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## EMT, CSCs, and drug resistance: the mechanistic link and clinical implications

Tsukasa Shibue<sup>1,2</sup> and Robert A. Weinberg<sup>1,2,3</sup>

<sup>1</sup>Whitehead Institute for Biomedical Research, 455 Main Street, Cambridge, Massachusetts 02142, USA

<sup>2</sup>Ludwig Center for Molecular Oncology, Massachusetts Institute of Technology

<sup>3</sup>Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

### Abstract

The success of anticancer therapy is usually limited by the development of drug resistance. Such acquired resistance is driven, in part, by intratumoural heterogeneity — that is, the phenotypic diversity of cancer cells co-inhabiting a single tumour mass. The introduction of the cancer stem cell (CSC) concept, which posits the presence of minor subpopulations of CSCs that are uniquely capable of seeding new tumours, has provided a framework for understanding one dimension of intratumoural heterogeneity. This concept, taken together with the identification of the epithelial-to-mesenchymal transition (EMT) programme as a critical regulator of the CSC phenotype, offers an opportunity to investigate the nature of intratumoural heterogeneity and a possible mechanistic basis for anticancer drug resistance. In fact, accumulating evidence indicates that conventional therapies often fail to eradicate carcinoma cells that have entered the CSC state via activation of the EMT programme, thereby permitting CSC-mediated clinical relapse. In this Review, we summarize our current understanding of the link between the EMT programme and the CSC state, and also discuss how this knowledge can contribute to improvements in clinical practice.

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The fact that most malignant tumours are composed of multiple phenotypically distinct subpopulations of neoplastic cells has been established by >60 years of research<sup>1-4</sup>. Nowadays, the phenotypic diversity of neoplastic cells within a tumour is considered a major driver of the development of resistance to therapy and, for this reason, is attracting increasing interest<sup>5</sup>. The biological heterogeneity arising through both genetic and non-genetic mechanisms contributes to the phenotypic differences between the distinct subpopulations of cancer cells residing within individual tumours<sup>6,7</sup>. Advances in genome-sequencing technologies are enabling rapid progress in delineating the types of genetic changes that underlie the phenotypic alteration and diversification of cancer cells<sup>8,9</sup>. In parallel, the ability of cancer cells to frequently interconvert between multiple alternative phenotypic states, doing so without concomitant mutational changes in their genomes, is

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Correspondence to R.A.W. [weinberg@wi.mit.edu](mailto:weinberg@wi.mit.edu).

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increasingly recognized; however, the nature of the non-genetic — that is, epigenetic — regulatory mechanisms that contribute substantially to the behaviours of cancer cells and the tumours that they form remains largely unexplored, revealing a critical need to uncover these mechanisms<sup>10,11</sup>.

The introduction of cancer stem cell (CSC) concept, which postulates the presence of minor populations of CSCs that are uniquely capable of seeding new tumours, has provided new insights into how epigenetic regulatory mechanisms can contribute to the phenotypic diversity of distinct subpopulations of cancer cells within a tumour<sup>12,13</sup>. This concept is based on the notion that various phenotypically distinct cancer cells residing within the same tumour mass are organized in a hierarchy, resembling the stem-cell hierarchy of the corresponding non-neoplastic tissue. Indeed, the functional parallels between CSCs and non-neoplastic stem cells are considered to be extensive, including the unique ability to entirely regenerate complex neoplastic and non-neoplastic tissues, respectively, under appropriate conditions<sup>14</sup>. Accordingly, in tumours, the epigenetically defined state of CSCs should, in principle, enable the tumour cells to self-renew in order to generate new CSCs, and to spawn progeny that differentiate into less-tumorigenic and non-self-renewing offspring — that is, the non-CSCs that are thought, in most cases, to form the bulk of the tumour. Implicit in this paradigm is the important hypothesis that continued tumour growth, metastasis formation, and recurrence after therapy can largely be attributed to the relatively rare subpopulations of CSCs within individual tumours<sup>15,16</sup>. Notably, discrete subpopulations of CSCs have now been identified in many types of human cancer, demonstrating the wide applicability of the CSC concept<sup>17</sup>.

The penetration of the CSC concept has caused some to argue that the often limited efficacy of conventional anticancer therapies is attributable to the targeting of the bulk population of non-CSCs within individual tumours, while not eliminating the rare subpopulation of CSCs<sup>18-20</sup>. Indeed, accumulating evidence has shown that CSCs are more resistant than non-CSCs to various types of conventional therapies<sup>21-23</sup>. Moreover, given their tumour-initiating capacity, the surviving CSCs are qualified to serve as the precursors of new tumour masses, ultimately leading to clinical relapse. These observations highlight the critical importance of understanding the epigenetic mechanisms that distinguish CSCs from the bulk population of non-CSCs; insights into these mechanisms hold the promise of yielding novel therapeutic strategies designed to eradicate CSCs, with the possible benefit of achieving extended, if not permanent, clinical remissions.

The epigenetic changes that account for the phenotypic differences between CSCs and non-CSCs are only beginning to be uncovered. In the case of carcinomas, the difference between CSCs and non-CSCs is likely to be attributable largely to the cell-biological programme termed epithelial-to-mesenchymal transition (EMT)<sup>24,25</sup>. This programme imparts heritable phenotypic changes to carcinoma cells through epigenetic modifications without introducing new genetic alterations. Upon activation of the programme, carcinoma cells lose many of their epithelial characteristics, including the presence of epithelial cell junctions and apical-basal polarity, and instead acquire mesenchymal attributes, such as an elongated, fibroblast-like morphology as well as an increased capacity for migration and invasion<sup>26</sup> (FIG. 1). In a number of carcinomas, only neoplastic cells within the CSC-enriched subpopulation exhibit

aspects of EMT-programme activation<sup>27-30</sup>. Of note, in certain experimental models of carcinoma, forced induction of an EMT programme in epithelial tumour cells substantially increases their capacity for tumour initiation<sup>27,31,32</sup>. In addition, EMT-programme activation confers on these tumour cells resistance to many types of therapeutic agents — another important attribute of CSCs<sup>33,34</sup>.

Perhaps ironically, we still possess relatively few insights into why these two clinically important cell-biological programmes — the CSC phenotype and EMT — are so intimately interconnected. Herein, we summarize our current understanding regarding the causes and effects of EMT-programme activation in carcinoma cells, with a particular focus on how this programme is linked to the CSC state. We also discuss the opportunities and challenges in targeting the EMT programme and, by extension, CSCs as a strategy for carcinoma treatment.

## Principles of the EMT programme

The roles of EMT and the reverse process, mesenchymal-to-epithelial transition (MET), were initially studied in the context of embryonic morphogenesis<sup>35,36</sup>. The findings revealed that during gastrulation in amniotes, the epiblast cells in a specific area of the primitive ectoderm — the first embryonic epithelial tissue — undergo EMT, thereby differentiating into primary mesenchyme cells, and ingress from the ectodermal layer, ultimately giving rise to mesodermal and endodermal cell layers. As a further example, neural crest cells within the dorsal neural tube — composed of neuroepithelial cells — also undergo EMT, migrate to various sites in the developing embryo, and differentiate into a variety of distinct cell types, including melanocytes, peripheral neurons, glial cells, and connective-tissue cells. By contrast, during the embryonic development of a kidney, cells in the metanephric mesenchyme undergo MET in order to form the renal epithelium and, ultimately, generate nephrons<sup>37</sup>. These examples comprise only a few of the instances in which epithelial–mesenchymal interconversions have key roles in embryogenesis.

In addition, the EMT programme plays a central part in various pathological processes, including wound healing, tissue fibrosis, and carcinoma progression<sup>38,39</sup>. In all of these processes, activation of the EMT programme elicits changes in multiple fundamental aspects of cellular physiology that include: alterations in the cytoskeletal organization and associated changes in cell morphology from squamous, cuboidal, or columnar shapes to spindle-like, elongated forms (FIG. 1); dissolution of epithelial cell–cell junctions, including tight, adherens, and gap junctions; loss of apical–basal polarity and concomitant gain of front–rear polarity; acquisition of motility, as well as an ability to degrade and reorganize the extracellular matrix (ECM), enabling cell invasion; and reconfiguration of the expression patterns of at least 400 distinct genes<sup>40</sup>. Of note, both non-neoplastic and neoplastic epithelial cells can activate the multifaceted EMT programme to varying extents, often acquiring many, but not all, of the traits associated with the ‘canonical’ programme.

Various extracellular stimuli and corresponding intracellular signalling pathways have been implicated in the induction of EMT. The central role of TGF $\beta$ –SMAD signalling in multiple different models of EMT has been well established<sup>41,42</sup>. In addition, other signalling

pathways, including the canonical or noncanonical Wnt, growth factor–receptor tyrosine kinase, and ECM–integrin signalling pathways, acting in various combinations, also seem to contribute critically to the induction of EMT in a variety of tissue contexts<sup>43</sup>. Importantly, the EMT process operating in carcinoma cells *in vivo* is often, and perhaps invariably, triggered by specific signals released by stromal cells into the tumour microenvironment, some of which are also involved in choreographing various inflammatory and hypoxic responses<sup>44</sup>.

In both non-neoplastic and neoplastic cells, these diverse signal-transduction pathways converge on the activation of a relatively small group of transcription factors that proceed to orchestrate the gene-expression changes associated with EMT<sup>45</sup>. These transcription factors, often referred to collectively as ‘EMT-inducing transcription factors’ (EMT-TFs), can typically be classified into one of three different protein families — namely, the Snail (including Snail and Slug; also known as SNAI1 and SNAI2, respectively), ZEB (including ZEB1 and ZEB2), and basic helix–loop–helix (including TWIST1, TWIST2, and TCF3) families<sup>45</sup>. In certain contexts, however, other transcription factors, such as PRRX1, YAP1/TAZ, and SOX4, also have critical roles in EMT. These master regulators of EMT programmes govern the transcription of EMT-associated genes via promoter activation or repression, often achieved through modification of chromatin structure<sup>45</sup>. The end results are suppression, to various extents, of genes associated with the epithelial phenotype, such as those encoding E-cadherin and cytokeratins, and upregulation of genes associated with the mesenchymal-cell phenotype, including those encoding N-cadherin, fibronectin, and vimentin<sup>45</sup>.

The EMT-TFs often act cooperatively to regulate the expression of common target genes and, in addition, frequently control the expression of one another<sup>39,45</sup>. For example, Snail is an upstream regulator that induces the expression of multiple other EMT-TFs, including SLUG, ZEB1, and TWIST1. On the other hand, TCF3 seems to function as a downstream effector whose expression is induced by various other EMT-TFs, such as Snail, Slug, and ZEB1 (REFS 40,46). Owing to these reciprocal interactions, experimental expression of one EMT-TF usually results in upregulation of certain other EMT-TFs, ultimately inducing, in many cases, the full spectrum of EMT-associated changes. Such coordinated, multifaceted shifts in cell phenotype can obscure the functional differences between the various individual EMT-TFs, which seem to be specialized for orchestrating distinct subprogrammes of the extensive canonical EMT programme. Moreover, the controls regulating the experimental expression of EMT-TFs rarely recapitulate those operating physiologically, resulting in phenotypic responses in cells that are not observed, for example, in spontaneously arising tumours. These disparities raise concerns regarding the interpretability of observations derived from experimental overexpression of an individual EMT-TF.

In addition to being regulated by signalling pathways, the expression and functions of EMT-TFs are controlled by other upstream regulatory mechanisms. In particular, studies have highlighted the important roles of noncoding microRNAs (miRNAs), which suppress the expression of specific proteins through a combination of mRNA destabilization and translational repression<sup>47</sup>. Among the best-characterized examples of miRNAs that regulate the EMT programme are members of the miR-200 family, which downregulate the

expression of ZEB1 and ZEB2 proteins; intriguingly, the expression of miR-200 miRNAs is repressed, reciprocally, by ZEB1 and ZEB2, establishing a double-negative feedback loop<sup>48,49</sup>. Similarly, the expression of Snail is attenuated by members of the miR-34 family, which, in turn, is suppressed by Snail, forming another double-negative regulatory feedback loop between EMT-TFs and miRNAs<sup>50,51</sup>.

The expression levels of several EMT-TFs are also regulated at the level of post-translational protein stability: Snail is phosphorylated by GSK-3 $\beta$  at two consecutive serine-rich motifs, which targets this EMT-TF for ubiquitylation and subsequent proteasomal degradation<sup>52</sup>. By contrast, TWIST1 can be phosphorylated at Ser68 by ERK1/2, p38, and JNK, which protects this EMT-TF from ubiquitin-mediated degradation<sup>53</sup>. In addition, the Siah family of RING finger ubiquitin E3 ligases has a critical role in mediating the ubiquitylation and thus the subsequent proteasomal degradation of ZEB1 (REF. 54). Hence, EMT-TFs are controlled at multiple levels and by diverse upstream regulatory mechanisms, which cooperatively regulate activation of the EMT programme.

EMTs are known to have essential roles in embryogenesis, and the various EMT-TFs are specialized to orchestrate distinct steps of this process<sup>55</sup>. The associations of particular EMT-TFs with distinct embryonic phases of development have been demonstrated most elegantly through gene-targeting studies in mice. Mice deficient in the *Snai1* gene (encoding Snail) displayed the most severe phenotype observed to date in such studies: the embryos of these mice develop a morphologically abnormal mesodermal layer. More specifically, the cells within this layer fail to undergo EMT and retain their epithelial properties, resulting in the death of embryos at around embryonic day 8.5 (E8.5)<sup>56</sup>. *Twist1*-deficient and *Zeb2*-deficient mouse embryos both exhibit failure of neural tube closure and die at around E11.5 and E9.5, respectively<sup>57,58</sup>. Mice deficient for *Snai2* (encoding Slug), *Zeb1*, *Twist2*, or *Tcf3* can survive to term, but have various severe developmental defects<sup>59-63</sup>. Hence, different EMT-TFs enable distinct processes in embryonic morphogenesis, indicating that they control different forms of the EMT programme.

While the mechanisms underlying EMT-programme activation are becoming increasingly clear, those responsible for the induction of the reverse process — MET — remain enigmatic. The prevailing hypothesis is that MET results simply from the deactivation of a previously active EMT programme, caused by reduced or fully absent expression of EMT-TFs<sup>64,65</sup>; however, evidence also supports the presence of specific mechanisms that selectively activate the MET programme via pathways that do not directly involve the well-characterized EMT-TFs. For example, the reprogramming of mouse fibroblasts into pluripotent stem cells involves MET-programme activation that is mediated, in part, by the induction of epithelial gene expression by the transcription factor KLF4 (REF. 66). Moreover, during kidney development in mice, Wnt9b secreted by the ureteric epithelium triggers the secretion of two other signalling proteins, Wnt4 and Fgf8, from pre-tubular aggregates; the latter two factors then contribute critically to the transition of these initially mesenchymal aggregates into the renal epithelium, in part, through the activation of the *Lhx1* transcription factor<sup>67</sup>.

## EMT during carcinoma progression

In contrast to the general agreement on the contribution of EMTs to embryonic development, the involvement of this programme in carcinoma progression has been a matter of debate until very recently<sup>68</sup>. This controversy was largely attributable to scepticism raised by pathologists, who had not seen clear evidence of EMT in human carcinoma samples frequently enough to assert the wide involvement of the EMT programme in the pathogenesis of many cancer types<sup>69</sup>.

Indeed, the occurrence of a ‘complete EMT’, involving the loss of all epithelial traits and concomitant gain of the full spectrum of mesenchymal attributes (as seen in certain EMT processes during embryogenesis), is rare in human carcinomas. The exception to this rule derives from carcinosarcoma, a rare cancer type consisting of distinct epithelial (carcinomatous) and mesenchymal (sarcomatous) compartments that are derived from a common precursor-cell population<sup>70</sup>. The fact that the EMT programme in human carcinomas is usually activated only partially has probably obscured the detection of EMT markers in clinical carcinoma samples. The identification of EMT-programme components in islands of carcinoma cells has also been complicated by the presence of nearby stromal cells, which naturally display a diverse array of EMT-associated mesenchymal markers, confounding any clear association of EMT programmes specifically with the carcinoma cells.

Nonetheless, a rapidly growing number of studies are now adding to a large corpus of evidence for the activation of the EMT programme in, and its contributions to, the pathogenesis of human carcinomas. For more than 20 years, loss of expression of the adhesion protein E-cadherin in carcinoma cells has been recognized to be associated with metastasis and a poor prognosis<sup>71,72</sup>. More direct evidence of the relationship between the EMT programme and carcinoma progression was presented in the early 2000s, when the role of Snail as a driver of carcinoma progression was demonstrated. Specifically, expression of Snail in carcinoma cells was found to induce EMT in part via direct transcriptional repression of the gene encoding E-cadherin (*CDH1*)<sup>73</sup>. Moreover, Snail expression was detected in both invasive carcinoma cell lines and tumour specimens from patients with breast cancer, and the latter was found to correlate closely with histological dedifferentiation and the development of metastases<sup>74</sup>. A few years later, Twist1 was shown to be critical to the metastasis of mouse mammary carcinomas<sup>75</sup>. Results of a number of sub-sequent studies demonstrated correlations between the presence of EMT markers in many carcinoma types and poor patient prognoses, including, but not limited to, those with prostate, lung, pancreatic, liver, colorectal, or bladder carcinomas<sup>76-81</sup>.

The incomplete nature of the EMT programmes activated in human carcinomas — usually referred to as ‘partial EMT’ — could be inferred from early observations that loss of E-cadherin expression, a hallmark of invasiveness in carcinomas, is usually partial<sup>71,72</sup>. More recently, an RNA *in situ* hybridization analysis of circulating tumour cells (CTCs) from women with breast cancer revealed the presence of cancer cells that concomitantly express epithelial and mesenchymal transcripts, and thus exhibit partial EMT activation<sup>82</sup>. Remarkably, these partial EMT cells were observed in samples from patients harbouring any



of the three major histological subtypes of invasive breast cancer (ER/PR-positive, HER2-positive, or triple-negative), but not in non-neoplastic breast tissues. This partial activation of the EMT programme results in extensive phenotypic diversity of the carcinoma cells within individual tumours.

In addition to being activated partially, the EMT programme in human carcinomas is often activated reversibly, permitting the cancer cells to revert via MET programmes to epithelial states during the course of carcinoma progression (FIG. 2). Thus, in many different types of carcinomas, cancer cells at the invasive front of the primary tumour frequently exhibit signs of EMT activation, including the reduced expression of E-cadherin, while cells that follow behind usually display many epithelial traits and maintain extensive cell–cell adhesions<sup>83,84</sup>. This observation, together with the dynamic interconversions between the ‘leaders’ and the ‘followers’ that have been shown to occur during the process of carcinoma invasion<sup>85</sup>, implies a plasticity of cell phenotypes and thus a reversibility of EMT programmes. Stated differently, carcinoma cells can flexibly activate EMT and MET programmes in the course of carcinoma invasion.

The behaviour of disseminated carcinoma cells that seed metastatic colonies is of great interest: founders of metastatic colonies, which are likely to have undergone an EMT before leaving the primary tumour, usually produce progeny at distant sites that regain their epithelial traits<sup>86,87</sup> (FIG. 2). Moreover, studies in mouse models of breast or skin cancer have demonstrated that activation of the EMT programme in the primary tumour is crucial for the dissemination of tumour cells to the lungs, whereas the disseminated cells must subsequently undergo MET in order to efficiently form macroscopic metastases<sup>88,89</sup>. Gene-expression profiling of single cells from patient-derived xenograft (PDX) models of metastatic breast cancer has demonstrated that, before the formation of macroscopic metastases, disseminated tumour cells display stem-like transcriptional signatures, including the activation of the EMT programme; however, those in macroscopic colonies lose signs of EMT-programme activation and instead exhibit luminal-like, epithelial differentiation signatures<sup>90</sup>. Together, these observations indicate that the formation of macroscopic metastases usually involves inactivation of a previously active EMT programme in carcinoma cells.

How reversion of the EMT programme contributes mechanistically to the process of metastatic colonization remains to be resolved. In addition, restoration of the entire suite of epithelial traits is not necessary for the outgrowth of all types of metastases. Thus, in most cases of invasive lobular carcinoma of the breast, E-cadherin expression in the carcinoma cells is completely lost owing to *CDH1* mutation<sup>91</sup>; however, these cancer cells frequently form macroscopic metastases in various organs. To summarize, although both clinical and pre-clinical observations clearly demonstrate that the EMT programme is regulated in a dynamic and reversible fashion during the progression of carcinomas, the functional significance of this reversibility awaits future study.

The timing of EMT-programme activation during the progression of carcinoma has been studied extensively using genetically-engineered mouse models, in which carcinoma cells were genetically labelled and thus could be distinguished from surrounding non-neoplastic cells. The findings revealed, intriguingly, that the EMT programme can be activated at pre-

malignant stages of carcinoma development: in a breast cancer model driven by transgenic expression of a constitutively active mutant form of rat HER2, a high level of Twist1 expression in mammary epithelial cells was detected at the stage of atypical ductal hyperplasia (ADH)<sup>92</sup>. Similarly, in another mouse breast cancer model, with transgenic expression of polyomavirus middle-T antigen (PyMT), Snail expression in mammary epithelial cells was observed at the ADH stage<sup>93</sup>. Moreover, in a pancreatic cancer model based on elimination of p53 expression and concomitant expression of the constitutively active G12D-mutated KRas, the pancreatic epithelial cells began to express Zeb1 at the stage of pancreatic intraepithelial neoplasia<sup>94</sup>. These results are consistent with clinical observations that systemic dissemination of tumour cells can start early in the disease course of certain types of carcinomas, including those of the breast and prostate<sup>95</sup>.

Together, these findings suggest that activation of an EMT programme and the resulting dissemination of pre-neoplastic cells can occur early in the multistep process of carcinoma progression; however, several key questions regarding the activation of the EMT programme early in carcinoma progression remain unanswered. For instance, the types of genetic alterations that predispose pre-malignant cells to activate previously silent EMT programmes are unknown. Moreover, whether and how disseminated preneoplastic cells with EMT-programme activation contribute to the eventual formation of clinically detectable metastases is unclear<sup>96</sup>.

The activation of EMT programmes in carcinomas seems to be regulated not only temporally, but also spatially. As discussed, the dedifferentiation of epithelial tumour cells is particularly evident at the invasive fronts of carcinomas<sup>83,84</sup>, and data from genetically driven mouse models of carcinoma pathogenesis have reinforced the notion that the EMT programme is activated specifically at the invasive fronts of a tumour. In a breast cancer model with transgenic expression of the oncogenic transcription factor Myc, epithelium-derived cells with mesenchymal-like morphological features (spindle shape, elongated nuclei, and poorly defined cell borders) were enriched at the interface between tumour and stromal tissues<sup>97</sup>. Moreover, in a pancreatic cancer model, elevated expression of Zeb1 in the carcinoma cells was found to be evident particularly at the periphery of the tumour tissue, where abundant inflammatory cells accumulated<sup>94</sup>. The commonalities in both spatial and temporal patterns of EMT, observed among multiple carcinoma types in both humans and mice, indicate that EMT-programme activation is a highly regulated process, which is strongly influenced by contextual signals that individual carcinoma cells experience at various locations within a tumour.

## Microenvironmental regulation of EMT

A large body of evidence points to the critical contributions made by resident and recruited non-neoplastic cell types within the tumour stroma to the regulation of carcinoma cell behaviours. These stromal cells can be categorized into three major classes: angiogenic vascular cells, infiltrating immune cells, and carcinoma-associated fibroblasts (CAFs)<sup>98</sup>.



## The role of carcinoma-associated fibroblasts

CAFs have been reported to arise from multiple sources, including tissue-resident fibroblasts, mesenchymal stem cells, stellate cells, and endothelial cells; a distinct profile of secreted signalling molecules distinguishes CAFs from non-neoplastic tissue fibroblasts. At a biological level, CAFs display an 'activated' phenotype, reminiscent of that observed in cells actively participating in wound healing. Moreover, the classification of CAFs can substantially overlap with myofibroblasts that are abundant in the stroma of many high-grade carcinomas as well as in areas of active inflammation<sup>99,100</sup>. Although the precise molecular definition of this apparently heterogeneous collection of cells remains a matter of debate, a growing body of evidence supports the important role of CAFs in carcinoma progression<sup>101</sup>. Indeed, CAFs are particularly well characterized with respect to their involvement in EMT-programme activation in carcinoma cells (FIG. 3a).

Intriguingly, the admixture of CAFs isolated from prostate cancer tissue enabled immortalized, but not transformed, human benign prostatic hyperplasia epithelial (BPH-1) cells to form tumours in athymic nude mice, whereas fibroblasts isolated from the corresponding non-neoplastic prostatic tissue did not<sup>102</sup>. Some of the resulting tumour cells had reduced E-cadherin expression and elevated vimentin expression, indicating the induction of EMT in BPH-1 cells by the co-inoculated CAFs, which might at least partially explain the increased tumour-initiating potential observed<sup>102,103</sup>.

Several subsequent reports identified potential mechanisms by which CAFs activate the EMT programme in nearby carcinoma cells. CAFs from human prostate cancers induced EMT in co-cultured PC-3 human prostate carcinoma cells via secretion of matrix metalloproteinases (MMPs)<sup>104</sup>. In addition, the conditioned culture medium of CAFs isolated from invasive breast tumours induces EMT-like changes in multiple human breast cancer cell lines; this effect has largely been attributed to TGF $\beta$  secreted at high levels by CAFs<sup>105</sup>. Finally, CAFs have been shown to be enriched at the invasive fronts of multiple types of human carcinoma<sup>106-108</sup>, suggesting that signals arising from bidirectional interactions between carcinoma cell and CAFs account for the spatial patterns of EMT activation described previously.

## The influence of inflammation

Several conditions specific to the tumour microenvironment seem to contribute to the activation of the EMT programme in carcinoma cells. Among these conditions, tissue inflammation comprises a pervasive and critical component of the tumour microenvironment. Diverse lines of evidence suggest that inflammation contributes to at least two stages of carcinoma pathogenesis. First, chronically inflamed tissues are fertile sites for the initiation of carcinomas. Thereafter, established carcinomas can undergo histopathological progression to states of high-grade malignancy, such as those associated with expression of EMT programmes, under the influence of inflammation-associated signals (FIG. 3b). Indeed, in this second stage involving established malignant tumours, inflammatory cells and soluble mediators of inflammation, such as cytokines and chemokines, are abundant within the tumour microenvironment. Thus, the RAS oncoproteins, products of the most frequently mutated oncogenes in human cancers, have

been demonstrated to induce cancer cells to produce proinflammatory cytokines and chemokines, such as IL-8 and the CXCL1 (REFS 109,110). The resulting RAS-induced inflammatory tumour microenvironment contributes reciprocally to disease progression through several distinct mechanisms, including EMT-programme activation<sup>109,111</sup>.

Studies have highlighted the contribution of specific cytokines released by tumour-associated inflammatory cells to the activation of the EMT programme. TNF $\alpha$  treatment resulted in NF- $\kappa$ B-dependent stabilization of the otherwise unstable Snail protein in multiple human cancer cell lines, thereby activating EMT, which in turn increased tumour-cell invasiveness and dissemination to distant sites in preclinical models<sup>112</sup>. Other prototypic proinflammatory cytokines, including IL-1 $\beta$  and IL-6, have also been demonstrated to activate the EMT programme in carcinoma cells<sup>113,114</sup>. These observations provide a mechanistic link between tissue inflammation, heterotypic signalling, and activation of EMT programmes in carcinoma cells.

The contributions of cellular components of inflammation, such as macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs), to cancer progression have been studied extensively. Experiments in mice that lack expression of the key macrophage-lineage cytokine Csf1 and therefore lack functional macrophages have revealed that these cells are dispensable for the initiation and subsequent growth of PyMT-driven primary mammary carcinomas, but are critical to the formation of lung metastases from these tumours<sup>115</sup>. Moreover, close negative correlations between the abundance of tumour-associated macrophages (TAMs) and patient prognosis have been reported for multiple cancer types, including lung, breast, prostate, and ovarian cancers<sup>116</sup>.

Analyses of the mechanisms underlying promotion of carcinoma progression have demonstrated that TAMs, like CAFs, can contribute to the activation of EMT programmes in carcinoma cells. Thus, depletion of macrophages from mice bearing F9 teratocarcinoma allo-grafts results in the epithelial differentiation of tumour cells, thus indicating the critical role of macrophages in maintaining the mesenchymal properties of the tumour cells. This EMT effect is mediated, in part, by the secretion of TGF $\beta$  by macrophages<sup>117</sup>. Moreover, activated macrophages induce EMT in MCF7 human breast cancer cells following co-culture *in vitro* or co-implantation to form xenograft tumours *in vivo*; this effect is attributable, in part, to secretion of the chemokine CCL18 by macrophages<sup>118</sup>.

In addition, critical roles of neutrophils and MDSCs in the establishment, growth, and progression of various cancer types have been documented<sup>119,120</sup>. Intriguingly, such pro-tumour effects of these cells are also attributable, in part, to their capacity for activating EMT programmes within neighbouring cancer cells<sup>121,122</sup>. Hence, various secreted proteins and host cell types present in the inflammatory tumour microenvironment contribute, perhaps in a coordinated fashion, to the induction of EMT in carcinoma cells.

### The effects of hypoxia

Hypoxia, which is often present in poorly vascularized regions of carcinomas, can also contribute to activation of the EMT programme (FIG. 3c). A direct connection between HIF-1 — a central transcriptional regulator of cellular responses to hypoxia — and the EMT

of carcinoma cells has been demonstrated in the case of clear-cell renal-cell carcinoma (RCC), a disease in which frequent loss-of-function of the von Hippel-Lindau (VHL) tumour-suppressor protein results in the constitutive activation of HIF-1. In this context, HIF-1 represses the expression of E-cadherin indirectly by inducing the expression of ZEB1, ZEB2, and TCF3, thereby conferring mesenchymal attributes on the carcinoma cells<sup>123,124</sup>. In addition, multiple human carcinoma cell lines undergo EMT upon exposure to hypoxia *in vitro*, via a mechanism that is dependent, in part, on HIF-1 activation<sup>125-127</sup>. Notably, direct activation of the *TWIST1* promoter by HIF-1 has also been demonstrated<sup>127</sup>. Hence, multiple distinct components of the tumour microenvironment and physiological conditions contribute to the activation of the EMT programme in carcinoma cells via diverse mechanisms. This complexity creates a serious challenge to the formulation of therapeutic strategies designed to effectively target the EMT programme.

### The relationship between EMT and CSCs

The EMT programme has been portrayed for two decades as a major mechanism underlying carcinoma-cell invasion from the primary tumour mass into the surrounding stroma — the very first step of the complex series of events that lead to the formation of metastases<sup>128</sup>. This invasion step is followed by the entrance of cancer cells into the circulation (intravasation); transportation in the blood to the capillary beds of distant organs, where they become physically trapped; escape from capillary lumina to the tissue parenchyma (extravasation); and, ultimately, the outgrowth of these micrometastases into macroscopic metastases in the foreign tissue (colonization). These various processes, together, are often termed the ‘invasion-metastasis cascade’ (REF. 129). The scope of biological processes driven by the EMT programme, however, has been expanded far beyond the initial step of carcinoma-cell invasion, owing to the observation that experimental activation of EMT, via either the over-expression of Twist1 or Snail EMT-TFs, or treatment with TGF $\beta$ , confers many of the properties of CSCs on otherwise epithelial carcinoma cells<sup>27,31</sup>. These include CSC-specific cell-surface marker expression — that is, elevated and reduced expression of CD44 and CD24 glycoproteins, respectively, an increased ability to form spheres in a suspension culture, and an enhanced ability to seed tumours in mice. These observations, together with those from a series of follow-up studies, have now demonstrated that activation of the EMT programme in neoplastic cells is closely linked to entrance into the CSC state, across a wide variety of human carcinoma types, revealing multifaceted effects of the EMT programme in driving the malignant phenotype of carcinoma cells<sup>130</sup> — and bringing this developmental programme to the heart of cancer biology (TABLE 1).

The association between the EMT programme and the CSC state indicates that the activation of this programme in non-CSCs enables their conversion into CSCs. In addition, CSCs, by definition, should have the ability to differentiate into non-CSCs, triggered presumably by the activation of the MET programme. These assumptions, taken together with the aforementioned reversibility of EMT and MET in carcinoma cells, suggest the involvement of substantial phenotypic plasticity, enabling these cells to transition back-and-forth between the CSC and non-CSC states.

Indeed, tumour cells in the non-CSC subpopulation can spontaneously undergo EMT-like changes and acquire CSC-like cell-surface marker expression, as well as an enhanced capacity to seed tumours in mice<sup>131</sup>. A similar dedifferentiation of non-CSCs to CSCs has also been observed in multiple experimental systems<sup>132,133</sup>, indicating that the unidirectional stem-cell hierarchy operating in non-neoplastic tissues — whereby only stem cells can repopulate the entire cell population of a differentiated, functional adult tissue, but not the reverse — does not apply to neoplastic tissues. Moreover, given their observed plasticity, carcinoma cells might interconvert between multiple alternative states characterized by different degrees of mesenchymal features, one of which comprises CSCs. Such behaviour implies that EMT-induced non-CSC-to-CSC conversion should be considered simply as a phenotypic shift within a range of fluctuation, rather than true dedifferentiation. This plasticity of the CSC programme also points to the importance of further characterizing the differences between the stem-cell programmes of non-neoplastic tissues and those of neoplastic tissues, as this understanding will be critical to designing therapeutic strategies to selectively target CSCs without damaging non-neoplastic tissue stem cells.

### **EMT, CSCs, and metastasis**

Given their unique property of being able to seed new tumours, CSCs — but not non-CSCs — should be capable of founding metastatic colonies after disseminating to foreign tissues<sup>134</sup>. This logic, when taken together with the aforementioned EMT–CSC connection, indicates that EMT-programme activation is necessary not only for the physical dissemination of carcinoma cells to distant tissues, but also for entrance into the CSC state that enables the disseminated cells to serve as founders of metastatic colonies and, thus, to succeed in colonizing foreign tissues. Indeed, activation of the EMT programme in carcinoma cells results in the formation of larger numbers of macroscopic lung metastases in mice, even when these cells are injected directly into the venous circulation, thereby bypassing the earlier steps of the invasion-metastasis cascade<sup>127,135,136</sup>. Moreover, as mentioned previously, disseminated carcinoma cells often exhibit signs of activation of the EMT programme before the formation of macrometastases<sup>90,137</sup>. These observations reinforce the notion that EMT-programme activation enables carcinoma cells to serve as founders of metastatic colonies.

More recently, while attempting to elucidate the molecular interactions of carcinoma cells infiltrating the lung parenchyma with ECM proteins, we and others discovered that CSC-like cells with high metastasis-seeding abilities develop mature adhesion plaques — macromolecular structures composed of clustered integrins — far more abundantly than do the related, poorly metastasis-seeding and thus non-CSC-like cells<sup>138,139</sup>. These mature adhesion plaques contribute functionally to the differing powers of metastasis seeding between these cell types, by enabling potent activation of focal adhesion kinase (FAK), the activity of which is critical to the initial proliferation of CSC-like cells within the lung tissue<sup>140,141</sup>. Further exploration revealed the mechanistic basis for the differing capabilities of CSC-like cells that have activated an EMT programme and non-CSC-like cells that have not done so to form mature adhesion plaques: this difference is attributable, in part, to the distinct potentials of these cell types, following their extravasation, to extend actin-rich,

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integrin-presenting protrusions<sup>141</sup>. These protrusions, which we named ‘filopodium-like protrusions’ (FLPs), are abundantly formed only by the EMT-activated cells; following formation of integrin–ECM connections, the FLPs contribute to the development of mature adhesion plaques, thereby enabling the potent activation of FAK signalling<sup>141</sup>. Intriguingly, in multiple carcinoma cell types, experimental activation of the EMT programme results in a remarkable increase in the ability of these cells to extend FLPs<sup>141</sup>. This ability, in turn, permits these EMT-activated cells to proliferate following extravasation, ultimately enabling them to seed metastases efficiently. This chain of causality provides one explanation for the link between the EMT programme and the enhanced metastasis-seeding ability of carcinoma cells (FIG. 4a).

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In truth, this depiction might oversimplify the complexity of the final, post-extravasation steps of metastasis formation: the EMT programme might enable physical dissemination of carcinoma cells to distant tissues and also confer on them an ability to initiate metastatic growths, but is unlikely to solve an additional and critical obstacle confronting these cells. This hurdle involves the adaptation of cells originating in one tissue to the microenvironment of a second, unfamiliar tissue. Most carcinoma cells are hypothesized to be poorly adapted to grow in foreign tissues, at least initially, and must contrive adaptive programmes in order to continue to expand. These adaptations are likely to be accompanied by, as mentioned, the reversion of the EMT programme (or activation of a MET programme) in carcinoma cells, and indeed robustly growing metastatic colonies are usually composed mostly of carcinoma cells that have regained their epithelial attributes<sup>86,87</sup>.

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In contrast to the prevailing view that the EMT programme contributes critically to cancer metastasis, two reports have presented evidence of the contrary. In one of these studies, a lineage-tracing approach was used to follow the fate of tumour cells that have expressed the EMT markers, S100A4 (also known as fibroblast-specific protein 1) and vimentin, and demonstrated that lung metastases in the PyMT-driven mouse mammary carcinoma model are formed predominantly by cells that have not previously expressed these markers<sup>142</sup>. In the other study, pancreas-specific deletion of either *Snail* or *Twist1* was found to have no substantial effect on metastasis formation in a mouse model of pancreatic ductal adenocarcinoma<sup>143</sup>. These studies have raised questions regarding the ubiquitous requirement for EMT in cancer metastases, although the observations made therein need to be interpreted with caution. As discussed previously, the EMT programme is usually activated only partially in carcinoma cells, resulting in aggressive cells that might not be fully mesenchymal and, thus, might not express the two markers specific for highly mesenchymal cells, S100A4 and vimentin. Moreover, given their extensively overlapping functions, the effects of genetic elimination of a single EMT-TF are likely to be masked by the compensatory upregulation of others.

### **The molecular mechanism behind the EMT–CSC link**

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The connection between EMT and CSC status is now supported by substantial experimental evidence; however, the mechanistic link between these two phenotypes remains largely elusive. One of the potential explanations for this link is that alterations in the spectrum of secreted proteins (that is, the ‘secretome’) of carcinoma cells induced by the EMT

programme establish autocrine signalling loops, which in turn are essential for the induction and maintenance of stem-cell properties. Indeed, results of a study analysing the secretome of transformed HMLER human mammary epithelial cells have revealed that the activation of the EMT programme induces autocrine signalling loops that are known to contribute critically to the ‘stemness’ of non-neoplastic and neoplastic cells; these include the TGF $\beta$  – SMAD and Wnt– $\beta$ -catenin pathways<sup>144</sup> (FIG. 4b). Consistent with this notion, the blockade of these auto-crine signalling loops is sufficient to prevent the acquisition of CSC properties by the HMLER cells following experimental activation of the EMT programme.

The EMT programme also seems to contribute to CSC phenotype through its effects on intracellular signalling pathways. For example, Snail has been reported to reduce the expression of the tumour-suppressor protein p53 in carcinoma cells via formation of a Snail–histone deacetylase 1 (HDAC1)–p53 ternary complex and subsequent deacetylation of p53, thereby promoting its protea-somal degradation<sup>145</sup>. Importantly, the tumour-initiating ability of neoplastic cells in the PyMT-driven mouse mammary tumour model is diminished by deletion of *Snail*, and this ability can be restored through concomitant deletion of *Tp53* (encoding p53)<sup>145</sup>. Hence, the EMT programme seems to confer CSC phenotypes on carcinoma cells through effects on both the extracellular and intracellular signalling machinery.

The importance of the EMT programme in driving the CSC phenotype itself requires further investigation. Indeed, whether activation of the EMT programme is necessary and/or sufficient for the acquisition of the CSC phenotype across various carcinoma types remains to be determined. In breast cancer, CSCs are demonstrated to be present in two alternative phenotypic states, namely, epithelial-like and mesenchymal-like, and cells in either state are capable of seeding tumours in mice<sup>146,147</sup>. Moreover, although the activation of the EMT programme markedly increases the tumour-initiating ability of carcinoma cells in many model systems, excessive activation of this programme is found to be detrimental for tumour initiation (FIG. 1b). This finding explains why increasing attention is being placed on the ‘partial EMT’ state, which seems to be critical for the maximal tumorigenic activity of CSCs<sup>148</sup>. More specifically, in a model of claudin-low breast cancer, in which most carcinoma cells display a highly mesenchymal phenotype, a subpopulation of cells with relatively epithelial characteristics exhibited greater tumorigenic activity than the bulk population of the cells<sup>149</sup>. Together, these observations indicate that the contribution of the EMT programme to the CSC phenotype is variable, most likely depending on cell type and/or coexisting genetic/epigenetic abnormalities. This variability underscores the complexity of the genetic/epigenetic mechanisms driving the CSC phenotype in carcinoma cells, an understanding of which will likely be critical to the future development of novel therapies directed against the (partially) mesenchymal cells in human carcinomas, including their subpopulations of CSCs.

## EMT and therapeutic resistance

Soon after the introduction of the CSC concept, some researchers postulated that CSCs are more resistant than non-CSCs within the same tumours to elimination following conventional chemotherapy and radiotherapy<sup>12,150</sup>. This idea originated, at least in part,



from early observations indicating that non-neoplastic tissue stem cells exhibit higher levels of resistance to chemotherapeutic agents than that of the differentiated cell types within the same tissues<sup>151</sup>. This resistance has been attributed to various mechanisms, including: elevated expression of antiapoptotic proteins; increased levels of ATP-binding cassette (ABC) transporters, transmembrane protein transporters that are known to mediate drug efflux and, thus, to confer multidrug resistance on cancer cells<sup>152-155</sup>; and the slow proliferation rate of stem cells<sup>156</sup>.

More recently, technical advances in the identification and isolation of CSC-enriched subpopulations of cells from various tumours types have made assessment of the relative sensitivity of CSCs and non-CSCs to therapeutic agents possible. These analyses have revealed, as anticipated, that chemotherapy and/or radiotherapy successfully eliminate the bulk population of non-CSCs, while leaving behind considerable numbers of CSCs in multiple cancer types, in both preclinical and clinical samples<sup>21,157,158</sup>. In addition, the survival of CSCs following certain kinds of molecularly targeted treatment and immunotherapy is greater than that of non-CSCs. For instance, the CSC-enriched subpopulation of chronic myeloid leukaemia (CML) cells was found to be far more resistant to treatment with the BCR–ABL1 kinase inhibitor imatinib than was the CSC-deprived subpopulation of the CML cells from the same patients<sup>159</sup>. In addition, in a mouse model of prostate cancer, therapeutic vaccination using a cDNA library derived from non-neoplastic prostate tissue results in the initial regression of the tumour followed by the recurrence of a more aggressive tumour composed of tumour cells with a much higher tumour-initiating ability than those of the original tumour<sup>160,161</sup>.

Nonetheless, further understanding of the connection between CSCs and therapeutic resistance had been hampered by the lack of reliable markers to identify cell populations highly enriched with CSCs, and by an insufficient understanding of the mechanisms that govern the phenotype of CSCs. In fact, the sets of markers used to identify cancer cell populations enriched for CSCs are being continuously updated, leaving room for further improvement. These difficulties greatly complicate attempts to predict the therapeutic resistance of 'pure' populations of CSCs. Nonetheless, the identification of the EMT programme as a common regulator of the CSC phenotype across various carcinoma types has provided us with an experimental opportunity to investigate, at the molecular level, how CSCs and therapeutic resistance are linked via EMT programmes (FIG. 5; TABLE 2).

Analyses of the relationships between gene-expression profiles of tumour samples and the clinical responses of the patients from which they were obtained have resulted in the identification of a strong correlation between an EMT-associated gene-expression signature and treatment resistance. For instance, an analysis of responses to chemotherapy in a large cohort of patients with breast cancer revealed a close association between therapeutic resistance and increased expression of genes that are usually expressed primarily by the stromal cells; this transcriptional upregulation seems to be caused, in part, by activation of the EMT programme within carcinoma cells<sup>162</sup>. In addition, findings of a study designed to interrelate gene-expression profiles and responses to EGFR or PI3K inhibitors in clinical samples and in various cell lines derived from patients with non-small-cell lung carcinoma

(NSCLC) have also identified a 76-gene EMT signature as a robust predictor of clinical resistance to these agents<sup>163</sup>.

Collectively, these observations strongly support the notion that the EMT programme contributes critically to the development of resistance to various types of therapeutic agents in multiple cancer types; they also illustrate how the identification of the functional link between the EMT programme and the CSC phenotype has enabled us to understand at the molecular level the otherwise enigmatic phenomenon concerning the resistance of CSCs to multiple therapeutic modalities. Further study of the connections between the EMT programme, the CSC phenotype, and therapeutic resistance will certainly be required for the development of more effective treatment strategies to eradicate CSCs.

## Targeting the EMT programme for therapy

The development of treatment strategies to directly target the EMT programme is both reasonable and promising, given the emerging importance of this programme in the malignant progression of carcinomas, as well as the frequent acquisition of therapeutic resistance by carcinoma cells. At least three different approaches might be pursued: preventing the induction of EMT; selectively targeting cells that have active components of an EMT programme; and/or reversing the process of EMT by forcing more-mesenchymal carcinoma cells to revert to an epithelial state via activation of the MET programme.

### Prevention of EMT induction

EMTs can be prevented by interfering with the signalling processes that are critical to the activation and subsequent maintenance of EMT programmes. TGF $\beta$  signalling is among the best-characterized pathways involved in EMT induction; however, this cytokine has multifaceted effects on cancer cells and, therefore, attempts at the therapeutic inhibition of this pathway require caution. Specifically, although TGF $\beta$  potently promotes the progression of the tumours via mechanisms including the activation of the EMT programme and resulting invasion of carcinoma cells into the surrounding non-neoplastic tissue, it also negatively controls the initial stages of tumour formation through its antiproliferative effects<sup>164,165</sup>. Indeed, SKI and the SKI-like protein SnoN, endogenous inhibitors of TGF $\beta$  signalling, are known oncoproteins; increased expression of these proteins has been associated with the development of many types of human cancers, including melanoma and oesophageal cancer<sup>166</sup>. Hence, inhibition of TGF $\beta$  signalling might actually be counterproductive by promoting carcinoma-cell proliferation in the early stages of the disease. In addition, TGF $\beta$  signalling has critical roles in controlling the behaviours of stromal and immune cells, making it difficult to specifically target the process of TGF $\beta$ -mediated induction of EMT in the cancer cells. Nevertheless, inhibitors targeting this signalling pathway are currently undergoing both preclinical and clinical testing<sup>167,168</sup>; owing to the complex and multifaceted effects of TGF $\beta$  signalling, appropriate stratification of the patients and optimization of drug-administration protocols will be critical to the effective use of these inhibitors.

Other potential therapeutic targets for preventing the induction of EMT include hepatocyte growth factor (HGF)–HGF receptor (HGFR; a proto-oncogene also known, confusingly, as

MET) signalling. Besides promoting cell proliferation, HGF–HGFR signalling also activates the EMT programme and induces cell motility<sup>169,170</sup>. This pathway contributes critically to cancer pathogenesis, as exemplified by its frequent activation, often by point mutation or amplification of the HGFR gene (*MET*), in many cancer types. Thus, HGF–HGFR signalling is a promising target of anticancer therapy, and substantial efforts have been made to develop antagonists of this pathway. In particular, a number of small-molecule inhibitors of the enzymatic activity of the HGFR tyrosine-kinase domain are being evaluated in clinical trials. Of note, two multikinase inhibitors with activity against this protein are already approved by the FDA: crizotinib, for the treatment of NSCLC; and cabozantinib, for the treatment of medullary thyroid cancer and RCC<sup>171</sup>. However, the extent to which HGFR inhibition contributes to the antitumour activity of these agents in these indications remains to be clarified.

In principle, blockade of EMT could also be accomplished by targeting the components of the tumour microenvironment that contribute to the activation of the EMT programme in carcinoma cells. Possible treatment strategies along this line include suppression of tumour-associated inflammation and hypoxia, as well as targeting of specific types of host cells, such as CAFs or TAMs<sup>172,173</sup>. As discussed, however, the manner in which each of these microenvironmental components triggers activation of the EMT programme in nearby carcinoma cells remains under investigation; a more detailed understanding of these mechanisms will be necessary in order to manipulate the microenvironmental factors in ways that will block EMT-programme activation.

### Selective targeting of cells that have undergone EMT

Substantial efforts have been made to selectively target cancer cells that have undergone EMT, starting with attempts to therapeutically block the functions of classic EMT-associated markers. For instance, the natural compound withaferin A binds to, among other things, vimentin intermediate filaments, a standard marker of EMT, ultimately causing the degradation of these filaments<sup>174</sup>. Withaferin A treatment has been demonstrated to block the *in vitro* invasion and *in vivo* metastasis formation by breast cancer cells<sup>175</sup>, and is, therefore, an attractive target for further development as a therapeutic anticancer agent. In addition, treatment with monoclonal antibodies against the ectodomain of N-cadherin, another important marker of EMT, had the same effects on multiple prostate cancer cell lines<sup>176</sup>. The therapeutic index of both agents, however, remains to be examined in depth. Potential complications might plausibly arise from the fact that these mesenchymal markers are widely expressed by non-neoplastic mesenchymal cells, such as fibroblasts, resulting in on-target, off-tumour toxicities.

Defining and interfering with signalling pathways that contribute critically to the behaviours of carcinoma cells that have undergone EMT is another potential approach to anticancer therapy. One study along this line has identified AXL, a receptor tyrosine kinase activated by the GAS6 ligand, as a signalling protein whose expression and function are linked tightly with expression of an EMT signature<sup>163,177</sup>. The expression of AXL is strongly induced by the experimental activation of the EMT programme in the immortalized MCF10A human mammary epithelial cell line<sup>178</sup>. In addition, short hairpin RNA (shRNA)-mediated

knockdown of AXL expression attenuates *in vitro* invasion as well as *in vivo* tumour initiation and metastasis formation by multiple mesenchymal-like breast cancer cell lines<sup>178</sup>. Similarly, in the aforementioned study that identified a strong correlation between the EMT-associated gene-expression signature and resistance to EGFR or PI3K inhibitors in NSCLC, the expression of an EMT-associated signature was closely accompanied by the elevated expression of AXL and its ligand, GAS6. Intriguingly, the combination of the EGFR inhibitor erlotinib and the AXL inhibitor SGI-7079 successfully overcame the resistance of mesenchymal-like NSCLC cells to erlotinib treatment in a mouse xenograft model<sup>163</sup>. Together, these experimental observations identify AXL as a critical mediator of EMT-associated phenotypes in cancer cells, including CSC-like properties, which suggests a strong potential of this receptor as a therapeutic target for the selective elimination of CSCs with an active EMT programme. The first AXL-specific small-molecule inhibitor, BGB324, entered clinical trials in 2013 (REF. 179). An initial clinical study in NSCLC has demonstrated that BGB324 can be safely administered to patients and a proportion of patients achieve durable disease stabilization for at least 6 months<sup>180</sup>.

High-throughput screening approaches have been used to identify therapeutic vulnerabilities in carcinoma cells that have an active EMT programme and thus have entered a CSC state. For example, a chemical compound screen of cells from the immortalized HMLE human mammary epithelial cell line, with or without prior experimental activation of the EMT programme, resulted in the identification of the potassium ionophore salinomycin as an agent with cytotoxic activity specifically in cells that have undergone EMT<sup>181</sup>. Findings of an analogous screen for kinase inhibitors demonstrated that inhibitors targeting PKCa selectively eliminate HMLE cells with an active EMT programme<sup>182</sup>. In both cases, the clinical utility of these and similar agents for targeting CSCs with active EMT programmes requires extensive testing and validation.

### Reversing the process of EMT

From a therapeutic standpoint, reversing the process of EMT — inducing MET — would be beneficial, considering the critical contribution of the EMT programme to multiple malignant traits of cancer. By doing so, cancer cells in the CSC state with an active EMT programme will be forced to differentiate into non-CSCs and regain epithelial traits, thereby losing both enhanced tumorigenic activity and resistance to various therapeutic agents. This strategy is reminiscent of differentiation therapy used in patients with acute promyelocytic leukaemia (APL), whereby treatment with all-*trans* retinoic acid (ATRA) induces terminal differentiation of leukaemic promyelocytes, eventually leading to apoptotic death. ATRA is a component of the first-line treatment of APL and its use has dramatically improved the prognosis of patients with this disease<sup>183</sup>.

As reported in 2016, a screen for agents that reactivate the promoter of the E-cadherin gene (*CDHI*) in mesenchymal cells identified cholera toxin and forsko-lin, both of which increase intracellular levels of cyclic AMP and thereby enhance signalling through protein kinase A (PKA)<sup>184</sup>. Treatment of the spontaneously arising mesenchymal derivatives of HMLE cells with either of these two agents, or forced expression of a constitutively active PKA mutant, resulted in the induction of MET and associated reductions in invasiveness and sphere-

forming potential<sup>184</sup>. Interestingly, this MET was accompanied by sensitization of these cells to therapeutic agents that included doxorubicin, paclitaxel, proteasome inhibitors, and EGFR inhibitors<sup>184</sup>. Additional studies will be needed to assess the general applicability and clinical utility of these findings. In particular, given the aforementioned requirement for reversion of the EMT programme at the last step of the invasion–metastasis cascade, therapeutic activation of the MET programme might adversely promote metastatic colony formation. Thus, the timing and strategy of MET-programme activation will need to be determined carefully, as a prerequisite to the success of this therapeutic approach. Nonetheless, such ‘differentiation therapy’ is an attractive avenue for the development of novel anticancer drugs.

## Conclusions

Advances in basic and translational cancer biology have enabled us to understand, in unprecedented detail, the actions and the outcomes of cancer therapies. In particular, major conceptual advances, including the introduction of the CSC concept and elucidation of the connection between the EMT programme and the CSC state, have provided us with new insights into clinically significant problems, such as the development of resistance to therapy. Conventional therapeutics usually eliminate the bulk non-CSC population while sparing the minor subpopulations of CSCs, thereby leaving open the possibility of future local disease recurrence and/or the development of metastases<sup>17,185</sup>. Moreover, EMT seems to be a major strategy used by carcinoma cells to acquire a CSC phenotype, making this programme an attractive novel target for cancer therapy<sup>24</sup>. Indeed, therapeutic strategies based on this new knowledge are currently being designed and tested, and will probably result in improvements in cancer therapy in the near future<sup>186</sup>.

Nevertheless, the deduction that activation of the EMT programme in carcinoma cells generates CSCs has been challenged by more recent findings. Thus, while EMT-programme activation in the otherwise-epithelial carcinoma cells generally results in an enhanced potential for tumorigenesis, full activation of the entire EMT programme — that is, complete transition to a mesenchymal cell type — has been found to be detrimental to tumorigenic activity<sup>88,148,187</sup>. Moreover, under certain conditions, CSCs can undergo phenotypic drift, thus losing mesenchymal traits while maintaining their tumorigenic activity<sup>146,147</sup>. Such observations indicated that the EMT programme and the CSC phenotype are closely associated, but can actually be uncoupled from one another under certain conditions. Further investigation of the association and distinction between EMT and the CSC state will be required in order to exploit the EMT–CSC link for the improvement of therapeutic practice.

Another important challenge related to EMT–CSC connection derives from the phenotypic plasticity of non-CSCs. Unlike their counterparts in non-neoplastic tissues (that is, terminally differentiated cells), non-CSCs in the tumour tissue can spontaneously undergo EMT and dedifferentiate into new CSCs, thereby regenerating tumorigenic potential<sup>188</sup>. Hence, initial eradication of CSCs alone will probably not preclude disease recurrence; consideration of the simultaneous targeting of CSCs and non-CSCs, as well as a better understanding of the process of dedifferentiation of non-CSCs, will be critical to the development of effective anticancer therapies.

Research advances have provided solid evidence for the connection between the activation of the EMT programme and the development by carcinoma cells of resistance to therapeutics, not only in the experimental models, but also in clinical settings. Indeed, residual carcinoma cells surviving after various types of therapy, including chemotherapy, molecularly targeted therapy, and immunotherapy, commonly display signs of EMT activation. Thus, the efficiency of these therapeutic modalities in generating durable clinical responses might be improved substantially by targeting cancer cells that have activated portions of the EMT programme. However, the mechanisms accounting for the maintenance and/or induction of the EMT programme in these residual carcinoma cells remain largely obscure. EMT in carcinoma cells seems to be governed by highly complex and redundant mechanisms, posing a serious challenge to the selective and truly effective targeting of this programme. Overcoming these difficulties will enable us to design treatment strategies that have a minimal risk of developing resistance, ultimately paving the way to curative treatments of cancer. Finally, as is apparent from the present discussions, many of the biological traits of cancer cells are dictated by non-genetic mechanisms, revealing the limits of cancer genome sequencing in providing insights into many aspects of cancer cell biology and necessitating the integrated use of complementary approaches, such as epigenomics and transcriptomics.

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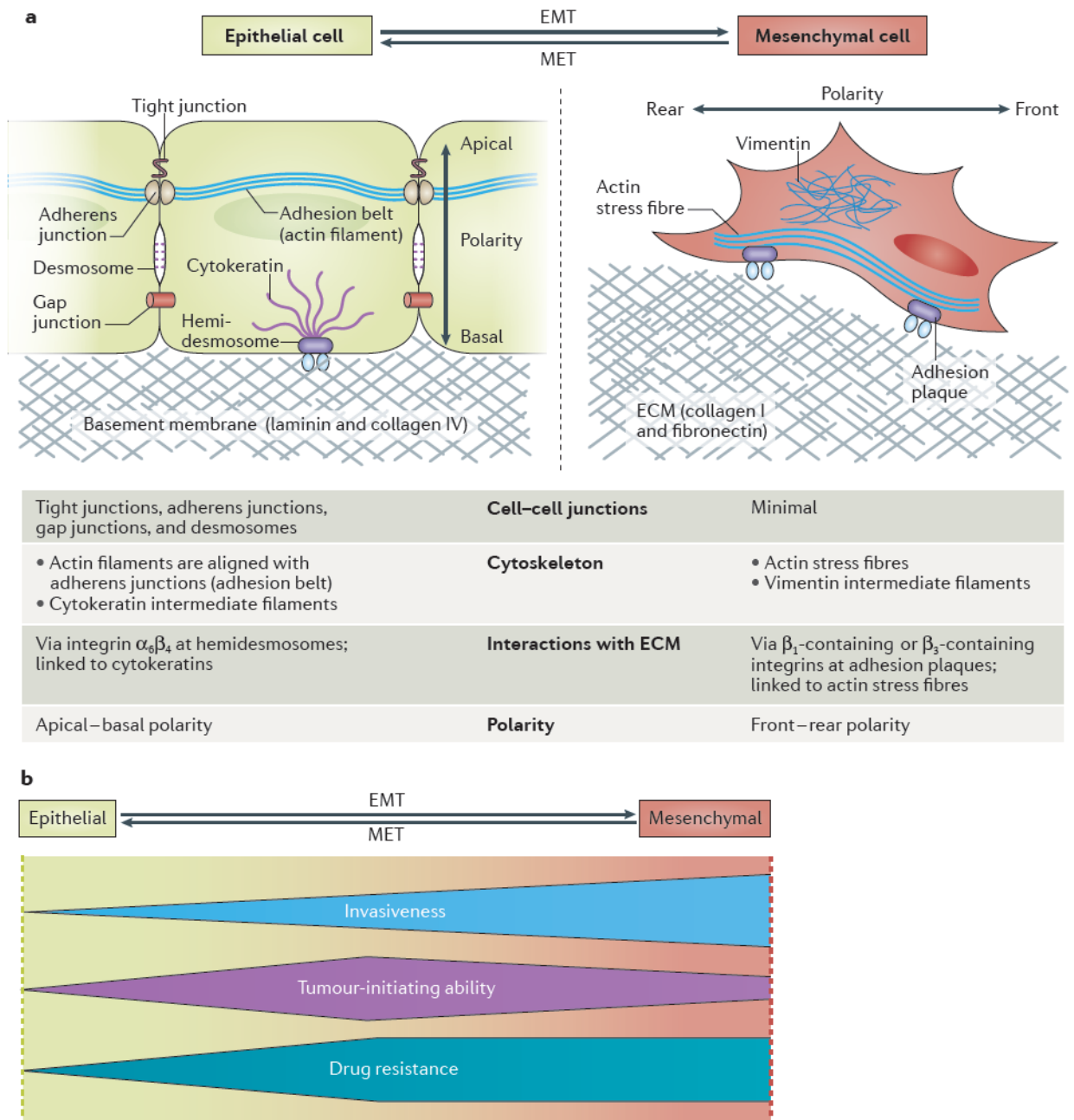
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### Key points

- The cancer stem cell (CSC) concept posits that a subpopulation of neoplastic cells with stem-cell properties — particularly the capacity to self-renew and give rise to various more differentiated cell types — lies at the apex of a tumour cell hierarchy and serves as a critical driver of tumour progression
- The phenotypic differences between CSCs and the bulk tumour cells that lack ‘stemness’ (that is, non-CSCs) seem to be attributable predominantly to epigenetic changes caused by the activation of an epithelial-to-mesenchymal transition (EMT) programme in the former
- Thus, the CSC paradigm provides an explanation for how epigenetic mechanisms can drive the phenotypic diversity of neoplastic cells — an attribute critical for the development of resistance to therapy
- Indeed, most conventional therapeutics are inefficient in eradicating carcinoma cells that have entered the CSC state via activation of the EMT programme, thereby permitting CSC-dependent disease relapse
- Targeting the EMT programme in order to eliminate CSCs offers a promising avenue for the improvement of cancer therapy; however, the success of this approach will require an increase in our mechanistic understanding of the EMT–CSC link



**Figure 1. Morphological and physiological changes associated with the epithelial-to-mesenchymal transition (EMT)**

**a** | A schematic overview of EMT-associated changes in cell physiology. Activation of the EMT programme induces profound changes in various aspects of cell morphology and physiology, most notably in cell cell junctions, cytoskeletal composition, cellular interactions with the extracellular matrix (ECM), and cell polarity. **b** | Summary of the physiological outcomes of EMT in carcinoma: the profile of the shapes illustrates how the extent of invasiveness, the tumour-initiating ability, and degree of drug resistance change across the spectrum of EMT-programme activation. Carcinoma cells invade surrounding

tissues either by individual-cell migration or multicellular migration (as cell cohorts). In general, migration of individual cells, which requires the strong activation of the EMT programme, results in faster tissue invasion than occurs by multicellular migration, the mode of migration that predominates when the EMT programme is only weakly activated<sup>189</sup>. The tumour-initiating ability of carcinoma cells is also affected by the level of EMT-programme activation, peaking at an intermediate level of EMT in these cells; extensive EMT activation is usually detrimental to tumour-initiating ability<sup>149</sup>. The drug resistance of carcinoma cells also seems to be maximal at an intermediate level of EMT-programme activation, but plateaus (rather than declines) with further activation of this programme<sup>149,184</sup>. MET, mesenchymal-to-epithelial transition.

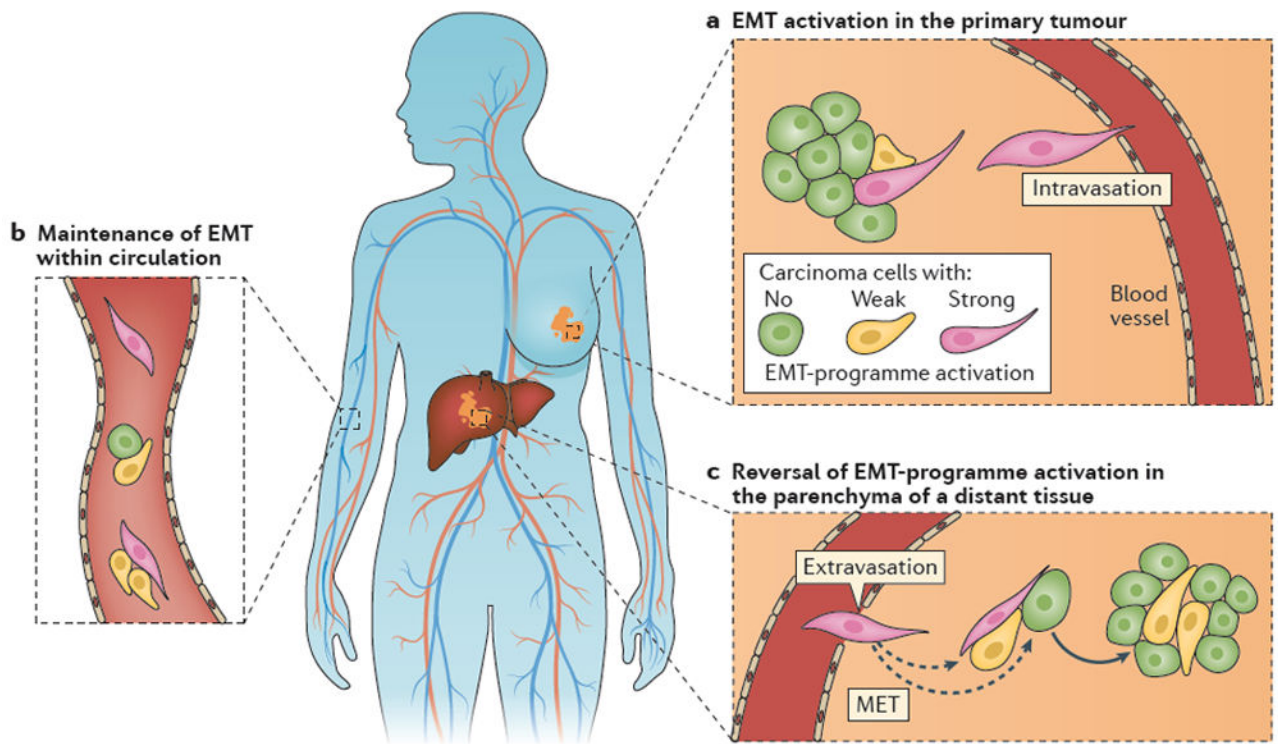
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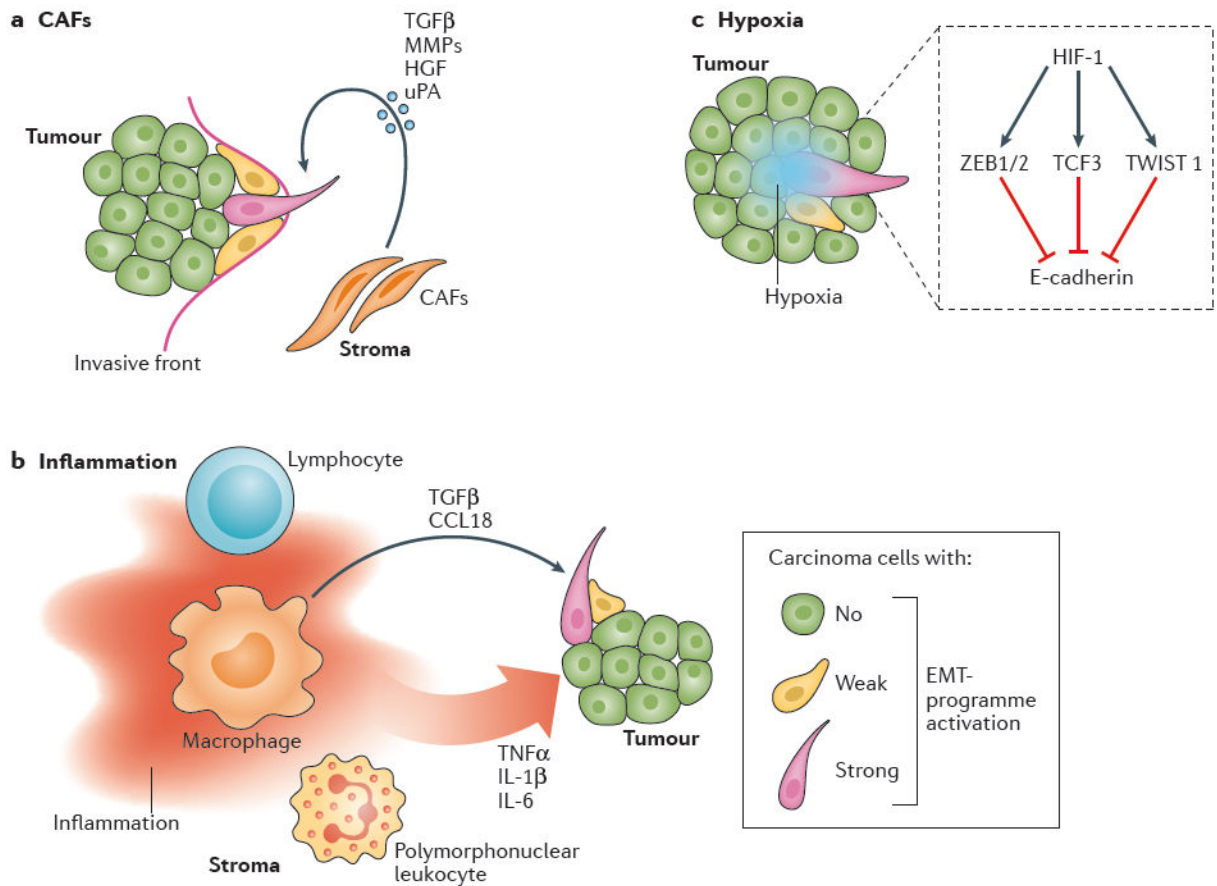
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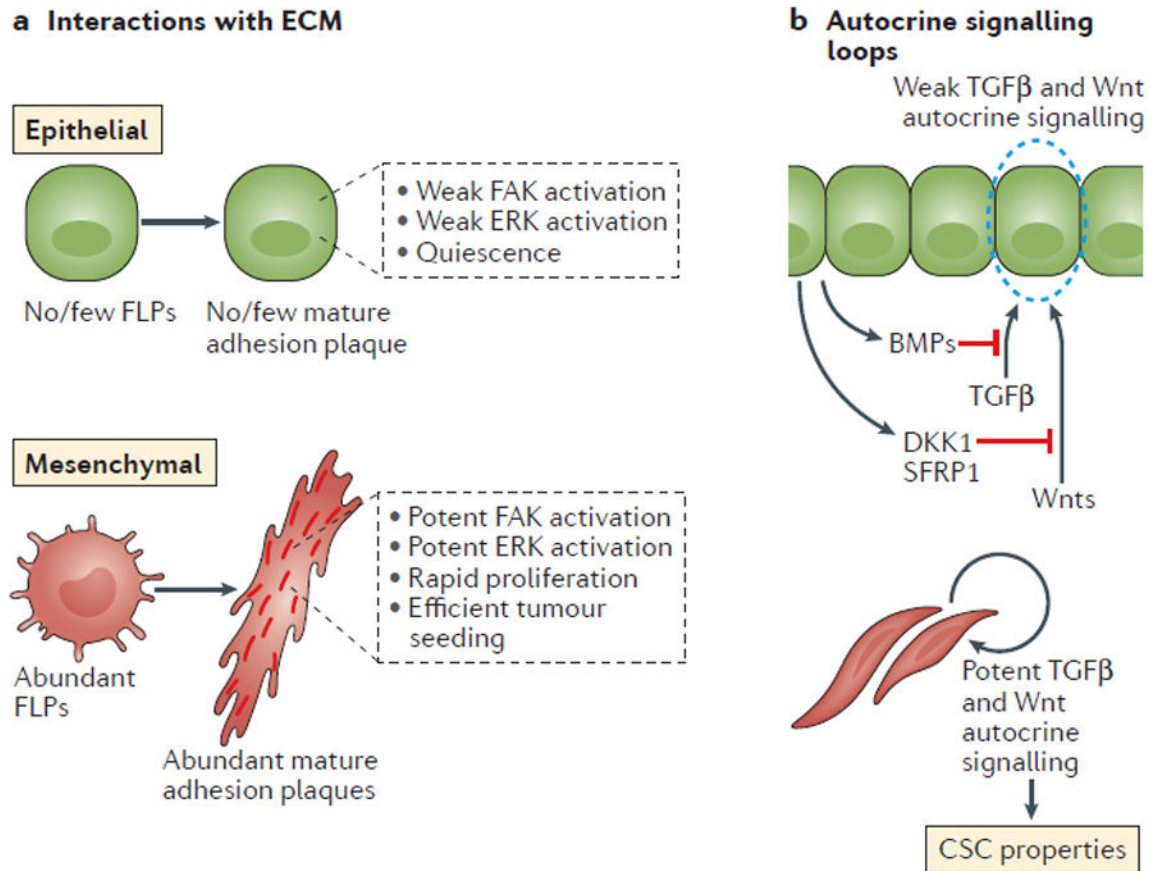
**Figure 2. The patterns of epithelial-to-mesenchymal transition (EMT)-programme activation during carcinoma progression**

**a** | In primary tumours, activation of the EMT programme enables particular carcinoma cells to invade the surrounding stroma; some of these cells eventually enter the systemic circulation. **b** | Circulating tumour cells can demonstrate epithelial and/or mesenchymal traits. Indeed, cancer cells in the circulation, whether solitary or in clusters, frequently exhibit signs of at least partial EMT<sup>82</sup>. **c** | Reversal of the EMT process — that is, activation of the mesenchymal-to-epithelial transition (MET) programme — following dissemination of carcinoma cells to distant tissues seems to be critical for the outgrowth of metastases from many types of carcinoma<sup>88,89</sup>.



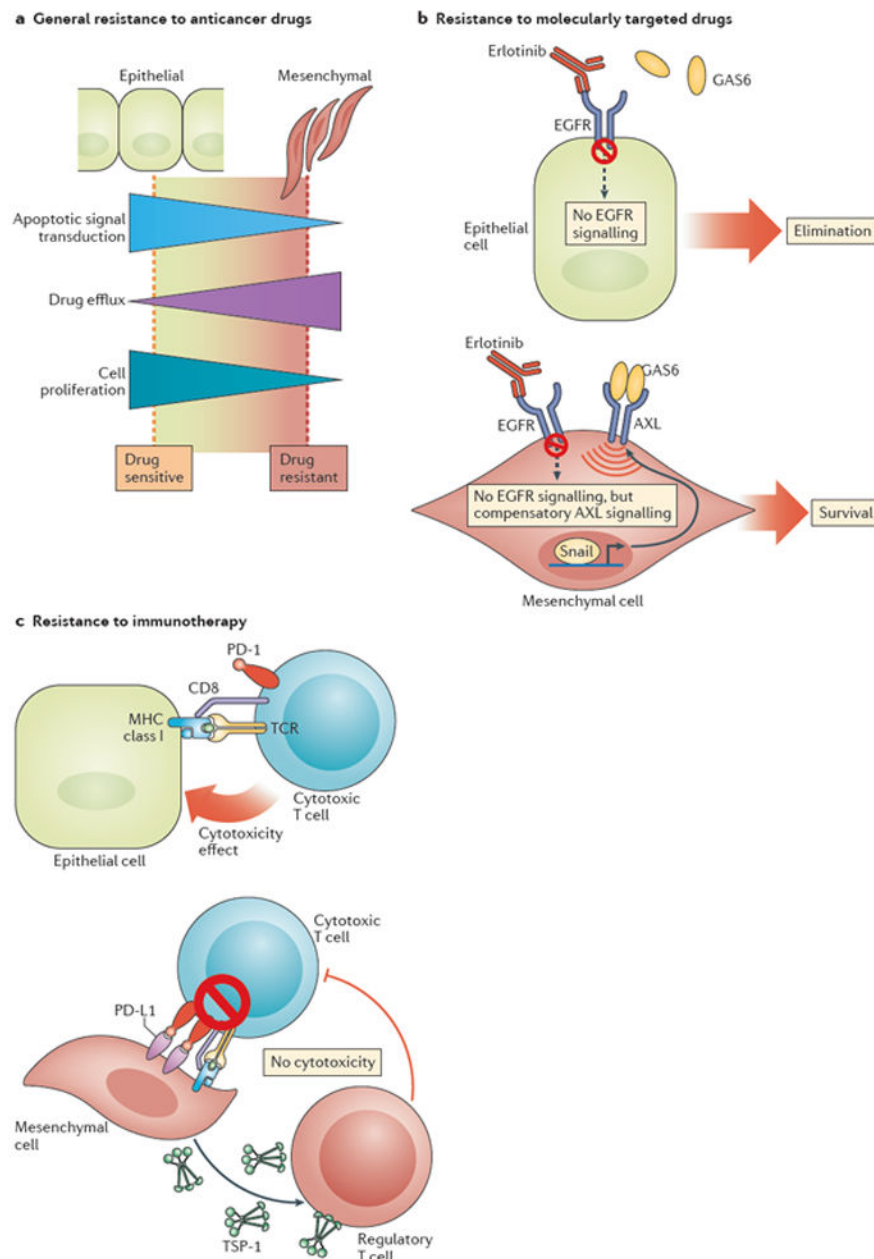
**Figure 3. The contribution of the tumour microenvironment to the activation of the epithelial-to-mesenchymal transition (EMT) programme**

**a** | Carcinoma-associated fibroblasts (CAFs) are frequently observed at the invasive front of tumours, and probably make an important contribution to the induction of EMT in nearby carcinoma cells by secreting various cytokines and enzymes. **b** | The tumour microenvironment is often characterized by chronic inflammation; both soluble and cellular mediators of tumour-associated inflammation can contribute to the induction of an EMT programme in carcinoma cells. **c** | Hypoxia is another common characteristic of the tumour microenvironment. HIF-1, the central mediator of the responses to tumour-associated hypoxia, has been demonstrated to trigger an EMT process, involving the prototypic transcription factors ZEB1/2, TCF-3, and TWIST1, in carcinoma cells. CCL18, C–C-motif chemokine 18; HGF, hepatocyte growth factor; HIF-1, hypoxia-inducible factor 1; MMP, matrix metalloproteinase; TGF $\beta$ , transforming growth factor  $\beta$ ; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; uPA, urokinase plasminogen activator.



**Figure 4. The mechanistic link between the epithelial-to-mesenchymal transition (EMT) programme and cancer stem cell (CSC) status**

**a** | The EMT programme enables carcinoma cells to interact productively with the surrounding extracellular matrix (ECM) proteins. Such changes in cell interactions with the ECM, in turn, reinforce the tumour-initiating ability of cancer cells with an active EMT programme. In particular, EMT enables the efficient development by carcinoma cells of integrin-containing mature adhesion plaques, and these plaques, once formed, trigger signalling pathways critical to the proliferation of cancer cells, such as those involving focal adhesion kinase (FAK) and extracellular signal-related kinase (ERK). **b** | The activation of the EMT programme results in the establishment of several autocrine signalling loops, including the transforming growth factor  $\beta$  (TGF $\beta$ ) and canonical and/or noncanonical Wnt pathways. These signalling loops contribute to the CSC properties of cells with an active EMT programme. BMPs, bone morphogenetic proteins; DKK1, Dickkopf-related protein 1; FLPs, filopodium-like protrusions; SFRP1, secreted Frizzled-related protein 1.



**Figure 5. The mechanism underlying epithelial-to-mesenchymal transition (EMT)-dependent acquisition of therapeutic resistance**

**a** | EMT-associated downregulation of multiple apoptotic signalling pathways, enhanced drug efflux, and slow cell proliferation all contribute to enhance the general resistance of carcinoma cells to anticancer drugs. **b** | In addition, the EMT-associated transcription factor Snail induces the expression of the AXL receptor tyrosine kinase on the surface of carcinoma cells. AXL signalling, triggered by the binding of its ligand growth arrest-specific protein 6 (GAS6), enables Snail-expressing carcinoma cells to override cytostatic effects of EGFR blockade with small-molecule inhibitors (such as erlotinib) or antagonistic monoclonal antibodies. **c** | The EMT programme also activates several processes that enable

carcinoma cells to evade the lethal effect of cytotoxic T cells. These changes include elevated expression of programmed cell death 1 ligand 1 (PD-L1), which binds to the programmed cell death protein 1 (PD-1) inhibitory immune-checkpoint receptor that is expressed by cytotoxic T cells and thereby diminishes their function; and increased secretion of thrombospondin-1 (TSP-1), which promotes the development of regulatory T cells within the tumour microenvironment that ultimately suppress the activity of cytotoxic T cells.

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Table 1

## EMT–CSC connections in various carcinoma types

Experimental system	Observations	Refs
<b>Breast carcinoma</b>		
HMLER human mammary epithelial cells	<ul style="list-style-type: none"> <li>Epithelial-to-mesenchymal transition (EMT)-programme activation in HMLER cells, induced either by the ectopic expression of TWIST1 or Snail, or by treatment with transforming growth factor <math>\beta</math> (TGF<math>\beta</math>), confers multiple cancer stem cell (CSC) properties; these include a CSC-like cell-surface marker expression profile (CD44<sup>high</sup>/CD24<sup>low</sup>) as well as enhanced <i>in vitro</i> tumour sphere formation and <i>in vivo</i> tumour-seeding abilities</li> <li>Naturally occurring CD44<sup>high</sup>/CD24<sup>low</sup> HMLER-cell subpopulation displays signs of EMT-programme activation, including reduced expression of <i>CDH1</i> (encoding E-cadherin) and increased expression of the <i>CDH2</i>, <i>VIM</i>, and <i>FN1</i> mRNAs (encoding N-cadherin, vimentin, and fibronectin, respectively).</li> </ul>	27,31
Clinical samples (metaplastic or claudin-low subtypes)	The clinical samples of metaplastic and claudin-low subtypes of breast cancer simultaneously display a CSC-like profile of cell-surface markers (CD44 <sup>high</sup> /CD24 <sup>low</sup> ) and signs of EMT-programme activation, which include reduced expression of <i>CDH1</i> mRNA	190
Clinical samples	The expression of miR-200c, an antagonist of the EMT programme, is reduced in the CSC fraction (CD44 <sup>high</sup> /CD24 <sup>low</sup> ) compared with the non-CSC population of clinical breast cancer samples	191
<b>Lung carcinoma</b>		
H1299 human non-small-cell lung cancer (NSCLC) cell line	The CD44 <sup>high</sup> subpopulation of H1299 cells exhibits signs of EMT-programme activation (including increased expression of <i>CDH2</i> and <i>VIM</i> mRNAs) and enhanced tumour-seeding ability in nude mice	192
LC31 human NSCLC cell line	TGF $\beta$ -induced EMT in LC31 cells confers elevated expression of the OCT4, NANOG, SOX2, KIT, and CD133 proteins, an increased potential for tumour sphere formation, and an enhanced tumour-seeding ability	193
<b>Prostate carcinoma</b>		
PC3 human prostate cancer cell line	The induction of EMT by the ectopic expression of platelet-derived growth factor D (PDGF-D) in PC3 cells increases their <i>in vitro</i> tumour sphere formation and <i>in vivo</i> tumour-seeding abilities	194
Tumour cells from a <i>Pten</i> -null and <i>Kras</i> <sup>G12D</sup> -driven mouse prostate cancer model	Mesenchymal cell subpopulation (epithelial cell adhesion molecule (EpCAM) <sup>low</sup> /CD24 <sup>low</sup> ) of these tumour cells exhibits an enhanced ability to form tumour spheres, compared with that of a more epithelial cell subpopulation	30
<b>Pancreatic carcinoma</b>		
Multiple human pancreatic cancer cell lines	The knockdown of zinc finger E-box-binding homeobox (ZEB1) expression in Panc1 and MiaPaCa2 human pancreatic cancer cell lines reduces their potentials for <i>in vitro</i> tumour sphere formation and <i>in vivo</i> tumour seeding	32
Clinical samples	Expression of the ZEB1 transcription factor is associated with tumour recurrence after surgery in pancreatic cancer clinical samples	32
<b>Head and neck carcinoma</b>		
Clinical samples	<ul style="list-style-type: none"> <li>The CSC populations (CD44<sup>high</sup>/CD24<sup>low</sup>/aldehyde dehydrogenase (ALDH)<sup>+</sup>) of head and neck cancer clinical samples exhibit signs of EMT-programme activation, including increased expression of Snail and vimentin, as well as reduced expression of E-cadherin</li> <li>Knockdown of Snail expression in these CSC subpopulations results in a reduced ability for tumour sphere formation</li> </ul>	28
<b>Colon carcinoma</b>		
Clinical samples	The CSC subpopulations (CD133 <sup>high</sup> /CD26 <sup>high</sup> ) of colon cancer clinical samples exhibit signs of EMT-programme activation, including increased expression of N-cadherin and vimentin, as well as reduced expression of E-cadherin	29



**Table 2**

Therapy resistance conferred by epithelial-to-mesenchymal transition (EMT)

Therapeutic agent	Observations	Refs
<b><i>Inhibition of apoptotic signalling</i></b>		
Cisplatin	Slug blocks p53-mediated transcriptional induction of <i>PUMA</i> (also known as <i>BBC3</i> , encoding Bcl-2-binding component 3) expression by directly repressing the <i>PUMA</i> promoter region; multiple lung adenocarcinoma cell lines acquire cisplatin resistance through this mechanism	195,196
Tumour necrosis factor $\alpha$ (TNF $\alpha$ ) treatment; $\gamma$ -irradiation	Snail confers resistance against multiple apoptosis-inducing stimuli, in part by promoting AKT activation, upregulating the expression of the pro-survival protein Bcl-X <sub>L</sub> , and delaying cell-cycle progression	197,198
TNF-related apoptosis-inducing ligand (TRAIL)	EMT-programme activation diminishes E-cadherin-mediated clustering of the TRAIL receptors DR4 and DR5, thereby making carcinoma cells resistant to TRAIL-induced apoptosis	199
<b><i>Enhancement of drug efflux</i></b>		
Doxorubicin	EMT-programme activation induces the expression of multiple members of the ATP-binding cassette (ABC) transporter family, thereby rendering these cells resistant to doxorubicin.	200
<b><i>Protection against molecular targeted agents</i></b>		
EGFR inhibitors	The activation of EMT and subsequent expression of AXL receptor tyrosine kinase confer resistance to EGFR inhibitors on <i>EGFR</i> -mutant non-small-cell lung carcinoma (NSCLC) cells	201,202
EGFR inhibitors; PI3K inhibitors	An EMT-associated gene-expression signature predicts the resistance of NSCLC cells to EGFR inhibitors and PI3K inhibitors	163
<b><i>Desensitization to immunotherapy</i></b>		
Dendritic cell (DC)-mediated immunotherapy (intratumoral injection of DCs pulsed with a tumour antigen)	Snail expression in melanoma cells contributes to resistance to DC-mediated and CTL-mediated immunotherapy via enhanced thrombospondin-1 expression and resultant induction of immunosuppressive regulatory T cells within the tumour tissue	203
Immune-checkpoint inhibition	Zinc finger E-box-binding homeobox (ZEB1)-mediated activation of EMT in NSCLC cells relieves miR-200-mediated repression of programmed cell death 1 ligand 1 (PD-L1) expression, a major inhibitory ligand for the programmed cell death protein 1 (PD-1) immune-checkpoint protein on CD8 <sup>+</sup> CTLs. This effect sensitizes these cells to immunotherapies targeting the PD-1/PD-L1 axis, while potentially conferring on them resistance to other strategies of activating antitumour immunity, such as the functional blockade of another immune-checkpoint protein, CTLA-4	204