# EMT transition states during tumor progression and metastasis

Ievgenia Pastushenko[1], Cédric Blanpain[1,2]*

[1]Université Libre de Bruxelles, Laboratory of Stem Cells and Cancer, Brussels, Belgium

[2]WELBIO, Université Libre de Bruxelles, Brussels, Belgium

*Correspondance: Cedric.Blanpain@ulb.ac.be (C. Blanpain)

Keywords: epithelial-mesenchymal transition, hybrid EMT, cancer, metastasis, stemness, plasticity

**Abstract**

Epithelial-to-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 trough the different EMT states.

**EMT transition states**

Epithelial to 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 co-expressed 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 co-expression 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 co-expression of epithelial and mesenchymal markers evaluated by immunostaining or enrichment of hybrid EMT RNA signature has been 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 TFs. Interestingly, cells exhibiting partial EMT were spatially localized at the leading edge of the tumor [38].

In this review 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 cancer associated fibroblasts. For these reasons, researchers had 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 result in oncogenic recombination and YFP expression exclusively in embryonic pancreatic epithelial cells, more than half of the tumors showed EMT features, characterized by the gain of mesenchymal markers *Zeb1* or *Fsp1* or the loss of E-Cadherin. A smaller proportion of tumor cells co-expressed 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 prostatic 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 peritoneal [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 carcino-sarcoma 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 6 distinct subpopulations. Immunostaining of 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 co-express 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 level of CD106, CD61 and CD51, was also found in *MMTV-PyMT* luminal and in metaplastic *Pik3ca/p53cKO* mammary tumors [9] (Table 3, 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* where maintained in the hybrid states and were completely lost only in the late stages of EMT (Figure 3) [9]. Similarly, mesenchymal markers exhibited different patterns of expression: some known EMT genes and transcription factors (TFs) such as *Cdh2*, *Vim*, *Snai1*, *Twist1/2*, *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 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 re-localization of epithelial proteins [47].

***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 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 5 fold increase in tumor propagation as compared to tumor epithelial cells [9]. However, this enhanced tumor propagation does 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 co-expression 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 cancer 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 micro-RNA 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 the tumor cells, the proportion of highly aggressive tumors and strongly inhibited metastasis, suggesting that whereas the deletion of *Twist1* or *Snai1* alone is not sufficient to suppress EMT, *Zeb1* deletion had 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. Surprisingly, 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 late EMT state and inhibit the capacity of tumor cells to undergo mesenchymal to 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 needs to undergo MET for metastatic colonization and growth, metastases in humans often present an epithelial morphology, possibly due to the re-acquisition 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 macro-metastasis express high levels of pancytokeratin and low levels of Vimentin, while micro-metastasis, 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]. While Prrx1b is associated with increased blood dissemination of tumor cells, Prrx1a promotes metastatic outgrowth after lung colonization and knockdown of both Prrx1a and Prrx1b isoforms suppressed 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 circulating tumor cells (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 presented increased lung metastasis as compared to full EMT populations when injected intravenously. Interestingly, while Epcam- EMT cells were 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 sates [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 re-localization of adhesion proteins in pancreatic tumors undergoing non-transcriptional EMT, could lead to the residual adhesion between tumor cells, contrasting with the single-cell migration observed during transcriptionally mediated EMT [47]. Clusters of circulating tumor cells (CTC) 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-b 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, co-expression 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 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 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 form 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 tumours, high matrix stiffness correlates with poor survival. Increasing matrix stiffness promotes nuclear translocation of Twist1, which promotes tumour 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 ATAC-seq in HF derived SCCs combined with transcriptional profiling allows to define the chromatin remodeling associated with EMT and infers the gene regulatory network 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 transcription factors 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 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 co-expression of Zeb1 and Ovol2 TFs [107, 108]. Mathematical modeling has been used to predict the gene regulatory networks that promote the epithelial, mesenchymal and hybrid states. These models usually predict that epithelial and mesenchymal TFs and micro-RNAs 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 non-transformed 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 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 degree 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. What is the sequence of events that drive carcinoma cell progression trough 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 response 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 on 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 Question Box**

- 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 states 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?
- Do particular EMT transition states is 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 maintain 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?

## References


1. MA, Nieto *et al.* (2016) - Emt: 2016. Cell 166 (1), 21-45.
2. T, Brabletz (2012) - To differentiate or not--routes towards metastasis. Nat Rev Cancer 12 (6), 425-36.
3. B, De Craene and G, Berx (2013) - Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 13 (2), 97-110.
4. A, Puisieux *et al.* (2014) - Oncogenic roles of EMT-inducing transcription factors. Nat Cell Biol 16 (6), 488-94.
5. RY, Huang *et al.* (2013) - An EMT spectrum defines an anoikis-resistant and spheroidogenic intermediate mesenchymal state that is sensitive to e-cadherin restoration by a src-kinase inhibitor, saracatinib (AZD0530). Cell Death Dis 7 (4), 442.
6. J, Zhang *et al.* (2014) - TGF-beta-induced epithelial-to-mesenchymal transition proceeds through stepwise activation of multiple feedback loops. Sci Signal 7 (345), 2005304.
7. T, Hong *et al.* (2015) - An Ovol2-Zeb1 Mutual Inhibitory Circuit Governs Bidirectional and Multi-step Transition between Epithelial and Mesenchymal States. PLoS Comput Biol 11 (11), 1004569.
8. MK, Jolly *et al.* (2016) - Stability of the hybrid epithelial/mesenchymal phenotype. Oncotarget 7 (19), 27067-84.
9. I, Pastushenko *et al.* (2018) - Identification of the tumour transition states occurring during EMT. Nature 556 (7702), 463-468.
10. NV, Jordan *et al.* (2011) - Tracking the intermediate stages of epithelial-mesenchymal transition in epithelial stem cells and cancer. Cell Cycle 10 (17), 2865-73.
11. MJ, Hendrix *et al.* (1997) - Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. Am J Pathol 150 (2), 483-95.
12. P, Bronsert *et al.* (2014) - Cancer cell invasion and EMT marker expression: a three-dimensional study of the human cancer-host interface. J Pathol 234 (3), 410-22.
13. VB, Sampson *et al.* (2014) - Wilms' tumor protein induces an epithelial-mesenchymal hybrid differentiation state in clear cell renal cell carcinoma. PLoS One 9 (7), 0102041.
14. MJ, Schliekelman *et al.* (2015) - Molecular portraits of epithelial, mesenchymal, and hybrid States in lung adenocarcinoma and their relevance to survival. Cancer Res 75 (9), 1789-800.
15. MSY, Hiew *et al.* (2018) - Incomplete cellular reprogramming of colorectal cancer cells elicits an epithelial/mesenchymal hybrid phenotype. J Biomed Sci 25 (1), 018-0461.
16. R, Strauss *et al.* (2009) - Epithelial phenotype confers resistance of ovarian cancer cells to oncolytic adenoviruses. Cancer Res 69 (12), 5115-25.
17. R, Strauss *et al.* (2011) - Analysis of epithelial and mesenchymal markers in ovarian cancer reveals phenotypic heterogeneity and plasticity. PLoS One 6 (1), 0016186.
18. CA, Livasy *et al.* (2006) - Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 19 (2), 264-71.
19. PA, Thomas *et al.* (1999) - Association between keratin and vimentin expression, malignant phenotype, and survival in postmenopausal breast cancer patients. Clin Cancer Res 5 (10), 2698-703.
20. R, Yagasaki *et al.* (1996) - Clinical significance of E-cadherin and vimentin co-expression in breast cancer. Int J Oncol 9 (4), 755-61.

21. K, Kolijn *et al.* (2015) - Morphological and immunohistochemical identification of epithelial-to-mesenchymal in clinical prostate cancer. Oncotarget 6 (27), 24488-98.
22. AD, Grigore *et al.* (2016) - Tumor Budding: The Name is EMT. Partial EMT. J Clin Med 5 (5).
23. C, Dmello *et al.* (2017) - Vimentin regulates differentiation switch via modulation of keratin 14 levels and their expression together correlates with poor prognosis in oral cancer patients. PLoS One 12 (2), 0172559.
24. M, Zacharias *et al.* (2018) - Bulk tumour cell migration in lung carcinomas might be more common than epithelial-mesenchymal transition and be differently regulated. BMC Cancer 18 (1), 018-4640.
25. JT, George *et al.* (2017) - Survival Outcomes in Cancer Patients Predicted by a Partial EMT Gene Expression. Cancer Res 77 (22), 6415-6428.
26. P, Bitterman *et al.* (1990) - The significance of epithelial differentiation in mixed mesodermal tumors of the uterus. A clinicopathologic and immunohistochemical study. Am J Surg Pathol 14 (4), 317-28.
27. W, DeLong *et al.* (1993) - Sarcomatoid renal cell carcinoma. An immunohistochemical study of 18 cases. Arch Pathol Lab Med 117 (6), 636-40.
28. S, Haraguchi *et al.* (1999) - Pulmonary carcinosarcoma: immunohistochemical and ultrastructural studies. Pathol Int 49 (10), 903-8.
29. D, Sarrio *et al.* (2008) - Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 68 (4), 989-97.
30. Y, Yabuuchi *et al.* (2018) - Carcinosarcoma of the esophagus with rapid morphological change. Am J Gastroenterol 113 (5), 018-0013.
31. A, Paniz-Mondolfi *et al.* (2014) - Cutaneous carcinosarcoma: further insights into its mutational landscape through massive parallel genome sequencing. Virchows Arch 465 (3), 339-50.
32. A, Paniz-Mondolfi *et al.* (2015) - Cutaneous carcinosarcoma and the EMT: to transition, or not to transition? That is the question. Virchows Arch 466 (3), 359-60.
33. JA, Somarelli *et al.* (2015) - Carcinosarcomas: tumors in transition? Histol Histopathol 30 (6), 673-87.
34. H, Koba *et al.* (2018) - Next-generation sequencing analysis identifies genomic alterations in pathological morphologies: A case of pulmonary carcinosarcoma harboring EGFR mutations. Lung Cancer 122, 146-150.
35. N, Yamashita *et al.* (2018) - Epithelial Paradox: Clinical Significance of Coexpression of E-cadherin and Vimentin With Regard to Invasion and Metastasis of Breast Cancer. Clin Breast Cancer 16 (17), 30629-8.
36. V, Fustaino *et al.* (2017) - Characterization of epithelial-mesenchymal transition intermediate/hybrid phenotypes associated to resistance to EGFR inhibitors in non-small cell lung cancer cell lines. Oncotarget 8 (61), 103340-103363.
37. A, Grosse-Wilde *et al.* (2015) - Stemness of the hybrid Epithelial/Mesenchymal State in Breast Cancer and Its Association with Poor Survival. PLoS One 10 (5), 0126522.
38. SV, Puram *et al.* (2017) - Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. Cell 171 (7), 1611-1624.
39. AD, Rhim *et al.* (2012) - EMT and dissemination precede pancreatic tumor formation. Cell 148 (1-2), 349-61.

40. M, Ruscetti *et al.* (2015) - Tracking and Functional Characterization of Epithelial-Mesenchymal Transition and Mesenchymal Tumor Cells during Prostate Cancer Metastasis. Cancer Res 75 (13), 2749-59.
41. M, Chanrion *et al.* (2014) - Concomitant Notch activation and p53 deletion trigger epithelial-to-mesenchymal transition and metastasis in mouse gut. Nat Commun 5 (5005).
42. Z, Zhao *et al.* (2016) - In Vivo Visualization and Characterization of Epithelial-Mesenchymal Transition in Breast Tumors. Cancer Res 76 (8), 2094-2104.
43. Y, Del Pozo Martin *et al.* (2015) - Mesenchymal Cancer Cell-Stroma Crosstalk Promotes Niche Activation, Epithelial Reversion, and Metastatic Colonization. Cell Rep 13 (11), 2456-2469.
44. A, Van Keymeulen *et al.* (2015) - Reactivation of multipotency by oncogenic PIK3CA induces breast tumour heterogeneity. Nature 525 (7567), 119-23.
45. S, Koren *et al.* (2015) - PIK3CA(H1047R) induces multipotency and multi-lineage mammary tumours. Nature 525 (7567), 114-8.
46. M, Latil *et al.* (2017) - Cell-Type-Specific Chromatin States Differentially Prime Squamous Cell Carcinoma Tumor-Initiating Cells for Epithelial to Mesenchymal Transition. Cell Stem Cell 20 (2), 191-204.
47. NM, Aiello *et al.* (2018) - EMT Subtype Influences Epithelial Plasticity and Mode of Cell Migration. Dev Cell 45 (6), 681-695.
48. D, Nassar and C, Blanpain (2016) - Cancer Stem Cells: Basic Concepts and Therapeutic Implications. Annu Rev Pathol 11, 47-76.
49. SA, Mani *et al.* (2008) - The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133 (4), 704-15.
50. AP, Morel *et al.* (2008) - Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 3 (8), 0002888.
51. A, Biddle *et al.* (2011) - Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res 71 (15), 5317-26.
52. S, Liu *et al.* (2013) - Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. Stem Cell Reports 2 (1), 78-91.
53. T, Celia-Terrassa *et al.* (2012) - Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. J Clin Invest 122 (5), 1849-68.
54. AM, Krebs *et al.* (2017) - The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer. Nat Cell Biol 19 (5), 518-529.
55. J, Yang *et al.* (2004) - Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117 (7), 927-39.
56. X, Zheng *et al.* (2015) - Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature 527 (7579), 525-530.
57. KR, Fischer *et al.* (2015) - Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature 527 (7579), 472-6.
58. NM, Aiello *et al.* (2017) - Upholding a role for EMT in pancreatic cancer metastasis. Nature 547 (7661), E7-E8.
59. X, Ye *et al.* (2017) - Upholding a role for EMT in breast cancer metastasis. Nature 547 (7661), E1-E3.
60. OH, Ocana *et al.* (2012) - Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. Cancer Cell 22 (6), 709-24.

61. S, Takano *et al.* (2016) - Prrx1 isoform switching regulates pancreatic cancer invasion and metastatic colonization. Genes Dev 30 (2), 233-47.
62. JH, Tsai *et al.* (2012) - Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 22 (6), 725-36.
63. F, Bocci *et al.* (2017) - Numb prevents a complete epithelial-mesenchymal transition by modulating Notch signalling. J R Soc Interface 14 (136).
64. S, Kuriyama *et al.* (2014) - In vivo collective cell migration requires an LPAR2-dependent increase in tissue fluidity. J Cell Biol 206 (1), 113-27.
65. MK, Jolly *et al.* (2018) - Hybrid epithelial/mesenchymal phenotype(s): The 'fittest' for metastasis? Biochim Biophys Acta 8 (18), 30064-7.
66. B, Huang *et al.* (2015) - Modeling the Transitions between Collective and Solitary Migration Phenotypes in Cancer Metastasis. Sci Rep 5 (17379).
67. TT, Dang *et al.* (2015) - DeltaNp63alpha Promotes Breast Cancer Cell Motility through the Selective Activation of Components of the Epithelial-to-Mesenchymal Transition Program. Cancer Res 75 (18), 3925-35.
68. J, Gao *et al.* (2014) - TGF-beta isoforms induce EMT independent migration of ovarian cancer cells. Cancer Cell Int 14 (1), 014-0072.
69. P, Friedl and D, Gilmour (2009) - Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol 10 (7), 445-57.
70. K, Campbell *et al.* (2016) - A common framework for EMT and collective cell migration. Development 143 (23), 4291-4300.
71. C, Revenu and D, Gilmour (2009) - EMT 2.0: shaping epithelia through collective migration. Curr Opin Genet Dev 19 (4), 338-42.
72. MK, Jolly *et al.* (2015) - Implications of the Hybrid Epithelial/Mesenchymal Phenotype in Metastasis. Front Oncol 5 (155).
73. N, Aceto *et al.* (2014) - Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 158 (5), 1110-1122.
74. YC, Chung *et al.* (2016) - Rab11 collaborates E-cadherin to promote collective cell migration and indicates a poor prognosis in colorectal carcinoma. Eur J Clin Invest 46 (12), 1002-1011.
75. XL, Gao *et al.* (2017) - Cytokeratin-14 contributes to collective invasion of salivary adenoid cystic carcinoma. PLoS One 12 (2), 0171341.
76. KJ, Cheung *et al.* (2013) - Collective invasion in breast cancer requires a conserved basal epithelial epithelial program. Cell 155 (7), 1639-51.
77. Z, Mu *et al.* (2015) - Prospective assessment of the prognostic value of circulating tumor cells and their clusters in patients with advanced-stage breast cancer. Breast Cancer Res Treat 154 (3), 563-71.
78. C, Wang *et al.* (2017) - Longitudinally collected CTCs and CTC-clusters and clinical outcomes of metastatic breast cancer. Breast Cancer Res Treat 161 (1), 83-94.
79. S, Jansson *et al.* (2016) - Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. BMC Cancer 16 (433), 016-2406.
80. AM, Larsson *et al.* (2018) - Longitudinal enumeration and cluster evaluation of circulating tumor cells improve prognostication for patients with newly diagnosed metastatic breast cancer in a prospective observational trial. Breast Cancer Res 20 (1), 018-0976.

81. A, Kulasinghe *et al.* (2018) - A Collective Route to Head and Neck Cancer Metastasis. Sci Rep 8 (1), 017-19117.
82. V, Murlidhar *et al.* (2017) - Poor Prognosis Indicated by Venous Circulating Tumor Cell Clusters in Early-Stage Lung Cancers. Cancer Res 77 (18), 5194-5206.
83. L, Klameth *et al.* (2017) - Small cell lung cancer: model of circulating tumor cell tumorospheres in chemoresistance. Sci Rep 7 (1), 017-05562.
84. I, Krol *et al.* (2018) - Detection of circulating tumour cell clusters in human glioblastoma. Br J Cancer 119 (4), 487-491.
85. M, Yu *et al.* (2013) - Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 339 (6119), 580-4.
86. M, Labelle *et al.* (2011) - Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell 20 (5), 576-90.
87. A, Lecharpentier *et al.* (2011) - Detection of circulating tumour cells with a hybrid (epithelial/mesenchymal) phenotype in patients with metastatic non-small cell lung cancer. Br J Cancer 105 (9), 1338-41.
88. JM, Hou *et al.* (2011) - Circulating tumor cells as a window on metastasis biology in lung cancer. Am J Pathol 178 (3), 989-96.
89. S, Wu *et al.* (2015) - Classification of circulating tumor cells by epithelial-mesenchymal transition markers. PLoS One 10 (4), 0123976.
90. AJ, Armstrong *et al.* (2011) - Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Mol Cancer Res 9 (8), 997-1007.
91. H, Polioudaki *et al.* (2015) - Variable expression levels of keratin and vimentin reveal differential EMT status of circulating tumor cells and correlation with clinical characteristics and outcome of patients with metastatic breast cancer. BMC Cancer 15 (399), 015-1386.
92. KA, Hyun *et al.* (2016) - Epithelial-to-mesenchymal transition leads to loss of EpCAM and different physical properties in circulating tumor cells from metastatic breast cancer. Oncotarget 7 (17), 24677-87.
93. A, Satelli *et al.* (2015) - Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression. Clin Cancer Res 21 (4), 899-906.
94. H, Ou *et al.* (2018) - Circulating Tumor Cell Phenotype Indicates Poor Survival and Recurrence After Surgery for Hepatocellular Carcinoma. Dig Dis Sci 21 (10), 018-5124.
95. D, Boral *et al.* (2017) - Molecular characterization of breast cancer CTCs associated with brain metastasis. Nat Commun 8 (1), 017-00196.
96. K, Kai *et al.* (2018) - CSF-1/CSF-1R axis is associated with epithelial/mesenchymal hybrid phenotype in in epithelial-like inflammatory breast cancer. Sci Rep 8 (1), 018-27409.
97. SC, Wei *et al.* (2015) - Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. Nat Cell Biol 17 (5), 678-88.
98. M, Debaugnies *et al.* (2018) - YAP and TAZ are essential for basal and squamous cell carcinoma initiation. EMBO Rep 19 (7).
99. DD, Shao *et al.* (2014) - KRAS and YAP1 converge to regulate EMT and tumor survival. Cell 158 (1), 171-84.
100. H, Yang *et al.* (2015) - ETS family transcriptional regulators drive chromatin dynamics and malignancy in squamous cell carcinomas. Elife 21 (4), 10870.
101. MR, Young *et al.* (1999) - Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc Natl Acad Sci U S A 96 (17), 9827-32.

102. R, Eferl and EF, Wagner (2003) - AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer 3 (11), 859-68.
103. F, Zanconato *et al.* (2015) - Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. Nat Cell Biol 17 (9), 1218-27.
104. F, Zhu *et al.* (2009) - Critical role of IkappaB kinase alpha in embryonic skin development and skin carcinogenesis. Histol Histopathol 24 (2), 265-71.
105. CS, Hoi *et al.* (2010) - Runx1 directly promotes proliferation of hair follicle stem cells and epithelial tumor formation in mouse skin. Mol Cell Biol 30 (10), 2518-36.
106. MK, Jolly *et al.* (2017) - Inflammatory breast cancer: a model for investigating cluster-based dissemination. NPJ Breast Cancer 3 (21), 017-0023.
107. K, Watanabe *et al.* (2014) - Mammary morphogenesis and regeneration require the inhibition of EMT at terminal end buds by Ovol2 transcriptional repressor. Dev Cell 29 (1), 59-74.
108. X, Ye *et al.* (2015) - Distinct EMT programs control normal mammary stem cells and tumour-initiating cells. Nature 525 (7568), 256-60.
109. MK, Jolly *et al.* (2015) - Coupling the modules of EMT and stemness: A tunable 'stemness window' model. Oncotarget 6 (28), 25161-74.
110. D, Jia *et al.* (2015) - OVOL guides the epithelial-hybrid-mesenchymal transition. Oncotarget 6 (17), 15436-48.
111. R, Gould *et al.* (2016) - Population Heterogeneity in the Epithelial to Mesenchymal Transition Is Controlled by NFAT and Phosphorylated Sp1. PLoS Comput Biol 12 (12), 1005251.
112. Bocci F, Tripathi SC, George JT, Casabar JP, Vilchez-Mercedes SA, Wong PK, Hanash SM, Levine H, Onuchic JN, Jolly MK (2018) NRF2 activates a partial Epithelial-Mesenchymal Transition and is maximally present in a hybrid Epithelial/Mesenchymal phenotype. BioRxiv http://dx.doi.org/10.1101/390237.
113. F, Bocci *et al.* (2018) - A mechanism-based computational model to capture the interconnections among epithelial-mesenchymal transition, cancer stem cells and Notch-Jagged signaling. Oncotarget 9 (52), 29906-29920.
114. M, Boareto *et al.* (2015) - Jagged-Delta asymmetry in Notch signaling can give rise to a Sender/Receiver can give rise to a Sender/Receiver hybrid phenotype. Proc Natl Acad Sci U S A 112 (5), 1416287112.
115. Bisogno LS, Friedersdorf MB, Keene JD (2018) Ras Post-transcriptionally Enhances a Pre-malignantly Primed EMT to Promote Invasion iScience (DOI:https://doi.org/10.1016/j.isci.2018.05.011).

*Tables*

**Table 1. Co-expression of epithelial and mesenchymal markers in different cancers and cancer cell lines**

| Experimental model | Cancer type | Markers used | Method | Ref |
|---|---|---|---|---|
| Cell line | Ovarian carcinoma | E-cadherin, N-Cadherin, Zeb1 | IF | [5] |
| Cell line | Breast carcinoma | E-cadherin, Vimentin | IF, FC | [6] |
| Cell lines | Oral SCC | Vimentin, Keratin5, 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<br>Luminal-like breast cancer<br>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] |

Table 1 summarizes the epithelial and mesenchymal markers reported to be co-expressed in different cancer cell lines, Patient Derived Xenografts (PDX) and primary human cancers.

SCC: Squamous Cell Carcinoma, ADC: Adenocarcinoma, IF: immunofluorescence, FC: Flow Cytometry, PDX: Patient Derived Xenograft.

**Table 2. EMT in mouse cancer models**

| Tumor type | Mouse models | Markers used to define epithelial and mesenchymal states | Reference |
|---|---|---|---|
| **Pancreatic cancer** | Pdx1CRE/KRasG12D/P53cKO/Rosa-YFP<br>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<br>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, Vim, 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, Vim, CD106, CD61, CD51 | [9], [46] |

Table 2 summarizes the mouse models describing the role of EMT during tumorigenesis in vivo. SCC: squamous cell carcinoma.

**Table 3. Characteristics of EMT transition states**

| 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 |

Table 3 summarizes the cell shape, the adhesion, the markers and the transcription factors specific for each EMT transition state. EMT: Epithelial to Mesenchymal Transition, TN: Triple Negative (CD106-CD51-CD61-), TP: Triple Positive (CD106+CD51+CD61+).

*Figure legends*

**Figure 1. Definition of tumor transition states occurring during 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 Vimentin. Cells in early hybrid EMT state co-express K14 and Vim, are more elongated but still cohesive. Cells in late hybrid EMT co-express 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 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.

**Figure 2. 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 below (+: low to ++++ very high).

**Figure 3. Gene regulatory network controlling EMT transition states.** (**A**) Examples of chromatin profiling using 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 enriched in ATAC-seq peaks that differ between EMT transition states [9].. Yellow color highlights the transcription factors common for all EMT transition states and orange color highlights transcription factors specific for each EMT transition state..

**Figure 4. Niches associated with EMT transition states**. (**A**) Schematic representation of the different niches associated with the different EMT transition states. Progression from hybrid EMT states to complete mesenchymal states is associated with progressive increase in the density of endothelial and lymphatic vessels, as well as macrophages [9]. (**B**) Immunofluorescence showing immune cell infiltration (CD45+) in late EMT states [9].

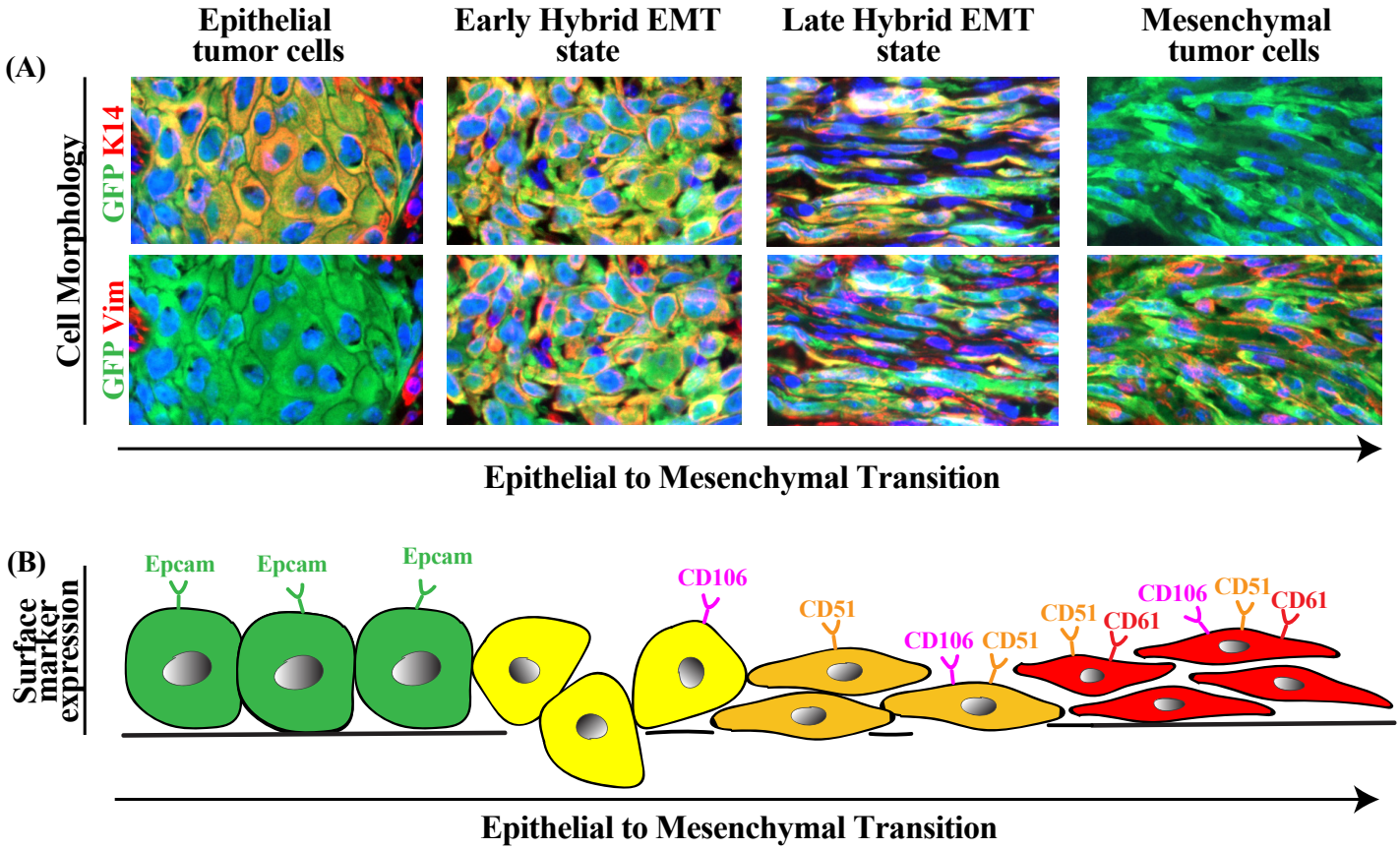
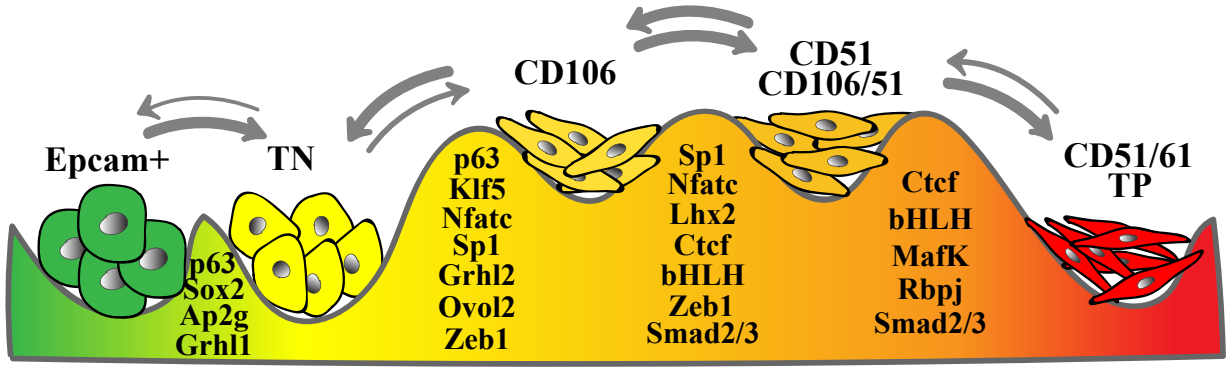

(A)
Epithelial tumor cells
Early Hybrid EMT state
Late Hybrid EMT state
Mesenchymal tumor cells
Cell Morphology
GFP K14
GFP Vim
Epithelial to Mesenchymal Transition
(B)
Surface marker expression
Epcam
Epcam
Epcam
CD106
CD51
CD106
CD51
CD51
CD61
CD106
CD51
CD61
Epithelial to Mesenchymal Transition
(C)
Single Cell RNA-Seq
Cdh1
Krt5
Vim
Mmp19
0 1 2 3
PC1
PC2
10
0
-10
-10 0 10 20

Figure 1




| | Epithelial Tumor cells | Early Hybrid EMT state | Hybrid EMT state | Late Hybrid EMT state | Mesenchymal Tumor cells |
|---|---|---|---|---|---|
| **Proliferation** | +++++ | ++++ | +++ | ++ | + |
| **Invasion** | + | ++ | +++ | ++++ | +++++ |
| **Plasticity** | + | ++ | +++ | ++++ | ++ |
| **Stemness** | + | +++ | +++ | +++ | +++ |
| **Metastasis** | + | ++++ | ++++ | ++ | + |

**Figure 2**

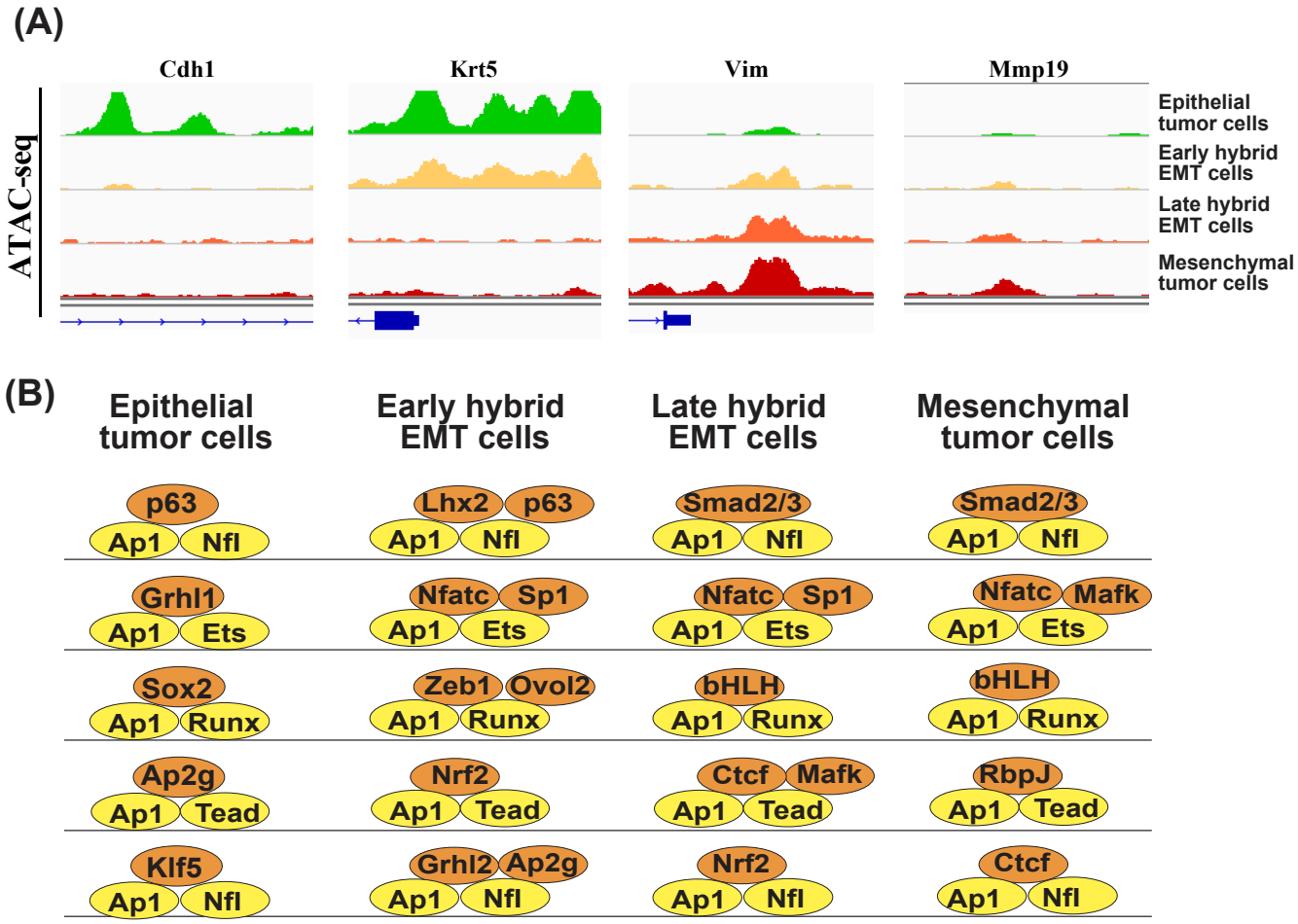

(A)
Cdh1
Krt5
Vim
Mmp19
ATAC-seq
Epithelial tumor cells
Early hybrid EMT cells
Late hybrid EMT cells
Mesenchymal tumor cells
(B)
Epithelial tumor cells
Early hybrid EMT cells
Late hybrid EMT cells
Mesenchymal tumor cells
p63 Ap1 Nfl
Lhx2 p63 Ap1 Nfl
Smad2/3 Ap1 Nfl
Smad2/3 Ap1 Nfl
Grhl1 Ap1 Ets
Nfatc Sp1 Ap1 Ets
Nfatc Sp1 Ap1 Ets
Nfatc Mafk Ap1 Ets
Sox2 Ap1 Runx
Zeb1 Ovol2 Ap1 Runx
bHLH Ap1 Runx
bHLH Ap1 Runx
Ap2g Ap1 Tead
Nrf2 Ap1 Tead
Ctcf Mafk Ap1 Tead
RbpJ Ap1 Tead
Klf5 Ap1 Nfl
Grhl2 Ap2g Ap1 Nfl
Nrf2 Ap1 Nfl
Ctcf Ap1 Nfl

Figure 3

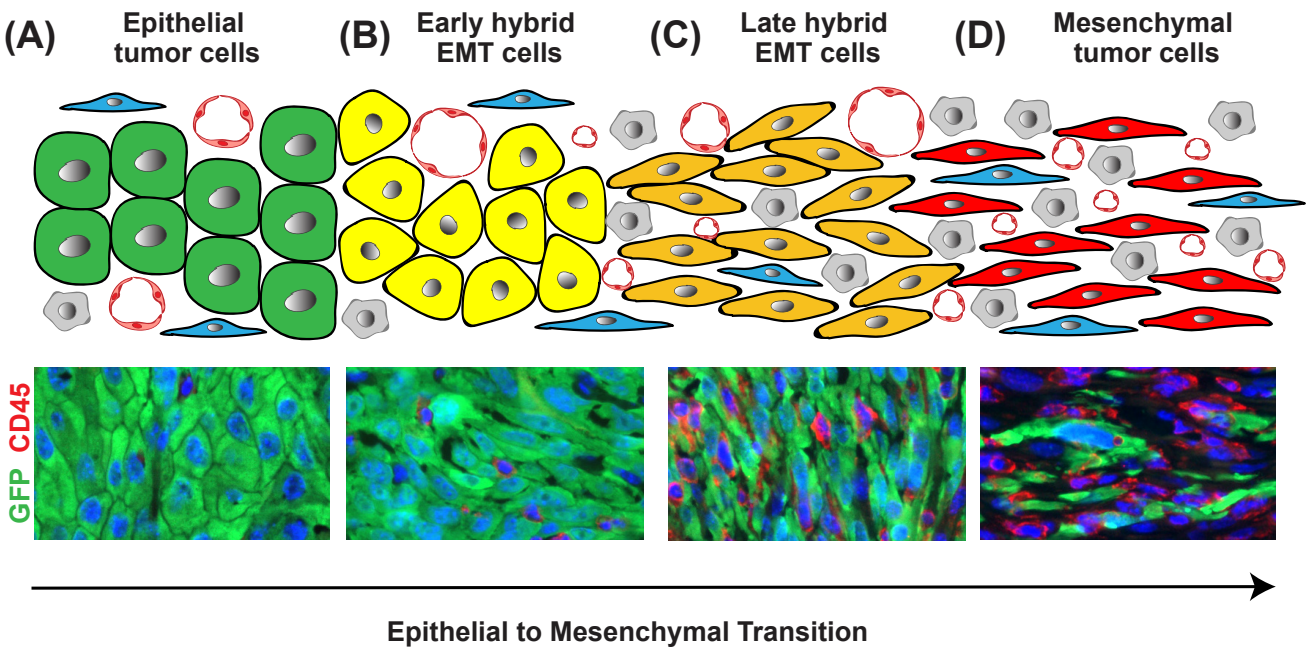

(A)
Epithelial tumor cells
(B)
Early hybrid EMT cells
(C)
Late hybrid EMT cells
(D)
Mesenchymal tumor cells
GFP CD45
Epithelial to Mesenchymal Transition

**Figure 4**

**EMT transition states during tumor progression and metastasis**

## Highlights

- EMT occurs through distinct intermediate states in vivo.
- Distinct EMT transition states can be identified using cell surface markers and single cell RNA-seq
- 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.