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Measuring and Modelling the Epithelial Mesenchymal Hybrid State in Cancer: Clinical Implications

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Key words: epithelial mesenchymal plasticity (EMP), epithelial mesenchymal transition (EMT), mesenchymal epithelial transition (MET), E/M Hybrid, partial-EMP, computational biology, molecular modelling, cancer

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Abstract

The epithelial-mesenchymal (E/M) hybrid state has emerged as an important mediator of the elements of cancer progression facilitated by epithelial mesenchymal plasticity (EMP). We review here the evidence for the presence and prognostic potential of E/M hybrid state in carcinoma, modelling predictions and validations studies to demonstrate stabilised E/M hybrid intermediates along the spectrum of EMP, and computational approaches for characterising and quantifying EMP phenotypes, with particular attention to the emerging realm of single-cell approaches through RNA sequencing and protein-based approaches.

1. Epithelial mesenchymal plasticity, E/M hybrids and cancer progression

E/M hybrid states in cancer

Epithelial–mesenchymal plasticity (EMP) defines a bi-directional axis of phenotypic change: epithelial–mesenchymal transition (EMT) and the reverse process of mesenchymal–epithelial transition (MET). These phenotypic changes have been implicated in embryonic and postnatal development, wound healing, and tissue repair, but are also exploited by cancer cells in avoidance of oncogene-induced senescence, immune-avoidance, invasive progression, survival during dissemination, generation of cancer stem cells, the creation of metastases, and therapeutic resistance [Derynck and Weinberg, 2019, Dongre and Weinberg, 2019, Williams et al., 2019].

Although it is likely that cancer cells can be found to exist at any point across the full EMP spectrum, it is becoming increasingly clear that carcinoma cells often undergo a partial EMT (pEMT) or incomplete EMT, which result in an E/M hybrid state, where cells co-express both epithelial and mesenchymal markers [Huang et al., 2013b, Zhang et al., 2014, Grosse-Wilde et al., 2015, Fustaino et al., 2017, Pastushenko et al., 2018a, Saitoh, 2018, Kroger et al., 2019, McFaline-Figueroa et al., 2019] (Figure 1). The presence of E/M hybrid phenotype is reported in several primary human cancers, including prostate, breast and lung cancer [Livasy et al., 2006, Kolijn et al., 2015, Wang et al., 2017, Zacharias et al., 2018].

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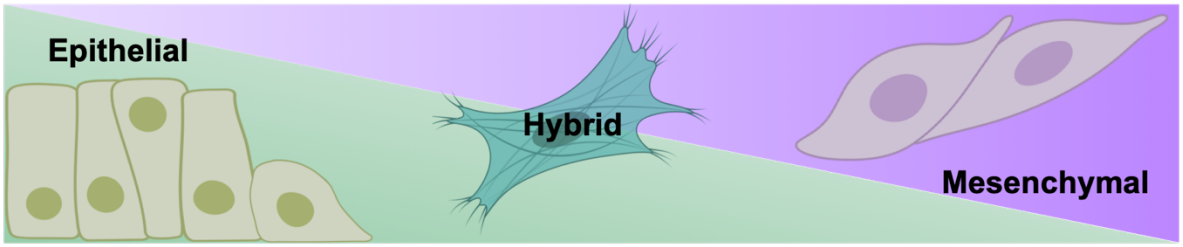

Figure 1: EMP and the E/M hybrid state. *Schematic representation of the Epithelial Mesenchymal Plasticity (EMP) continuum, along which E/M hybrid cells are commonly found in carcinoma tissues. Evidence is growing for the existence of stable intermediates held in check by complex molecular circuitry.*

The E/M hybrid phenotype has been increasingly evident in studies showing coexpression of epithelial (e.g. cytokeratins, E-Cadherin, beta-catenin) and mesenchymal (e.g. vimentin, N-Cadherin, fibronectin) markers in various carcinoma types [Sarrio et al., 2008, Kolijn et al., 2015, Yamashita et al., 2018, Meyer et al., 2019, Navas et al., 2020]. With the advent of molecular profiling, the EMP continuum (and implicit E/M hybrid states) is increasingly well characterised. EMP scores show a spectrum of values derived by composite analysis of expression levels for a range of epithelial and mesenchymal genes in both cell lines and tumour samples, across a large range of cancer origins (e.g. [Tan et al., 2014]). Good concordance exists amongst these different scoring systems, each of which showed that cells can undergo varying extents of EMT across tumour types [Chakraborty et al., 2020a]. These scores, and scRNA-seq across a range of cancer cell lines, indicate a continuum in the gene expression landscape of EMP in both tumours and cell lines [Cursons et al., 2018, Karacosta et al., 2019a, Cook and Vanderhyden, 2020]. Given that EMP often involves changes in cellular morphology too, an EMP continuum in the morphological landscape is also being increasingly characterized recently [Leggett et al., 2016, Devaraj and Bose, 2019]. The trajectories of individual cells in these EMP landscape(s) need not be a cell-autonomous phenomenon; they can be influenced by various biophysical and biochemical modes of cell-cell communication [Mandal et al., 2020, Tripathi et al., 2020b]. Novel analytical tools with single cell RNA-sequencing data are able to align cells along a "pseudospatial" trajectory between these two states [McFaline-Figueroa et al., 2019].

The term metastable state was coined to describe the instability of these E/M hybrid cells [Klymkowsky and Savagner, 2009, Jia et al., 2018], although it may not apply equally to all E/M hybrids. Certain E/M hybrid states appear to be favoured, and these have (i) relative stability / increased proportionality [Biswas et al., 2019], and (ii) enhanced malignant potential, with several studies now having demonstrated a higher malignant potential in E/M hybrid states than more frankly mesenchymal variants [Celia-Terrassa et al., 2012, Huang et al., 2013b, Bierie et al., 2017a, Celia-Terrassa et al., 2018, Pastushenko et al., 2018a, Kroger et al., 2019]. E/M hybrid subpopulations that

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remain more epithelial, with limited additional mesenchymal features, appear to have the greatest malignant and metastatic potential [Jolly et al., 2015, Pastushenko et al., 2018a, Gupta et al., 2019]. Thus, the development of robust and quantifiable methods for estimating the type and extent of E/M hybrid state is becoming an important clinical challenge.

The stability of certain E/M hybrid states is also implied by dynamics of EMP-positive cancer cell systems. Separate analysis of epithelial and mesenchymal transcriptomal components of breast cancer cell lines has shown that high epithelial gene expression often accompanies mesenchymal gene expression in cells designated as mesenchymal, reflecting a predominantly E/M hybrid phenotype, which was also seen in TCGA breast cancer specimens ([Foroutan et al., 2018]; see section 2. Computational approaches, below). The observation of stability states across the continuum also parallels modelling predictions based on plasticity stabilisation factors ([Jolly et al., 2018]; see Section 3. Mechanism-based mathematical models below).

The E/M hybrid phenotype in experimental systems

EMP-related changes occupy a nonlinear landscape in the multi-dimensional space of cancer progression. As mentioned above, physical and functional heterogeneity in cancers resulting from plasticity and changes across the EMP spectrum make it essential to study the E/M hybrid phenotype in cancer studies, for which model systems including cell line systems, preclinical models (e.g. syngeneic mouse cancers), and patient-derived xenografts (PDX) have been employed. For example, tumours arising in a mouse model of metastatic castration-resistant prostate cancer (mCRPC), in which the PI3K/AKT and RAS/MAPK pathways are co-activated in the prostate epithelium, exhibit EMT as detected by a GFP reporter driven off the vimentin promoter [Ruscetti et al., 2016]. Three sub-populations of cells were identified: epithelial ($EpCAM^{+}Vim^{-}$), E/M hybrid ($EpCAM^{+}Vim^{+}$), and mesenchymal ($EpCAM–Vim^{+}$). When cultured, the isolated epithelial and mesenchymal cells remained in their initial cell state, while the E/M hybrid tumour cells exhibited plasticity, transitioning into either epithelial or mesenchymal states within 24 hrs of plating. It has been shown that EMP state subpopulations isolated from systems with inherent EMP tend to revert to a stable equilibrium, as seen with EpCAM-low subpopulations of MMTV-PyMT [Beerling et al., 2016] and PMC42-LA [Bhatia et al., 2019] cells, and either epithelial ($EpCAM^{high}$) or mesenchymal ($EpCAM^{low}$) subpopulations of the HCC-38 human breast cancer cell line [Yamamoto et al., 2017], all of which revert to a tightly controlled epithelial: mesenchymal ratio [Beerling et al., 2016]. In SCC9 cells, $pEMT^{high}$ and $pEMT^{low}$ subpopulations remained distinct at 4 hours and 24 hours of culture after cell sorting, but resembled parental SCC9 cells after 4 days [Puram et al., 2017]. In HNSCC cells, segregated subpopulations returned to unsorted cell proportions over time, with a more rapid plasticity seen in E/M hybrid

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subpopulations [Pastushenko et al., 2018a]. These data comprehensively suggest that complex dynamics exists amongst the subpopulations of EMP-positive carcinoma cells, supporting the hypothesis of stable E/M hybrid states.

Indeed, evaluation of cancer cell lines in vitro has revealed E/M hybrid states in lung, breast, colorectal, ovarian, prostate and renal cancer cells [Hendrix et al., 1997, Huang et al., 2013a, Bronsert et al., 2014, Andriani et al., 2016, Bierie et al., 2017b, George et al., 2017]. E/M hybrid phenotypes have also been observed in PDX models of human lung, breast and colorectal cancer. In the context of PDX models, serial passages allow the human cancer stroma to be replaced with that of mouse, which provides advantages in clearly distinguishing the changes in the epithelial markers [Chao et al., 2017, Pastushenko et al., 2018b, Mizukoshi et al., 2020]. Aiello *et al*, 2018, using a lineage-labelling strategy in the KPCY mouse model of pancreatic ductal adenocarcinoma (PDAC), reported that pEMT cells were more predominant/proficient in generating epithelial cells compared to cells from tumours that had undergone full EMT [Aiello et al., 2018a]. A more comprehensive picture of EMT spectrum states is provided by the study from Pastushenko *et al* (2018), where they investigated the intermediate states using combinations of the CD106, CD51, and CD61 biomarkers, and found enhanced metastatic competence in relatively stable E/M hybrid states that retained more epithelial features. Further transplantation assays, scRNA seq and methylome analysis from the subpopulations revealed the diversity in EMT features, tumour seeding, dissemination and metastatic capability [Pastushenko et al., 2018b].

E/M hybrid circulating tumour cells

Circulating tumour cells (CTCs) represent a window into the metastatic process and the numbers of CTCs correlate strongly with prognosis and therapy response [Saxena et al., 2019, Burr et al., 2020]. The evidence of E/M hybrid phenotype or partial EMT states from CTC and CTC clusters as part of liquid biopsy assays is gaining hold in terms of their metastatic competence, stemness and therapeutic resistance. The existence of E/M hybrid phenotype has been observed in CTCs of clinical cancer patients at advanced stages [Theodoropoulos et al., 2010, Armstrong et al., 2011]. The E/M hybrid state has been particularly evident in CTCs, in part due to the utilisation of epithelial markers to isolate CTCs from a large background of non-epithelial blood cells, and the subsequent analysis of mesenchymal markers using various methods. Pioneering studies by Yu *et al* (2013), from the Haber lab, employed RNA *in situ* hybridisation of epithelial versus mesenchymal pools of mRNA to show that E/M hybrid EMP states were more common than fully epithelial or mesenchymal states [Yu et al., 2013]. Interestingly, the CTC clusters with E/M hybrid phenotype undergo collective migration, in which cells migrate with retained contact junctions. The leading cells showed increased mesenchymal

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characteristics and actin-mediated mobility, while central cells within the cluster maintained polarity and intercellular junctions, and migrated along with the traction forces generated by leader cells [Revenu and Gilmour, 2009, Aceto et al., 2014, Aceto et al., 2015, Jolly et al., 2015]. These clusters of cancer cells ready to disseminate are often reported in the invasive front of tumours [Yang et al., 2008]. Numerous studies have since confirmed such E/M hybrid enrichment in CTCs (reviewed in [Francart et al., 2018, Saxena et al., 2019]), and CTC phenotype analysis has shown an association between E/M hybrid CTCs and survival of PDAC patients [Sun et al., 2019]. These findings are not universal; a similar study using the CanPatrol RNA-ISH methodology in colorectal cancers found only mesenchymal CTCs to associate with shorter progression free survival, while both total CTCs and mesenchymal CTCs were significantly associated with unfavourable overall survival [Hou et al., 2020]. Nonetheless, CTCs are an abundant source of E/M hybrid cells with strong overall clinical implications.

As detailed more thoroughly in the next section, the last five years has seen a surge in single-cell RNA-sequencing technologies, which more precisely identify the partial EMT or E/M hybrid states from cancer datasets [Sarioglu et al., 2015]. Dong *et al,* 2018 reported the presence of E/M hybrid states in scRNA-seq datasets of primary breast cancer and lung adenocarcinoma patient-derived xenografts [Dong et al., 2018]. Puram *et al,* 2017 compared the scRNA-seq data from the primary and metastatic head and neck squamous cell carcinomas (HNSCCs) and identified a subset of malignant cells with a partial EMT signature [Puram et al., 2017]. *In situ*, these E/M hybrid cells were seen at the leading edge of tumours with cancer-associated fibroblasts. In addition, the E/M hybrid signature correlated with a malignant basal HNSCC subtype and lymph node metastasis, signifying that partial EMT promotes loco-regional invasion [Puram et al., 2017]. The advent of more combinatorial single cell approaches utilising NGS and mass cytometry are beginning to wholly portray integrated genome, transcriptome, DNA methylome [Barros-Silva et al., 2018], and proteome [Abouleila et al., 2019] information from individual cancer cells in relation to their EMT phenotype state [Dey et al., 2015, Macaulay et al., 2017]. It is indeed an exciting time for the E/M hybrid state, attested by these numerous excellent studies and the growing armamentarium of technologies, analytical tools and clinical implications.

Clinical utilisation of the E/M hybrid state

A number of studies have associated evidence of mesenchymal change (gain of mesenchymal markers and/or aberrant epithelial markers) in the tumour parenchyma with poor outcomes, especially prevalent in basal-like breast cancers, many of which are 'triple-negative breast cancers' lacking all 3 predictive receptors (ER, PR, HER2) [Thomas et al., 1999, Sarrio et al., 2008, Jeong et al., 2012, Karihtala et al., 2013]. The unique nature of the E/M hybrid state

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provides opportunity in assessing EMP from a prognostic / predictive clinical perspective, in terms of both malignant potential and therapy resistance. Advantages of the focus on the E/M hybrid state are two-fold; (i) evidence emerging as described above that the E/M hybrid cells associate better with carcinoma progression than frankly mesenchymal cells, and (ii) the increased certainty that the cells being identified are indeed carcinoma cells exhibiting EMP, since such bi-phenotypic cells are quite restricted outside of malignancy, unlike the abundance of cells exhibiting a mesenchymal phenotype, such as carcinoma-associated fibroblasts, endothelial cells, various immune cells. Several studies have identified basal-like breast cancers co-expressing classical epithelial (e.g. cytokeratin 5/6) and mesenchymal markers (e.g, vimentin) [Thomas et al., 1999, Cakir et al., 2012], and such co-expression has also been seen in colon cancer [Meyer et al., 2019], high grade serous ovarian cancer (HGSOC; [Gonzalez et al., 2018]), and lung carcinoma by novel single cell techniques such as DepArray and CyToff, respectively. High resolution, sub-cellular microscopy has been used to assess E/M hybrid state in a variety of tumours.

Immunohistochemical analysis of co-expression of epithelial and mesenchymal markers provided a more specific assessment of the E/M hybrid phenotype. Such an approach allowed us to identify a trend towards associating co-expression of cytokeratin (CK)-19 and vimentin in cells located in the dense connective tissue stroma with poorer overall survival [Raviraj et al., 2012]. A similar scenario of tumour budding has been reported in the stroma of colon carcinoma and shown to comprise partial EMT [Brabletz et al., 1998, Grigore et al., 2016, Meyer et al., 2019]. Grosse-Wilde *et al*, 2015 [Grosse-Wilde et al., 2015] hypothesised that stemness was associated with E/M hybrid cells, and employed E, M and E/M signatures derived from stable, clonal HMLER sublines, and single cell qPCR analysis to link a mixed EM signature with stemness in i) individual cells, ii) luminal and basal cell lines, iii) in vivo xenograft mouse models, and iv) in all breast cancer subtypes. Co-expression of E and M signatures was associated with poorest outcome in luminal and basal breast cancer patients as well as with enrichment for stem-like cells in both E and M breast cell-lines. Importantly, the prognostic potential of the shared E/M signatures could be related to both intercellular (E:M) cooperativity, as well as enhanced malignancy of the E/M hybrid cells, each of which were proposed as novel therapeutic targets independent of breast cancer-subtype. As detailed in the Modeling section below, an inferential model built on NCI-60 cell line gene

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expression data screened multiple E/M hybrid predictors against TCGA data across multiple tumour types [George et al., 2017]. The VIM:CDH1 gene expression ratio combined with the expression of CLDN7 provided the best assignment of various tumour cells into three phenotypes — epithelial, E/M hybrid and mesenchymal. Using this approach, metastatic breast tumours could be categorized as either having an epithelial or E/M hybrid phenotype, with the E/M hybrid score, which has tumour type-specific predictive power.

Several recent studies have further demonstrated potential prognostic significance of the E/M hybrid state with various methodologies. Gonzalez et al, 2018, identified clades of E-Cadherin / vimentin co-positive cells in HGOSC, which correlated with metastatic trajectory, and provided a link to a rarer subset of vimentin/cMyc/HE4 co-positive cells that correlated with poor prognosis [Gonzalez et al., 2018]. Karacosta *et al*, 2019 used mass cytometry time-course analysis in experimental systems to resolve lung cancer EMT and MET states [Karacosta et al., 2019b]. They constructed a lung cancer reference map of EMT and MET states referred to as the EMT-MET PHENOtypic STAte MaP (PHENOSTAMP), allowing them to characterise the EMT-MET profile of clinical lung cancers with single-cell resolution. They identified 3 epithelial states and 3 partial EMT states, one mesenchymal state and one MET state, and identified a 'continuous sweep' of these states when assessing clinical NSCLC samples, which resonated with different NSCLC cell lines. A further study [Navas et al., 2020] used high-resolution digital microscopic immunofluorescence analysis (IFA) of β-catenin to quantitate and colocalize E-cadherin and vimentin at subcellular resolution, in an assay they dubbed EMT-IFA. EMT-IFA analysis of core-needle biopsies from various advanced metastatic carcinomas identified E/M hybrid cells, the proportions of which were affected by different carcinoma-specific therapies. The EMT-IFA was proposed as a method for clinical monitoring of tumor adaptation to therapy. Most recently, Godin *et al*, 2020 reported a sequential chromogenic immunohistochemical multiplex (SCIM) technique to quantify E/M hybrid status through pan-CK and VIM analysis in urothelial carcinomas, in relation with cancer aggressiveness [Godin et al., 2020]. Using this methodology, they demonstrated E/M hybrid phenotypes in urothelial carcinomas at the time of diagnosis, and found them to be strongly associated with poor prognosis independent of standard clinicopathological features. Although presence of the E/M hybrid was associated with worse overall and disease-free survival, quantitation of the E/M hybrid did not offer any stratification.

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Collectively, the E/M hybrid state appears to offer considerable promise in terms of being (i) a somewhat unique state for which specific assays can be developed using different technologies and (ii) a state with prognostic potential, and also the potential to monitor therapy response, which ultimately may possibly be predictive of therapy response.

2. Computational approaches to reveal regulatory mechanisms underpinning epithelial-mesenchymal plasticity and the E/M hybrid phenotype

Deriving signatures with DE

Plastic phenotypes are often quantified using a workflow that combines a perturbation experiment *in vitro* with a differential expression analysis. To generate an EMT gene expression signature, an EMT program is stimulated using an EMT inducer such as TGFβ [Taube et al., 2010, Du et al., 2016, Cursons et al., 2018] or EGF [Cursons et al., 2015] to enable the identification of a group of genes that are differentially expressed in response to the stimulus, and in correspondence with morphological changes. These differentially expressed genes describe a stimulus-specific gene set for EMT, which can also be separated into "epithelial" and "mesenchymal" components representing the up- and down- regulated genes. Along these lines, Figure 2 displays Cancer Cell Line Encyclopedia (CCLE) breast cancer cell lines and The Cancer Genome Atlas (TCGA) tumour samples across an epithelial-mesenchymal landscape. The landscape shows that Basal and Claudin-low cell lines have lower epithelial scores but higher mesenchymal scores, while the luminal cell lines are less mesenchymal and more epithelial. We would expect that samples expressing an E/M hybrid phenotype would be found in the top right quadrant, with both a high mesenchymal and high epithelial score. Importantly, many Basal and even some of the more fully mesenchymal Claudin-low cell lines exhibit a E/M hybrid transcriptome, as do the vast majority of tumours.

Most experimental studies reviewed here can be used to derive epithelial and mesenchymal gene signatures, however very few have developed gene signatures specific to the E/M hybrid state. Some approximation of this has been made by combining "epithelial" and "mesenchymal" components into one signature. Grosse-Wilde *et al*, 2015 produced a E/M hybrid signature by combining 30 of each of the most extremely expressed genes in the E and M signatures from clonal HMLER human breast cancer cells, and found it to predict

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poor overall and relapse-free survival in both luminal and basal breast cancer subtypes [Grosse-Wilde et al., 2015]. They found considerable overlap between the HMLER EM signature and a Lung E/M signature also compiled from combining “epithelial” and “mesenchymal” components derived from a microarray dataset of 93 lung cancer cell lines [Loboda et al., 2011]. This Lung E/M signature was found to best differentiate two major unsupervised subpopulations of colon cancers [Loboda et al., 2011], and also associated with poor overall survival in both basal and luminal subtypes of breast cancer. These studies indicated that co-expression of epithelial and mesenchymal markers derived from multiple systems had prognostic value in breast cancer. Signatures from scRNA-seq are discussed below.

Methods for applying signatures

The higher-level biological information captured by gene expression signatures can be mapped onto new datasets using gene set analysis methods, revealing the extent to which they express similar molecular phenotypes. Gene set analysis methods (reviewed and benchmarked recently by Geistlinger and colleagues [Geistlinger et al., 2020]) can assess specific hypotheses, such as “Does this group of samples express an EMT program?”, or competitively test a group of gene signatures i.e. “Which molecular program is upregulated in these samples?”. When applied to differential analysis workflows, EMT gene expression signatures can evaluate the differential enrichment for an EMT program between two groups of samples. Alternatively, single-sample gene set analysis methods such as *ssGSEA* [Barbie et al., 2009] or *Singscore* [Foroutan et al., 2018, Bhuva et al., 2020] can be used to identify individual samples that are concordant with gene expression signatures, and estimate the EMP phenotype of a single sample.

Alternative scoring of EMT phenotypes

Similarly, various scoring methods have been developed for the quantification of EMT phenotypes in samples independent of a differential expression workflow. These approaches provide scores along a continuous phenotype based on the weighted sum [Byers et al., 2013, Guo et al., 2019] or distribution of gene expression values [Tan et al., 2014, Chakraborty et al., 2020a] for genes that are pro-mesenchymal or pro-epithelial. To

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place samples on a spectrum inclusive of an E/M hybrid state, a multinomial logistic regression (MLR)-based method was developed using a small number of key regulators to predict a score between 0 and 2 (epithelial to mesenchymal), where a middle value of 1 indicates the E/M hybrid state [Chakraborty et al., 2020a] . Other approaches [Tan et al., 2014, Chakraborty et al., 2020a] assume that the epithelial and mesenchymal states are at two ends of a continuum, and that E/M hybrid states are those that occur between the two extremes. Maximum variability in terms of EMT is associated with increased E/M hybrid signatures [Chakraborty et al., 2020b]. Further, the multinomial logistic regression (MLR)-based metric was suggested to have advantages over the 76 gene signature score and the Kolmogoriv-Smirnov test, as it was capable of distinguishing between the “pure” individual E/M hybrid rather than mixtures of epithelial and mesenchymal cells and uses a small number of predictors to calculate the E/M score.

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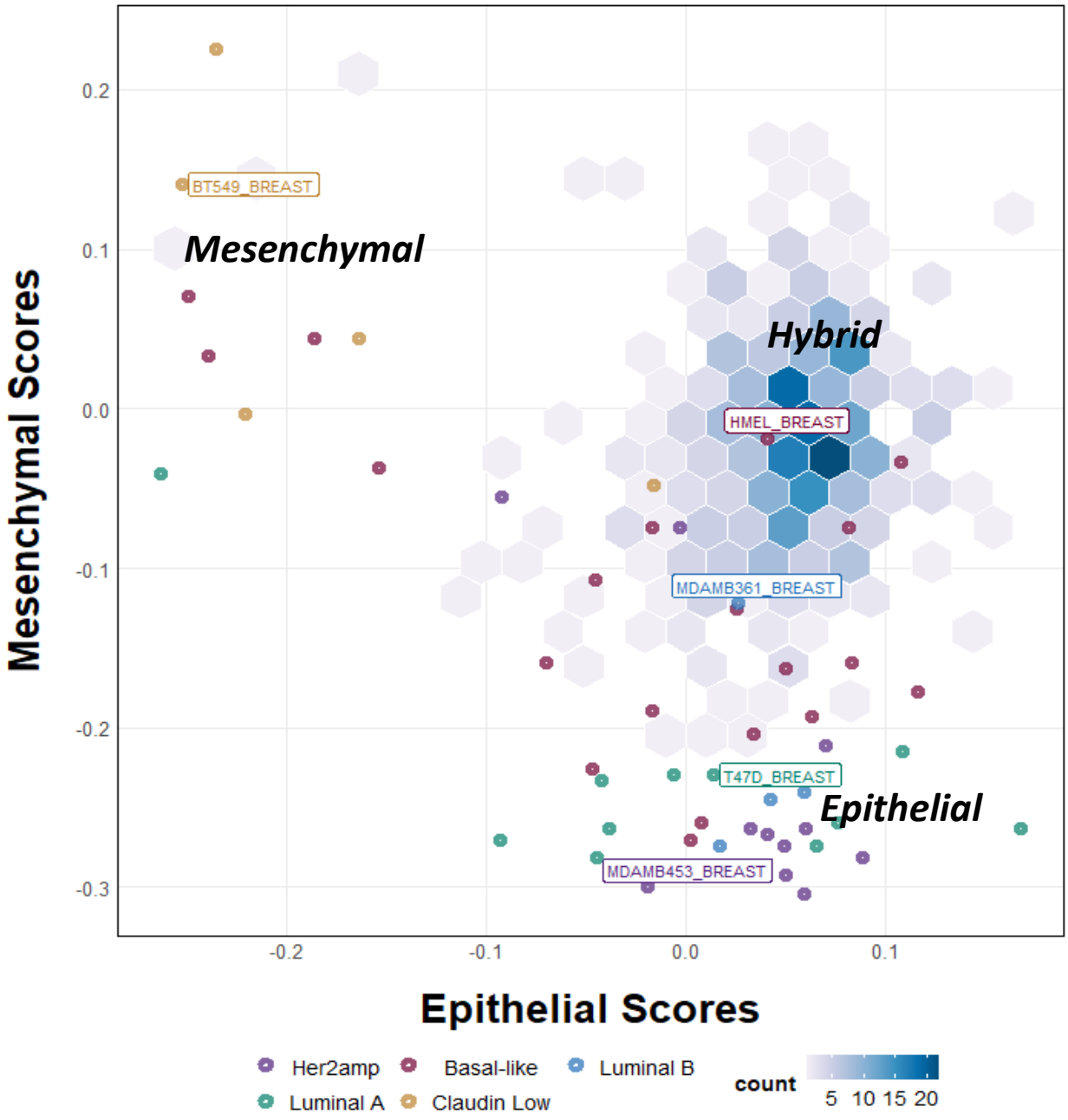
Figure 2: An epithelial-mesenchymal landscape. *Epithelial and mesenchymal scores for the CCLE breast cancer cell lines (circle markers, coloured by subtype) overlaying a landscape of breast cancer tumour sample scores from TCGA (hexbin density plot) [Barretina et al., 2012, Cancer Genome Atlas, 2012]. Epithelial and mesenchymal signatures were obtained from Tan et al. for both cell lines and tumour samples separately [Tan et al., 2014]. Scores were calculated using singscore and ranked using 5 stable genes [Foroutan et al., 2018, Bhuva et al., 2020].*



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Gene set scoring in single-cell data

Single cell RNA sequencing (scRNA-seq) offers a powerful tool to identify epithelial, mesenchymal and E/M hybrid states in mixed cell populations. Gene set testing methods to characterise molecular phenotypes are well established for bulk analyses [Khatri et al., 2012, Geistlinger et al., 2020], however the knowledgebases and gene signature databases that were developed for bulk sequencing may not be appropriate for the application to single-cell data. Instead, to phenotypically characterise groups of cells in scRNA-seq data, various reference-based cell type annotation methods have been developed. These methods bypass comparison to bulk-derived gene sets by projecting individual cells into a single-cell reference datasets using similarity metrics such as cosine similarity [Kiselev et al., 2018, Srivastava et al., 2018] or Spearman's correlation [Aran et al., 2019], or by applying transfer learning [Lieberman et al., 2018]. However, many of these approaches are dependent upon clustering performance, which introduces a selection bias [Zhang et al., 2019], and may not necessarily infer cell states [Chen et al., 2019]. Alternative methods, such as marker-based phenotyping, rely on existing knowledge of gene expression garnered from protein measurement or bulk RNA sequencing. Additionally, marker-based approaches may not be appropriate for quantifying E/M hybrid phenotypes due to the problem of drop out in scRNA-seq data, where lowly expressed genes may be undetected and falsely quantified as 'unexpressed' [Stegle et al., 2015].

Mapping to references in single-cell data

Lahnemann *et al*, 2020 has recently reviewed single cell "mapping" or cell type annotation [Lähnemann et al., 2020], where the authors argue in favour of reference-based methods. Here, unsupervised clustering followed by cell type annotation is considered "reference-free". The marker genes used in these "reference-free" methods are found on a correlative basis, where the functional relevance of the genes in specific phenotypes is not guaranteed [Yuan et al., 2017]. As discussed by Lahnemann *et al*, we need to enable quantification of the uncertainty in mapping cell type or cell state, however, this requires more sophisticated statistical methods. Although pseudo-bulk analyses [Lun et al., 2016] enable the application of the methods established for bulk sequencing [Tung et al., 2017, Crowell et al., 2019],

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these generalise large, heterogeneous populations that could otherwise reveal cell-to-cell variation and novel phenotypes.

Single-sample gene set testing in single cell data

Single sample gene set testing methods have been developed for bulk analyses that provide continuous phenotype scores for individual samples. If applied to scRNA-seq data these methods would phenotypically characterise individual cells, independent of clustering. However, previously developed bulk methods do not address transcript drop out and consequential sparsity of single cell data [Hicks et al., 2018]. Imputation methods have been developed [Lähnemann et al., 2020] but can introduce errors in downstream analyses [Andrews and Hemberg, 2018]. Current single sample methods cannot be directly applied to single-cell data, also due to the nuances of scRNA-seq such as the synchronisation of cell cycle stages [Stegle et al., 2015, Chen et al., 2019]. To overcome these challenges many single-cell specific enrichment methods are starting to emerge.

A handful of single-cell single-sample gene set testing methods have been developed that model the count overdispersion and technical noise observed in single-cell data, such as *PAGODA* [Fan et al., 2016] or *VAM* [Frost, 2020]. Alternatively, *Vision* calculates signature scores by summing the expression of "up" genes minus the expression of "down" genes, then standardising based on the cell's expression [DeTomaso et al., 2019] . Although *Vision* does not seem to penalise or account for gene drop out, the aggregate expression score avoids the per-gene technical noise problem. These methods have attempted to adjust gene set testing for scRNA-seq data, however, no benchmarking or comparison between methods has been performed. There is work being done to identify cell-type specific drop out patterns [Qiu, 2020], suggesting that drop out perhaps cannot be modelled during cell type discovery, and that we need an approach for characterising phenotype that is independent of drop out patterns.

Gene expression signature resources and the Hallmarks gene set

Gene expression signatures for a range of molecular phenotypes are available through the C2 collection of gene sets in the Molecular Signature Database (MSigDB) [Liberzon et al., 2015], which contain thousands of experimentally-curated gene sets from the literature.

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Knowledgebases such as the Gene Ontology [Ashburner et al., 2000, Consortium., 2019], KEGG [Kanehisa and Goto, 2000] or Reactome [Fabregat et al., 2018] are also available through MSigDB and can provide sets of genes associated with particular pathways or molecular programs. To summarise the large number of gene sets available in MSigDB and reduce redundancy, Liberzon *et al*, 2015 performed consensus clustering on > 8,000 gene sets from the C1-C6 collections to identify 50 clusters of gene sets [Liberzon et al., 2015] . The authors assigned biological themes to each cluster, then curated and refined clusters to produce 50 "Hallmark" gene sets. This includes a 200-gene EMT signature that is representative of 107 individual EMT-related gene sets in MSigDB, including experimentally-derived expression signatures as well as pathways from knowledgebases.

Curated EMT gene signatures

Other EMT gene signatures have been defined using different meta-analysis methods and curation approaches [Groger et al., 2012, Tan et al., 2014, Foroutan et al., 2017]. For example, Foroutan *et al* used two meta-analysis techniques to obtain a stimulus-specific EMT signature using 10 cell line microarray datasets that provided evidence for TGFβ-induced EMT across a range of cell lines [Foroutan et al., 2017]. The signature was then used to assess cell lines from CCLE and patient samples from TCGA for evidence of a TGFβ-driven EMT. Byers *et al* [Byers et al., 2013] curated a 76 gene EMT signature using a lung cancer cell line panel based on gene-gene correlations with one of four key EMT genes (CDH1, VIM, FN1 and CDH2). Byers validated their expression signature on independent datasets where their signature successfully identified cell lines that had undergone EMT. To obtain a directional cancer-specific EMT signature (with epithelial and mesenchymal components), Tan et al stratified pan-cancer cell lines and patients according to known epithelial and mesenchymal markers [Tan et al., 2014]. By applying single-sample gene set enrichment using EMT gene sets from MSigDB, the authors identified EMT signatures that were associated with the most epithelial and most mesenchymal samples.

Need for new gene expression signatures

Many of these EMT gene signatures have been derived from microarray datasets and may not best represent the data we now obtain from modern RNA sequencing platforms [Wang

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et al., 2014]. High-throughput RNA-sequencing of FACS sorted populations [Fustaino et al., 2017], or even new single-cell sequencing technologies, offer an opportunity for new EMT signatures to be derived. These experiments may better represent the datasets currently being used for EMT scoring, and more accurately reflect the true biology. For example, McFaline-Figueroa and colleagues used single-cell sequencing to demonstrate regulatory checkpoints along the EMT continuum [McFaline-Figueroa et al., 2019]. The authors were able to spatially segregate cells that had undergone EMT from epithelial colonies, and by applying their method *Monocle2,* were able to align cells along a "pseudospatial" trajectory between these two states. Single-cell RNA sequencing experiments enable a finer characterisation of phenotypes and can reveal expression patterns of cells along an EMT continuum, making them suitable for deriving gene expression signatures in rarer, novel states such as the E/M hybrid state, and potentiallly in stable intermediates.

Single-cell sequencing lacks the methodology for signature derivation

Method development for single cell data analysis remains an active area of research, where identifying expression patterns in individual cells has been particularly challenging due to the low number of genes detectable per cell [Hicks et al., 2018]. Consequently, there are no established workflows for deriving gene signatures from single cell data. Using scRNA-seq approaches, E/M hybrid states have been identified based on the presence of both pro-epithelial and pro-mesenchymal markers in skin cancer [Dong et al., 2018], ovarian cancer [Gonzalez et al., 2018], HNSCC [Puram et al., 2017, Karacosta et al., 2019b], and lung, mammary and skin cancer [Pastushenko et al., 2018c]. Despite observations of E/M hybrid states, or partial EMT programs, there is a lack of gene expression signatures for this phenotype.

To instead identify expression signatures of cells in an unsupervised way, Puram *et al* applied non-negative matrix factorisiation (NMF) clustering to reveal a partial EMT gene expression program that expressed both ECM genes and EMT markers in a subset of cells [Puram et al., 2017]. To establish the independence of this partial EMT signature, the authors confirmed the lack of correlation with known EMT signatures and used a highly expressed marker from this signature, TGFβ-induced (TGFBI), to sort SCC9 cells into $pEMT^{high}$ and $pEMT^{low}$ cells and validate the findings. The cells that expressed the partial EMT

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signature were more invasive, but less proliferative, and were found using immunohistochemistry analysis for the top 10 genes (plus the p63 HNSCC marker) near the leading edge of the tumour adjacent to cancer associated fibroblast (CAF) populations.

Single cell sequencing offers the opportunity to characterise the heterogeneity of EMP and to uncover the particular molecular signatures that regulate the E/M hybrid state. Deriving robust signatures for the E/M hybrid state requires analysis methods that are sensitive to drop out and can address the challenge of mapping between bulk and single-cell data. To ensure the quality and reproducibility of both the signature-derivation methods and gene expression signatures, it is of importance that these are accessible and well communicated, for them to be eventually benchmarked. The application of single cell-derived signatures to bulk-sequencing data will also need to be validated and be able to distinguish E/M hybrid subpopulations from heterogeneous epithelial and mesenchymal populations.

Methods relating to phenotype quantification in single cell

Name	Type	Method	Single Sample	Reference
SingleR	Cell type annotation	Cluster reference + Spearman's correlation	Y	[Aran et al., 2019]
CaSTLe	Cell type annotation	Transfer learning between dataset and reference	N?	[Lieberman et al., 2018]
Cell BLAST	Cell type annotation	Autoencoder	Y	[Cao et al., 2019]
cellHarmony	Cell type annotation	Merges KNN graphs across datasets (Louvain)	Y	[DePasquale et al., 2019]
CellAtlasSearch	Cell type annotation	Cosine similarity to pre-processed reference	Y	[Srivastava et al., 2018]
CHETAH	Cell type annotation	Hierarchical classification tree *	Y	[de Kanter et al., 2019]
CellAssign	Cell type annotation	Probabalistic model	Y	[Zhang et al., 2019]
PAGODA	Gene set testing	PCA projection	Y	[Fan et al., 2016]
Vision	Signature scores	Aggregate expression	Y	[DeTomaso et al., 2019]
iDEA	DE and GSE	Uses summary statistics?	N?	[Ma et al., 2020]
LandSCENT	Cell type annotation	Define 'states' using entropy, then infer cell types of states	N	{Chen, 2019 #9527}

* Decision in branch traversal is based on expression profile correlations, where feature selection finds genes that discriminate between branches (includes "unassigned" or "intermediate" classes)

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3. Mechanism-based mathematical models to characterise signatures and implications of E/M hybrid phenotypes

Role of mathematical modelling in identifying the E/M hybrid state(s)

Mathematical models have played a major role in our understanding of the E/M hybrid state. As experiments typically tend to be performed at a single spatial scale (cell-level or population-level) it can be challenging to interpret results from these different spatial scales when processes also occur over multiple overlapping time scales. Mathematical models are a powerful tool with which to integrate the observations from different spatial and time scales, to not only interpret these experimental results, but to also generate and test new hypotheses that can be experimentally tested. This approach has proven extremely insightful in transforming the perception of EMT from a binary (all-or-none) process to a multistable process. Significantly, mathematical modelling studies were among the first to predict the existence of a stable E/M hybrid state characterised by stabilised co-expression of epithelial and mesenchymal markers [Lu et al., 2013, Tian et al., 2013]. This was later confirmed experimentally, both *in vitro* [Bao et al., 2013, Zhang et al., 2014, Pankova et al., 2016] and *in vivo* [Puram et al., 2017, Aiello et al., 2018b, Pastushenko et al., 2018b].

The two mathematical models, by Lu et al. [103] and Tian et al. [141], which initially predicted the existence of the stable E/M hybrid state, focused on signalling pathways within a single cell and built upon decades of observational and functional work by developmental and cancer biologists. Multiple signalling pathways have been implicated in EMT over many studies, but the activities of these pathways can be considered to converge to a core regulatory circuit composed of two families of transcription factors, SNAIL and ZEB, with two families of micro-RNAs, miR-34 AND miR-200, respectively [Nieto et al., 2016]. These two mathematical models used ordinary differential equations (ODEs) to characterise the dynamical behaviour of the core EMT regulatory circuit and observed that a stable E/M hybrid state, distinct from the epithelial and mesenchymal states, was possible. Further extensions to these models have identified factors to stabilise E/M hybrid phenotypes and have since been experimentally validated [Hong et al., 2015, Jolly et al., 2016]. For instance, knockdown of these “phenotypic stability factors” such as OVOL1/2 can drive E/M hybrid cells to a “complete EMT” phenotype during mammary morphogenesis [Watanabe et al.,

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2014] and in lung cancer too [Jolly et al., 2016]. These models tend to display non-linear responses and hysteresis whereby cells *en route* through EMT and MET may take different paths.

Different models of hysteretic patterns in EMT dynamics have been presented. Here, for illustrative purposes, let us consider an example presented in Figure 3 (adapted from Jolly et al. [83]) where ZEB1 mRNA levels vary in response to an inducing signal, in this case the protein SNAIL. In Figure 3A, for a proposed miR-200/ZEB/SNAIL circuit, if a cell starts in an epithelial phenotype and its endogenous SNAIL levels are increased, then it first transitions to the E/M hybrid state, and if SNAIL is further increased the cell transitions to the mesenchymal state. In the reverse direction, by decreasing SNAIL, the cell directly transitions to the epithelial state from the mesenchymal state. In Figure 3B, by including OVOL, a cell initially in the epithelial state transitions to the E/M hybrid state, and then the mesenchymal state with increasing TGFβ. When decreasing TGFβ, the cell first transitions to the E/M hybrid state and then the epithelial state. However, the path followed in both directions is still different – a phenomenon called as 'hysteresis'. Celia-Terrassa et al. [33] further demonstrated mathematically and validated experimentally by applying TGFβ treatment and then TGFβ withdrawal, that most but not all normal and tumour mammary epithelial cells exhibit hysteretic patterns in TGFβ-driven EMT. Statistically significant evidence of hysteresis is also found in TGFβ-treated lung cancer cells [Karacosta et al., 2019b].

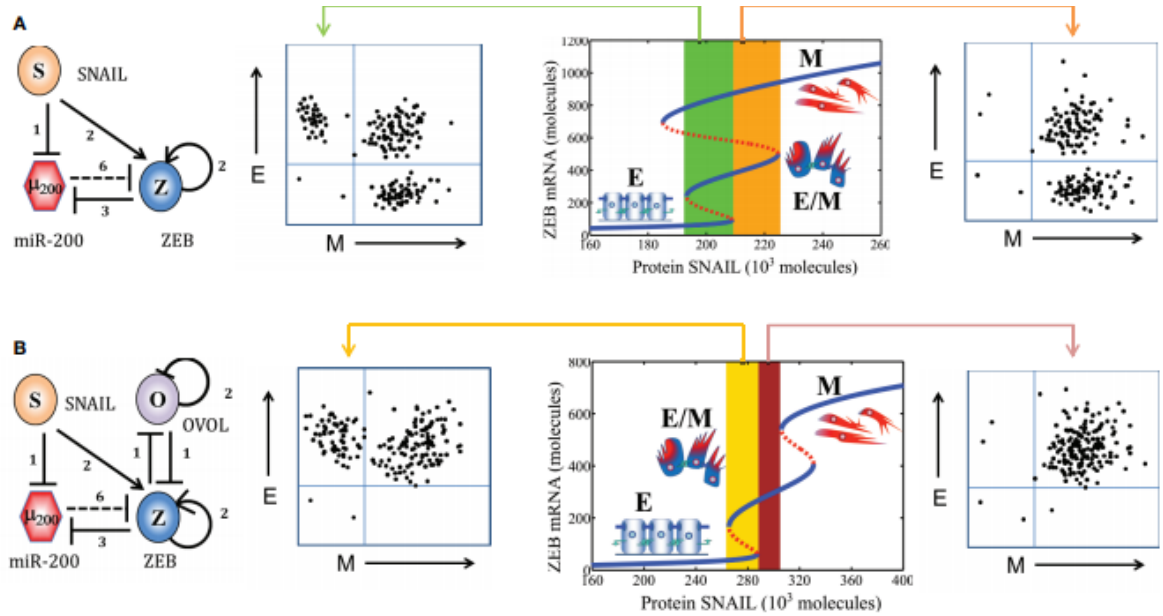

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Figure 3: Hysteresis in models of EMT. *(A) Left – miR-200/ZEB/SNAIL circuit. Middle – bifurcation of ZEB mRNA levels in response to protein SNAIL. In the green region, corresponding to a range of SNAIL, cells can be any of three states, E – epithelial, E/M hybrid, M – mesenchymal corresponding to a trimodal distribution as shown in the FACS (fluorescence-activated cell sorting) plot. For the orange region, corresponding to a different range of SNAIL, cells can either be in the E/M hybrid state or mesenchymal state corresponding to a bimodal distribution in the FACS plot. Cells with a value of SNAIL less than in the green region adopt the epithelial state. Cells with a value of SNAIL greater than the orange region adopt the mesenchymal state. (B) Left- miR-200/ZEB/SNAIL/OVOL circuit. Middle – bifurcation of ZEB mRNA levels in response to protein SNAIL. In the yellow region cells can either be in the epithelial or E/M hybrid state. In the red region cells occupy the E/M hybrid state. FACS plots are shown for both yellow and red regions. (Adapted from Figure 5 Jolly et al. [83]).*

Alternative single cell models have focused on larger networks describing the multiple signalling pathways implicated with EMT. While continuous models for large networks can provide information regarding temporal network dynamics, such as phenotypic plasticity and transition rates, it can be experimentally intractable to determine all of the relevant parameters, therefore Boolean-logic approaches, where a gene is considered either active or inactive, have been considered. Using this approach, a study by Steinway et al. [134] identified two stable fixed points corresponding to the epithelial and mesenchymal phenotypes. A subsequent study identified new fixed points that exhibit features of both epithelial and mesenchymal phenotypes thought to be associated to E/M phenotypes and identified putative targets that supress hepatocellular carcinoma EMT. *In vitro* analysis indicated many of the predictions do supress the TGFß-driven EMT, and thus are potential therapeutic targets [Steinway et al., 2015]. Later work by Font-Clos et al. [56] extended the work of Steinway et al. [134] to identify multiple E/M fixed points between the more stable epithelial and mesenchymal fixed points. Hari et al. [74] have since compared the Boolean-logic approach with *RACIPE* (Random Circuit Perturbation), where an ensemble of continuous models is generated with random chosen kinetic parameters for a given network topology and steady state solutions are clustered to identify robust dynamical features of the given network. They show that multistability correlates with the net number of positive feedback loops. In contrast to traditional approaches where nodes in a network are targeted to control EMT plasticity, Hari et al. [74] propose breaking the feedback loops by targeting their links rather than nodes as a possible route to restrict the EMT plasticity.

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Population dynamics of EMP

Experiments at the population scale have observed epithelial-mesenchymal heterogeneity where cells in the same tissue/population could be either epithelial, mesenchymal, or in an E/M hybrid state, and are able to switch between these states. Recent studies have demonstrated the ability of cells to switch states in the absence of EMT-inducing factors such as TGFß. Ruscetti et al. [127] show that an initial population of E/M hybrid cells can quickly generate a mixture of epithelial, E/M hybrid, and mesenchymal cells. Further, Bhatia et al. [18] demonstrates that an initial population consisting of either epithelial or mesenchymal cells has the ability to regain the phenotypic equilibrium of the parental population. Mathematical models at the population scale for EMT dynamics have tended to be phenomenological. Markov models, where the states of cells in the population at a future time depend only on the states on the current time, have been popular in describing experimental data [Gupta et al., 2011, Mandal et al., 2016, Risom et al., 2018, Chapman et al., 2019, Karacosta et al., 2019b]. However, these models lack integration with the core regulatory network models previously discussed. Therefore, recent work has explored how to link the well-characterised dynamics of EMT regulatory networks to experimentally observed population-level behaviour. In an example of integrating experimental data with known interaction and regulatory networks, Cursons et al. [44] used an experimental model of EMP driven by the well-known ZEB1-miR200 network [Gregory et al., 2011]. Exploring plasticity across an EMP landscape, they integrated experimental data with a known miR-mRNA interaction network and known protein interaction networks to characterise a co-targeted regulatory network regulating EMT and MET.

One approach to incorporate core regulatory networks is to consider a population of cells where each cell contains a copy of the core EMT regulatory circuit [Tripathi et al., 2020a]. The dynamics of the EMT circuit can be calculated for each cell independently to determine the phenotype: epithelial, E/M hybrid, or mesenchymal. Motivated by spontaneous EMT observed in Ruscetti et al. [127], Tripathi et al. [142] developed a model which included cell proliferation, and show that noise in partitioning of signalling pathways during division could be a possible mechanism by which epithelial-mesenchymal heterogeneity can be maintained over extended periods of time involving multiple generations and passages.

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Spatiotemporal dynamics and heterogeneity in EMP: Role of cell-cell communication and cell-ECM crosstalk

An alternative approach to link the cell-level EMT behaviour is to explicitly consider spatial structure. Early evidence of EMT in clinical samples arose from differences in spatial localisation of ß-catenin and E-cadherin across different parts of the tumour [Brabletz et al., 2001]. Related mathematical models tend to be multi-scale models where dynamics of intracellular E-cadherin and ß-catenin signalling drive cellular level properties including cell-cell adhesion. This has been explored using both an off-lattice framework [Ramis-Conde et al., 2008] and an on-lattice cellular Potts model, by integrating CompuCell3D and Bionetsolver [Andasari et al., 2012]. Both models demonstrate properties associated with EMT where cells lose cell-cell adhesion, break off from a primary tumour body, and migrate through and invade surrounding tissues as part of the metastatic cascade. Tumour budding, where numerous finger-like projections, or buds, extend from the primary tumour towards neighbouring stroma in various cancers, has also been identified as marker of invasive behaviour and a possible clinical readout of partial EMT [Bronsert et al., 2014, Grigore et al., 2016]. Other models include features associated with EMT states, such as the different survival times, migration speeds, and interactions with the environment, to develop a novel multi-organ model that explicitly accounts for EMT processes in individual cancer cells in the context of the invasion-metastasis cascade, and are able to produce biologically realistic outcomes regarding tumour shape and metastatic distribution [Franssen and Chaplain, 2019]. Bocci et al. [24], by coupling EMT and cell migration, consider one part of this process – the migration from the primary tumour - to explain individual and collective cancer cell migration. Their model robustly recapitulates circulating tumour cell cluster fractions and size distributions experimentally observed across several cancer types. They identify that mechanisms that increase the population of the E/M hybrid state, rather than a complete EMT, are required to generate large clusters of five to ten cells.

Mesenchymal states and epithelial states of breast cancer stem cells have been observed located at the tumour invasive front and more centrally, respectively [Liu et al., 2014]. By integrating the core EMT regulatory circuit into each cell in a two-dimensional hexagonal grid lattice, spatio-temporal models can be developed to explore the role of cell-cell communication, through Notch-Delta or Notch-Jagged signalling, to show that a gradient of

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EMT-inducing signal, for example TGF-beta, can recapitulate the experimentally observed spatial segregation of different EMT phenotypes [Bocci et al., 2019a]. Mechanosensing through cell-extracellular matrix crosstalk can also influence EMT through an EMT-ECM stiffness feedback loop. Stiffer substrates can results in cells becoming more mesenchymal-like [Matte et al., 2019] and EMT can alter the stiffness of the ECM [Peng et al., 2017]. Kumar et al. [95] use a system of ODEs to describe these relationships and illustrate that the scattering of a heterogeneous population depends on both the ECM density and fraction cells which have undergone EMT. By considering a cell contractility-ECM feedback loop, [Ahmadzadeh et al., 2017], in experimental results and modelling, also find that the driving force underlying EMT is directly proportional to matrix stiffness and identify intermediate ECM stiffness as being optimal for cell invasion. Further experiments by [Margaron et al., 2019] investigate the mechanical properties of single cells in different EMT states and interestingly find that the mechanical properties of E/M hybrid cells are not in between those of epithelial and mesenchymal cells. Instead, they find that E/M hybrid cells produce lower forces and as a consequence of their lower contractility move faster and have a higher invasive potential. Using these observations they identified that triple-negative breast cancer cells had E/M hybrid characteristics rather than mesenchymal characteristics. Their results suggest that the mechanical and structural aspects of the E/M hybrid state are important to understand tumour-cell dissemination.

In summary, mathematical models have served as a powerful tool to unravel new biological insights into EMT/MET, and have driven the next set of experiments, for instance, those characterising the features of stable E/M hybrid state(s). Further development of these models, with close integration and validation with experimental data, will improve understanding of stability of the E/M hybrid states, their spatial patterns, and how they contribute to disease progression.

4. Conclusions, state of play, and potential needs

Clearly there is growing evidence for a preponderance of E/M hybrid states in carcinoma systems, and that these states often are associated with more aggressive malignant / metastatic capacity. As detailed above, a growing number of computational approaches have been developed to illustrate and characterise E/M hybrid states, and theoretical prediction

and support for the stability of particular intermediates has been gleaned from mathematical modelling around some of the well characterised molecular feedback systems. The E/M hybrid state, in a carcinoma context, is somewhat unique, such that opportunities exist in the prognostic and predictive biomarker setting that have started to emerge and show promise, and new technologies are being developed to assess this. To our knowledge, whether these E/M hybrid states show specific sensitivity/resistance profiles to cancer therapies remains to be seen, however it is likely that many of the studies implicating EMT in therapy resistance have been carried out with cells that exhibit an E/M hybrid phenotype. Studies that shed further light on the reasons behind the metastatic advantages of the E/M hybrid state, their biomarker potential based on unique co-expression of epithelial and mesenchymal markers, and opportunities for specificity in therapeutic targeting, will be helpful in determining and achieving the exploitation of EMP in cancer care.

Figure Legends

Figure 1: EMP and the E/M hybrid state. Schematic representation of the Epithelial Mesenchymal Plasticity (EMP) continuum, along which E/M hybrid cells are commonly found in carcinoma tissues. Evidence is growing for the existing of stable intermediates held in check by complex molecular circuitry.

Figure 2: An epithelial-mesenchymal landscape. Epithelial and mesenchymal scores for the CCLE breast cancer cell lines (circle markers, coloured by subtype) overlaying a landscape of breast cancer tumour sample scores from TCGA (hexbin density plot) [Barretina et al., 2012, Cancer Genome Atlas, 2012]. Epithelial and mesenchymal signatures were obtained from Tan et al. for both cell lines and tumour samples separately [Tan et al., 2014]. Scores were calculated using *singscore* and ranked using 5 stable genes [Foroutan et al., 2018, Bhuva et al., 2020].

Figure 3: Hysteresis in models of EMT. (A) Left – miR-200/ZEB/SNAIL circuit. Middle – bifurcation of ZEB mRNA levels in response to protein SNAIL. In the green region, corresponding to a range of SNAIL, cells can be any of three states, E – epithelial, E/M hybrid, M – mesenchymal corresponding to a trimodal distribution as shown in the FACS (fluorescence-activated cell sorting) plot. For the orange region, corresponding to a

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Acknowledgements:

MKJ and EWT were supported by a SPARC grant awarded by MHRD, Government of India (SPARC/2018-2019/P303/SL). MEL and EWT received foundational support from the National Breast Cancer Foundation (CG-10-04). MJD is funded by Cancer Council Victoria APP1187825, Cure Brain Cancer and the National Breast Cancer Foundation (CBCNBCF-19-009), and the Betty Smyth Centenary Fellowship in Bioinformatics. SB was supported by a QUT Postgraduate Research Award scholarship.

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