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The EMT spectrum and therapeutic opportunities

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Abstract

Carcinomas are phenotypically arrayed along an EMT spectrum, a developmental program currently exploited to understand the acquisition of drug resistance through a re-routing of growth factor signalling. This review collates the current approaches employed in developing therapeutics against cancer-associated EMT, and provides an assessment of their respective strengths and drawbacks. We reflect on the close relationship between EMT and chemoresistance against current targeted therapeutics, with a special focus on the epigenetic mechanisms that links these processes. This prompts the hypothesis that carcinoma-associated EMT shares a common epigenetic pathway to cellular plasticity as somatic cell reprogramming during tissue repair and regeneration. Indeed, their striking resemblance suggests that EMT in carcinoma is a pathological adaptation of an intrinsic programme of cellular plasticity that is crucial to tissue homeostasis. We thus propose a revised approach that targets the epigenetic mechanisms underlying pathogenic EMT to arrest cellular plasticity regardless of upstream cancer-driving mutations.

1 The EMT spectrum

Recent evidence has advanced and broadened the definition of epithelial-mesenchymal transition (EMT) in human pathologies. Whilst earlier studies relied on the use of key epithelial and mesenchymal markers to detect its aberrant activation during pathogenesis, it now becomes clear that this is a not a simple binary decision to acquire either an epithelial or a mesenchymal state. Rather, pathological EMT manifests dynamic transitional states punctuated by metastable intermediates (Nieto et al., 2016). This chapter collates the current knowledge of the molecular mechanisms underlying this phenomenon, and discusses current efforts in the deployment and development of therapeutic interventions.

EMT is orchestrated by a core set of transcription factors (EMT-TFs), each having the ability to drive EMT via largely analogous genetic programmes. These include SNAI1/2, TWIST, and ZEB, among others. As reviewed elsewhere, a myriad of growth factor and developmental signals activate these EMT-TFs (Thiery et al., 2009). However, the precise reasons for why this highly controlled programme is aberrantly triggered at times are varied and often obscured. This is compounded by the inherent difficulty in quantifying the extent of the so-called "partial EMT" in each disease state—just exactly how stable is metastable? Such complexities present a formidable challenge in rational drug design. Indeed, with such variations, what works in one context or in a particular patient could be futile or harmful in another. Nevertheless, with fresh knowledge and the benefit of hindsight, certain principles have emerged.

Like with other examples of heterogeneity encountered in biology, there is also heterogeneity following the execution of the EMT programme. One explanation is that EMT heterogeneity results from a diverse mix of populations undergoing EMT at different rates and downstream to various cues. For example, circulating tumor cells (CTCs) isolated from breast cancer patients display a spectrum of epithelial-mesenchymal hybrid features (Khoo et al., 2015; Yadavalli et al., 2017; Yu et al., 2013a), the composition of which varies significantly among patients and is greatly dominated by the underlying biology of the primary tumor. Along the clinical course, the epithelial-mesenchymal hybrid features of CTCs continue to evolve, further illustrating that the metastable state itself exists as a dynamic range of equilibrium. With this appreciation of EMT as a spectrum of different states, broader perspectives of how to manipulate the metastable state within each context can thus be provided.

2 EMT Drug Discovery Platforms

At the heart of each drug discovery platform is a cohesive concept. In the development of EMT-targeting therapeutics, the following approaches have been adopted: 1) killing cells that have undergone EMT; and 2) reversing EMT in metastable cells. It is worth noting here that while these approaches share a common purpose, the rationale for each is distinct.

2.1 Targeting EMT-induced Cancer Stem Cells

In addition to greater chemoresistance, cells that have undergone EMT bear increased stem-like traits *in vitro* (Mani et al., 2008; Morel et al., 2008) and *in vivo* (Guo et al., 2012); this observation raised the hope that targeting EMT could eradicate the rare

self-renewing and multipotent "cancer stem cells" (CSCs) that persist following conventional chemotherapy. EMT is also associated with increased cell migration and resistance to anoikis, properties that are associated with tumor invasion and metastasis. Thus, the specific killing of cells that have undergone EMT is an attractive therapeutic strategy against CSCs.

To date, the most extensive and prominent EMT-targeting screen was performed on the HMLE series of immortalized human mammary epithelial lines. These lines have been well characterized in studies of cellular transformation (Elenbaas et al., 2001). This model system led to the discovery of the EMT-induced, tumor-initiating CSC, typified by their $CD44^{high}/CD24^{low}$ phenotype (Mani et al., 2008; Morel et al., 2008). The production of these cells was shown to be achieved either through the forced expression of EMT-TFs (SNAI1, TWIST1 and ZEB1) or through a combination of growth factors and RNAi (shEcad) (Mani et al., 2008; Scheel et al., 2011).

A high-throughput screen in a 384-well format was conducted using an HMLE derivative line that was induced to undergo EMT by expressing shEcad. This screen identified the selective cytotoxic effects of salinomycin, a potassium ionophore hitherto known as an antibiotic, on the CSC subpopulation >100-fold relative to paclitaxel (Gupta et al., 2009). Subsequent studies revealed that salinomycin promotes the degradation of the Wnt co-receptor LRP6 (lipoprotein receptor related protein 6) by inhibiting its phosphorylation, thereby attenuating Wnt signaling (Lu et al., 2011). The HMLE plateform was further deployed in expanded screens identifying other candidate compounds, most notably ML239, which appears to target NF-κB signaling (Carmody et al., 2012). More recently, a synthetic derivative of salinomycin was shown to kill breast cancer stem cells by sequestering iron in the lysosome, thereby triggering ferroptosis (Mai et al., 2017).

However, despite these advances, there are potential drawbacks to the cytotoxic killing of carcinoma cells undergoing an EMT. First, the endpoint of their transition is often not a permanent mesenchymal state but rather a metastable intermediate state, thus rendering them difficult to target. Indeed, the spectrum of intermediate states exhibited by CTCs (Khoo et al., 2015; Yadavalli et al., 2017; Yu et al., 2013a) likely means that they are not an effective target. Second, cytotoxicity exerts a selective pressure, that may hasten the evolution of CSCs into alternative metastable states not sensitive to the drug.

2.2 Reversing EMT in metastable cancer cells

In using an EMT reversal approach, mesenchymal-like carcinoma cells are reverted to their epithelial-like (original) phenotype, thereby restricting the (acquired) self-renewal and invasive properties of these cancer cells. However, few suitable models exist for testing non-cytotoxic, EMT-reversing agents. One platform used the NBT-II rat bladder carcinoma line to screen for compounds that could reverse growth factor-induced cell scattering (Chua et al., 2012). Though modest in scale, this screen identified non-cytotoxic compounds that target ALK5/TGFβR1, MAPK, Src, and PI3K to reverse the scattering phenotype without impacting cellular proliferation. Two of these compounds, PD0325901 and Saracatinib, enhanced mesenchymal to epithelial transition (MET) when used in combination in NSCLC lines (Chua et al., 2015). Two other pre-clinical studies have reported the anti-EMT activity of Src

kinase inhibitors in ovarian and breast carcinoma cell lines (Huang et al., 2013; Vultur et al., 2008).

A mesenchymal derivative of the HMLE cell model has also been used to identify compounds that promote MET (Pattabiraman et al., 2016; Tam et al., 2013). In a high-throughput screen with a firefly reporter linked to the *Cdh1/E-cadherin*, the authors found that forskolin and cholera toxin effectively induced MET by activating protein kinase A (PKA) through elevating intracellular cyclic AMP. This, in turn, activates PHD finger protein 2 (PHF2), which demethylates histone H3K9me2 and H3K9me3 to de-repress epithelial markers and permanently reverse EMT driven by epigenetic mechanisms. Importantly, the resultant MET strongly abrogates the tumor-initiating capacity and increases the drug sensitivity of EMT-prone carcinoma lines of various tissue origins. A similar platform also utilized an epithelial marker promoter induction (EpI) screen to identify histone deacetylase inhibitors (HDACi) as a potent class of EMT reversing agents (Tang et al., 2016; Yun-Ju Huang and Yo-Yan Huang, 2016).

An inherent shortcoming of the conventional cell-based platforms is their inadequacy to model the complex tissue microenvironment in which EMT occurs *in vivo*. To mimic this, a co-culturing system employing modern microfluidics has been developed incorporating tumor spheroids in a 3-dimensional hydrogel scaffold (Aref et al., 2013). This model also allows for assessing the contribution of endothelial cells in the system. One could expect that, with continual advances in methodology, new facets of the EMT process and, therefore, new strategies of intervention, will be uncovered.

Several candidate EMT reversing agents are already available clinically, such as Saracatinib. Initially developed for the treatment of cancer, saracatinib is a dual-kinase inhibitor, targeting Src and Bcr-Abl tyrosine kinases. Although saracatinib is well tolerated in humans and showed promising results in animal studies, its efficacy in clinical trials has been disappointing either alone or in combinatorial treatments (Kim et al., 2009; Puls et al., 2011). In view of this, the functionally related focal adhesion kinase (FAK) could be tested for EMT reversal properties, as an inhibitor PF-00562271 has shown encouraging signs in early clinical trials (Infante et al., 2012).

A further application of these EMT reversing inhibitors would be in combination with other drugs to generate synthetic lethality. Along these lines, small chemical inhibitors of various signaling pathways are currently being used in clinical trials for their anti-EMT activities. Amongst these, inhibitors targeting the TGF-β pathway—a classical activator of EMT—have shown the most promise. Of note, the TGF-β inhibitor, LY2157299 (Galunisertib), is in Phase II studies against glioblastoma and hepatocellular carcinoma (Brandes et al., 2016; Giannelli et al., 2016; Rodon et al., 2015). Activation of the AXL receptor is reported to aberrantly phosphorylate SMAD3 to induce EMT in HCC progression in collaboration with TGF-β (Reichl et al., 2015). As such, the concurrent targeting of AXL and TGF-β may prove superior to monotherapy aimed at interfering with TGF-β signaling, and this warrants further investigation, especially given the current availability of AXL inhibitors in the clinic (Antony et al., 2016; Byers et al., 2013; Feneyrolles et al., 2014; Giannelli et al., 2016; Nieto, 2013).

Broadly speaking, inhibitors targeting the major cellular signaling pathways often have an impact on the EMT status of the carcinomas, as these pathways are intimately linked with EMT during development (Thiery et al., 2009; Voon and Thiery, 2017). It is worth noting, too, the potential hazards of reversing EMT in disseminated tumor cells, as MET is already employed by these metastasized cells as a strategy to promote colonization at distal sites (Beerling et al., 2016; Nieto, 2013; Ocana et al., 2012; Tsai et al., 2012). Therefore, precautions should be observed in the use of EMT-reversing agents in the clinic and only within a clear therapeutic window.

While these drugs may have anti-EMT activities, they were developed to target cancer-driving mutations within these pathways (Table 1). In other words, their clinical benefits are seldom benchmarked against their overall contribution to EMT-associated tumorigenicity and plasticity. Ironically, their inability to completely abrogate EMT may eventually become a driving force behind chemoresistance against these drugs.

2.3 EMT, epigenetics and chemoresistance

Numerous studies have reported the presence of residual resistant cells following chemotherapy, and these cells have been associated with an EMT phenotype in clinical settings as well as in animal models (Byers et al., 2013; Fischer et al., 2015; Kitai et al., 2016; Manchado et al., 2016; Shao et al., 2014; Zheng et al., 2015). EMT-associated chemoresistance may also be accompanied with a switch to compensatory pathways, so that carcinoma cells can regain cellular homeostasis (Kitai et al., 2016; Manchado et al., 2016). Whilst the precise basis for the correlation between EMT and cell survival remains obscure, it is likely that intermediate EMT states offer attractive "safe havens" in which cell signalling can be re-wired to become independent of the targeted pathway. Here, the capacity to shift to an alternate and viable phenotype relies on the cell's EMT-endowed plasticity, often termed epithelial mesenchymal plasticity (EMP) (Byers et al., 2013; Nieto, 2013).

It has been proposed that intermediate states represent quasi-discreet epigenetic states, which are characterized by altered histone modifications on key loci such as *E-cadherin/Cdh1* and *miR-200* (Nieto et al., 2016; Tam and Weinberg, 2013). Accordingly, the same epigenetic machineries that mark these intermediate states are often implicated in the acquisition of chemoresistance. An important class of such histone modifiers are the polycomb group (PcG) repressor complexes, PRC1 and -2. During EMT, the PRC2 complex is recruited to the *CDH1* promoter by the EMT-TF SNAI1, whereby it catalyzes the trimethylation of histone H3K27 to repress E-cadherin expression (Herranz et al., 2008). The same complex is also responsible for the trimethylation and silencing of miR-200, which gives rise to chemoresistance (Ceppi et al., 2010; Lim et al., 2013; Sato et al., 2017; Tryndyak et al., 2010). PRC1 components, such as BMI1, are considered stem cell factors that support normal stem cells and their transformed counterparts (Park et al., 2004; Valk-Lingbeek et al., 2004). The upregulation of BMI1 during carcinogenesis was reported to induce EMT and the invasive phenotype, and this was mediated via its cooperative actions with TWIST1 on *Cdh1* and *INK4A* (Song et al., 2009; Yang et al., 2010).

Acetylation is another histone modification associated with EMT and chemoresistance. During cancer metastasis, the histone deacetylases (HDAC) 1 and 2—as part of the Mi-2–nucleosome remodeling and deacetylase (NuRD) repressive complex—are recruited by Snail and TWIST to the *Cdh1* and *Foxa1* promoters, leading to their repression, respectively (Fu et al., 2011; Peinado et al., 2004; von Burstin et al., 2009; Xu et al., 2017). However, various components of the NuRD complex, and specifically, the HDACs, will confer drug resistance to cancer cells (Fu et al., 2011; Li et al., 2014; Sakamoto et al., 2016). Consequently, HDAC inhibitors like vorinostat, mocetinostat, and valproic acid are currently being evaluated as anti-EMT agents (Bruzzese et al., 2011; Caponigro et al., 2016; Lan et al., 2016; Meidhof et al., 2015; Sakamoto et al., 2016; Schech et al., 2015; Schobert and Biersack, 2017).

A similar correlation between EMT and chemoresistance is also observed for lysine-specific demethylases, such as LSD1, an emerging class of epigenetic modulators (Bennani-Baiti, 2012; Lei et al., 2015; Nagasawa et al., 2015). LSD1 modulates gene expression by removing methyl groups on lysine 4 or lysine 9 of histone H3 to repress or activate target promoters, respectively (Shi et al., 2004). In the context of EMT, the induction of EMT in mammary epithelial cells involves the recruitment of LSD1 by SNAI1 to promoters of E-cadherin, claudin and cytokeratin family genes, which targets them for repression (Lin et al., 2010a; Lin et al., 2010b). In recent years, the association of LSD1 expression with malignancy, chemoresistance, and poor survival has raised interest into the therapeutic potential of its inhibitors (Lv et al., 2012; Nagasawa et al., 2015; Yu et al., 2013b; Zhao et al., 2012).

In addition to histone modification, DNA methylation patterns are altered during persistent, mutation-driven EMT during carcinogenesis (McDonald et al., 2011; Tam and Weinberg, 2013). A key mediator of these aberrations appears to be the ten-eleven translocation 1 (TET1) methylcytosine dioxygenase, which initiates the demethylation of DNA and is associated with tumorigenesis in many cancers (Fu et al., 2014; Song et al., 2013; Sun et al., 2013; Tsai et al., 2014). However, there is opposing evidence as to the role of TET1 in EMT-induced chemoresistance: TET1 has been reported to promote cisplatin-resistance through its induction of EMT in ovarian cancer (Han et al., 2017), but act as a barrier against EMT in mammary epithelial cells by de-repressing the *miR-200* promoter (Song et al., 2013).

Finally, it warrants highlighting that the epigenetic states of the EMT intermediates are cooperatively maintained at multiple levels of epigenetic regulation, with all the usual regulatory elements and limitations of a complex network. For example, just as miR-200 is a target of PRC2-mediated repression, the PRC2 component Suz12 is conversely targeted by miR-200 (Iliopoulos et al., 2010; Lim et al., 2013). Moreover, a functional cross-talk between TET1 and NuRD during EMT is also likely, given their cooperation in vitamin C-induced MET during somatic cell reprogramming (Chen et al., 2013).

2.4 A better mousetrap beyond the EMT spectrum?

From a clinical perspective, the resistance of cancer cells by virtue of their EMT state necessitates targeting the compensatory pathways employed by the cells for their eradication. However, it is just as likely that the very same mechanisms will later give rise to resistance to a new drug. Hence, rather than targeting the ever-shifting compensatory growth factor pathways, it would seem a better idea to shutdown cellular plasticity. A major obstacle in this approach is that we have an incomplete grasp of the molecular underpinnings of this plasticity. Nevertheless, some cues can be drawn from the field of tissue stem cells, where recent data reveal a genetic programme in differentiated cells that promotes cellular plasticity. Modern lineage tracing studies have demonstrated that some differentiated epithelial cells possess an innate ability to de-differentiate *in vivo*, and gain multipotency under specific circumstances (Rios et al., 2016; van de Moosdijk et al., 2017). This phenomenon is most clearly seen during injury and tissue regeneration, but also during inflammation and at certain stages during post-natal development, such as in the mammary gland during pregnancy. Indeed, in specific instances, the induction of stemness is reliant on the co-activation of the EMT programme (Guo et al., 2012; Ye et al., 2015). And, although the precise reason for this association is not known, it is clear that the capacity for somatic cell reprogramming—which was dramatically demonstrated in the generation of induced pluripotent stem cells (iPSc) from terminally differentiated fibroblasts—is integral to tissue homeostasis (Gregorieff et al., 2015; Smith et al., 2016; Takahashi and Yamanaka, 2006; Tetteh et al., 2016; van Es et al., 2012). In this light, it is possible that our current investigation of EMT-associated plasticity and induction would converge on common molecular mechanisms. In other words, disease-associated EMT may be a pathological manifestation of aberrantly activated normal somatic reprogramming of differentiated cells into functional stem cells (Ye et al., 2015).

Such a model of common epigenetic pathways governing EMP and induced pluripotency (iP) indeed has the capacity to accommodate common observations between the two phenomena. A prime example of this would be the role of p53 as a barrier, whereby the loss of its function lowers the threshold for entrance into EMP just as it would enhance the iP efficiency (Ansieau et al., 2008; Austin et al., 2013; Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009; Mu et al., 2017). A significant part of this is mediated through the p53-miR-200 regulatory network, which features prominently in the regulation of EMP and iP (Chang et al., 2011; Hu et al., 2014; Kim et al., 2011; Song et al., 2013). A further common feature is the repressive effects exerted by lineage-determining transcription factors, such as BRIGHT/ARID3A, RUNX3, GRHL2 and PAX5 (Chung et al., 2016; Hanna et al., 2008; Hikichi et al., 2013; Popowski et al., 2014; Voon et al., 2012). Of relevance, both processes are governed by cell extrinsic factors, such as growth factors (Lluis et al., 2008; Thiery et al., 2009; van Es et al., 2012; Vidal et al., 2014), and intrinsic epigenetics elements, such as the TET/miR-200 axis (Hu et al., 2014; Song et al., 2013) and the NuRD repressor complex (Chen et al., 2013; dos Santos et al., 2014; Ebrahimi, 2015; Fu et al., 2011).

Despite these parallels, there are obvious differences between the induction of EMP in carcinoma and somatic reprogramming, specifically during the generation of iPSc from fibroblasts. Most notably, the induction of pluripotency in the case of the latter

is preceded by MET. It reverts fibroblasts into an epithelial phenotype similar to that of embryonic stem cells (Li et al., 2010). Consistent with this, pro-EMT signals like TGF-β (Ichida et al., 2009; Qin et al., 2014; Vidal et al., 2014), Wnt/β-catenin (Ho et al., 2013; Lluis et al., 2008), and Hippo (Qin et al., 2012) pathways act as barriers against iP in a context-specific manner. At the same time, inhibitors of these pathways, such as the aforementioned anti-EMT TGF-β inhibitors, strongly enhance the efficiency of somatic reprogramming (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Overall, it seems EMP and iP each requires a phenotypic shift along the EMT spectrum (albeit, in opposite directions) towards an intermediate metastable state en route to dedifferentiation/reprogramming. If so, then it is imperative that the innate molecular barriers—such as oxidative and methylation states of the chromatin and their regulators, which safeguard against phenotypic slippage—are thoroughly elucidated. Ultimately, the promise of a plasticity-centric paradigm is its amenability to the precise targeting of EMT-associated plasticity in carcinomas irrespective of the upstream driver mutations, and invulnerable to the re-routing of the signalling circuit observed in current strategies. Accordingly, the development of these next-generation therapeutics will require discovery platforms that assay the functional output of the involved epigenetic machineries rather than, for example, the activation of a particular marker gene.

3 Concluding remarks

EMT has emerged in recent years to be a major driver of chemoresistance to anti-cancer therapies in the clinic. This is closely linked to phenotypic plasticity in the form of metastable intermediates over the EMT spectrum. The biological reason for this phenomenon is currently unclear, but it is possible that aberrant EMT in carcinoma cells unlocks an innate dedifferentiation programme integral to tissue repair, development, and homeostasis. Importantly, such an engine of plasticity would also fuel tumor heterogeneity, progression, and immune escape. Despite the clear need, targeting EMT in cancer therapy has proven challenging due to conceptual difficulties in the design of viable screens. Conventional screening approaches that focus on interfering with specific molecular interactions are unsuitable or have yielded inconsistent results. In this review, we surveyed the current efforts to develop and deploy anti-EMT therapeutics and discussed their relative effectiveness. By way of this evaluation, a novel concept is put forth to selectively inhibit low-order epigenetic mechanisms that promote plasticity. In doing so, the phenotypic flexibility that enables cancer cells to be “moving targets” will be greatly restricted, thereby enhancing the efficacies of current therapeutics.

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