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Controversies around epithelial–mesenchymal plasticity in cancer metastasis

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Abstract

Experimental evidence accumulated over decades has implicated epithelial-mesenchymal plasticity (EMP), which collectively encompasses epithelial-mesenchymal transition and the reverse process of mesenchymal–epithelial transition, in tumour metastasis, cancer stem cell generation and maintenance, and therapeutic resistance. However, the dynamic nature of EMP processes, the apparent need to reverse mesenchymal changes for the development of macrometastases and the likelihood that only minor cancer cell subpopulations exhibit EMP at any one time have made such evidence difficult to accrue in the clinical setting. In this Perspectives article, we outline the existing preclinical and clinical evidence for EMP and reflect on recent controversies, including the failure of initial lineage-tracing experiments to confirm a major role for EMP in dissemination, and discuss accumulating data suggesting that epithelial features and/or a hybrid epithelial-mesenchymal phenotype are important in metastasis. We also highlight strategies to address the complexities of therapeutically targeting the EMP process that give consideration to its spatially and temporally divergent roles in metastasis, with the view that this will yield a potent and broad class of therapeutic agents.

Epithelial–mesenchymal transition (EMT) has well established roles in developmental programmes involved in generating new tissues and organs, and is followed, in most cases,

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by the reverse process of mesenchymal-epithelial transition (MET)[1–3]. The EMT and MET processes also have instrumental roles in placentation[4], endometrial function[5] and fibrosis[6]. The dynamic combination of these processes is collectively encompassed by the term 'epithelial-mesenchymal plasticity' (EMP), which we and others advocate as a term of preference[7–13] over 'epithelial plasticity'[14,15], a more general term indicating flexibility in the epithelial state. By contrast, the terms EMT and MET are used to indicate the transitional directionality that is addressed in specific studies. The regulatory framework of EMP is well described, incorporating multiple pathways at numerous levels[16,17]. These processes are evolutionarily conserved with both common core elements and context-dependent molecular specializations in different species and in specific biological scenarios[1,2]. Moreover, EMP provides cells, tissues and organs with a range of mechanisms to influence growth and repair and handle diverse environmental stressors.

Cancer cells exploit EMP processes by manipulating a range of involved control mechanisms (FIG. 1). Consequently, EMP can then contribute directly or indirectly to several of the classical hallmarks of malignancy[18,19], many of which manifest as an enhancement of the cancer stem cell (CSC) phenotype and increased metastatic potential[20–22]. The core evidence supporting a role for EMP in metastasis stems from observations and functional evidence of the enhanced escape of mesenchymally shifted carcinoma cells from the primary tumour, together with their elevated survival, stemness and metastasis-initiation capacity relative to tumour cells with epithelial characteristics[3]. These observations are contrasted by evidence that experimental induction of enforced or stable mesenchymal features abrogate metastatic outgrowth in preclinical models, and that metastases display similar or enhanced epithelial properties relative to their primary tumours[22–25]. Although much of the work on EMP in cancer focuses on carcinomas specifically, related plasticity programmes are described in other cancer types, including sarcomas[26] and haematogenous tumours[27]. Changes in transcriptional programmes that are consistent with EMP have also been identified in stromal cells, likely contributing to the pathobiology of the tumour microenvironment[28–30].

The role of EMP in cancer progression has not been universally accepted for multiple reasons, including the paucity of robust evidence for a process that is likely to be transient and episodic[31,32], the relative scarcity of data supporting the occurrence of MET at the metastatic site, and observations that tumour cells can retain complete metastatic capability whilst maintaining an epithelial phenotype[33–36]. The literature is further confounded as several terms have been used to describe different aspects of the EMT process, including partial EMT (pEMT) and incomplete EMT, both of which refer to cells that have not fully transitioned along the epithelial-mesenchymal axis. We prefer to use the term pEMT, as this indicates that the positioning is deliberate with respect to the cell potentially harnessing beneficial properties from both polar states, whereas incomplete EMT implies that cells try to fully transition to a mesenchymal end point but are unable to do so. pEMT results in a hybrid state (with co-expression of both epithelial and mesenchymal markers in the same cell) or a metastable state (referring to the degree to which cells are fixed at their current position along the epithelial-mesenchymal axis)[37–40]. Evidence from the past 2 years has shown that, among hybrid subpopulations, those that retain more epithelial features with less mesenchymal conversion have the greatest malignant and metastatic potential[40,41]. The

relative expression of epithelial and mesenchymal markers might hypothetically be lower in these hybrid cells than in fully epithelial or mesenchymal cells, respectively, further challenging their identification in vivo and possibly compromising lineage-tracing efforts.

Elucidating the exact involvement of EMP in cancer progression, especially in metastasis, has been challenging, but therapies that target this novel and potentially unique cell population might demonstrate substantial anticancer efficacy with relatively low toxicity[42]. Furthermore, therapeutic control of EMP holds potential as a strategy by which to increase the sensitivity of tumours to a range of existing treatment modalities, including endocrine[43], chemotherapeutic[44], growth factor-targeted[45], anti-angiogenic[46] and radiotherapy-based strategies[47] (FIG. 2). This approach likely extends to a broad spectrum of non-epithelial cancers, in which similar principles might apply under the banner of de-differentiation and re-differentiation.

Following a timely Viewpoint article addressing the knowledge gaps around EMP in cancer[20], we emphasize herein the issues related to achieving clinical utility for EMP axis manipulation, with the aim of identifying the critical hurdles preventing its translation to improve patient outcomes. Accordingly, in this Perspectives article, we summarize the existing preclinical and clinical evidence for EMP in cancer, reflect on the controversies surrounding EMP, and describe therapeutic strategies to address the complexities of targeting a process with spatially and temporally divergent roles in metastasis.

EMT and metastasis

Functional and clinical evidence

Extensive in vitro evidence has delineated the contributions of EMT to tumour metastasis[1,24], particularly in relation to tumour cell migration and invasion. In vivo studies also support a role for EMT in promoting metastasis, having correlated EMT status with the ability of malignant cells to escape the primary tumour. Genetically engineered mouse models (GEMMs) are an important preclinical resource that enable modelling of autochthonous cancers caused by known genetic drivers in an immuneintact syngeneic environment[48] (BOX 1). Trimboli et al.[49] surveyed a number of breast cancer GEMMs and found a high incidence of EMT events (25–64% of tumours) in *Myc* oncogene-driven tumours, which model basal-like and claudin-low breast cancer, in contrast to the observation of infrequent EMT (0–5% of tumours) in tumours from mouse mammary tumour virus (MMTV) promoter-driven polyoma middle T oncogene (MMTV-PyMT) and MMTV promoter-driven ERBB2 (MMTV-Neu) mice, which respectively model metastatic luminal breast cancer and HER2-amplified breast cancer. Interestingly, EMT frequency in the primary tumour, although dependent on the oncogenic driver, did not correlate with metastasis formation, as EMT^{high} tumours from MMTV promoter-driven MYC (MMTV-Myc) mice did not give rise to lung metastasis, whereas EMT^{low} tumours from MMTV-PyMT and MMTV-Neu mice reliably developed lung metastases[49]. Lineage-tracing in PTEN/p53-deficient and carcinogen-induced (medroxyprogesterone acetate (MPA) or 7,12-dimethylbenz(a) anthracene (DMBA)) mouse mammary cancer models showed consistent evidence of pEMT tumour clones in the context of mostly epithelial tumour clones[50]. Consistent with this observation, in clinical tumours, advances in single-cell sequencing

technology have revealed that, although pEMT is clearly evident in some cells, most tumour cells largely preserve the epithelial features seen in cells of their specific origin[51]. In our own preliminary clinical study in locally advanced breast cancers, the presence of EMT before neoadjuvant chemotherapy was associated with improved survival outcomes, whereas its presence after neoadjuvant chemotherapy was associated with poorer survival[52], indicating that EMT does not automatically correlate with clinically significant metastasis. By contrast, patients with primary prostate, breast or lung cancers that exhibit the full range of EMP (that is, transcriptional evidence of both EMT and MET) have the poorest outcomes[12]. The cellular context and the degree of EMT might explain these important differences, with considerable importance being attributed to the hybrid state[42,53]. In addition, the degree of plasticity and the extent of EMT seem to be important, with the highest metastatic potential having been demonstrated in tumour cell subpopulations retaining both epithelial and mesenchymal features whilst lacking the epithelial marker epithelial cell adhesion molecule (EpCAM)[12,40].

Evidence for the prognostic importance of EMT in multiple carcinoma types abounds[52] (TABLE 1), including associations between inferior patient outcomes and positive expression of mesenchymal markers (for example, vimentin, N-cadherin, α-smooth muscle actin (a-SMA) and S100A4)[54,55], a lack of epithelial protein expression (for example, E-cadherin[56] and EpCAM[57]), expression of core EMT-activating transcription factors (EMT-TFs; such as SNAIL1/2, ZEB1/2 and TWIST1/2)[58] expression of epithelial-mesenchymal axis position stabilizers (for example, grainyhead-like protein 2 homologue (GRHL2) and Ovo-like 2 (OVOL2))[59,60], and expression of microRNAs (miRNAs) that regulate these EMP processes (for example, miR-200 family members and miR-34)[61]. Specific core drivers of MET have also been described, such as carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5; also known as CEA)[62] and cellular communication network factor 6 (CCN6)[63], although the relationship of these drivers with clinical outcome remains to be determined. In epithelial malignancies such as breast tumours, EMT is more prevalent in subtypes that are prone to distant dissemination such as basal-like[55] and claudin-low[64] malignancies. Consistent with this observation, tumours derived from tissues of mesenchymal origin are frequently highly invasive (for example, glioma) or characterized by early metastasis (for example, melanoma)[65].

Beyond its direct contributions to tumour dissemination, EMP is also intertwined with the stemness properties of tumour cells, which confers abilities conducive to the successful expansion of metastatic deposits. Breast CSCs frequently exhibit EMT, the abrogation of which impinges on their stemness[66–69]. Although many examples support this relationship (reviewed in REF.[70]), the two processes are not inextricably linked and several studies demonstrate an uncoupling of stemness from the mesenchymal state. In particular, knockdown of *PRRX1,* which encodes an EMT inducer, reversed EMT and promoted metastasis in breast cancer cells[71]; however, the carcinoma cells resulting from the MET retained elements of stemness. Furthermore, in human mammary epithelial (HMLER) cells, induction of a mesenchymal state by constitutive ectopic expression of ZEB1 led to loss of the epithelial-mesenchymal hybrid state (defined as CD104+/CD44^{high} in this study) and the corresponding adult stem cell programme, along with acquisition of enhanced tumorigenicity[39]. In summary, whilst EMT does not always correlate with metastasis and

poor clinical outcomes, the hybrid state, in particular, has been implicated in a variety of carcinoma types. It will be critical to further refine approaches that enable the identification and quantification of tumour cells that are cycling through rounds of EMT and MET in clinical material to examine clinical utility.

EMT timing and context

Tumour cells might undergo EMT in the early stages of tumour initiation. Indeed, endogenous expression of EMT-TFs is observed in many normal tissues during pathological processes related to cancer pathogenesis. For example, EMT can be observed during carcinogenesis-accelerating inflammation, evidenced by the presence of EMT changes in normal colonic and pancreatic tissues during inflammatory bowel disease[72] and chronic pancreatitis[73], both of which are associated with increased risk of subsequent malignancy. Similarly, EMT occurs during inflammation arising from chronic oral infections linked to oral carcinomas[74] and in hepatitis B-driven inflammation associated with hepatocellular carcinoma (HCC)[75]. Furthermore, carcinogens such as tobacco smoke and alcohol induce EMT in lung tissue[76] and breast cells[77], respectively.

EMT is also implicated in the development of malignancy through the suppression of oncogene-induced senescence[78]. Common pathological phenotypes of many tumours, such as the loss of a polarized single layer of epithelium, are consistent with EMT[6]. However, mutations in genes encoding EMT-TFs are not sufficient to drive ab initio tumorigenesis in mice, despite causing a range of developmental defects[79–81]. Rather, it is the pathological spatial and temporal regulation of EMT-TF expression that influences tumour progression.

EMT activation in tumour cells can be triggered by specific features of the local microenvironment in the rapidly growing primary tumour, such as hypoxia[82–84], metabolic reprogramming[85–90] and matrix stiffness[91,92] (FIG. 1). Consequently, it is unsurprising that EMT is commonly observed at the tumour invasive front, where tumour cells directly interact with stromal cells and the extracellular matrix, or surrounding necrotic regions, where cells can be exposed to adverse microenvironmental conditions. Epithelial marker loss has been reported in budding tumour cells at the invasive front of primary colorectal cancers (CRCs), suggestive of EMT[93–95], and further work has reported E-cadherin loss, increased nuclear ZEB1 staining and the acquisition of spindle cell morphology in these tumour buds[94,96]. Furthermore, a pEMT signature was characterized in a subset of head and neck squamous cell carcinomas (HNSCCs) using single-cell RNA-sequencing technology, particularly in cells at the invasive front of primary tumour nodules[51]. The pEMT tumour cells appeared to be directly in contact with cancer-associated fibroblasts (CAFs), with ligand-receptor analyses indicating the emission of pro-EMT signals from these stromal cells. Observations using intravital microscopy and lineage-tracing models for breast cancer have also provided direct evidence that a small population of primary tumour cells undergo EMT, often localized at the border area of tumour lobules adjacent to the vasculature-enriched stroma[33,97]. Similarly, tumour cell EMT is required to stimulate the appropriate metastatic niche, and this process is amplified via a positive feedback loop through which CAFs promote EMT[98].

Heterogeneity in EMT status exists between tumour cells within a single primary tumour[51,99,100], and this spectrum of states has led to speculation that EMT programmes occurring later in cancer pathogenesis might be different from those involved in tumour initiation. In this context, many studies suggest that only a minor subpopulation (~1–10%) of the total primary tumour cell population harbours EMT events[33,34,100,101]. Although in vivo evidence from GEMMs did not support the hypothesis that the minority of EMT populations in the primary tumour were metastasis-initiating cells, it did not rule out the possibility of intercellular cooperation; thus, tumour cells that have undergone EMT could still have a key role in metastasis formation (BOX 2). In these contexts, cells that have undergone EMT might influence the behaviour of non-EMT neighbour cells, enabling them to utilize some of the benefits of the mesenchymal state without experiencing the drawbacks of EMT such as reduced proliferative capacity. Under hypoxic conditions, which commonly induce EMT[51,82,84], exosomal transfer of WNT4 from CRC cells drove translocation of β-catenin to the nucleus in normoxic CRC cells, with a consequent increase in migration and invasion[102]. In addition, extracellular vesicles from mesenchymally shifted 22RV1 human prostate cancer cells have been reported to induce mesenchymal changes in neighbouring cells through transforming growth factor-β (TGFβ) signalling and induce resistance to anti-androgens[103], and exosomes from 30KT human bronchial epithelial cells following oncogene-induced EMT promoted chemoresistance in parental 30KT cells[104].

Although much of the focus on the roles of EMP has been in advanced tumour scenarios, it has also been implicated in very early malignancy events, such that targeting EMT might have a broader application than first anticipated.

EMT in circulating and disseminated tumour cells

Analysis of circulating tumour cells (CTCs) can provide important insights into the molecular and cellular features of cancer cells in transit during the metastatic cascade. Observations of higher levels of mesenchymal gene expression in CTCs relative to tumour cells from both primary tumours and metastases provide a potential answer to the quandary that EMT is rarely observed in either primary tumours or metastases[7,9,100]. Indeed, RNA profiling of CTCs has shown a spectrum of states along the EMP axis[100]. In this seminal study, RNA in situ hybridization using pooled probes specific for epithelial or mesenchymal markers demonstrated a dynamic range of EMP states in breast cancer CTCs derived from an index patient, with shifts towards a predominantly mesenchymal state generally correlating with each episode of disease relapse[100]. In a study by Wu et al.[105], classification of CTCs according to EMT marker expression in 12 patients with early-stage breast cancer and 6 patients with metastatic breast cancer revealed increased levels of mesenchymal CTCs as well as circulating tumour microemboli with a mesenchymal phenotype in metastatic cases Multi-marker assessment of early-stage breast cancer CTCs has also uncovered more frequent expression of mesenchymal markers *(VIM, SNAI1* and *UPAR)* in lymph node-positive patients compared with lymph node-negative patients[106] and has been reported to provide prognostic information[107].

Over the past 5 years, single-cell RNA-sequencing has been used to assess isolated CTC populations and CTC clusters[108]. Studies using xenografts of the LM2 variant of MDA-

MB-231 human breast cancer cells have shown that CTC clusters are oligoclonal and have a more robust metastatic capacity than single CTCs, consistent with their association with poor prognosis in breast and prostate cancer, and have implicated the epithelial cell junction component plakoglobin (JUP) in CTC cluster formation[109]. Furthermore, a single-cell RNA-sequencing study of CTCs in a mouse model of pancreatic cancer showed an overall loss of expression of the epithelial markers *Chd1* (which encodes E-cadherin) and *Muc1* (which encodes mucin-1) in CTCs compared with the primary xenograft tumours, with mixed and heterogeneous expression of mesenchymal markers in the same cells[110]. The authors also reported enriched expression of the pancreatic stem cell markers *Aldh1a1* and *Aldh1a2* in CTCs, but these events were not associated with an epithelial or mesenchymal state. Our group has seen enriched, but dysregulated, EMP (increases in both epithelial and mesenchymal gene expression) in CTCs compared with primary breast cancer xenograft tumours[13]. A further study reported enrichment for cell adhesion-related and proliferation-related genes, along with the proliferative marker Ki-67, in CTC clusters compared with single CTCs from patients with breast cancer and from xenograft models[111]. In 2018, CTCs with CSC and pEMT phenotypes co-expressing cytokeratin, aldehyde dehydrogenase 1 (ALDH1) and nuclear TWIST1 ($CSC^+/pEMT^+$ CTCs) were shown to have significant prognostic value in metastatic breast cancer compared with non-$CSC^+/pEMT^+$ CTCs, CTCs with CSC and epithelial phenotypes (CSC^+/epithelial-like CTCs), or non-CSC^+/epithelial-like CTCs[112]. Furthermore, the incidence of $CSC^+/ pEMT^+$ CTCs was increased after first-line chemotherapy and, therefore, these might represent a chemoresistant subpopulation. In a breast cancer model, Padmanaban et al.[36] showed that inducible knockdown of *CDH1* expression reduced the proliferation and survival of tumour cells as well as CTC numbers. Furthermore, our group has also reported reduced proliferation, both in vitro and in vivo, in MDA-MB-468 breast cancer cells after *CDH1* knockdown[51]. Consistently, in another study, $EpCAM^{low}$ CTCs, whilst present in patients with breast and prostate cancer, lacked prognostic significance compared to their $EpCAM^{high}$ counterparts[113].

EMT is also frequently observed in disseminated tumour cells (DTCs) found in the bone marrow of patients with cancer[7,10,114,115]. This is an important observation, as a subpopulation of DTCs are believed to be precursors to macrometastases, but it is not yet possible to predict which specific cells will survive and escape dormancy to form a clinically significant tumour. The bone marrow is a relatively hypoxic tissue, raising the possibility that hypoxia-induced EMT processes in local stromal cells and arriving tumour cells promote the successful colonization of this niche followed by a period of dormancy[116].

Given the position of CTCs and DTCs along the metastatic cascade, extensive data showing the enrichment of EMP in these cells compared with the tumours from which they originated is generally considered to be highly supportive of the role of EMP in metastasis. The observation that prognostic relationships are stronger for epithelial CTCs supports the notion that MET might be important prior to intravasation at the metastatic site, and might challenge the long-standing association between the mesenchymal phenotype and enhanced survival under adverse conditions.

MET and metastatic outgrowth

Epithelial characteristics in metastases

The earliest indications of MET in the clinical setting were in colon adenocarcinomas, where cells at the invasive front were found to be mesenchymally shifted (as evidenced by nuclear β-catenin localization), whereas distal liver metastases were epithelial (as evidenced by cytoplasmic β-catenin localization)[93]. Subsequently, equal or stronger expression of the epithelial marker E-cadherin has been reported in distant metastases compared with matched primary breast and prostate cancer specimens[117–121]. Moreover, all metastatic tumours of invasive ductal carcinoma origin expressed E-cadherin, irrespective of the E-cadherin status of the primary breast tumours[120]. Similarly, E-cadherin expression has been seen in bone metastases originating from E-cadherin-negative, poorly differentiated primary breast carcinomas[122]. These observations are supported by transcriptional studies, in which metastases often cluster with their respective primaries[123]. In bladder cancer, the presence of EMT (loss of *CDH1* expression and elevated expression of mesenchymal markers *ZEB1, ZEB2* and *VIM*) was associated with muscle-invasive bladder cancers but not with non-muscle invasive bladder cancers[124]. However, among muscle-invasive bladder tumours, epithelial markers correlated with decreased survival, implying that mesenchymal and epithelial states are associated with different phases of tumour progression[124].

These data collectively reinforce the epithelial requirements for metastases, which, when combined with the strong implications for mesenchymal capacity in earlier stages of the metastatic cascade, have led to the hypothesis regarding the requirement for MET following EMT.

Metastatic capacity of epithelial variants

A number of in vivo studies support a requisite role for EMT reversal in metastases. Using a series of T24 bladder cancer cell lines selected in vivo for increasing metastatic ability (via two rounds of intracardiac inoculation and outgrowth from resultant bone lesions, which is a relevant metastatic site), our group reported an association between macrometastatic competence and epithelial phenotype when cells were subsequently introduced either systemically or directly to bone[125]. We showed that the epithelial T24 bladder cancer variants (TSU-B1/B2) formed more metastases than the mesenchymal parental cells after intra-cardiac inoculation, albeit with a lower capacity to escape from the primary tumour when cells were implanted orthotopically. This observation supports the notion that mesenchymal attributes are important in initial escape from the primary tumour, whereas epithelial characteristics are required for the establishment of macrometastases. Consistent with this hypothesis, Celia-Terrassa et al.[126] showed that stable mesenchymal variants of both T24 bladder cancer and PC-3 prostate cancer cell lines were unable to form metastases following intracardiac and orthotopic inoculation, respectively. Similarly, only cells with an epithelial phenotype (E-cadherin^{high}, CK18^{high}) gave rise to lung and liver metastases following orthotopic inoculation in a panel of isogenic mouse breast cancer (4T1-derived) cell lines[127]. TWIST1-overexpressing breast cancer cells require E-cadherin for dissemination from organoids in vitro[128], where they retain adherence junctions and proliferation[129]. Additionally, overexpression of miR-200 family miRNAs in the otherwise

weakly metastatic 4T07 cells increased metastasis from the orthotopic site[130]. Furthermore, a 2018 analysis implicated tightly balanced feedback loops producing bi-stability of cellular states, known as hysteresis, in control of metastasis[131].

Manipulation of EMT signalling provides further evidence of the importance of MET in successful metastasis. The continuous activation of EMT signalling was shown to inhibit metastasis formation. Indeed, in an immunocompetent mouse breast cancer model (MMTV-neuNT), mesenchymal tumour cells induced by SNAIL1 overexpression were unable to form macrometastatic lesions, although DTCs were readily observed[132]. Moreover, TWIST-induced EMT in squamous cell carcinoma cells promoted dissemination to the vasculature and distant organs in a mouse model, but TWIST had to be switched off for the formation of macrometastases[133]. Similarly, loss of PRRX1-driven EMT was required for in vivo metastasis of BT-549 breast cancer cells despite the retention of stemness attributes throughout the process[71]. In another mouse model, induction of MET through overexpression of the transcription factor inhibitor of differentiation 1 (ID1) in HMLER cells promoted the conversion of micrometastases from TWIST-expressing HMLER tumours to macrometastases[134]. This was not seen with SNAIL1-expressing HMLER cells, consistent with the selectivity of ID1 in binding basic helix-loop-helix transcription factors.

Transient induction and reversal of EMT by inducible expression of SNAIL1 and SNAIL2 in prostate cancer cell lines has identified a specific MET-reversing gene signature that differed from the original epithelial transcriptional state[12]. This signature was enriched in clinical metastatic tissue, and its presence in primary prostate cancers was predictive of poor outcome, potentially due to an enhanced capacity of those primary tumours to undergo MET during metastasis. Interestingly, epithelial cells that had previously passed through the mesenchymal state were altered at the transcriptional level relative to cells permanently held in an epithelial state, showing enrichment of ‘solid tumour’, ‘cell movement’ and ‘invasion of cells’ gene sets as well as androgen and anti-androgen responsiveness. These findings imply that mesenchymal properties conducive to tumour spread and therapeutic resistance are retained. These data also highlight a level of EMT-MET cycling in the primary tumour, consistent with the observation of cells that have undergone EMT at the tumour invasive front in other studies[33,93–97,135–137]. Such cycling has been documented in a number of cancer types[11,138], and the incremental molecular impact has been revealed through both modelling and measurement[131].

The collective observations of the epithelial nature of metastases and the failure of artificial EMT induction to promote tumour spread to the macrometastatic stage support the notion that MET has an important role in metastasis, at least in metastatic establishment at the secondary site, by enabling cohesive growth[23,25]. It is apparent that the incidence of MET likely varies depending on the metastatic site and is dependent on extrinsic microenvironmental signals received by the tumour cells at the secondary site. Along these lines, Pastushenko et al.[40] showed that, although subcutaneously injected $EpCAM^{-}$cells did not give rise to $EpCAM^{+}$ cells in secondary tumours, all $EpCAM^{-}$subpopulations led to $EpCAM^{+}$ deposits when colonizing the lung microenvironment. In a mouse model, MDA-MB-231 breast cancer cells required preconditioning of the MET-promoting microenvironment in the lung for metastasis formation, which led to upregulation of E-

cadherin expression in MDA-MB-231 cells and the development of lung metastases[139]. In an experimental study, co-culture of DU145 prostate cancer cells with hepatocytes, modelling the respective metastatic niche in the liver, resulted in upregulation of E-cadherin[140]. Such comparisons might need to be extended to bone, brain and/or liver metastases from the same patients or experimental animals, and could ultimately lead to selective tailoring of treatment of metastatic cancers based on their metastatic site. Aiello et al.[141] found a shift from mesenchymal to epithelial histology as metastases in an autochthonous model of pancreatic ductal adenocarcinoma became larger. Furthermore, they also accumulated a desmoplastic stroma and became hypovascular, recapitulating their primary tumours.

The potential for MET has also been documented in cultured cell systems. Indeed, a dynamic flux between epithelial and mesenchymal states has been elegantly demonstrated in Dunning rat carcinoma cells[11] and HCC-38 human breast cancer cells[116]. In addition, the PMC42-LA subline of the PMC42-ET human breast cancer cell line[142] is stably epithelially shifted but shows dynamic plasticity. EMT marker expression is upregulated by epidermal growth factor (EGF) treatment in both lines, and sustained EMT induction in human mammary epithelial cells can become epigenetically stabilized[143]. Although the extent to which this inherent plasticity exists across cell lines is unclear, it does not seem to be universal, with only a minority of breast cancer cell lines showing such subpopulations[138].

These studies further reinforce the requirement of epithelial features for metastatic competence across numerous models, despite the advantages associated with mesenchymal attributes in earlier stages of metastasis. A key point is the multitasking potential of cells exhibiting a hybrid phenotype, possibly explaining the otherwise heroic metastatic competence of epithelial variants despite major penalties in early steps such as migration, invasion, intravasation and survival as CTCs.

EMP-independent metastasis

Controversy regarding the role of EMP in metastasis stems from difficulties in generating evidence that cells exhibiting a predominantly epithelial phenotype in metastases actually underwent EMT[34,144]. Although EMT blockade by individually targeting crucial EMT-TFs does not necessarily impair metastasis, questions can be raised as to whether the right EMT-TFs were targeted and/or whether other EMT-TFs compensated. In mice, genetic knockout of *Snail1* or *Twist1* did not alter the emergence, dissemination or liver metastasis of invasive pancreatic cancer[101], yet a similar approach of tissue-specific *Zeb1* silencing resulted in a marked reduction in metastasis[35,145]. Moreover, breast tumour cell-specific knockout of *Twist1* impaired lung metastasis formation in a mouse model[146]. However, inhibiting EMT by overexpression of miR-200s, which directly target ZEB1 and ZEB2, did not impair lung metastasis formation in an orthotopic breast tumour GEMM[34,147]. By contrast, miR-200 levels in clinical breast tumours were positively associated with increased risk of metastatic spread[130]. These studies support the requirement of MET for secondary tumour outgrowth and/or the dispensability of EMT for tumour cell escape from the primary site. Indeed, these authors showed a marked reduction in the number of CTCs in mice bearing miR-200-transfected tumours, despite a dramatic stimulation of metastasis[130]. These inconsistent

observations when targeting different EMT-TFs in different tumour models suggest a tumour-specific involvement of the EMT programme.

To precisely evaluate the contributions of EMT to tumour progression in vivo, lineage-tracing strategies using GEMMs have been developed (BOX 1). Using novel reporters that integrate both transcriptional and post-transcriptional regulation to test whether MET is required for metastasis in cancer models, Somarelli et al.[148] showed that metastasis of human uterine carcinosarcoma CS-99 cells from the subcutaneous site occurred via a MET-dependent pathway, whereas metastatic colonization appeared to be MET independent in two prostate carcinoma models (rat AT3 cells inoculated subcutaneously and human DU145 cells inoculated intravenously). Using pancreatic epithelium-specific Pdx1-Cre to mediate both oncogenic *Kras*^{G12D}/*Tp53*$^{flox/flox}$ and yellow fluorescent protein (YFP) marker expression, EMT activations were observed in premalignant lesions, as evidenced by gain of ZEB1 and fibroblast-specific protein 1 (FSP1) expression and/or the loss of E-cadherin expression in tumour cells[149]. These EMT tumour cells (YFP$^+$/E-cadherin$^-$) from the premalignant lesions exhibited enhanced tumorigenic capacity relative to their non-EMT (YFP$^+$/E-cadherin$^+$) counterparts in vivo. However, tumour cells isolated from late-stage pancreatic tumours did not show such differential tumorigenic abilities. Both EMT and non-EMT cells gave rise to invasive pancreatic tumours and distant metastases regardless of their initial phenotypes when engrafted into recipient mice. Interestingly, tumours in both groups were histologically similar and contained a comparable prevalence of EMT events, suggestive of substantial EMP in these tumour cells[149].

Given the EMP of tumour cells, it is challenging to demonstrate the direct contribution of EMT to metastasis in vivo. For this purpose, further lineage-tracing models have been developed using mesenchymal-specific promoters (for example, using the *Fsp1* and *Vim* promoters) to mediate a fluorescent marker switch (from red fluorescent protein (RFP) to green fluorescent protein (GFP) expression) only in tumour cells that have undergone EMT[34] (BOX 1). These models enable the tracing of the EMT history of a cell with permanent markers that distinguish cells that revert to an epithelial phenotype after metastatic seeding from tumour cells that never underwent EMT. Unexpectedly, analysis of lung metastases from these breast cancer models did not demonstrate the presence of GFP$^+$ cells. Rather, the metastases were chiefly composed of RFP$^+$ cells with an epithelial phenotype, indicating that they were derived from tumour cells that had not transitioned through EMT. By contrast, EMT events (GFP$^+$) were detected in 1–2% of primary tumour cells, which exhibited advantages in invasion, and were enriched among CTCs relative to primary tumour cells that were not marked as undergoing an EMT[34]. However, the primary tumour cells that had undergone EMT were vastly outnumbered by non-EMT-marked tumour cells; furthermore, these latter cells also resembled the majority of tumour cells in the metastatic outgrowth. It remains unclear how many cells that have undergone EMT would be necessary or sufficient to induce metastasis.

These findings evoked another round of vigorous debate about the real contributions of EMT in metastasis[1,24,147]. Specifically, concerns have been raised that certain mesenchymal-specific promoters might have limitations in their ability to fully describe the complex and diverse EMT process, that EMT lineage-tracing models might not be sensitive enough to

report possible pEMT programming (given that, in this instance, both epithelial and mesenchymal features might be less strongly regulated relative to cells undergoing full EMT), and that cells with a strong enough EMT to activate the reporter system (which rarely occurs in the primary tumour but is enriched in CTCs) might not be able to reverse the EMT and subsequently colonize metastatic sites (BOX 1). Importantly, these studies did not deny the involvement of EMT in tumour progression, and EMT tumour cells clearly exhibited chemoresistant features that led to their contribution to metastasis after chemotherapy[34,101]. Thus, to fully understand the involvement of EMT in metastasis, novel and highly sensitive EMT lineage-tracing models that rely on multiple EMT markers and can report the dynamic change of phenotypes, ideally at the single-cell level, will be necessary.

One potential rationale for the requirement of MET in metastasis is the loss of proliferation observed with mesenchymal changes in many, but not all, systems. In support of this hypothesis, highly mesenchymal variants of T24 human bladder carcinoma cells and PC-3 human prostate cancer cells were unable to metastasize in immunocompromised mice as effectively as their more epithelial counterparts, and were compromised in their proliferative capacity[126]. Our group has confirmed a loss of proliferative capacity in MDA-MB-468 human breast cancer cells in which E-cadherin expression has been supressed[51], and this was also seen in two different mammary cancer GEMMs[36]. Furthermore, a 2018 study showed that IL-1β-expressing innate immune cells that were systematically invoked by the primary tumour maintained metastasis-initiating cancer cells at the metastatic site in a ZEB1-positive differentiation state, preventing them from becoming proliferative E-cadherin-positive progeny[150]. Nevertheless, this relationship is not universal and examples of EMT systems that retain proliferative capacity have been reported[151,152]. However, ZEB1/2-driven EMT seems to be particularly associated with a decrease in proliferation[153–155]. Thus, the specific driver of EMT likely contributes to the variations in the apparent requirement for MET in the formation of metastases. Furthermore, the concomitant presence of independent oncogenic drivers of proliferation might override the otherwise inhibitory influence of EMT on proliferation.

The hybrid state

Hundreds of EMT-related genes have been characterized and evaluated to quantify the EMT status of cells and transcriptional metrics for quantification of EMT have been developed[65,156]. Among all predictors, the *VIM:CHD1* gene expression ratio combined with the expression of *CLDN7* (which encodes claudin 7) was shown to be the best approach for the assignment of various tumour cells into three phenotypes — epithelial, hybrid and mesenchymal[156]. Using this approach, metastatic breast tumours were categorized as either having an epithelial or hybrid phenotype. Another study evaluating EMT status — as defined by an alternate EMT quantification algorithm — showed that cell lines and cancers from different origins had different EMT phenotypic ranges, but the EMT status did not correlate with either tumour aggressiveness or poor survival outcomes in patients[65]. Segregation of the epithelial and mesenchymal components of this score showed that breast cancer specimens in The Cancer Genome Atlas were predominantly hybrid in nature, and that many of the mesenchymally classified breast cancer cell lines (basal B subgroup) also

exhibited a high expression of both epithelial and mesenchymal markers and, therefore, also had a hybrid phenotype[157].

With the exception of CTCs, the hybrid EMT state of human cancers has been mostly characterized using bulk tumour transcriptomes. Thus, the hybrid EMT status might be due to the contamination of stromal components[158]. Of course, the differential components in stroma might also provide independent clues to tumour subtypes[159]. In this instance, single-cell RNA sequencing results should be more informative, as it would enable distinction between mesenchymal stromal cells and EMT tumour cells. Indeed, a pEMT programme including upregulation of mesenchymal genes and genes encoding extracellular matrix proteins in conjunction with downregulation of certain epithelial genes was confirmed in a subset of HNSCC cells[51]. This programme did not include genes encoding the classical EMT-TFs SNAIL1, TWIST1/2 or ZEB1/2, although this finding could be due to the currently limited ability of this technique to detect lowly expressed genes, leading to an incomplete characterization of EMT status. Metastatic breast cancer cells with activation of an EMT-related stem cell programme have also been characterized at the single-cell level[160]. However, there was very limited overlap among the EMT markers identified in these two studies[51,160]. Nevertheless, whether the hybrid state represents a stable phenotype or is a snapshot of the dynamic and fluctuating EMT status of individual tumour cells remains an open question, although very new and preliminary (not yet peer reviewed) single-cell RNA sequencing data suggests a continuum[161]. In SCC9 human HNSCC cells, cultured $pEMT^{high}$ and $pEMT^{low}$ subpopulations remained distinct at 4 h and 24 h after cell sorting, but became indistinguishable after 4 days of culture, with both cultures recapitulating the distribution of marker expression in unsorted SCC9 cells[51]. Similarly, distinct subpopulations of HNSCC cells returned to the proportions seen in unsorted cells over time, although hybrid mesenchymal subpopulations were more plastic than either the most highly mesenchymal or epithelial subpopulations[40]. Similar reversion of an $EpCAM^{low}$ subpopulation of MMTV-PyMT[33] and PMC42-LA[162] cells has also been reported, and mutually regulated epithelial ($EpCAM^{+}$) and mesenchymal ($EpCAM^{-}$) subpopulations of the HCC-38 human breast cancer cell line have been shown to revert to a tightly controlled epithelial to mesenchymal ratio[33]. Moreover, relatively stable hybrid cell populations with greater tumorigenicity than epithelial or mesenchymal cells have been described. In these populations, the hybrid state was maintained by the EMT-TF SNAIL1 in concert with canonical WNT signalling, whereas constitutive ectopic expression of ZEB1 led to a fully mesenchymal shift, non-canonical WNT signalling, and loss of tumorigenicity in these cells[39].

A degree of finesse to EMT control has emerged with the identification of phenotypic stability factors (such as GRHL2 and OVOL2), which can interact with the standard EMT control machinery to stabilize the hybrid state[59,60]. In the context of cancer, phenotypic stability factors have been shown to promote the dissemination of tumour cell clusters and their expression is associated with an inferior prognosis, whereas a fully mesenchymal signature correlated with improved survival outcomes[163]. Similarly, a bivalent chromatin configuration was identified in the promoter of the EMT-TF ZEB1 (REF.[164]), allowing rapid on-off cycling of ZEB1 expression in response to microenvironmental signals and control of tumorigenesis and/or outgrowth of secondary tumours.

Collectively, the hybrid EMT state in tumour cells describes the presence of both epithelial and mesenchymal markers in the same tumour cells. It might reflect a stable state of tumour type or a transiting phase of tumour cells in switching their phenotype. Its correlation with aggressiveness and metastasis further enforces the crucial role of EMP in tumour progression.

Therapeutic implications of EMP

Therapeutic resistance and EMT

Highly pertinent to the clinical relevance of EMP is the relationship between therapeutic resistance and EMT, which has been well established in numerous studies[70,165]. Unlike the intertwined and sometimes conflicting literature associating EMT with the metastatic process, evidence supporting a role for EMT in treatment resistance is consistent and compelling. Enrichment of EMT markers at the RNA, protein and phenotypic levels has been described after exposure to a broad spectrum of therapeutic modalities, including hormonal therapies, chemotherapies, radiotherapy and many targeted therapies (FiG. 2). However, it is important to not simply confirm induction of EMT per se but to also to demonstrate its impact on treatment sensitivity, including in clinical cohorts.

In prostate cancer specimens, an EMT gene expression profile, which was only observed after neoadjuvant docetaxel and hormonal therapy, correlated with decreased time to relapse[44]. Specifically, multivariate analysis revealed that low *CDH1* expression was predictive of reduced time to prostate-specific antigen relapse and high *ZEB1* expression correlated with rapid radiological progression. Furthermore, in prostate cancer, low tumour E-cadherin expression, which was mediated by reduced miR-200c and miR-205 expression, has additionally been linked to increased relapse rates after chemotherapy but not after surgery alone[166]. In breast cancer, EMT was enriched after docetaxel treatment[167] and, in a second cohort, induction of EMT after chemotherapy correlated with increased expression of the ABC subfamily G member 2 (ABCG2) and ABC subfamily B member 1 (ABCB1; also known as MDR1) drug efflux proteins[168]; however, neither study reported patient outcomes. Our own study confirmed EMT induction after neoadjuvant chemotherapy in 24% of patients with breast cancer, which was associated with worse disease-free survival[165]. In another study, patients with late-stage breast cancer whose CTCs showed a pEMT phenotype (defined by expression of cytokeratin, ALDH1 and nuclear TWIST1) after chemotherapy had inferior progression-free survival, which was particularly marked in patients with HER2-negative cancers[112]. In patients with non-small-cell lung cancer who were treated with chemoradiotherapy, expression of mesenchymal markers in pretreatment specimens did not correlate with outcomes, but patients with evidence of EMT in their post-treatment resection specimen had inferior disease-free survival[47]. In patients with oesophageal cancer who had received chemotherapy, both increased SNAIL1 and decreased E-cadherin expression were predictive of poor chemotherapy response and inferior overall survival[169]. In patients with rectal cancer, reduced E-cadherin expression, nuclear β-catenin expression, reduced miR-200c expression and the presence of tumour budding were all associated with non-response to neoadjuvant chemoradiotherapy, whereas reduced expression of E-cadherin and miR-200c were both associated with reduced cancer-specific

survival[170]. Beyond the aforementioned studies addressing the combination of chemotherapy and surgical treatments, radiotherapy has been shown to induce EMT in patients with CRC, which correlated with an increased recurrence rate[171], and EMT-positive CTCs from patients with non-small-cell lung cancer have been shown to be enriched after radiotherapy[172,173].

For endocrine treatment, despite extensive preclinical evidence for EMT as a driver of resistance to hormonal therapy, few clinical studies have been performed and have generally omitted direct survival correlations. In a breast cancer study, neoadjuvant letrozole induced EMT changes similar to the aforementioned chemotherapy-induced EMT, a finding that was confirmed in two independent cohorts; however, associations with patient outcomes were not explored[167]. In further research into metastatic disease, SLUG (also known as SNAI2) expression in two independent cohorts of patients with oestrogen receptor-positive metastatic breast cancer treated with endocrine therapy was correlated with decreased progression-free survival[174]. There is also considerable preclinical evidence that androgen deprivation therapy, including luteinizing hormone-releasing hormone (LHRH) agonists and oral androgen receptor-binding anti-androgens, induce EMT in prostate cancer, leading to consequent anti-androgen resistance; however, clinical correlates are again lacking. For example, E-cadherin expression was decreased and expression of vimentin, N-cadherin and ZEB1 were increased in clinical prostate cancer samples after development of insensitivity to combined androgen blockade relative to pretreatment specimens[175]. In a previously cited study[44], combined androgen blockade and chemotherapy led to induction of EMT, which correlated with poor outcomes in early-stage prostate cancer.

A range of targeted therapies beyond endocrine blockade could also be affected, with evidence of EMT induction across multiple tumour types treated with agents against a wide spectrum of targets. In HER2-positive breast cancer cell lines, some clones underwent spontaneous EMT in culture, which led to trastuzumab resistance[45], although the clinical significance has yet to be investigated. An EMT signature showed the strongest correlation with resistance to the EGF receptor inhibitor gefitinib in both lung and head and neck cancer cell lines[176]. Furthermore, EMT in *ALK*-translocated lung cancer cell lines led to resistance to the ALK inhibitor crizotinib[177].

In addition to treatment-induced EMT, stimulation of EMT through various means prior to exposure to therapeutic agents has also been shown to make tumour cells less responsive to treatment relative to controls and could, therefore, have a clinical impact. Elements of the tumour microenvironment, such as hypoxia (which induces hypoxia-inducible factor-α (HIF1α) activity)[178–181] and acidosis[182,183], might attenuate chemo-responsiveness through EMT-related mechanisms. In keeping with these findings, induction of MET through inhibition of EMT-TFs can reverse therapeutic resistance (reviewed in REF.[8]) (FIG. 1).

Of additional interest, and intricately linked to the aforementioned EMT-associated therapeutic resistance, is the persistence of EMT or EMT-related changes that increase tumour aggressiveness after treatment. Beyond the impact of EMT in promoting resistance to the EMT-inducing treatment, EMT in tumours after therapy might result in increased proliferation, angiogenesis, immunosuppression and metastatic dissemination as well as

suppression of apoptosis and induction of CSCs (reviewed in REF.[165]). Our own group has noted that, for virtually all therapies that have been established to induce EMT, eventual patient survival is shortened relative to the control period, implying accelerated progression, whereas for therapies known to reverse EMT, survival tends to be prolonged in excess of the duration of benefit from the treatment, implying that EMT reversal during treatment leads to less aggressive progression after treatment cessation[165]. Furthermore, considering the broad spectrum of cellular features affected by shifts along the epithelial-mesenchymal axis, it is unsurprising to find substantial changes in the parameters that govern the success of immunotherapy[184,185] (BOX 3).

Targeting EMP

The involvement of EMT in the metastatic process, treatment resistance and accelerated progression post-therapy indicates that the ability to reverse or otherwise manipulate EMT could have therapeutic potential across a number of scenarios. The area warranting the greatest caution is that of direct EMT reversal with the aim of preventing metastatic spread, considering the aforementioned demonstration of the importance of the epithelial state in many contexts for the full establishment of macrometastases. In reality, the majority of treatment scenarios across the spectrum of cancer management involve directly treating, rather than preventing, existing metastases, be it micrometastases in the adjuvant setting or macrometastases in established disseminated malignancy. However, care is still required in the treatment of locally confined but unresectable cancers, lest dissemination be facilitated (FIG. 3). Careful context-specific experiments will be required to fully delineate the impact of such interventions on tumour pathogenesis, progression and dissemination.

In contrast to the dichotomous results regarding promotion of metastasis, the extensive preclinical data and generally supportive clinical data regarding the role of EMT in resistance to a broad spectrum of treatment modalities indicates the potential therapeutic value of EMT-reversing therapies in overcoming resistance and generates less concern regarding potential detrimental effects (FIG. 2). The many additional studies implicating EMT in accelerating progression after cessation of many such therapies strengthen this case. A wide range of agents that directly or indirectly target EMP processes have been reviewed elsewhere[8,186]. Considering this therapeutic potential, different approaches have been developed to manipulate the epithelial-mesenchymal axis, which can be classified into therapies that prevent or revert mesenchymal transition, target cells in the mesenchymal or hybrid states, inhibit transitions, fix cells to a position along the epithelial-mesenchymal axis, or target unique factors induced in cells after cycling through epithelial-mesenchymal-epithelial states.

To date, the major initiatives have addressed the first therapeutic scenario — prevention or reversal of mesenchymal transition. In this space, a number of methods have been developed to harness various aspects of native cellular control of the EMT process. Non-coding miRNAs have established roles in the physiological repression of EMT (reviewed in REF.[187]); consequently, their use to prevent or reverse EMT is a rational consideration. The miR-200 family has emerged as having a fundamental place in suppression or reversal of EMT, with upregulation of miR-200s having been shown to render both chemoresistant

prostate carcinoma cells[166] and pancreatic cancer cells[188,189] sensitive to chemotherapy. As an example of feasibility, clinical trials employing mimics of the tumour-suppressive miR-34 (REF.[190]) and miR-16 (REF.[191]) have now entered phase I clinical trials for the treatment of liver cancer and mesothelioma, respectively, with promising early results. The converse approach to delivery of such EMT-suppressive miRNAs is the therapeutic suppression of EMT-TFs. Extensive laboratory studies support the therapeutic potential of EMT-TF inhibition to increase treatment response across a range of therapeutic scenarios, including hormonal therapy for breast cancer[192], chemotherapy for ovarian malignancy[193] and targeted therapy for lung cancer[194]. Although EMT-TFs were previously considered undruggable due to a lack of functional domains targetable by small molecules, the rise of methods such as CRISPR-Cas9 and TALEN technology, which enable targeting of specific genes through genomic and epigenomic manipulation, has provided the tools to allow exploration of EMT-TF modulation; however, there are still considerable hurdles before clinical use is possible[195–197]. Modulation of upstream pathways that control or trigger EMT is also a compelling approach[17,198,199], particularly as many pathways have either established agents or agents under development. In this regard, a number of systems have been successfully targeted to improve therapeutic activity using such concepts, including through targeting of WNT[200], nuclear factor-κB[192] and ERK1/2 signalling[201]. Further consideration should be given to trials evaluating such therapeutic combinations with standard therapies across the treatment spectrum to harness the EMT-reversing properties of these approaches.

Beyond targeting tumour cells directly, there is mounting evidence that non-malignant stromal cells and non-cellular elements might drive EMT, implying that their manipulation could reverse the process. Indeed, evidence of this EMT-driving effect exists for tumour-associated macrophages[202,203], neutrophils[204], fibroblasts[205], dense collagen[206] and fibronectin[207]. As a proof-of-principal for the potential of targeting stromal factors, suppression of the M2 macrophage phenotype reversed EMT in tumour cells and resensitized lung tumours to paclitaxel in a human lung cancer cell line A549 xenograft mouse model[208]. In a co-culture model, antibody-mediated neutralization of neutrophil-produced IL-17a reversed neutrophil-induced EMT changes in gastric cancer cells and inhibited their migration and invasion[204]. Depletion of CAFs using a fibroblast-targeted immunotoxin inhibited TGFβ signalling, reduced tumour progression and enhanced chemosensitivity in the syngeneic 4T1 breast cancer mouse model, which is consistent with inhibition of EMT, although the process was not directly assessed[209]. Furthermore, knockout of the gene encoding collagen VIII was reported to reduce EMT in renal cells in the context of experimental diabetic nephropathy[210], although such studies have not been performed in cancer models.

Although many cytotoxic agents induce EMT, inhibitors of microtubule assembly, including eribulin and the vinca alkaloids, might exert the reverse effect (FiG. 2). Indeed, eribulin has been shown to reverse EMT in cancer cell lines and animal models[211] and vinca alkaloids were reported to inhibit EMT-associated growth in lung cancer in vitro and in vivo[212]. Similarly, vinflunine reversed EMT and induced cell death in bladder cancer cell lines[213]. Hinting at this potential, phase III clinical trials of eribulin in both breast cancer and liposarcoma have demonstrated modest initial responses yet, unexpectedly, substantial

survival benefits, which has led to the licencing of the drug for both indications[214,215]. Beyond single-agent use, these drugs could also be considered as part of combination regimens with chemotherapeutic agents known to drive EMT in the course of developing resistance.

The concept of transdifferentiation is related to the process of EMT reversal. During MET, malignant cells redifferentiate to reassume more of the characteristics of their epithelial cell of origin. During the transdifferentiation process, cells that have gained malignant potential through dedifferentiation to a mesenchymal form are driven along an alternative pathway involving differentiation to assume the properties of a different cell type. In a seminal example of this process, breast cancer cells that had undergone EMT were driven to differentiate into adipocytes using a combination of the peroxisome proliferator-activated receptor-γ (PPARγ) agonist rosiglitazone combined with the MEK inhibitor trametinib, losing the ability to invade and metastasize in the process[216].

Regarding the therapeutic targeting of cells in the mesenchymal state, a high-throughput molecular screening approach was performed for agents with activity against mesenchymally shifted cancer lines, which identified three such agents — salinomycin, etoposide and abamectin[217]. Preclinical confirmation of ZEB1 downregulation and, consequently, chemosensitization of lymphoma cells in response to salinomycin treatment[218], have led to the planning for clinical development of this agent. Following initial observations of improved survival rates among patients with breast cancer and comorbid diabetes who are being managed with metformin, this oral hypoglycaemic agent was subsequently found to reverse EMT and increase the sensitivity of chemoresistant breast cancer cells to chemotherapy[219]. To our knowledge, no initiatives have specifically targeted the partially transitioned cellular form, which might require further work to molecularly characterize key features of this hybrid state. However, the approach has conceptual merit.

Finally, arguably the most novel therapeutic concept is that of fixing cells at a given position on the epithelial-mesenchymal axis to prevent access to the range of states that might be required to facilitate different stages of the metastatic cascade. A mechanistic example of this process is the transcription factor OVOL, which interacts with the miR-200-ZEB1 regulatory axis to expand the hybrid epithelial-mesenchymal compartment and can influence the epithelial-mesenchymal axis bidirectionally[220]. If interventions could be developed to stabilize the hybrid state and restrict EMP, this approach could hypothetically prevent multiple elements of cancer progression.

Conclusions and outstanding questions

Controversies regarding the contribution of EMT to tumour progression stem primarily from the failure of some lineage-tracing experiments to identify cells in the metastatic site that have undergone EMT and, subsequently, MET[34,101,147]. By contrast, a plethora of data — which are still accruing — have incriminated EMP in the metastatic process[35,71,133,144,145,148,149]. As described in this Perspectives article, there are possible technical reasons for these contrasting findings related to the sensitivity of detection of relevant epithelial-mesenchymal hybrid cells, which are also increasingly implicated in

metastatic potential, whereby the lineage-tagging systems might not be strong enough to provide a readout of subtle changes in gene expression in an individual EMT target gene; accordingly, we await the development of more sensitive systems. At the same time, there are several studies that illustrate the arrival and establishment of carcinoma cells with epithelial traits at metastatic sites, which seems to confer maximal metastatic potential, at least in some scenarios, and might be necessary for macrometastatic outgrowth[33,36,98,125,131]. The degree to which these outwardly epithelial cells partially access elements of the mesenchymal phenotype during dissemination and whether an accentuating ability to shift between states would further increase the dissemination potential, remains to be determined.

In addition to these controversies, there are complexities arising from different interpretations of the EMT concept in various contexts. From primary tumour initiation to progression to metastasis formation, discrete forms of the EMT programme might be involved, such that considering the process as a single unvarying entity could be misleading. EMT is an enormous programme, with approximately one-third of the transcriptome regulated, and with both duplicity and diversity in the many mechanisms that regulate it, such that attempts to subclassify EMT forms might also illuminate current controversies between different experimental models. For example, EMT arising in tumours as a result of two distinct stimuli, EGF activation or hypoxia, was found to involve different effector pathways and behaviours and, importantly, resulted in differential changes in sensitivities to therapies[221]. Due to these substantial contextual differences, it is important to specify the EMT programme according to different tumour types, stages, local stressors and previous exposure to therapies.

EMT events in primary tumours are relatively rare. Nevertheless, certain EMT-related features could be involved in most tumour developmental steps. EMP bestows, on tumour cells, the ability to adapt to the changing microenvironment during secondary tumour formation and in the context of therapeutic treatment (FIGS 1, 2), and might provide a unique opportunity for the development of new targeted therapies. Current therapeutic concepts mostly consider either the epithelial or mesenchymal phenotype, trying to target either state. This approach could limit the application of such therapies due to the ability of tumour cells to transition along the EMP axis to alternative non-sensitive states, and leads to additional considerations of assessing potential responders and therapeutic timing issues. However, targeting EMP to control the transitioning phase of tumour cells could restrict this route of escape and is, therefore, a promising direction for future research (FIG. 3). The detrimental impact of therapy-induced EMP adds breadth to the potential advantages that success in this endeavour could bring, adding reversal of resistance of established therapies to the achievable benefits. A combined therapy that blocks this avenue might, therefore, enhance the efficacy of conventional chemotherapies. Considering existing candidates, the EMT-reversal attributes of eribulin have been suggested to underpin its greater survival benefits in head-to-head comparisons with other therapies, relative to those promoting EMT[222]. Expanding this concept, our own broader assessment of the survival benefits associated with a wide range of therapies confirms the disproportionately larger survival benefits for those therapies known to reverse EMT[39].

In addition to targeting EMT processes in tumour cells directly, the manipulation of stromal cells implicated in contributing to EMT, such as CAFs or tumour-associated macrophages[98,209,223], might represent another strategy. As they are not malignant cells, these targets have the advantage that clonal selection and acquisition of new mutations are not routes of resistance. Finally, having entered the era of efficacious immunotherapy, where vast resources are currently being committed to finding ways to extend the benefits of treatment from a modest proportion of patients to many, extensive evidence that EMT ties to immune suppression justifies efforts to expand our understanding of the effect of manipulating EMT in this context.

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Box 1 |

EMT lineage-tracing strategies in genetically engineered mouse models

Different strategies have been developed to trace epithelial-mesenchymal transition (EMT) in vivo, addressing three major challenges, including distinguishing post-EMT tumour cells from tumour-associated stromal cells that also exhibit mesenchymal phenotypes, the identification of post-EMT tumour cells that might have reversed back to an epithelial phenotype, and reporting on the dynamic EMT status or partial EMT phenotype of tumour cells. Multiple genetically engineered mouse models have been applied in these EMT lineage-tracing strategies (as depicted below), each of which have a number of advantages and disadvantages.

Tumour-specific fluorescent markers

Tumour-specific promoter (Tum Pro)-driven Cre is crossed with ubiquitous promoter (Ubi Pro)-driven fluorescence reporter alleles[33,40,49,148,188,224].

Marked genes

- *Pdx1*-Cre (pancreatic ductal adenocarcinoma)[149]
- MMTV-Cre (breast cancer)[33,49]
- *Wap*-Cre (breast cancer)[49]
- *Krt14*-CreER (skin cancer)[224]
- *Lgr5*-CreER (skin cancer)[40,224]

Advantages

- Distinguishes EMT tumour cells from tumour-associated stromal cells that exhibit mesenchymal phenotypes

Disadvantages

- Evaluation of EMT status relies on the analysis of EMT marker expression
- *Lgr5*-Cre might also be activated in non-epithelial lineages in the *Lgr5*-CreER skin cancer model

Mesenchymal-specific fluorescent markers

Mesenchymal-specific promoter (Mes Pro)-driven Cre is crossed with a Ubi Pro-driven fluorescent marker switch model[34,49,96,223].

Marked genes

- *Fsp1*-Cre (breast cancer and pancreatic ductal adenocarcinoma)[34,49,97,225]
- aSMA-Cre (pancreatic ductal adenocarcinoma)[225]
- Vimentin-CreER (breast cancer)[34]

Advantages

- Distinguishes tumour cells that have undergone a cycle of EMT and mesenchymal-epithelial transition (MET) from those that persist with an epithelial phenotype

Disadvantages

- Only one EMT-related marker can be analysed at a time
- Irreversible; can only trace one-time activation of the EMT programme
- Tracks only the transcriptional regulation of the mesenchymal marker
- Relies on sufficiently strong expression of the mesenchymal marker

Fluorescence-tagged EMT marker genes

EMT marker gene promoter (EMT Pro; for example, *Cdh1, Snail1* or *Snail2*) is used to directly drive fluorescence expression[226,227].

Marked genes

- E-cadherin-CFP (pancreatic ductal adenocarcinoma)[227]
- Snail-GFP (breast cancer)[226]
- Slug-GFP (breast cancer)[226]

Advantages

- Reports dynamic changes in EMT status and the ‘partial EMT’ phenotype during tumour progression

Disadvantages

- Additional markers are required to distinguish tumour cells from neighbouring stromal cells
- Post-EMT events could be reported as negative if cells reversed back to epithelial phenotype

Clonal lineage-tracing

A lineage-specific promoter (Lin Pro) is used to induce the random recombination of fluorescent markers (such as *Krt5* and *Elf5* for the basal and luminal lineages, respectively)[50].

Marked genes

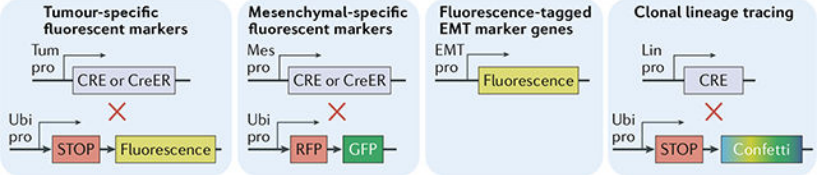
- *Krt5*-rtTA/*TetO*-cre (breast cancer, basal origin)[50]
- *Elf5-rtTA*/*TetO*-cre (breast cancer, luminal origin)[50]

Advantages

- Reports the origin and clonal evolution of tumour cells

Disadvantages

- Elucidation of EMT status requires additional analyses of EMT marker expression
- Tracing limit on the number of tumour cell colonies



Box 2 |

Alternatives to intrinsic EMP in metastasis

Several alternatives to the role of intrinsic epithelial-mesenchymal plasticity (EMP) in metastasis have been proposed, including phenotypic, genotypic, heterotypic and intercellular cooperativity as well as other putative mechanisms that enable mesenchymally shifted tumour cells and/or other cell types to facilitate epithelial tumour cell dissemination.

Phenotypic and genotypic cooperativity

Cooperativity between epithelial and mesenchymal tumour cell populations has been reported to drive metastasis in multiple cancer models.

In a hamster model of oral carcinoma established from epithelial and/or mesenchymal tumour cells, tumours from the mesenchymal variant were enriched for local invasion, yet tumours established from the mesenchymal or epithelial variants alone could not form metastases[228]. Co-inoculation of both epithelial and mesenchymal variants, either subcutaneously or intravenously, led to metastasis of the epithelial variant.

In a PC-3 prostate cancer tumour xenograft mouse model, co-inoculation of mesenchymal PC-3 variants promoted metastasis of epithelial PC-3 variants[126].

In a xenograft mouse model of breast cancer, co-injection of epithelial-mesenchymal transition (EMT)-induced HMLER cells (through enforced expression of EMT-activating transcription factor genes such as *TWIST1, SNAI1* or *SIX1)* promoted the metastasis of non-EMT-induced HMLER cells[229].

In the mouse mammary tumour virus promoter-driven polyoma middle T oncogene (MMTV-PyMT) model[33], the combined presence of both epithelial and mesenchymal populations was observed in the vasculature following orthotopic inoculation of E-cadherin^{low} cells isolated from a mammary tumour. Although both epithelial and mesenchymal variants reached the lungs, E-cadherin^{low} cells converted to an E-cadherin^{hgh} phenotype within one to two cell divisions. Whether the more migratory and/or invasive E-cadherin^{low} cells cooperated to help E-cadherin^{hgh} cells reach the lungs is unclear.

Heterotypic cooperativity

Heterotypic cooperativity arising from clonal heterogeneity has also been reported to drive metastasis[230,231], challenging the original dogma that metastases arise from a single tumour cell. Evidence of widespread polyclonality has been observed in metastases[109,232,233], consistent with polyclonality in earlier stages (such as invasion and circulating tumour cell clusters). These studies do not distinguish epithelial-mesenchymal heterogeneity — rather, they represent heterogeneity in general.

Intercellular cooperativity

Cues provided by cancer-associated fibroblasts, haematopoietic cells and other cells of the pre-metastatic niche have been reported to drive metastasis[139], and might also promote the survival of epithelial cells during metastasis formation.

Alternative mechanisms

Potential mechanisms invoked to enable mesenchymally shifted tumour cells and/or other cell types to assist the spread of epithelial tumour cells include cluster dissemination[109], heterotypic signalling[126,228–231] and transfer of components of the mesenchymal transcriptome or proteome to epithelial cells (via exosomes, extracellular secretion or other processes)[104,229,234,235].

Box 3 |

Immunotherapy and eMP

Phenotypic shifts along epithelial–mesenchymal axis, particularly epithelial–mesenchymal transition (EMT), have been reported to influence the likely success of cancer immunotherapy strategies via altered expression of immunomodulatory factors, an unsurprising finding given the wide range of cellular features that are influenced by epithelial–mesenchymal plasticity (EMP).

eMT increases immune checkpoint expression

- Co-culture with mesenchymal oral squamous cell carcinoma cells increased programmed cell death 1 ligand 1 (PD-L1) expression on dendritic cells and tumour-associated macrophages, which was blocked by inhibiting antigen presentation; EMT reduced PD-L1 expression on tumour cells[236].
- Melanomas lacking ESRP1 had increased levels of EMT, programmed cell death 1 (PD-1), PD-L1, PD-L2, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and immune cytolytic activity[237].
- In mouse and human melanoma cells, EMT induction through SNAIL1 expression caused progression by enhancing both invasion and immunosuppression; regulatory T cells were induced and dendritic cells were inhibited, with increased CTLA-4 and PD-1 expression[238].
- Activation of ZEB1 or repression of microRNA miR-200 induced EMT in human and murine non-small-cell lung cancer cell lines, leading to upregulation of PD-L1 (a miR-200 target), with consequent $CD8^+$ lymphocyte suppression[239].
- In oesophageal squamous cell carcinoma cell lines, inhibition of the known EMT suppressor GSK3β induced EMT and upregulated PD-L1 expression; EMT-positive tumour cells could induce T cell apoptosis[240].

Immune checkpoint expression induces eMT

- PD-L1 expression promoted EMT in oesophageal cancer cells, and PD-1-mediated stimulation of PD-L1 enhanced this effect[241].
- PD-L1 strongly activated EMT programmes in glioblastoma cells via RAS signalling, resulting in increased proliferation and invasion[242].
- In nasopharyngeal carcinoma, PD-L1 expression activated EMT via PI3K–AKT signalling, leading to invasion and metastasis in vitro and in vivo, respectively[243].
- EMT correlated with increased PD-L1 expression, decreased $CD8^+$ T cell numbers and increased $FOXP3^+$ cell numbers in pancreatic cancer. Both EMT and PD-L1 expression could be stimulated by interferon-γ in pancreatic cancer cell lines[244].

- In osteosarcoma, PD-L2 signalling promoted invasion and migration in vitro and in vivo, respectively, via EMT induction[245].

Associations between eMT and immune checkpoint expression

- In lung adenocarcinoma tissues, EMT positively correlated with tumour cell PD-L1 expression and levels of $CD8^+PD\text{-}1^+$ lymphocyte infiltrates[246].
- In lung adenocarcinoma data sets, EMT was co-incident with increased expression of PD-L1, PD-L2, PD-1, TIM3, B7-H3, B and T lymphocyte associated (BTLA) and CTLA-4, along with $CD4^+FOXP3^+$ regulatory T cells[247].
- EMT was associated with increased PD-1 and PD-L1 expression in tumour cells, increased $FOXP3^+$ lymphocytic infiltrates, and poor prognosis in hepatocellular carcinoma cases[248].
- Fibroblast growth factor 2 (FGF2)-overexpressing bladder tumours in the The Cancer Genome Atlas showed increased frequency of EMT and increased expression levels of CTLA-4, PD-1 and PD-L1[249].
- In hepatocellular carcinoma, PD-L1 expression in high-risk tumours was closely associated with EMT marker expression and poor survival[250].
- PD-L1 expression correlated with an EMT phenotype and poor outcomes in lung adenocarcinoma but not in lung squamous cell carcinoma[251].
- In thymic carcinoma tissues, PD-L1 expression correlated with EMT, and levels of EMT, PD-L1 and transforming growth factor-β (TGFβ) were all increased by immunotherapy[252].
- Although epidermal growth factor receptor (EGFR) stimulation in salivary adenoid cystic carcinoma cells induced both EMT and PD-L1 expression, EMT was suppressible by *SNAI1* knockdown and PD-L1 by *MYC* knockdown, but not the reverse[253].

EMT modulates immunotherapy efficacy

- In melanomas with high EMT (as indicated by low *ESRP1* expression), immune checkpoint molecule expression was increased with a trend towards improved overall survival[237].
- Immune suppression was seen after induction of EMT in mouse melanoma cells by SNAIL1 overexpression, and these cells were not responsive to activated dendritic cell immunotherapy in mice; resistance was due to low immune reactivity, as well as resistance to cytolysis, and could be reversed using *SNAI1*-specific siRNA[238].
- In patients with metastatic urothelial cancers and high T cell infiltrates treated with nivolumab, a high mRNA-based EMT signature in tumours corresponded with lower response rates and shorter survival; these tumours might correspond to the immune-excluded phenotype[254].

Immune surveillance and metastasis

Only a very small minority of disseminating cells survive and metastasise, the bulk of which are cleared by the immune system. The increase in EMT in circulating cells could potentially assist dissemination by allowing escape from immune-based killing in the circulation.

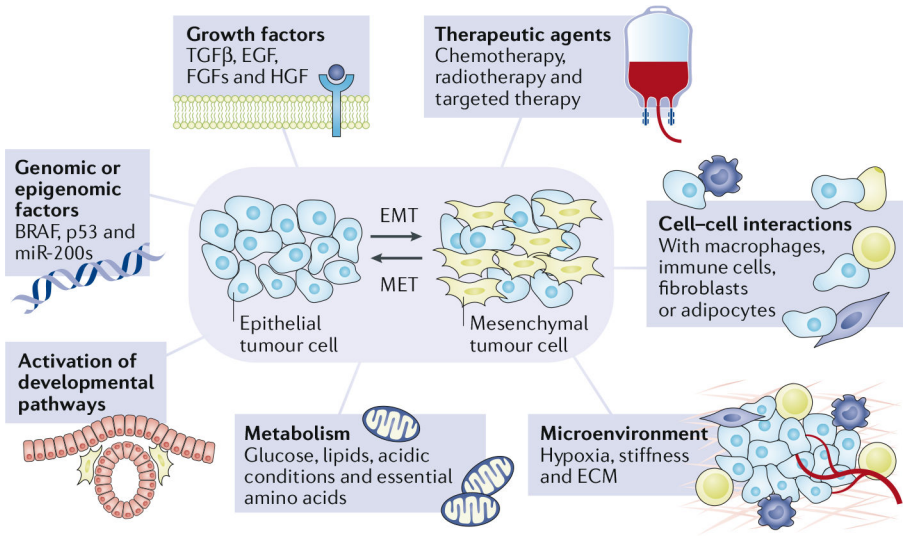


Fig. 1 |. Types of EMP stimuli.

Many categories of factors are known to induce epithelial-mesenchymal transition (EMT), the inhibition or removal of which might promote the reverse process of mesenchymal-epithelial transition (MET). Microenvironmental cells (for example, tumour-associated macrophages, hypoxic adipocytes and other inflammatory cells) produce EMT-promoting factors such as transforming growth factor-β (TGFβ), epidermal growth factor (EGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), tumour necrosis factor, IL-6 (REF.[225]) and leptin[85,86]. Through activation of the nuclear factor-κB (NF-κB) pathway, these cells invoke crosstalk with EMT-activating transcription factors[255,256]. Alterations of the metabolic microenvironment induced by rapid primary tumour growth might also induce EMT[87–90], and hypoxia, through the action of hypoxia-inducible factor 1α (HIF1α), can directly drive the expression of EMT-activating transcription factors in various tumour types[51,82,84]. Matrix stiffness has also been shown to stimulate EMT[91,92,257]. Therapeutic agents have primarily been shown to promote EMT in association with drug resistance[43–47,52,70,165–175], although some are associated with MET, and these cause significant improvements in disease-free survival and overall survival[165]. Developmental pathways, which might be activated by genomic and/or epigenomic regulators, have also been implicated in epithelial-mesenchymal plasticity (EMP)[1,2]. ECM, extracellular matrix.

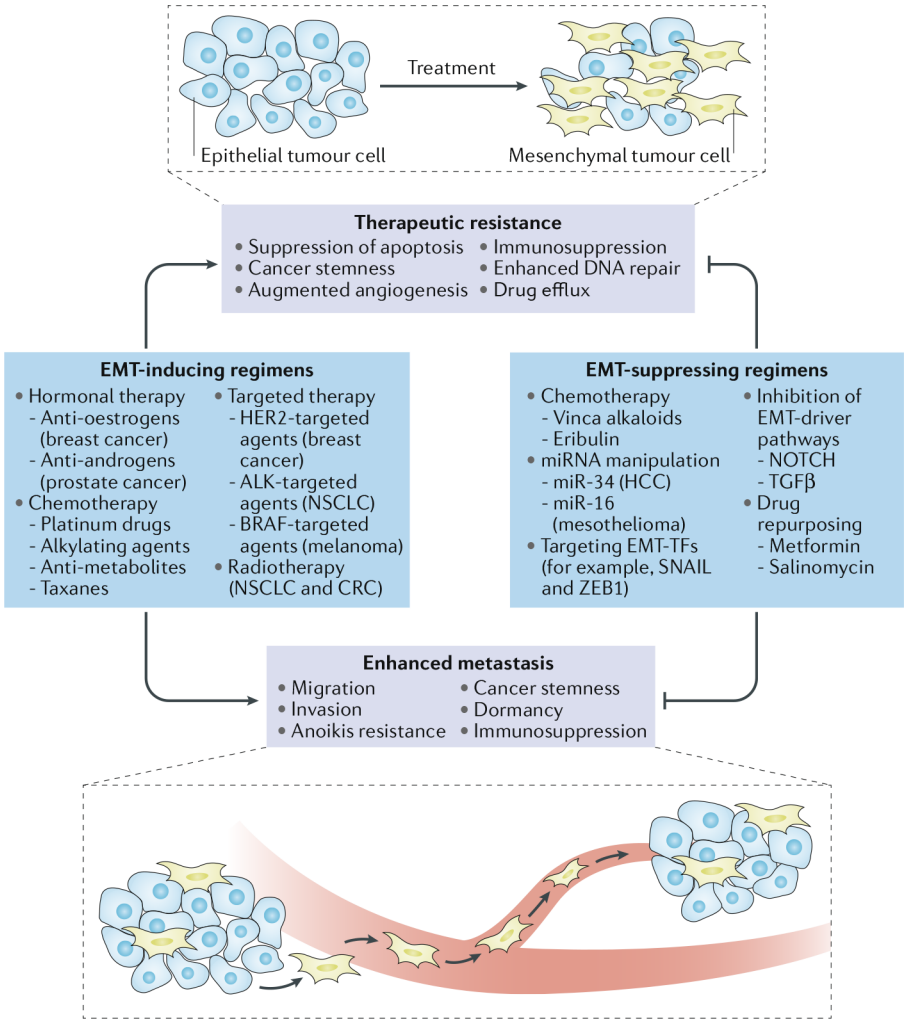


Fig. 2 |. Therapy-induced EMT and potential EMT-suppressing regimens.

Epithelial-mesenchymal transition (EMT) induced by a spectrum of therapeutic agents and modalities has consequences for treatment resistance and/or metastasis across many cancer types. Potential mechanisms through which EMT might contribute to therapeutic resistance include reducing the sensitivity to proapoptotic signals (reviewed in REF.[258]), acquisition of stemness features[66–69], stimulation of angiogenesis[259], upregulating expression of immune checkpoint molecules[41] and increasing immune suppression by altering the balance of infiltrating immune cells[185,237], reducing DNA damage in concert with enhancing DNA repair[260,261], and upregulating expression of export pumps that actively eliminate cytotoxic drugs from cells[262–264]. Furthermore, cells undergoing therapy-induced EMT might proliferate at decreased rates and, therefore, have decreased sensitivity to chemotherapeutic agents[34,36,51], and migration of cancer cells to a microenvironment that is poorly accessible to drugs (for example, through the blood-brain barrier) might reduce the impact of therapeutic interventions. For example, in human epidermal growth factor receptor 2 (HER2)-positive breast cancer, continued treatment with HER-targeted therapy (for example, trastuzumab) can trigger EMT[265] and relapse can occur in the brain alone despite an ongoing good response elsewhere in the body[266]. Treatment with existing or novel

therapies (for example, eribulin or vinca alkaloids)[211,212] might minimize or revert EMT-associated features and, therefore, reduce the emergence of therapeutic resistance. CRC, colorectal cancer; EMT-TF, EMT-activating transcription factor; HCC, hepatocellular carcinoma; miRNA, microRNA; NSCLC, non-small-cell lung cancer; TGFβ, transforming growth factor-β.

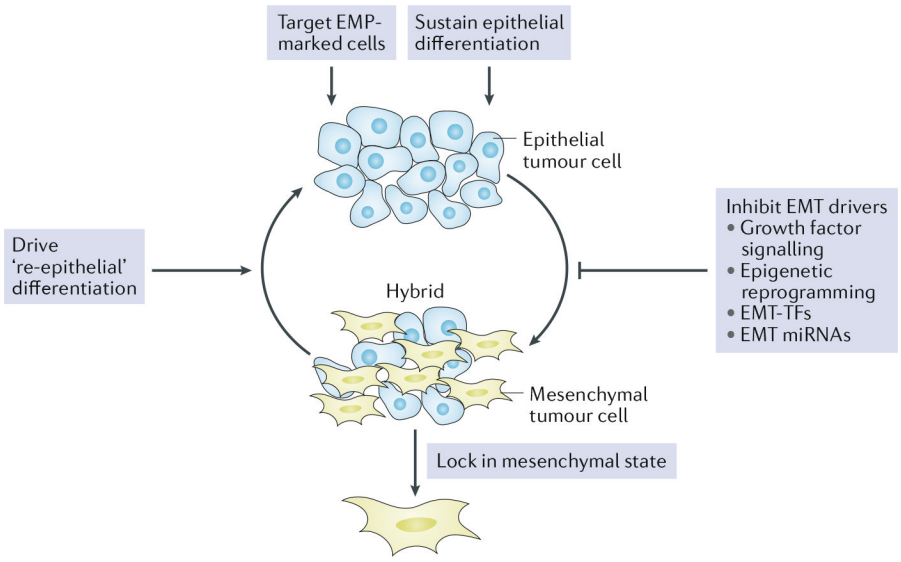


Fig. 3 |. Therapeutic opportunities to address EMP.

The various states produced during epitheLial— mesenchymal plasticity (EMP) provide a number of opportunities to influence cancer progression via different strategies. The supporting rationale for this concept is the notion that targeting or preventing epithelial-mesenchymal transition (EMT) to sustain epithelial differentiation or selectively targeting the mesenchymal state by inhibiting EMT-driving targets might be most effective as part of adjuvant therapy for early-stage cancers, where the invasive outgrowth of cells from established deposits as well as quiescent, stem-like, mesenchymally shifted cells that have disseminated could be addressed therapeutically. Late-stage bulky metastases, for which we have very limited effective treatment options, might respond best to therapies that reverse or prevent mesenchymal-epithelial transition (MET) and drive re-epithelial differentiation or selectively target the uniquely EMP-marked epithelial tumour cells that emerge following the EMT-MET cycle process. Further options across the treatment continuum might be to develop therapies that specifically target the unique aspects of the hybrid epithelial-mesenchymal phenotype or fix cells in the mesenchymal state to deprive cancers of the progression mechanisms associated with plasticity. EMT-TF, EMT-activating transcription factor; miRNA, microRNA.

Table 1 |

Detection of EMP markers in clinical samples

Modality or assay type	Comments	Refs
Protein markers		
Single protein markers	Can be detected in tumour parenchyma; difficult to be certain of cell origin	267,268
Multiplexed immunohistochemistry or immunofluorescence assays with epithelial and mesenchymal markers	More precise than single protein markers; excludes cells undergoing full transition	206,269
RNA markers		
RT-PCR (using epithelial, mesenchymal or E^M markers)	Issues with cellular contamination if not single-cell RT-PCR	6,12
RNA-Seq (using signatures, single-cell barcoding RNA-Seq or EMT scores)	Suitable for data mining; issues with cellular contamination if not single-cell RNA-Seq; single-cell barcoding RNA-Seq can provide a more comprehensive picture but on a population basis; use of EMT scores (for example, Tan et al.[65] and Foroutan et al.[157]) can inform on EMP-specific markers (for example, E^M)	51,65,157,270,271
Spatial RNA analysis	Provides morphological context; molecular crowding within cells can lead to spatial overlap of fluorescence signals	Not available
RNA-ISH or RNAscope (CTCScope)	Limited number of probes; for example, the combination of 7 epithelial and 3 mesenchymal probes has been reported by Yu et al.[100]; ensures viability of CTCs[272]	100,272
NanoString DSP	More probes possible compared with other RNA-based approaches due to barcoding	273

CTCs, circulating tumour cells; DSP, digital spatial profiling; E^M, epithelial cells that have been through an EMT–MET cycle; EMP, epithelial–mesenchymal plasticity; EMT, epithelial–mesenchymal transition; RNA-ISH, RNA in situ hybridization; RNA-Seq, RNA sequencing; RT-PCR, reverse transcription polymerase chain reaction.