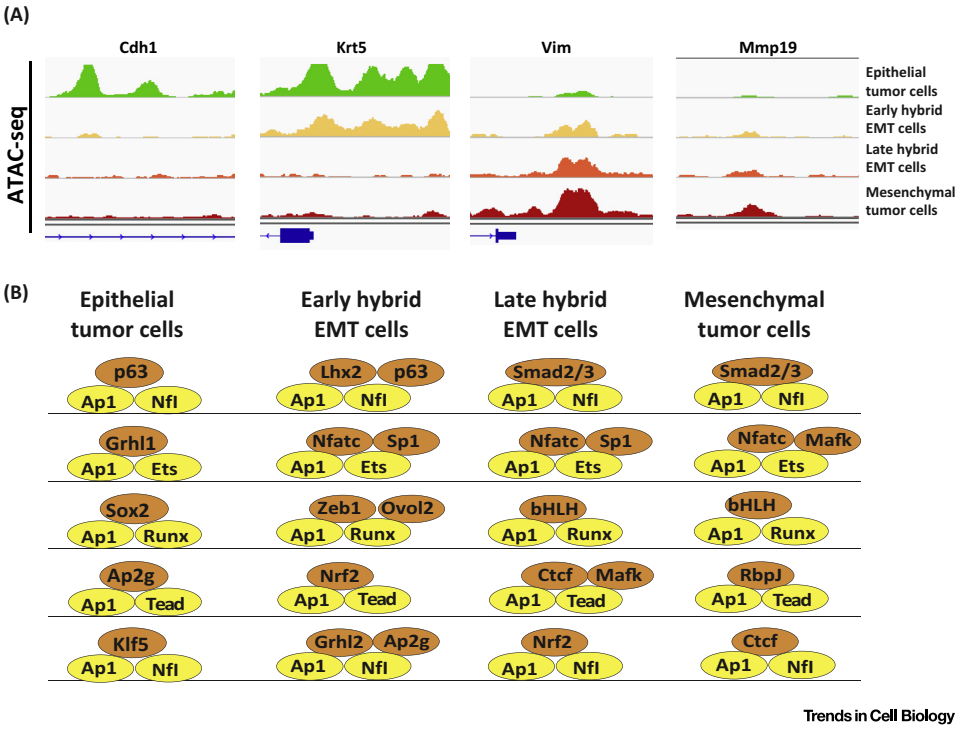

Figure 3. Gene Regulatory Network Controlling Epithelial–Mesenchymal Transition (EMT) Transition States. (A) Examples of chromatin profiling using an assay for transposase-accessible chromatin using sequencing (ATAC-seq) showing changes in chromatin accessibility in the different EMT transition states. Green color denotes chromatin profile from tumor epithelial cells, yellow color denotes chromatin profile from early hybrid EMT cells, orange color denotes chromatin profile from late hybrid cells, and red color denotes chromatin profile from fully mesenchymal cells. (B) Representation of chromatin remodeling and their associated transcription factors (TFs) enriched in ATAC-seq peaks that differ between EMT transition states [9]. Yellow color highlights the TFs common for all EMT transition states and orange color highlights TFs specific for each EMT transition state.

have tumor propagating potential, albeit slightly reduced, supporting the notion that tumor cells can possess cancer stem cell features independently of EMT [46,51–53].

In some models, such as ovarian cancer, a hybrid EMT phenotype is associated with increased tumor stemness, whereas fully epithelial or fully mesenchymal phenotypes were associated with loss of stem cell markers and tumorigenicity [17].

In HF derived SCCs, EMT tumor mesenchymal cells presented increased tumor propagating potential. Whereas $EpCAM^{+}$ epithelial tumor cells give rise to epithelial cells and mesenchymal tumor cells upon subcutaneous transplantation, $EpCAM^{-}$ tumor cells only give rise to $EpCAM^{-}$ mesenchymal tumor cells, indicating that tumor epithelial cells can be more plastic than tumor mesenchymal cells [46]. In this model, hybrid EMT populations displayed a fivefold increase in tumor propagation as compared to tumor epithelial cells [9]. However, this enhanced tumor propagation did not further increase in tumor cells that underwent complete EMT and lost the expression of epithelial markers [9]. Although all EMT subpopulations presented a certain degree of plasticity upon subcutaneous transplantation, the early hybrid EMT subpopulation was relatively primed towards a hybrid EMT phenotype, while the most mesenchymal subpopulation was primed towards a mesenchymal phenotype and did not revert spontaneously to a more epithelial phenotype. The intermediate EMT subpopulations were the most plastic,

giving equal rise to the other populations [9]. In pancreatic tumors driven by the same genetic alterations, *KrasG12D/p53cKO*, tumor propagation of epithelial and hybrid EMT cancer cells defined by E-cadherin and vimentin coexpression was increased when compared to mesenchymal cells [54].

Altogether these studies reveal that EMT is frequently associated with increased tumor propagation as compared to epithelial tumor cells, and sometimes hybrid EMT populations are more clonogenic as compared to late EMT cells. In addition, the different EMT subpopulations, depending on the microenvironment, have the ability to give rise to all the other populations, although some populations are biased to give rise to particular subpopulations. These data suggest that EMT occurs in a sequential manner and that the tumor cells progress from epithelial state to mesenchymal state by passing through different intermediate states. However, it is also possible that some tumor epithelial cells directly give rise to highly mesenchymal states or that tumor mesenchymal cells give rise to tumor epithelial cells without passing through intermediate states.

EMT Transition States and Metastasis

The role of EMT in metastasis has been recently debated and there are cancers that seem to metastasize without EMT. EMT was initially shown to promote metastasis by the demonstration that Twist1 silencing in breast cancer cell lines decreases lung metastasis [55]. In contrast, it was suggested that EMT was dispensable for metastasis due to the presence of metastasis in a mouse model of pancreatic tumors in which either Twist1 or Snai1 were deleted [56], or in a mouse mammary tumor model with overexpression of mir200, a microRNA that targets Zeb1 and Zeb2 and inhibits EMT [57]. However, these studies assumed, without experimental demonstration, that deletion of Twist1 or Snai1 or overexpression of mir200 is sufficient to completely inhibit EMT in these mouse models [58,59]. In contrast, deletion of *Zeb1* in the same pancreatic mouse cancer model significantly decreased invasiveness of highly aggressive tumor cells and strongly inhibited metastasis, suggesting that deletion of *Twist1* or *Snai1* alone is not sufficient to suppress EMT and that *Zeb1* deletion has a much greater impact on the tumor phenotype and metastasis formation [54].

Overexpression of Prrx1 TF induces EMT in kidney epithelial cells [60] and makes the cells more invasive in human cancer cell lines. Both kidney epithelial cells and human breast cancer cells overexpressing Prrx1 fail to give rise to lung metastasis after intravenous injection, while Prrx1 silencing in these cell lines promotes efficient lung colonization, suggesting that suppression of EMT is important for lung colonization [60]. Continuous overexpression of Prrx1 may lock tumor cells in a late EMT state and inhibit the capacity of tumor cells to undergo mesenchymal–epithelial transition (MET), thereby limiting the capacity to give rise to lung colonization and the growth of metastasis. Consistent with the notion that tumor cells need to undergo MET for metastatic colonization and growth, metastases in humans often present an epithelial morphology, possibly due to the reacquisition of epithelial features by tumor cells that underwent partial or complete EMT to leave the primary tumors. Similarly, in *probasin-CRE/Pten cKO/KRasG12D* model of prostate cancer, lung proliferating macrometastasis express high levels of pancytokeratin and low levels of vimentin, while micrometastasis, which remain small, dormant lesions express high levels of vimentin and low levels of pancytokeratin, further suggesting that reversion to an epithelial phenotype through MET promotes growth of metastasis [40]. Two Prrx1 isoforms have been described to have an opposite impact on EMT [61]. While overexpression of Prrx1a was associated with increased expression of E-cadherin and decreased invasion, overexpression of Prrx1b decreased E-cadherin expression, increased invasion, and associated with a poorly differentiated phenotype [61]. Although Prrx1b is associated with

increased blood dissemination of tumor cells, Prrx1a also promotes metastatic outgrowth after lung colonization, and knockdown of both Prrx1a and Prrx1b isoforms suppresses blood dissemination and metastasis in this model [61]. Twist1 overexpression in mouse skin SCC promotes tumor invasion and intravasation of tumor cells into blood circulation, and these CTCs display an EMT phenotype. However, downregulation of Twist1 is required for efficient lung metastasis formation [62]. Altogether, these studies suggest that EMT is important for initiating the metastatic cascade in some tumors, its downregulation is required for metastatic outgrowth.

In HF-derived EMT skin SCC, tumor mesenchymal cells are more efficient than tumor epithelial cells to induce lung metastasis following IV injection [46]. However, hybrid EMT tumor cells present increased lung metastasis as compared to full EMT populations when injected intravenously. While $EpCAM^{-}$ EMT cells are not able to revert completely to epithelial phenotype following subcutaneous transplantation, both hybrid and full EMT tumor cells can undergo complete MET when metastasized to the lung [9], further underscoring the importance of the microenvironment in the regulation of EMT and MET. Interestingly, CTCs detected in the blood of EMT SCCs were $EpCAM^{-}$ tumor cells enriched in early hybrid EMT states [9], demonstrating that tumor cells with hybrid EMT phenotype not only exhibit increased lung colonization ability *in vivo*, but also intravasate blood circulation more efficiently [9].

Hybrid EMT phenotype has been associated with collective cell migration during development, wound healing, and cancer, where migrating cells acquire mesenchymal features such as loss of apical–basal polarity, increasing their motility, while maintaining cell–cell adhesion with neighboring cells [12,14,47,63–72]. The relocalization of adhesion proteins in pancreatic tumors undergoing nontranscriptional EMT, could lead to the residual adhesion between tumor cells, contrasting with the single-cell migration observed during transcriptionally mediated EMT [47]. Clusters of CTCs were shown to arise from oligoclonal tumor cell aggregates and not from intravascular aggregation of tumor cells [73], and are associated with increased metastatic capacity and poor patient outcome as compared to single CTC [69,74–84]. CTC clusters detected in the blood of patients with breast cancer are strongly positive for mesenchymal markers and weakly positive for pancytokeratin [85], supporting the role of hybrid EMT in metastatic dissemination of tumor cells. The mesenchymal features found in CTC clusters could be mediated by the release of TGF-β by the platelets frequently associated with CTC clusters [85,86].

Hybrid EMT phenotype has been also detected in CTCs in the blood of human patients with non-small cell lung cancer [87–89], prostate [90], breast [85,89,91], liver [89], colorectal [89], gastric [89], and nasopharyngeal [89] cancers. Interestingly, coexpression of epithelial and mesenchymal markers rather than fully epithelial or mesenchymal phenotype, has been associated with poor clinical prognosis in these cancers [85,87,89,91–95].

Microenvironment Associated with EMT Transition States

The phenotypic plasticity by which epithelial tumor cells that initially undergo EMT are able to revert to epithelial phenotype by MET at the distant site has been suggested to be regulated by the microenvironment [2]. Supporting this hypothesis, the different EMT populations are localized in distinct tumor regions associated with particular microenvironment in skin SCC and mammary tumors [9]. The composition of the different stromal components changes as tumor cells progress towards EMT, with a major increase in immune infiltrate particularly enriched for monocytes and macrophages, as well as an increase in the density of blood and lymphatic vessels (Figure 4A–D) [9]. Interestingly, *in vivo* depletion of macrophages increased the proportion of $EpCAM^{+}$ epithelial

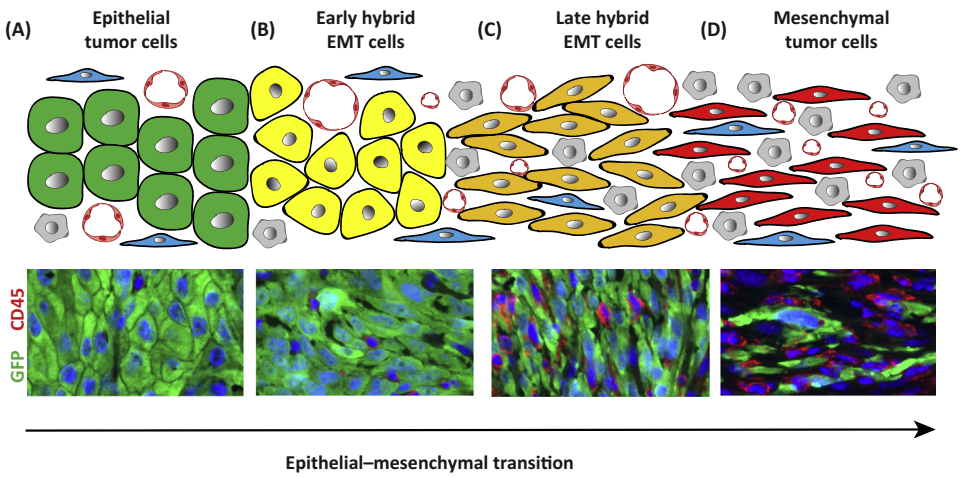

Figure 4. Niches Associated with Epithelial–Mesenchymal Transition (EMT) Transition States. (A–D) Schematic representation of the different niches associated with the different EMT transition states. Progression from hybrid EMT states (B and C) to complete mesenchymal states (D) is associated with progressive increase in the density of endothelial and lymphatic vessels, as well as macrophages [9].

tumor cells and early hybrid EMT states, and prevented further EMT progression towards fully mesenchymal state. In addition, when the TC subpopulations with different degree of EMT were isolated from their natural niche and subcutaneously transplanted into immunodeficient mice, they lost this spatial organization, and the tumor populations with different degree of EMT were distributed more randomly [9]. These observations suggest the importance of the microenvironment in controlling EMT progression.

Breast cancer cell lines acquire hybrid EMT phenotype under conditions rich in extracellular matrix. Tumor cells significantly upregulated the expression of Csf-1 and angiopoietin, and downregulated the expression of epithelial genes such as Krt18. Targeting Csf1/Csf1r axis prevented EMT in these settings [96]. In breast tumors, high matrix stiffness correlates with poor survival. Increasing matrix stiffness promotes nuclear translocation of Twist1, which promotes tumor invasion and metastasis [97]. High matrix stiffness also promotes nuclear localization of Yap1 [97], which is increased in SCCs presenting EMT [98], supporting the notion that Yap1 promotes EMT by the nature of the tumor microenvironment [98,99]. Interestingly, the mechanisms regulating the nuclear translocation of Twist1 and Yap1 upon increased matrix stiffness are different. Yap1 localization is responsive to changes in cell shape, that occur upon changes in matrix stiffness, while Twist1 localization was not affected by changes in actin cytoskeleton, thus supporting the existence of distinct Twist1 and Yap mechanotransduction pathways [97].

Gene Regulatory Network of EMT Transition States

The different EMT transitional states are associated with changes in the chromatin and transcriptional landscape of the cells that are mediated by gene regulatory networks (GRNs) that control the gene expression program specific of each state. Recent progresses have been made to define the enhancer logic and GRN that control the different EMT states.

Chromatin profiling using assay for transposase-accessible chromatin using sequencing (ATAC-seq) in HF derived SCCs combined with transcriptional profiling allows to define the chromatin

remodeling associated with EMT and infers the GRN that regulates the different EMT transition states. Interestingly, tumor specific active enhancers of epithelial and mesenchymal tumor cells are both enriched for AP1, Ets, Nfi, Tead, Runx, and Nfkb TF binding sites, suggesting that the same core of TFs is required to induce chromatin remodeling in the different EMT transition states [9,46] and consistent with the major defect of skin tumor development following the deletion of these TFs in skin SCCs [98,100–105]. In addition to these core TFs, different transition states were associated with specific epithelial and mesenchymal specific TFs. Zeb1, Trp63, Twist 1/2, and Lhx2 were predicted to be involved in promoting the early hybrid EMT states, whereas Smad2 was enriched in the latter stages of EMT. Supporting this notion, sustained expression of ΔNp63 or blocking Tgf-β/Smad2 pathway decrease the transition from $EpCAM^{+}$ to $EpCAM^{-}$ and increase the proportion of early hybrid state at the expense of full EMT [9]. Likewise, ΔNp63 promotes a hybrid EMT state in basal like breast cancer through simultaneous increases in Slug and Axl expression, which activate the EMT program and miR-205, which silence Zeb1/2 and prevents the loss of epithelial features [67,106].

Despite the important advances in our understanding of the mechanisms by which different TFs can induce EMT or MET, the specific regulatory elements that can stabilize the hybrid EMT phenotype in cancer cells, or to promote the transition from the hybrid state to complete EMT, or to induce MET remains poorly understood. In ovarian carcinoma cell line with hybrid EMT phenotype Src kinase inhibitor induced restoration of E-cadherin, that is associated with a decrease in Snai1 and Snai2 levels, while Zeb1, Zeb2, and Twist1 levels remained stable, suggesting that Src kinases can be involved in stabilization of hybrid EMT phenotype [5]. Willms' tumor TFs (WT1) exert dual function by transcriptionally activating Snai1 expression and, at the same time, preventing repression of E-cadherin by Snai1, thus contributing to the maintenance of a hybrid EMT state in renal cancer [13].

During mammary gland development, cells of the terminal end buds were stabilized in a hybrid EMT state through the coexpression of Zeb1 and Ovol2 TFs [107,108]. Mathematical modeling has been used to predict the GRNs that promote the epithelial, mesenchymal, and hybrid states. These models usually predict that epithelial and mesenchymal TFs and microRNAs repress the expression of each other, forming a mutually inhibitory loop, for example, miR34/snai1 or miR200/Zeb loops have been proposed. Such a mutually inhibitory loop leads to bistable switches, which promotes two distinct fates. However, when mutual repression is not strong enough, or when one TF strongly promotes its own expression, an intermediate state can be induced, leading to the formation of a third fate. Epithelial TFs, such as Ovol2 or Grhl2, by acting as a molecular brake on EMT were predicted to promote a hybrid EMT state with high tumor initiating potential [8,109,110]. Higher levels of Grhl2 and Ovol2 were predictive of poor patient outcome [8].

Similarly, using a computational approach, Nfatc1 and Sp1 were proposed to act as master regulators controlling EMT, and when acting together, to promote a hybrid EMT phenotype. This bioinformatic prediction was validated in nontransformed mammary gland cells and colorectal cancer cells, where upon simultaneous Nfatc1 and Sp1 expression, almost half of the cells acquired hybrid EMT phenotype [111]. Nfatc1 promotes EMT and migration in breast and lung cancers [106,107], and is predicted to regulate the chromatin landscape and GRN of EMT transitional states in skin cancers [9,46].

Recently, using a mathematical modeling approach, NRF2 was proposed to stabilize the hybrid EMT state and prevent progression towards a complete EMT [112]. Similarly, Numb was predicted to prevent a complete EMT by stabilizing hybrid EMT through Notch signaling.

Numb-KD in lung adenocarcinoma cell line with stable hybrid EMT phenotype promoted progression to full EMT [63]. Using a similar mathematical modeling approach, Notch-Jagged signaling has also been predicted to stabilize hybrid EMT phenotype [113,114].

These studies suggest that computational approaches can be very useful in modeling EMT and identifying new factors or combinations of regulatory elements that control the different EMT transition states. However, careful experimental approaches are needed to validate these predictions. It is also important to keep in mind that EMT is not always transcriptionally regulated, as recently illustrated by the post-transcriptional promotion of hybrid EMT phenotype by Ras [115], and the post-translational regulation of EMT in pancreatic tumors [47].

Concluding Remarks

The studies summarized in this review demonstrate that EMT is not a binary process, and different tumor cell populations presenting different degrees of EMT can be found in different cancers. These different populations present different functional properties and the hybrid EMT state is associated with increased metastatic potential.

Despite the progresses in the identification of the different EMT states and understanding the mechanisms regulating cell fate transition in tumors, there are still many questions unanswered (see Outstanding Questions). What is the sequence of events that drive carcinoma cell progression through the different EMT states? Which are the molecular players that control each transition and how these different cellular states can be stabilized? Does stabilization of specific EMT phenotype or switching between epithelial and mesenchymal states promote cancer progression and metastasis? Can specific genetic events like somatic mutations or epigenetic modifications contribute to maintain a specific EMT phenotype? Which are the precise mechanisms by which microenvironment influence cell fate decision during EMT? Do the different EMT subpopulations present different responses to chemotherapy, radiotherapy, or immunotherapy? If so, by which molecular mechanisms?

The combination of computational approaches and novel technologies such as single-cell sequencing, chromatin profiling, or *in vivo* intravital microscopy, should help to better understand the dynamics and the molecular mechanisms controlling EMT related cancer heterogeneity.

Finally, the basic understanding of the mechanisms controlling EMT should be used to develop new therapeutic strategies to prevent tumor progression, metastasis, and resistance to therapy in human cancers.

Outstanding Questions

Which is the first molecular event that triggers EMT?

Which are the temporal and spatial sequences of molecular events that regulate the different EMT transition states?

Which factors inhibit late EMT transition states in most human cancers?

Does EMT occur through the activation of common pathways across different tumor types, or does EMT exhibit tissue-specific features?

Which are the molecular players that control each EMT transition state and how these different cellular states can be stabilized?

Does stabilization of specific EMT transition states promote cancer progression and metastasis?

Is partial EMT required for metastasis?

What is the role of MET for metastasis growth?

What extrinsic factors in the metastasis microenvironment promote MET?

Are particular EMT transition states associated with resistance to therapy?

What are the mechanisms by which EMT promote resistance to therapy?

Can specific genetic events, such as somatic mutations or epigenetic modifications contribute to maintaining a specific EMT phenotype?

Which are the precise mechanisms by which inflammatory cells influence cell fate decision during EMT in each transition state?

Does tumor angiogenesis or hypoxia regulate EMT?

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