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# Hybrid epithelial/mesenchymal phenotypes promote metastasis and therapy resistance across carcinomas

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ABSTRACT

Cancer metastasis and therapy resistance are the major unsolved clinical challenges, and account for nearly all cancer-related deaths. Both metastasis and therapy resistance are fueled by epithelial plasticity, the reversible phenotypic transitions between epithelial and mesenchymal phenotypes, including epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET). EMT and MET have been largely considered as binary processes, where cells detach from the primary tumor as individual units with many, if not all, traits of a mesenchymal cell (EMT) and then convert back to being epithelial (MET). However, recent studies have demonstrated that cells can metastasize in ways alternative to traditional EMT paradigm; for example, they can detach as clusters, and/or occupy one or more stable hybrid epithelial/mesenchymal (E/M) phenotypes that can be the end point of a transition. Such hybrid E/M cells can integrate various epithelial and mesenchymal traits and markers, facilitating collective cell migration. Furthermore, these hybrid E/M cells may possess higher tumor-initiation and metastatic potential as compared to cells on either end of the EMT spectrum. Here, we review *in silico*, *in vitro*, *in vivo* and clinical evidence for the existence of one or more hybrid E/M phenotype(s) in multiple carcinomas, and discuss their implications in tumor-initiation, tumor relapse, therapy resistance, and metastasis. Together, these studies drive the emerging notion that cells in a hybrid E/M phenotype may occupy 'metastatic sweet spot' in multiple subtypes of carcinomas, and pathways linked to this (these) hybrid E/M state (s) may be relevant as prognostic biomarkers as well as a promising therapeutic targets.



**Contents**



*Abbreviations:* CTC, Circulating Tumor Cell; EMT, Epithelial-Mesenchymal Transition; MET, Mesenchymal-Epithelial Transition; TGFβ, Transforming growth factor beta; EpCAM, Epithelial cell adhesion molecule; EMT-TFs, EMT-inducing transcription factors; E/M, epithelial/mesenchymal; IBC, Inflammatory breast cancer; SCLC, Small cell lung cancer; NSCLC, Non-small cell lung cancer; HGSOC, High grade serous ovarian carcinoma; FACS, fluorescence activated cell sorting; SCC, squamous cell carcinoma; PSF, phenotypic stability factor; CRPC, castration-resistant prostate cancer; PDAC, Pancreatic ductal adenocarcinoma; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; FSP1, Fibroblast Specific Protein 1.

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## 1. Introduction

The epithelial-mesenchymal transition (EMT) is a fundamental cell biological process implicated in embryonic development, tissue repair, and cancer progression. EMT comprises a compendium of intricately interconnected molecular (biochemical) and morphological (biophysical) changes such as decreased cell-cell adhesion due to breakdown of cell-cell junctions, reorganization of apico-basolateral polarity into front-rear polarity, and emergence of migratory and invasive traits usually associated with a mesenchymal phenotype (Savagner, 2015). Initially characterized as a developmental biology phenomenon (Greenburg & Hay, 1982; Hay, 1995), EMT soon caught the attention of cancer biologists and was proposed as a key mechanism that can facilitate the invasion of tumor cells through the basement membrane and their entry into the circulation to drive metastatic dissemination (Thiery, 2002). A subset of these circulating tumor cells (CTCs) can survive the invasion-metastatic cascade and undergo a mesenchymal-epithelial transition (MET) – the reverse of EMT – to colonize distant organs (Kalluri & Weinberg, 2009; Thiery, Acloque, Huang, & Nieto, 2009). EMT has been largely viewed as a binary process, such that the final cell state was viewed as an individually migrating cell with many, although possibly not all, characteristics of mesenchymal lineage such as fibroblasts (Arnoux, Côme, Kusewitt, Hudson, & Savagner, 2005; Chaffer & Weinberg, 2011; Kalluri & Weinberg, 2009; Lee, Dedhar, Kalluri, & Thompson, 2006; Scheel & Weinberg, 2012; Thiery, 2002; Thiery et al., 2009; Thiery & Sleeman, 2006). Thus, any partial state (s) were considered to be 'metastable' or transient snapshots encountered during EMT. However, recent *in silico*, *in vitro* and *in vivo* studies have demonstrated that cancer cells may stably acquire one or more hybrid epithelial/mesenchymal (E/M) phenotype(s) that can display a mixture of epithelial and mesenchymal traits at molecular and/or morphological levels (Burger, Danen, & Beltman, 2017; Jolly et al., 2015a; Jolly & Levine, 2017; Nieto, Huang, Jackson, & Thiery, 2016). In other words, it is possible in many cases that one or more hybrid E/M phenotype(s) can be a stable endpoint of the transition (Jolly et al., 2018a). This more nuanced view of EMT is more commonly referred to as epithelial plasticity or epithelial-mesenchymal plasticity (Bhatia, Monkman, Toh, Nagaraj, & Thompson, 2017; Nieto et al., 2016).

Interestingly, cells co-expressing epithelial and mesenchymal markers have been observed across multiple cancer subtypes in cancer cell lines, primary tumors, animal models, individual CTCs, clusters of CTCs, and metastases (Jolly, Boareto, Huang, et al., 2015a). This hybrid E/M state exhibits properties reminiscent of cellular behavior during branching morphogenesis in multiple organs and wound healing (Bhatia et al., 2017; Jolly, Boareto, Huang, et al., 2015a; Jolly, Ward, et al., 2018a; Micalizzi, Farabaugh, & Ford, 2010; Nieto et al., 2016; Revenu & Gilmour, 2009). Whether this hybrid E/M state represents a stable phenotype or is simply a snapshot in time of a cell transitioning from purely epithelial to purely mesenchymal remains a challenging question to answer, and it is very likely to be context-dependent. Part of the challenge is measuring and characterizing simultaneously a large set of genes and phenotypes (e.g. migration, invasion etc.) at multiple time-points and at a single-cell level. This lack of dynamic, single-cell data obscures the underlying nonlinear dynamics of EMT and the variability in the execution of this program across cells in a clonal population. Thus, we are restricted to rely on a few molecular and morphological traits to discern whether cells are undergoing a full EMT or attaining hybrid E/M phenotype(s). However, recent studies, discussed later in this review, have made progress on this question and enabled the experimental identification of many distinct hybrid E/M states (Biddle, Gammon, Liang, Costea, & Mackenzie, 2016; Grosse-Wilde et al., 2015; Jolly et al., 2016; Pastushenko et al., 2018; Ruscetti et al., 2016).

Another challenge is defining the spectrum of hybrid E/M phenotype (s). EMT is a multi-dimensional process with changes not only in the genomic, epigenomic and morphological aspects described above, but also in a host of other cellular traits, such as metabolism, epigenetics, proliferative index, immune evasion, and tumor-initiation potential (Li & Li, 2015) (Fig. 1A). Thus, different hybrid E/M phenotypes may occupy different areas of this multi-dimensional space that may or may not overlap; consequently, the regulatory machinery driving these different aspects of EMT may or may not share common drivers (Jolly, Ware, Gilja, Somarelli, & Levine, 2017a; Saunders & McClay, 2014) (Fig. 1B). Indeed, this non-overlapping multi-dimensionality of both molecular drivers and resulting phenotypes is likely to be responsible, at least in part, for some of the misunderstandings among the cancer research community about the relevance of EMT to metastasis (Brabletz, Kalluri, Nieto, & Weinberg, 2018; Fischer et al., 2015; Krebs et al., 2017; Zheng et al., 2015).

In this review, we focus on hybrid E/M cells that co-express epithelial and mesenchymal markers, and/or exhibit a combination of biophysical traits typically indicative of both epithelial and mesenchymal phenotypes, such as cell-cell adhesion and cell migration, thereby enabling collective cell migration. Here, we collate and discuss evidence across cancer types in identifying the existence and plasticity of hybrid E/M phenotype(s) and various associated traits, such as anoikis-resistance, stemness, migration, and therapy resistance. We discuss the ramifications of hybrid E/M phenotype(s) for cancer metastasis, therapy resistance, and tumor relapse – the major unsolved clinical challenges that together claim over 90% of all cancer-related deaths (Ben-Jacob, Coffey, & Levine, 2012; Gupta & Massague, 2006).

## 2. Complex molecular networks regulate cellular phenotype and plasticity

EMT can be induced by a multitude of microenvironmental biochemical and/or mechanical cues, such as TGFβ and Notch pathways, hypoxia and matrix stiffness (Kumar, Das, & Sen, 2014; Rice et al., 2017; Wei et al., 2015), that converge on a set of transcription factors (termed as EMT-TFs), such as ZEB1/2, SNAIL, SLUG, TWIST1/2, PRRX1, FOXC2, and GSC (De Craene & Berx, 2013). EMT can also be induced by potent oncogenes and stem cell regulators such as c-MYC (Bin, Cho, Lee, & Kang, 2010; Larsen et al., 2016; Yin, Cheryan, Xu, Rishi, & Reddy, 2017), or by loss of tumor suppressors such as BRCA1 or p53 (Chang et al., 2011; Dong et al., 2013; Sengodan, Sreelatha, Nadhan, & Srinivasan, 2018). Similarly, MET can be induced by overexpression of MET-TFs, such as OVOL1/2, GRHL2, ELF3, and ELF5 (De Craene & Berx, 2013; Mooney et al., 2017; Roca et al., 2013; Somarelli et al., 2016a), or the removal of an EMT-inducing signal (Jo et al., 2009; Saxena, Kalathur, Neutzner, & Christofori, 2018a). These EMT-TFs typically repress the transcription of epithelial marker E-cadherin and/or induce its delocalization from the cell membrane (Schmalhofer, Brabletz, & Brabletz, 2009) – a hallmark of EMT (De Craene & Berx, 2013). Typically, EMT also involves loss of other epithelial markers such as epithelial-cell adhesion molecule (EpCAM), dissolution of zonal occludens-1 (ZO-1) from cell-cell contacts, and/or gain of one or more mesenchymal markers such as N-cadherin, vimentin, and alpha-smooth muscle actin (αSMA). Thus, EMT-TFs can repress the epithelial markers and/or induce the mesenchymal ones (De Craene & Berx, 2013). Conversely, some MET-TFs can transcriptionally activate E-cadherin (Shimamura et al., 2011; Varma et al., 2012). Besides transcriptional control, EMT/MET is also regulated by multiple post-transcriptional mechanisms, including microRNAs (Lu, Jolly, Levine, Onuchic, & Ben-Jacob, 2013), long non-coding RNAs (Xu et al., 2016), alternative splicing (Brown, Reinke, Damerow, Perez, & Chodosh, 2011; Jolly et al., 2018b; Preca et al., 2015), epigenetics (Tam & Weinberg, 2013), and post-translational protein stability (De Herreros, Peiró, Nassour, & Savagner, 2010). Moreover, EMT can influence and, in turn, be influenced by metabolic reprogramming in cancer cells (Bhowmik et al., 2015; Kanska et al., 2017). To further complicate the picture, these mechanisms can also be unique to the cell type or tissue of origin, as we have shown for carcinomas (cancers of epithelial origin) versus sarcomas (cancers of mesenchymal origin)

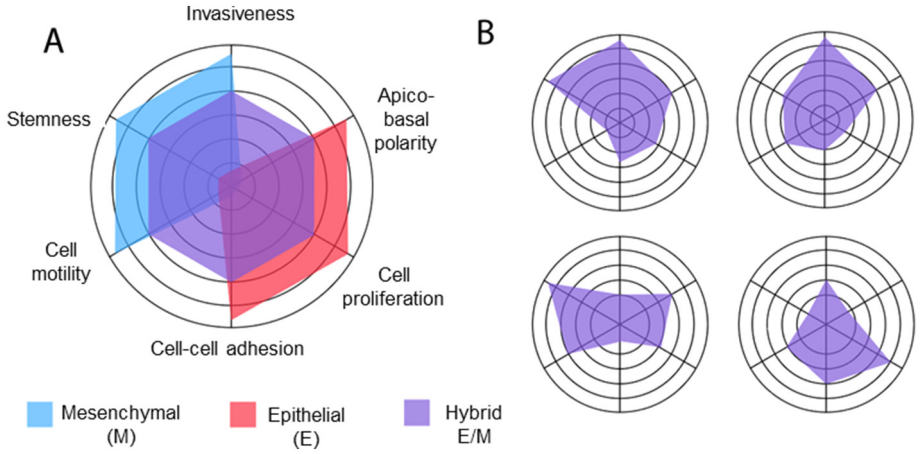


**Fig. 1.** Spectrum of epithelial-mesenchymal plasticity, as represented by spider plots A) EMT is a multi-dimensional process, involving changes in many biophysical and molecular parameters, a subset of which are represented here. Mesenchymal (M) cells are typically more motile and invasive (blue shaded area), while epithelial (E) cells are typically more adherent, proliferative, and polarized in apico-basal manner (red shaded area), hybrid E/M cells tend to contain both E and M properties, albeit to varied extents. B) Distinct hybrid E/M phenotype(s) may occupy different regions of this multi-dimensional landscape, highlighting the spectrum of epithelial-mesenchymal plasticity and the inherent difficulty in both defining a given cell's true phenotype, and targeting these diverse phenotypes clinically.

(Somarelli, Shetler, et al., 2016a). In some rare tumor types such as carcinosarcomas, both the epithelial and mesenchymal phenotypes may co-exist simultaneously due to divergent phenotypic evolution from a common clone (Somarelli, Boss, Epstein, Armstrong, & Garcia-Blanco, 2015; Zhao et al., 2016).

Within the underlying regulatory networks, interconnections and feedback loops among EMT-inducing factors can often give rise to emergent, non-linear and non-intuitive dynamics (Fig. 2A). Computational models representing these diverse interactions can be helpful in elucidating this inherent non-linear network dynamics, and can generate testable hypotheses that can pinpoint useful experiments (Jolly, Tripathi, Somarelli, Hanash, & Levine, 2017b). For example, we developed a computational model based on the mutually inhibitory feedback loop involving members of the microRNA (miR)-200 family and ZEB1/2. In this feedback loop, the miR-200 family – guardians of the epithelial phenotype – inhibits the translation of ZEB1/2 mRNA; also, the EMT-TFs ZEB1/2 repress the transcription of miR-200 family members (Bracken et al., 2008; Burk et al., 2008; Park, Gaur, Lengyel, & Peter, 2008). This feedback loop, also referred to as 'the motor of cellular plasticity'(Brabletz & Brabletz, 2010), controls cell-cell adhesion, cell-matrix adhesion, cell motility and invasion (Sigloch, Burk, Biniossek, Brabletz, & Schilling, 2015; Sundararajan, Gengenbacher, Stemmler, Kleemann, & Brabletz, 2015; Ungewiss et al., 2015), and other traits associated with EMT such as stemness (Jolly et al., 2014; Wellner et al., 2009), immunosuppression (Chen et al., 2014; Dongre et al., 2017; Mak et al., 2016; Tripathi et al., 2016), and tumor angiogenesis (Pecot et al., 2013). Our computational model predicted that this feedback loop can not only drive transitions between epithelial (E; high miR-200, low ZEB) and mesenchymal (M; low miR-200, high ZEB) states, but also may enable cells to attain a stable hybrid E/M phenotype (medium miR-200, medium ZEB) (Lu et al., 2013) (Fig. 2B). Indeed, experimental validation in H1975 non-small cell lung cancer (NSCLC) cells revealed that the H1975 cells display a stable hybrid E/M phenotype *in vitro* over multiple passages (Jolly et al., 2016). Also, the levels of ZEB in H1975 are intermediate relative to that in other epithelial or mesenchymal NSCLC cell lines (Jia et al., 2017a).

Interestingly, the stable existence of one or more hybrid E/M phenotype(s) has been a robust prediction of many computational models that have been developed for different EMT regulatory networks using varying modeling schemes, further emphasizing that EMT is rarely an 'all-or-none' process (Font-Clos, Zapperi, & La Porta, 2018; Gould, Bassen, Chakrabarti, Varner, & Butcher, 2016; Hong et al., 2015; Huang et al., 2017; Jia et al., 2015; Jolly et al., 2016; Joo, Zhou, Huang, & Cho, 2018; Lu et al., 2013; Steinway et al., 2014; Steinway et al., 2015; Tian, Zhang, & Xing, 2013). These studies have identified network motifs stabilizing epithelial, mesenchymal or hybrid E/M phenotypes and unraveled the role of GRHL2, ΔNP63α and OVOL2, and simultaneous treatment with EMT and MET inducers in modulating EMT /MET dynamics. Future modeling efforts can include dynamic epigenetic effects such as bivalent/poised chromatin of many regulators that can facilitate

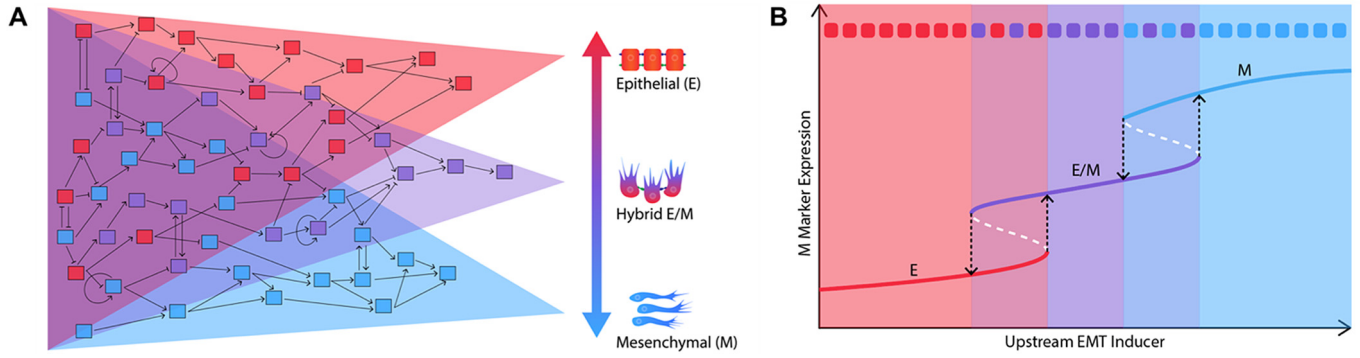


**Fig. 2.** Interconnected regulatory networks govern E, M, and hybrid E/M phenotypes. A) Representative cellular circuitry showing that the non-linear emergent behavior of this network can lead to different cellular phenotypes – E, M, and one or more hybrid E/M phenotype(s). B) A bifurcation diagram showing how EMT can be induced in a step-wise process with an external signal as the input (x-axis). Solid curves denote phenotypic (stable) states, dotted lines represent unstable states. Arrows showing transitions among various phenotypes reveal an asymmetry – the value of the signal that drives the transition from E to E/M (first upward arrow) is not the same at which cells transition from E/M to E (first downward arrow). Moreover, one or more cellular phenotypes can co-exist (second and forth shaded rectangles from the left).

cell plasticity, as seen in the context of both EMT and stem cells (Bernhart et al., 2016; Bernstein et al., 2006; Chaffer et al., 2013; Taube et al., 2017).

These computational models have made two other key predictions. First, EMT and MET need not be symmetric processes, i.e. the trajectory that cells take while undergoing EMT need not be the same when they undergo MET. (Fig. 2B). Detailed single-cell experiments capturing EMT dynamics will be valuable in testing this prediction. Second, the models predict that cells in a clonal population can spontaneously switch between multiple phenotypes (E, M, hybrid E/M). Consequently, isogenic cells can display non-genetic heterogeneity in terms of their EMT status. This prediction has been validated *in vitro* in multiple cancer subtypes, where fluorescence activated cell sorting (FACS) analysis of clonal/isogenic populations based on varied epithelial and mesenchymal cell surface markers have identified cells in epithelial, mesenchymal and hybrid E/M phenotypes (George, Jolly, Xu, Somarelli, & Levine, 2017; Grosse-Wilde et al., 2015). Perhaps the most compelling evidence strengthening this notion was gathered recently through *in vitro* studies in prostate cancer (Ruscetti et al., 2016), and *in vivo* mouse models for squamous cell carcinoma and human mammary tumors (Pastushenko et al., 2018). In these experiments, that are discussed in detail below, the authors not only identified multiple distinct hybrid E/M subpopulations, but also observed spontaneous inter-conversion among these different phenotypes.

It is important to note that a cell population exhibiting either epithelial or mesenchymal markers in different sub-populations need not always translate to the existence of hybrid E/M cells co-expressing markers at the single cell level. For example, among a panel of non-small cell lung cancer cell lines, while both H1975 and H2291 were categorized as 'intermediate' based on bulk proteomic measurements (Schliekelman et al., 2015). However, follow-up immune-fluorescence staining and single cell analysis revealed that while the H1975 cells were almost exclusively single-cell hybrid E/M, the H2291 cells contained mixtures of E and M sub-populations and no hybrid E/M cells (Jolly et al., 2016). Interestingly, these two possibilities need not be mutually exclusive – epithelial, mesenchymal and hybrid E/M phenotypes can also co-exist within a single population (Jolly, Tripathi, et al., 2017b). Similarly, studies identifying different clusters of tumor subtypes based on bulk transcriptomics data may report a co-expression of canonical epithelial and mesenchymal genes; whether those samples truly contain any individual hybrid E/M cells needs to be followed up via single-cell experiments.

Most investigations into EMT and its implications have been conducted in carcinomas, and more specifically in adenocarcinomas - tumors formed from glandular epithelium, i.e. cells synthesizing substances such as hormones for release into the bloodstream. Most cancers originating in breast (Makki, 2015), colorectal (Thrumurthy, Thrumurthy, Gilbert, Ross, & Haji, 2016), pancreatic (Bond-Smith, Banga, Hammond, & Imber, 2012), and prostate (Nadal, Schweizer, Kryvenko, Epstein, & Eisenberger, 2014) tissues are adenocarcinomas; however, only 40% of lung cancer cases are adenocarcinomas (Zappa & Mousa, 2016). Besides adenocarcinomas, epithelial cancers can also be squamous cell carcinomas, arising from squamous epithelium that perform barrier and absorptive functions in the body. EMT has been implicated in squamous cell carcinomas as well as other non-epithelial tumors such as brain tumors, sarcomas, carcinosarcomas (biphasic tumors comprised of elements of carcinomas and sarcomas), melanomas, and hematopoietic malignancies (Kahlert, Joseph, & Kruyt, 2017; Pang, Carbini, Moreira, & Maki, 2018; Somarelli et al., 2015).

In this review, we focus on the emerging existence of hybrid E/M phenotypes and their relevance in the context of carcinomas. Table 1 summarizes the studies in various carcinomas that have identified hybrid E/M phenotype(s) using multiple markers. Table 2 presents the data on stemness, plasticity, tumor-initiation potential, and/or metastatic propensity for hybrid E/M phenotype(s) in various carcinomas. It is intriguing to note that many markers used independently for identifying hybrid E/M phenotypes have been reported as regulates of adult and/or cancer stem cells, particularly in breast cancer, such as SLUG and ΔNP63α (Guo et al., 2012; Maria, Giulia, Giacobbe, Peschiaroli, & Frezza, 2015; Nassour et al., 2012). This congruence further strengthens our predicted association of hybrid E/M phenotype with stemness, as discussed later. The identification and characterization of hybrid E/M phenotype(s) has witnessed a surge in recent years, however, those reports still account for a small percentage of the EMT literature (Fig. 3A-B). It is noteworthy that computational models of EMT have played a key role in driving many ideas related to existence and implications of these hybrid E/M phenotype(s) (Fig. 3C).

## 3. Evidence for the existence of hybrid E/M phenotype(s)

### 3.1. Breast cancer

A majority of breast cancers are carcinomas, and their histological stratification is based primarily on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Triple negative breast cancers (TNBCs) are defined by lack of all these receptors, and are quite aggressive (Pareja et al., 2016). Seminal work in breast cancer demonstrated clearly that while post-EMT cells were more invasive and had heightened tumor-initiation ability (stemness) (Mani et al., 2008; Morel et al., 2008; Yang et al., 2004), MET was crucial in reawakening proliferation after post-EMT cells had extravasated and seeded (Ocaña et al., 2012). These important studies viewed EMT/ MET largely as a binary process.

However, in contrast to these studies, several recent studies in breast cancer have identified various manifestations of hybrid E/M phenotypes – both in *in vitro* experiments and in patient-derived CTCs. For example, induction of SNAIL can alter the levels of E-cadherin and vimentin, but does not elicit a motile phenotype in multiple breast cancer cell lines (Lundgren, Nordenskjöld, & Landberg, 2009). Similarly, TWIST1 induction coupled with TGFβ treatment of the epithelial fraction of HMLE cells was shown to induce molecular changes associated with EMT, but the epithelial morphology persisted, suggesting a partial EMT response (Dragoi et al., 2016). Thus, 'molecular EMT' and 'morphological EMT' need not always co-occur (Cheung & Ewald, 2014). Another manifestation of hybrid E/M phenotype(s) can be a co-expression of various canonical epithelial and mesenchymal markers (Armstrong et al., 2011; Yu et al., 2013). Such heterogeneity in the extent of EMT induction can depend on the dose and duration of EMT-inducing signals such as TGFβ treatment (Gao, Zhu, Nilsson, & Sundfeldt, 2014; Gregory et al., 2011; Keshamouni et al., 2009; Lenferink et al., 2010; Milano et al., 2016; Tanaka & Ogishima, 2015; Tian et al., 2018; Xu et al., 2018; Zadran, Arumugam, Herschman, Phelps, & Levine, 2014; Zhang et al., 2014). One additional factor that can influence the extent of EMT induction is inherent non-genetic heterogeneity in a clonal population, emerging from different epigenetic states 'poised' to varying extents to undergo a transition and/or phenotypic heterogeneity resulting from nonlinear dynamics of the underlying regulatory networks (Frank & Rosner, 2012; Jia, Jolly, Kulkarni, & Levine, 2017b; Jolly, Kulkarni, Weninger, Orban, & Levine, 2018c; Lee et al., 2014). Single-cell RNA-seq data of human mammary epithelial cells HMLE induced with TGFβ reveal such non-genetic heterogeneity, where the rates of progression of cells towards a mesenchymal state are highly varying (van Dijk et al., 2018). Put together, breast epithelial cells induced with TGFβ may display one or more hybrid E/M phenotype(s).

In addition, around 75% of CTCs from women with metastatic breast cancer co-expressed cytokeratin (epithelial marker), and vimentin and N-cadherin (both mesenchymal markers) (Armstrong et al., 2011). Similarly, another study showed that 48% of CTCs from twenty metastatic breast cancer patients co-expressed vimentin and keratins 8, 18 or 19 (Polioudaki et al., 2015). Furthermore, based on relative co-expression of various epithelial (E) and mesenchymal (M) markers, five categories of CTCs ranging from fully epithelial to intermediate (E < M, E=M, E >

**Table 1**
Evidence for the existence of hybrid E/M phenotype(s) across cancer types. 'Scale' column denotes whether the co-existence of markers has been demonstrated at a single-cell level or not; while the former possibility indicates specifically the existence of individual hybrid E/M cells, the latter represents potentially a mixture of E and M cells, but does not rule out the existence of individual hybrid E/M cells. Different sets of markers and model systems used in various carcinomas are included.

| Cancer type | Cancer subtype | Specimen | E/M markers used | Experiments done | Scale | Reference |
|---|---|---|---|---|---|---|
| Breast | | ALDH+ nonpathogenic mammary cells | Keratins vimentin | RNA seq, IF | Single cell | Colacino et al., 2018 |
| | | Basal MECs | EpCAM, Slug, Zeb1 | FACS, PCR, IF | Single cell | Ye et al., 2015 |
| | | CTCs | E-cad, N-cad, vimentin, EpCAM | IF | Single cell | Armstrong et al., 2011 |
| | | | Keratins, EpCAM, E-cad, N-cad, fibronectin | IF-based RNA-ISH | Single cell | Yu et al., 2013 |
| | | HMLER cells | CD44, CD24, and others | PCR, FACS | Single cell | Grosse-Wilde et al., 2015 and 2018 |
| | | MCF7, T47D and BT474 cells | E-cad, vimentin | PCR, Western blot, IF | Population | Saxena et al., 2018b |
| | | Metastases | E-cad, vimentin, Connexins 26 and 43 | IHC | Population | Chao et al., 2012 |
| | | MMTV-PyMT tumors | EpCAM, vimentin, keratin 14 | PCR, immunostaining, FACS | Single cell | Pastushenko et al., 2018 |
| | | Primary tumors | Keratin, vimentin | IHC | Single cell | Thomas et al., 1999 |
| | | T-47D, MCF-7, MDA-MB-468 and MDA-MB-231 cells | Snail, vimentin, E-cad | Western blot, Immunocytochemistry | Population | Lundgren, Nordenskjöld, & Landberg, 2009 |
| | IBC | SUM149 and SUM190 cells | E-cad, vimentin | IF | Single cell | Kai et al., 2018 |
| | | SUM149, Mary-X, FC-IBC-01, and FC-IBC-02 cells; MDA-IBC-3 cells | CD24, CD44; E-cad, ZEB1, vimentin | Flow cytometry, cDNA microarray | Single cell | Roberston et al., 2012 |
| | Non-tumorigenic epithelial | HMLE cells | E-cad, vimentin, fibronectin, etc. | RNA seq | Single cell | van Dijk et al., 2018 |
| | | | ZEB1, E-cad, β-catenin | IF | Single cell | Dragoi et al., 2016 |
| | | MCF10A cells | E-cad, N-cad | Western blot | Population | Milano et al., 2016 |
| | | | E-cad, vimentin | Flow cytometry | Single cell | Hong et al., 2015 |
| | | | | IF, Western blot, PCR | Single cell | Zhang et al., 2014 |
| | Triple negative (TNBC) | MDA-MB-231 and SUM159 cells | CD44, CD24, ITGB4 | FACS, RNA seq, Western blot | Single cell | Bierie et al., 2017 |
| | | MDA-MB-231 cells | CD44, CD24 | IF | Single cell | Boareto et al., 2016 |
| | | | Keratins 8 and 18, vimentin | IF | Single cell | Hendrix et al., 1997 |
| | Varying ER, HER2 status | Drug tolerant MDA-MB-231, SUM159, 4T1, MCF7, and SKBr3 cells | CD44, CD24 | IHC, FACS | Single cell | Goldman et al., 2015 |
| Prostate | | 22Rv1 cells | E-cad, N-cad, vimentin, fibronectin | Western blot | Population | Shiota et al., 2015 |
| | | CTCs | E-cad, N-cad, vimentin, EpCAM | IF | Single cell | Armstrong et al., 2011 |
| | | Docetaxel resistant DU145 | E-cad, ZEB1 | Western blot | Population | Hanrahan et al., 2017 |
| | | DU145 cells | E-cad, vimentin | Flow cytometry, IF, Western blot | Single cell | George et al., 2017 |
| | | DU145 subline xenografts | E-cad, ZEB1 | Western blot | Population | Putzke et al., 2011 |
| | | Enzalutamide resistant LNCaP95 cells | Snail, E-cad | PCR | Population | Ware et al., 2016 |
| | | Metastases | E-cad | IHC | Population | Chao et al., 2012 |
| | | OPCT-1 cells | E-cad, vimentin, CD24, CD44, others | IF, PCR, FACS, Western blot | Single cell | Harner-Foreman et al., 2017 |
| | | PC-3/Mc cells | CD44, CD24 | FACS | Single cell | Celià-Terrassa et al., 2012 |
| | | Primary CPKV murine tumors | EpCAM, vimentin | FACS, IHC, IF | Single cell | Ruscetti et al., 2015 |
| | | | | FACS, PCR | Single cell | Ruscetti et al., 2016 |
| Lung | | CTCs, CTM | E-cad, N-cad, vimentin, cytokeratins | IHC | Single cell | Hou et al., 2011 |
| | NSCLC | A549 cells | E-cad, vimentin | Flow cytometry, IF, Western blot | Single cell | George et al., 2017 |
| | | | | PCR, Western blot, IF | Population | Saxena et al., 2018b |
| | | | Many | Transcriptional expression profile | Population | Zadran et al., 2014 |
| | | A549, LT73, and H460 cells | E-cad, Slug | PCR, IF | Single cell | Andriani et al., 2016 |
| | | AC and SCC primary tumors | Many | IHC | Population | Zacharias et al., 2018 |
| | | H1975 cells | E-cad, vimentin | IF | Single cell | Bocci et al., 2017<br>Jolly et al., 2016<br>Jolly, Preca, et al., 2018b |
| | | | ZEB1, E-cad | IF, PCR, Western blot | Single cell | Jia et al., 2017a |
| | | H1993, H1385, H1975 cells | E-cad, vimentin | IF, Western blot | Population | Schliekelman et al., 2015 |
| | | HCC827 erlotinib-resistant cells | E-cad, vimentin | Flow cytometry, IF | Single cell | Fustaino et al., 2017 |
| | | NSCLC CTCs | vimentin, Keratin | IF | Single cell | Lecharpentier et al., 2011 |
| | | Squamous cell carcinoma lesions | E-cad, vimentin | IHC | Single cell | Aruga et al., 2018 |
| | SCLC | DMS 79 xenografts | CK18, CK19, EpCAM, E-cad, N-cad, FSP1 | IHC, PCR | Population | Meredith et al., 2016 |
| | | SCLC26A, NCI-H526, NCI-H417, | E-cad, vimentin, β-catenin | Western blot | Population | Hamilton, Hochmair, Rath, |

(continued on next page)

**Table 1** (*continued*)

| Cancer type | Cancer subtype | Specimen | E/M markers used | Experiments done | Scale | Reference |
|---|---|---|---|---|---|---|
| | | GLC16, GLC14 | | | | Klameth, & Zeillinger, 2016a |
| Ovarian | | 26/43 cell lines of SGOCL library | E-cad, pan-cytokeratin, vimentin | PCR, IF | Single cell | Huang et al., 2013 |
| | | CAOV3, PEO14, OV90, and OVCA420 cells | E-cad, vimentin, Slug, Tcf21 | IF, PCR, Western blot, FACS | Single cell | Varankar et al., 2018 |
| | | HGSOC primary tumors | E-cad, vimentin | CyTOF | Single cell | Gonzalez et al., 2018 |
| | | OVCAR3 and OvCa432 cells | E-cad, N-cad | IF, PCR | Single cell | Klymenko, Johnson et al., 2017b |
| | | OvCar3 cells, CTCs | EpCAM, CK5/7, N-cad, vimentin, Snail | Multiplex RT-PCR | Single cell | Blassl et al., 2016 |
| | | Primary ovarian cancer cultures | E-cad, vimentin, Laminin | IF | Single cell | Strauss et al., 2011 |
| | | Primary tumors | E-cad, N-cad, vimentin, keratin | IHC, Western blot | Population | Hudson et al., 2008 |
| | | TCGA gene expression data | Tcf21, Slug, fibronectin (FN1), others | Clustering | Population | Gardi et al., 2014 |
| SCC | | Murine model tumor cells | EpCAM, vimentin, keratin 14 | PCR, immunostaining, FACS | Single cell | Pastushenko et al., 2018 |
| | | Primary tumors and SCC99 cells | Vimentin, Slug, and others | RNA-seq, FACS, IHC | Single cell | Puram et al., 2017 |
| | Esophageal (ESCC) | Primary tumors and metstatic lymph nodes | E-cad, N-cad, vimentin | IHC | Population | Wen et al., 2016 |
| | Head & Neck (HNSCC) | p-16 positive CERV196 cells | E-cad, β-catenin, vimentin | ICC, Western blot | Population | Umbreit et al., 2014 |
| | Oral | BCl2-induced HSC-3 cells | N-cad, E-cad | Western blot, flow cytometry | Population | Zuo et al., 2010 |
| | Oro-Esophageal (OESCC) | OESCC tissue samples, 5 OESCC cell lines | E-cad, Claudins 1 & 7, vimentin | IHC, Westen blot | Population | Usami et al., 2008 |
| Pancreatic | | BxPC-3 and AsPC-1 cells | E-cad, vimentin, ZEB1 | RT-PCR, Western blot | Population | Gao et al., 2017 |
| | | | E-cad, vimentin, β-catenin | IF, PCR | Population | Rice et al., 2017 |
| | | KPC mouse model | E-cad, claudin-7, EpCAM, β-catenin | Western blot, IF, FACS, RT-PCR | Single cell | Aiello et al., 2018 |
| | | KPC mouse model- PanINs and circulating pancreatic cells | ZEB1, EpCAM | IF | Single cell | Rhim et al., 2012 |
| | | PANC-1 and BxPC-3 cells | E-cad, vimentin | IF, Western blot, RT-PCR | Population | Chen et al., 2018 |
| | | | E-cad, vimentin, Snail, Gli1 | IF, Western blot, RT-PCR | Population | Shan et al., 2017 |
| | | PANC-1 and KP-1NL cells | E-cad, vimentin, Snail | IF, Western blot, RT-PCR | Population | Kabashima et al., 2009 |
| | | PANC-1 cells | Many | Transcriptional expression profile | Population | Zadran et al., 2014 |
| | | Xenografts from human pancreatic tumor | CD24, CD44, EpCAM | FACS | Single cell | Li et al., 2007 |
| Other | Colorectal | HCT-15 and SK-CO-1 cells | E-cad, vimentin, occludin, Snail | Westen blot, RT-PCR | Population | Hiew et al., 2018 |
| | | SW480 and SW620 cells | E-cad, vimentin | Flow cytometry, IF, Western blot | Single cell | George et al., 2017 |
| | Liver | PLC/PRF/5 and HepG2 cells | E-cad, vimentin | IF, PCR, Western blot | Population | Steinway et al., 2015 |
| | Many carcinomas | Human metastatic tumors in ascites and pleural fluids | vimentin, keratin | IF | Single cell | Ramaekers et al., 1983 |
| | Renal cell carcinoma | SN12C and ACHN cells; canine kidney MDCK | E-cad, Snail | IF, Western blot | Single cell | Sampson et al., 2014 |

M) to fully mesenchymal were identified (Yu et al., 2013). The relative frequency of these phenotypes can change dynamically during response to systemic therapy or disease progression (Yu et al., 2013). Furthermore, primary tumors from TNBC samples were enriched for cells co-expressing epithelial and mesenchymal markers (Yu et al., 2013), suggesting a correlation between a hybrid E/M state and aggressive behavior. Strikingly, recent observations found that when cultured in suspension, the transcriptomes of both epithelial and mesenchymal sub-clones of the HMLER cell line displayed hybrid E/M signatures, suggesting that both epithelial and mesenchymal populations had undergone a partial phenotypic transition (Grosse-Wilde et al., 2015). Single-cell qPCR analysis of the mammospheres generated from epithelial and mesenchymal sub-clones revealed the existence of individual $CD24^{hi}$ /$CD44^{hi}$ cells that co-expressed comparable levels of canonical epithelial and mesenchymal genes, confirming their hybrid E/M status. This study suggests that some cancer cells are primed to transition toward a hybrid E/M state when appropriate environmental conditions (e.g. anchorage-independent growth) are provided (Grosse-Wilde et al., 2015).

Besides $CD24^{hi}$/ $CD44^{hi}$, various markers have been proposed for 'metastable' hybrid E/M state(s) in breast cancer: P-cadherin (Ribeiro & Paredes, 2015), SLUG (van Dijk et al., 2018), and ITGB4 (integrin-beta 4) (Bierie et al., 2017). Both P-cadherin and EMT-TF SLUG can drive collective cell migration (Hudson et al., 2009; Leroy & Mostov, 2007; Plutoni et al., 2016; Savagner et al., 2005) – a proposed hallmark of partial EMT (Micalizzi et al., 2010). SLUG has also been reported to induce a hybrid E/M phenotype in mammary epithelial cells co-expressing various epithelial and mesenchymal markers (Ye et al., 2015). Similarly, overexpression of ΔNP63α in breast cancer cells can drive collective invasion and induce a hybrid E/M state in basal-like breast cancer by tuning ZEB through simultaneous SLUG-induced ZEB activation and miR205-induced ZEB downregulation (Dang, Esparza, Maine, Westcott, & Pearson, 2015; Jolly et al., 2017c). Furthermore, P-cadherin can be regulated by ΔNP63α (Ribeiro & Paredes, 2015), highlighting a potential interplay and reinforcement of factors that can induce and/or maintain hybrid E/M state(s).

One breast cancer subtype that reflects a prevalent hybrid E/M phenotype is inflammatory breast cancer (IBC) (Jolly, Boareto, et al., 2017c). IBC is a highly aggressive locally advanced breast cancer with unusually poor patient prognosis (Jolly, Boareto, et al., 2017c). Multiple IBC cell lines – MDA-IBC-3, SUM190, FC-IBC-02 and SUM149 – express intermediate levels of ZEB1, E-cadherin and vimentin (Kai et al., 2018; Robertson et al., 2012). Further, FACS analysis of IBC cell lines and mouse models SUM149, Mary-X, FC-IBC-01, and FC-IBC-02 reveal that a large percentage

**Table 2**
Evidence for the stemness/tumor-initiation potential of hybrid E/M phenotype(s) across cancer types.

| Cancer type | Specimen | Experiments done | Condition | Reference |
|---|---|---|---|---|
| Breast | HMLE cells | Mammosphere formation, 3D collagen culture | In vitro | Schmidt et al., 2015 |
| | HMLER cells | Mammosphere formation | In vitro | Grosse-Wilde et al., 2018<br>Grosse-Wilde et al., 2015 |
| | HMLER-Twist, HMLER-Snail and MCF7 cells | Invasion assay; orthotopic injection | Both | Neelakantan et al., 2017 |
| | MDA-MB-231, SUM159, 4T1, MCF7, and SKBr3 cells | Enrichment of stem cell markers; dilution assay in mice | Both | Goldman et al., 2015 |
| | MDA-MB-231, SUM159, and NAMECR cells | Orthotopic xenograft | In vivo | Bierie et al., 2017 |
| | Primary and metastatic tumors | Xenograft, mammary pad injection | In vivo | Al-Hajj et al., 2003 |
| Prostate | CPKV mice with PTEN deletion | Mammosphere formation, enrichment of stem cell markers; subcutaneous implantation | Both | Ruscetti et al., 2015 |
| | DU156 sublines | Colony formation assays; tibial xenograft | Both | Putzke et al., 2011 |
| | OPCT-1 clone P4B6 | Sphere formation, enrichment of stem cell markers; subcutaneous injection | Both | Harner-Foreman et al., 2017 |
| | PC-3 sublines | Orthotopic implantation | In vivo | Celia-Terrassa et al., 2012 |
| | PKV cells | Plasticity assay | In vitro | Ruscetti et al., 2016 |
| Lung | A549, LT73, and H460 cells | Enrichment of stem cell markers; subcutaneous injection | Both | Andriani et al., 2016 |
| | Erlotinib-resistant HCC827 cells | Spheroid formation, wound healing assay | In vitro | Fustaino et al., 2017 |
| | Mouse model | Serial transplantation, spheroid cultures, xenograft | Both | Zheng et al., 2013 |
| Ovarian | Modified SKOV3 and OvCa433 cells | Hanging drop MCA formation | In vitro | Klymenko, Johnson, et al., 2017b |
| | Primary ovarian cancer cultures | Xenograft | In vivo | Strauss et al., 2011 |
| | SKOV3 cells | Spheroid formation, anoikis, invasion, and migration assays; subcutaneous xenograft | Both | Huang et al., 2013 |
| SCC | CA1, MET1, and MET2 cells | Orthotopic injection; single-cell cloning followed by FACS | Both | Biddle et al., 2011 |
| | Various mouse models | Cell transplantation, intravenous and intraperitoneal injection | In vivo | Pastushenko et al., 2018 |
| Pancreatic | PANC-1 and KP-1NI SP cells | Invasion assay; intra-splenic injection | Both | Kabashima et al., 2009 |
| | Primary tumors | Patient-derived xenograft, orthotopic pancreatic tail injection | In vivo | Li et al., 2007 |
| Other | Immortalized keratinocyte HaCaT cells | Subcutaneous injection | In vivo | Tsuji et al., 2008 |

of cells are $CD24^{hi}$ $CD44^{hi}$ (Robertson et al., 2012), a proposed signature of the hybrid E/M phenotype (Grosse-Wilde et al., 2015). Consistently, IBC typically metastasizes via clusters or tumor emboli, and knockdown of E-cadherin was shown to drastically reduce tumor growth *in vivo* for multiple IBC cell lines (Jolly, Boareto, et al., 2017c).

### 3.2. Prostate cancer

The predominant form of prostate cancer is prostate adenocarcinoma, with varying grade and molecular subtypes. Most prostate adenocarcinoma is androgen receptor driven, although cellular plasticity can promote a transition towards an EMT-like state (Byrne et al., 2016; Sun et al., 2012) or to an androgen receptor-independent, neuroendocrine-like state (Ku et al., 2017; Mu et al., 2017), most often in response to treatment with novel hormonal therapies that target androgen receptor signaling. Indeed, multiple investigations have identified the presence of hybrid E/M cells in cell lines, animal models, and clinical samples. *In vitro* studies of PC-3 revealed two subpopulations: PC-3/S and PC-3/Mc. While PC-3/S was post-EMT, PC-3/Mc was relatively more epithelial-like. Most of the PC-3/Mc cells were $CD24^{hi}$/$CD44^{hi}$ (Celià-Terrassa et al., 2012), a proposed signature for a hybrid E/M phenotype (Grosse-Wilde et al., 2015). Further analysis of gene expression data – based on a statistical

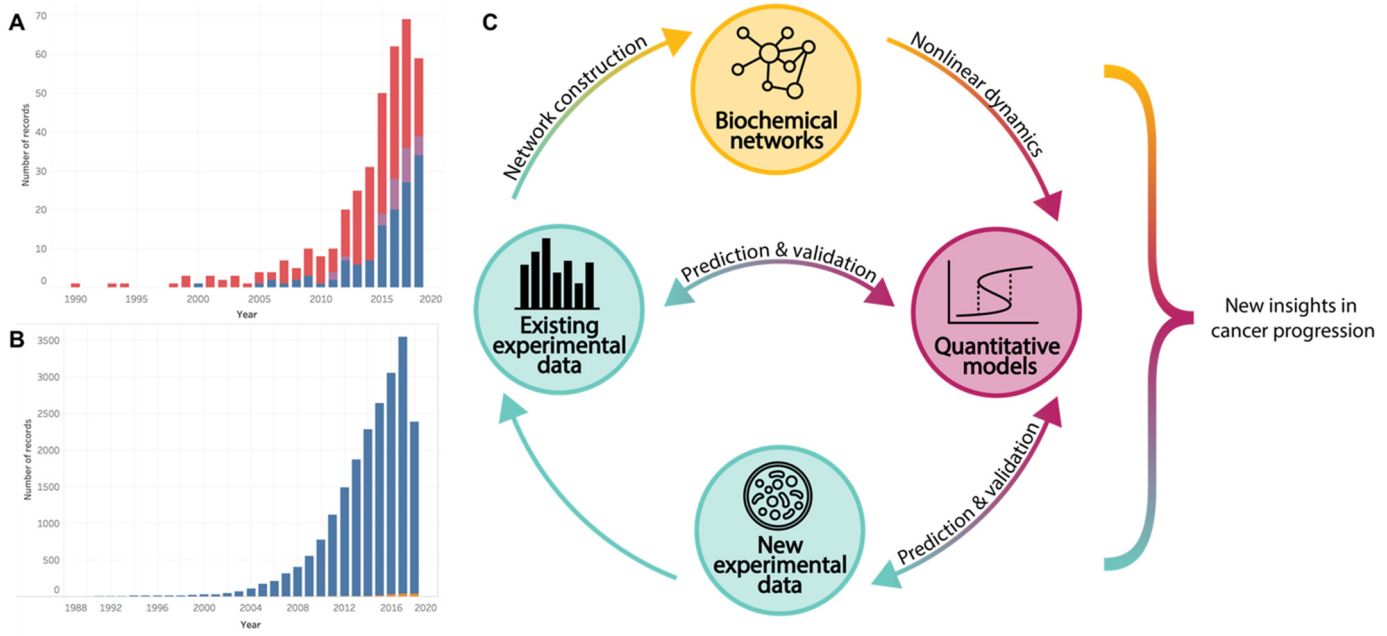


**Fig. 3.** Status of hybrid E/M phenotypes in EMT literature. A) No. of PubMed entries over the years containing the terms "hybrid E/M", "partial EMT", "incomplete EMT", "intermediate EMT", "weak EMT" or equivalent (blue), those containing "epithelial plasticity" (red), and those containing both "epithelial plasticity" and one of the synonyms of "partial EMT" B) Those shown in A) shown relative to the total no. of PubMed entries for "EMT", highlighting that the theme of partial EMT state(s) is still relatively unexplored (see the barely visible orange at the bottom of the bars). C) Integrated computational-experimental approach that has helped drive gaining insights into characterizing hybrid E/M state(s).

model that can quantify the extent of EMT for a given transcriptomic signature – confirmed a hybrid E/M status of PC-3/Mc cells (George et al., 2017). The EMT scoring metric discussed above also predicted DU145 to lie midway on the EMT spectrum. Flow-cytometry and ImageStream analysis of E-cadherin and vimentin co-expression on these cells revealed the existence of distinct DU145 subpopulations – epithelial, mesenchymal, and hybrid E/M (George et al., 2017). Similarly, the P4B6 clone generated from a human prostate cancer cell line (OPCT-1) contained individual cells co-expressing E-cadherin and vimentin, ZEB1, and displayed a $CD24^{hi}/CD44^{hi}$ profile (Harner-Foreman et al., 2017). P4B6 clones also contained both CD44s and CD44v – the splice variants of CD44 receptor that are found typically exclusively in mesenchymal or epithelial cells respectively (Brown et al., 2011; Hernandez et al., 2015). Their simultaneous presence further argues for the description of P4B6 clones as a hybrid E/M phenotype (Jolly, Preca, et al., 2018b).

*In vivo*, a prostate cancer mouse model harboring PTEN deletion and conditional activation of $KRAS^{G12D,}$ (CPKV mouse model) contained epithelial, hybrid E/M and mesenchymal subpopulations both in primary tumor cells and CTCs (Ruscetti, Quach, Dadashian, Mulholland, & Hong, 2015). A cell line derived from this mouse model also contained all 3 subpopulations – E, M, and hybrid E/M – as revealed by flow cytometry analysis (Ruscetti et al., 2016). The subpopulations within the cell line also spontaneously interconverted between the three states, validating the mathematical models that predict the existence of multistability in EMT networks and consequent phenotypic switching, even in the absence of an EMT/MET inducer (Burger et al., 2017; Jolly & Levine, 2017). In clinical samples, a hybrid E/M phenotype was reported in prostate cancer CTCs metastases from men with metastatic castration-resistant prostate cancer (CRPC) co-expressing epithelial and mesenchymal biomarkers such as EpCAM, cytokeratins, E-cadherin, N-cadherin, and vimentin (Armstrong et al., 2011). Likewise, hybrid E/M cells have been observed in prostate cancer metastases, where circulating mesenchymal cells only underwent a partial MET: metastases showed an upregulation of membranous E-cadherin without a significant loss of mesenchymal biomarkers vimentin and fibroblast specific protein 1 (FSP1) (Chao, Wu, Acquafondata, Dhir, & Wells, 2012).

### 3.3. Lung cancer

Lung cancer has two major histological subtypes: 80-85% tumors belong to non-small cell lung cancer (NSCLC), and the remaining 15-20% belong to small cell lung cancer (SCLC). Lung adenocarcinoma is frequently observed among NSCLC. A recent study of 38 lung adenocarcinoma cell lines found distinct molecular and morphological features across these lines – epithelial, mesenchymal and hybrid E/M (Schliekelman et al., 2015). A subset of these hybrid E/M cell lines exhibited high ratios of CDH1_S/VIM (ratio of cell surface localized E-cadherin to whole cell lysate vimentin), migrated and invaded collectively, and showed upregulated levels of cytoskeletal and actin-binding proteins, pointing towards an aggressive phenotype (Schliekelman et al., 2015). Later, single-cell investigation of hybrid E/M cell lines revealed several scenarios, including i) cases where individual cells co-expressed E and M markers (H1975), ii) cases containing mixtures of epithelial and mesenchymal cells but not individual hybrid E/M cells (H2291) (Jolly et al., 2016), and iii) a combination of these two possibilities. H1975 maintained a stable hybrid E/M phenotype over multiple passages *in vitro*; knockdown of the transcription factors OVOL2 or GRHL2 destabilized the hybrid E/M phenotype and drove these cells towards a complete EMT state (Jolly et al., 2016). These factors that appear to contribute to stabilizing the hybrid E/M phenotype have been referred to as 'phenotypic stability factors' (PSFs) (Yaswen, 2015). Similar to these reports in NSCLC, in physiological contexts, GRHL2 can stabilize key epithelial traits during embryonic development of lung and other epithelial tissues (Werth et al., 2010). GRHL2 activates E-cadherin during the development of lung airways and alveolar epithelium at all stages (Varma et al., 2012). In lung epithelium, GRHL2 and NKX2-1 can activate each other, both of which are reported as among the top activators of E-cadherin (Shimamura et al., 2011; Varma et al., 2012). Besides GRHL2 and OVOL2, NUMB – an inhibitor of Notch signaling (Flores, McDermott, Meunier, & Marignol, 2014) – was identified as another PSF for H1975 cells, as it prevented the cells from undergoing a Notch signaling-driven EMT (Bocci et al., 2017). Consistently, the role of NUMB as a PSF was also reported for other NSCLC adenocarcinoma cell lines A549 and PC9 (Kikuchi, Sakakibara-konishi, Furuta, & Kikuchi, 2018).

Flow cytometry and immunofluorescence analyses identified hybrid E/M phenotype(s) in NSCLC, including: a) HCC827- and HCC4006- derived drug-resistant subpopulations that co-express E-cadherin and vimentin (Fustaino et al., 2017), b) A549 cells co-expressing E-cadherin and vimentin (George et al., 2017), and c) A549, LT73, and H460, co-expressing E-cadherin and SLUG (Andriani et al., 2016). Co-expression of vimentin and keratin was also reported in patients with metastatic NSCLC (Lecharpentier et al., 2011). Similarly, E-cadherin and SLUG were concomitantly expressed in many primary NSCLC tumors (Andriani et al., 2016). In addition, a recent study showed co-expression of E-cadherin, N-cadherin, Twist, vimentin, cytokeratins and vimentin in lung adenocarcinomas and squamous cell carcinomas, and noted the predominance of bulk cancer cell migration in these cases (Zacharias, Brcic, Eidenhammer, & Popper, 2018), reminiscent of the reports on tumor budding across multiple cancer types (Bronsert et al., 2014). Put together, cells in a hybrid E/M phenotype have been observed in NSCLC cell lines, primary tumors, and CTCs.

SCLC has also been proposed to be categorized as a hybrid E/M phenotype. SCLC cells typically grow as multi-cellular aggregates in suspension (Krohn et al., 2014; May, Crawford, & Nedelcu, 2018), and metastasize early, often via clusters of cancer cells (Salgia et al., 2018). Moreover, SCLC cells lack many signs of a post-EMT phenotype, including cell individualization, prominent actin stress fiber formation, and marked expression of vimentin and αSMA (Ito et al., 2017). Multiple SCLC cell lines such as GLC14, GLC16, NCI-H526, NCI-H417, and SCLC26A express varying levels of E-cadherin, vimentin, β-catenin, and stemness markers, such as SOX2, indicating a ubiquitous presence of different degrees of EMT-like phenotypes in SCLC (Hamilton et al., 2016a). Single-cell investigations are needed to verify if these cell lines contain individual hybrid E/M cell rather than subpopulations of E and M cells.

Heterogeneous expression of epithelial markers E-cadherin and EpCAM, and mesenchymal marker vimentin was also observed in individual CTCs and clusters of CTCs for both SCLC and NSCLC patients (Hou et al., 2011), and for two cell lines established from SCLC CTCs BHGc10 and BHGc7 (Hamilton, Rath, Klameth, & Hochmair, 2016b). BHGc10 and BHGc7 cells are also capable of inducing macrophage differentiation *in vitro*; this locally induced inflammation may enable passive shedding of CTCs without the need for a full-blown EMT (Hamilton & Rath, 2016), as also postulated for the case of IBC (Jolly, Boareto, et al., 2017c).

### 3.4. Ovarian cancer

A majority of ovarian tumors are epithelial in nature, and can be categorized as type I and type II carcinomas. Type I are slow-growing indolent neoplasms, while type II carcinomas are clinically aggressive that may develop *de novo* from the tubular and/or ovarian surface epithelium (Koshiyama, Matsumura, & Konishi, 2017). A subgroup of type II carcinomas – high-grade serous ovarian cancer (HGSOC) – is the most lethal subtype of ovarian cancer (Koshiyama et al., 2017). In HGSOC, SLUG and SNAIL were shown to be associated with altered cellular morphology, through downregulation of multiple components of cell junction components such as E-cadherin and ZO-1 (Kurrey, Amit, & Bapat, 2005). A hybrid E/M phenotype was first reported in ovarian cancer primary cultures, where a total of 28% of all ovarian tumors and 33% of serous ovarian carcinoma tumors analyzed displayed simultaneous

immunoreactivity for both E-cadherin and N-cadherin (Hudson, Zeineldin, & Stack, 2008). Flow cytometry analysis of ovarian cancer xenografts, biopsies, and multiple cell lines revealed a significant proportion of EpCAM and/or E-cadherin positive cells co-expressing mesenchymal markers vimentin and/or CD44 (Strauss et al., 2009; Strauss et al., 2011). This co-expression is reminiscent of a 'mixed' E/M phenotype reported in multiple neoplastic progenitors of epithelial ovarian carcinoma, including ovarian surface epithelium that can express both vimentin and keratin (Strauss et al., 2011), highly-differentiated fallopian tube epithelium, and from other regions of the ovary (Klymenko, Kim, & Stack, 2017a). Whether this 'mixed' phenotype suggests admixtures of E and M cells and/or individual hybrid E/M cells remains to be determined. A recent study, however, using single-cell mass spectrometry, identified tumors containing individual cells co-expressing E-cadherin and vimentin (Gonzalez et al., 2018).

Several groups have stratified HGSOC tumors into distinct groups based on their association with EMT/MET networks and a corresponding phenotype, at least at a population level. Similar to the aforementioned study in NSCLC (Schliekelman et al., 2015), an analysis of a panel of ovarian carcinoma cell lines for E-cadherin, vimentin, and pan-cytokeratin expression revealed four subgroups – epithelial (E), mesenchymal (M), intermediate E, and intermediate M. Intermediate E cells were E-cadherin$^{+}$/Vimentin$^{+}$, while intermediate M ones were E-cadherin$^{-}$/Vimentin+/Pan-cytokeratin$^{+}$. Strikingly, 26 out of these 43 cell lines were characterized as an intermediate E or M phenotype (Huang et al., 2013), recapitulating the extremely heterogeneous phenotypic cell states observed *in vivo* in primary tumors. Clustering of transcriptomic signatures in HGSOC tumors revealed three distinct functional classes, highlighting differential mechanisms of transformation (Gardi, Deshpande, Kamble, Budhe, & Bapat, 2014): Class 1 exhibited more epithelial features, Class 2 was EMT-driven in extensive cross-talk with extracellular matrix (ECM) components, while Class 3 represented mixed E-M features. This study also identified 13 transcription factors associated with these classes; further analysis based on promoter occupancies among these transcription factors suggested a mutually inhibitory loop between TCF21 and SLUG that may underlie phenotypic interconversions among epithelial, mesenchymal, and hybrid E/M phenotypes (Varankar et al., 2018). As compared to epithelial and mesenchymal states, hybrid E/M phenotypes co-expressing TCF21 and SLUG at an individual cell level exhibited greater plasticity in response to positive/negative growth regulators, indicating an enhanced 'poised' or fluid behavior in hybrid E/M phenotype(s) when exposed to varying microenvironments (Varankar et al., 2018).

### 3.5. Squamous cell cancer

Squamous cell carcinomas (SCCs) – the predominant cancer of the oral cavity – constitute up to 90% of head and neck cancer cases (Sanderson & Ironside, 2002), 20% of skin cancer cases (Stratigos et al., 2015), 30% of NSCLC cases (Rooney, Devarakonda, & Govindan, 2013), and most cancers in the upper part of the oesophagus (Pennathur, Gibson, Jobe, & Luketich, 2013). Unlike adenocarcinomas, SCCs arise from squamous epithelium, and lack separate basal and luminal cell lineages. *In vitro* studies in SCCs have identified multiple manifestations of hybrid E/M state(s). For instance, p16- positive head and neck SCC cells CERV196 showed an incomplete EMT upon induction with epidermal growth factor (EGF) where cell scattering and the levels of β-catenin was increased, while the levels of E-cadherin and vimentin changed only modestly (Umbreit et al., 2014). Similarly, an oral cavity derived cell line SCC9 exhibited a partial EMT response (Puram et al., 2017). Furthermore, overexpression of the anti-apoptotic factor Bcl-2 in HSC-3 cells induced cell scattering and N-cadherin expression, but did not entirely abrogate the levels of E-cadherin or induce higher levels of canonical EMT-TFs (Zuo et al., 2010). Whether these observations involve a partial EMT response at a single-cell level remains to be tested. A recent study using a $KRas^{G12D}$ and $p53^{-/-}$ mouse model of skin SCC generated tumors that have undergone spontaneous EMT, leading to many subpopulations of EpCAM-negative tumor cells. Single-cell transcriptional, epigenetic, and surface marker analysis of these subpopulations showed that they corresponded to different degrees of EMT (Pastushenko et al., 2018). This study suggested that an exogenous upregulation of any EMT-TF need not be necessary for inducing EMT in a subset of cells. It also provided the first *in vivo* evidence of multiple potentially stable hybrid E/M phenotypes at a single-cell level (Pastushenko et al., 2018).

Besides abovementioned *in vitro* and *in vivo* studies, multiple human SCC primary tumors can display hybrid E/M phenotypes. For example, based on immunohistochemical analysis of the levels of E-cadherin, vimentin, and the adherens junctions components claudin-1 and claudin-7, esophageal SCC samples were categorized into epithelial, complete EMT and incomplete EMT (Usami et al., 2008). In another study, approximately 43% of primary tumors and 53% of metastatic lymph nodes in esophageal SCC were classified as hybrid E/M, based on levels and localization of E-cadherin, N-cadherin and vimentin (Wen et al., 2016). Furthermore, most SCC tumors were reported to display continued EpCAM expression, despite enhanced expression of many mesenchymal genes, suggesting a highly heterogeneous induction of EMT (Natsuizaka et al., 2017). However, these studies reflect ensemble properties, limiting the conclusions that can be drawn about the involvement of individual cells in one or more hybrid E/M phenotype (s). Overcoming this limitation, recently, a detailed single-cell analysis of over 6,000 cells from 18 head and neck squamous cell carcinomas revealed a subset of cells exhibiting a partial EMT program, with upregulation of Vimentin and integrin-α5, without downregulation of their epithelial components (Puram et al., 2017). These cells were located at the leading edge in close proximity to cancer-associated fibroblasts (CAFs) and also expressed SLUG (SNAI2) - a regulator of partial EMT response during wound healing and mammary morphogenesis (Leroy & Mostov, 2007; Savagner et al., 2005).

### 3.6. Pancreatic cancer

Approximately 90% of pancreatic cancers are pancreatic ductal adenocarcinomas (PDAC), one of the most aggressive solid malignancies with an abysmally low 5-year survival rate of 4% (Gaianigo, Melisi, & Carbone, 2017). Multiple reasons underlie aggressive disease course of PDAC: limited symptoms at early stage, rapid progression and early dissemination (Gaianigo et al., 2017). In a mouse model harboring pancreas-specific mutations in *Kras* and *p53* (KPC model), the pancreatic intraepithelial neoplasia cells – precursor to PDAC – expressed ZEB. These cells that had undergone EMT were identified in circulation even before any histological evidence for PDAC (Rhim et al., 2012), suggesting a role of EMT in early dissemination. Moreover, 18% of these circulating pancreatic cells co-expressed ZEB1 and EpCAM, thus denoting a hybrid E/M phenotype *in vivo* (Rhim et al., 2012). Upregulation of multiple EMT-TFs in PDAC tissue and in undifferentiated cell lines Panc-1 and MiaPaCa-2 (Hotz et al., 2007), and increased metastasis *in vivo* upon knockdown of E-cadherin (von Burstin et al., 2009) suggested the potential contribution of EMT in PDAC metastasis. More recent studies have also highlighted hybrid E/M phenotypes co-expressing E-cadherin and vimentin in multiple PDAC cell lines BxPc-3 and Panc-1 (Gao et al., 2017; Viotti et al., 2018), particularly under increased matrix stiffness (Rice et al., 2017), hypoxia (Chen et al., 2018), or upon co-culture with cancer associated fibroblasts (Shan et al., 2017). A recent report also connected epithelial plasticity to organotropic metastatic in PDAC: liver metastasis, but not lung metastasis, required p120catenin/E-cadherin driven epithelial identity (Reichert et al., 2018).

Despite this evidence, the contribution of partial or complete EMT to PDAC metastasis remains debatable. Genetic knockdown of Twist or Snail in the KPC mouse model for PDAC did not significantly reduce the number of metastases (Zheng et al., 2015), but that of Zeb1 led to a 60% reduction (Krebs et al., 2017), suggesting potential tissue-

specificity and/or redundancies among the EMT-TFs in enabling a repertoire of cellular traits required for metastasis (Brabletz et al., 2018). A follow-up study using the same mouse model showed that cancer cells can either migrate as single cells utilizing the traditional EMT program, or migrate collectively as clusters in a hybrid E/M phenotype, defined as the cytoplasmic re-localization of E-cadherin (instead of transcriptional repression of E-cadherin) and relatively low levels of EMT-TFs (Aiello et al., 2018). This study, however, does not conclusively address the question whether this hybrid E/M phenotype is a transitional cell state or an end point of a transition. Moreover, while initial attempts in quantifying varying extents of EMT in PDAC cell lines have been made (Arumugam et al., 2009; Li et al., 2009), distinguishing between individual hybrid E/M cells vs. mixtures of epithelial and mesenchymal cells needs further work.

### 3.7. Other cancer types

A hybrid E/M phenotype has also been observed in other cancer types also such as colorectal cancer (Hiew et al., 2018), hepato-cellular carcinoma (Parveen, Vedagiri, Nair, Parthasarathy, & Harshan, 2017), renal carcinoma (Sampson et al., 2014) and epithelioid peritoneal mesothelioma (Bozzi et al., 2016). Colorectal cancer cells HCT-15 and SK-CO-1 express various epithelial and mesenchymal markers such as E-cadherin, occludin, vimentin, and Snail, at least at a population level, as assessed by ensemble mRNA and protein measurements (Hiew et al., 2018), when the Yamanaka factors OCT4, SOX2, KLF4 and c-MYC are retrovirally transduced. Similarly, Wilms' tumor 1 (WT1) induces a partial EMT in clear cell renal cell carcinoma cells by upregulating SNAIL but still preserving epithelial junctions (Sampson et al., 2014). Last but not the least, a co-expression of keratin and vimentin has been observed in many metastatic carcinoma cells present in the ascites and pleural fluid (Ramaekers et al., 1983).

## 4. Hybrid E/M phenotype(s) often promote metastasis and tumor relapse

### 4.1. Breast cancer

In breast cancer, Al-Hajj *et al.* identified a subpopulation of cells capable of initiating tumors efficiently when injected in limiting dilutions into immunocompromised host mice – as a $CD44^{+}/CD24^{-/low}$ population (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). This subpopulation was later popularized as cancer stem cells (CSCs), reminiscent of observations in liquid tumors (Reya, Morrison, Clarke, & Weissman, 2001). The concept of post-EMT cells being more stem-like was first demonstrated in breast cancer (Mani et al., 2008; Morel et al., 2008). Further, post-EMT cells and/or CSCs were reported to exhibit resistance to multiple therapies (Singh & Settleman, 2011) through mechanisms such as upregulation of ATP-binding cassette (ABC) transporters associated with multidrug resistance (Balaji, Udupa, Chamallamudi, Gupta, & Rangarajan, 2016; Saxena, Stephens, Pathak, & Rangarajan, 2011). With a growing appreciation for multiple intermediate states of EMT, recent studies have demonstrated that partial activation of EMT can be sufficient, or perhaps even more potent than a complete EMT, in promoting metastasis and therapy resistance. A comparison of stemness across HMLER subpopulations – epithelial ($CD44^{lo}$/ $CD24^{hi}$), mesenchymal ($CD44^{hi}/CD24^{lo}$), and hybrid E/M ($CD44^{hi}/CD24^{hi}$) – revealed that the hybrid E/M cells formed ten times more mammospheres as compared to epithelial and mesenchymal ones (Grosse-Wilde et al., 2015). A similar report in head and neck SCC suggests that CD24+/CD44+ cells formed larger tumors in nude mice compared to CD24-/CD44+ ones (Han, Fujisawa, Husain, & Puri, 2014).

It is also worth noting that in the seminal identification of breast CSCs, the post-EMT cells that behaved as CSCs - identified by $CD44^{+}$ $CD24^{-/low}$ – also retained expression of the epithelial marker EpCAM, suggesting a hybrid E/M phenotype of CSCs (Al-Hajj et al., 2003). Further, transient, but not sustained, activation of EMT-TF TWIST1 led to invasive growth, indicating that cells pushed to an extreme end of the 'EMT axis' may lose their stemness (Schmidt et al., 2015). Similarly, in multiple triple negative breast cancer cells lines MDA-MB-231, SUM159, and NAMECR, highly tumorigenic CSCs marked by integrin-beta 4 (ITGB4) reside in a hybrid E/M state (Bierie et al., 2017). This study concluded that residence in a highly epithelial or mesenchymal cell state is less compatible with tumor-initiating ability, and thus a forced entrance of epithelial cells to a full-blown EMT state can be counterintuitive for the production of CSCs (Bierie et al., 2017). This behavior is also mimicked in the case of IBC, where overexpression of ZEB1, knockdown of E-cadherin, or prolonged exposure to TGFβ – all of which can induce a full-blown EMT response – reduces the *in vivo* growth of primary and metastatic tumors (Chu, Boley, Moraes, Barsky, & Robertson, 2013; Lehman et al., 2013). Consistently, a 'quasi-mesenchymal' state of basal cells expressing keratins, E-cadherin, and αSMA may underlie their multi-lineage reconstitution potential (Sikandar et al., 2017).

Multiple mechanisms may underlie the association between hybrid E/M phenotypes and disease aggressiveness. First, cells in a hybrid E/M phenotype are likely to undergo collective cell migration (Watanabe et al., 2014) that may lead to formation of clusters of circulating tumor cells (CTCs). Recent *in vivo* studies that the clusters of CTCs, although very rare, can contribute to forming over 90% of metastases; these CTC clusters are enriched for cell junction components including plakoglobin, and for Notch-Jagged signaling (Aceto et al., 2014; Cheung et al., 2016; Jolly, Boareto, et al., 2017c). These clusters are formed before cells are launched into the bloodstream (Aceto et al., 2014), and Notch-Jagged signaling may contribute to the formation of these clusters through a phenomenon known as lateral induction, where adjacent cells acquire the same cell fate (Boareto et al., 2016; Boareto, Jolly, Ben-Jacob, & Onuchic, 2015; Owen, Sherratt, & Wearing, 2000; Saravanamuthu, Gao, & Zelenska, 2009). Frequent enrichment of Notch-Jagged signaling pathway in IBC further endorses its potential role in forming and/or maintaining the clusters of CTCs and in mediating stemness (Xiao, Ye, Yearsley, Jones, & Barsky, 2008). Cells in these clusters can provide survival signals to one another through junctional adhesions (Shen & Kramer, 2004), as evidenced by absence of apoptotic markers in clusters of CTCs but not in individual CTCs (Hou et al., 2011).

Second, given the high plasticity and stem-like behavior of hybrid E/M or partial EMT phenotypes (Font-Clos et al., 2018; Grosse-Wilde et al., 2015; Jolly et al., 2014), these cells may give rise to epithelial and mesenchymal subpopulations, while maintaining a repertoire of hybrid E/M cells (Grosse-Wilde et al., 2018; Ruscetti et al., 2016). These epithelial and mesenchymal subpopulations can then cooperate to accelerate tumor progression. For instance, a mixture of these subpopulations in a 1:1 ratio from HMLER cells form many more mammospheres *in vitro* as compared to either subpopulation alone (Grosse-Wilde et al., 2018). In addition, HMLER cells overexpressing Twist or Snail (HMLER-Twist/ HMLER-Snail) cells can impart metastatic traits to control HMLER cells without any explicit induction of EMT, through paracrine Hedgehog signalling (Neelakantan et al., 2017). Such cooperation has also been demonstrated *in vivo* (Tsuji et al., 2008). In this study, a subcutaneous xenograft inoculation of either EMT or non-EMT cells led to primary tumor establishment from both cell types, invasion into local tissue only by EMT cells, but no lung metastases by either of them. Conversely, intravenous injection of both cell types led to lung metastases formation only from non-EMT cells. Intriguingly, when a mixture of EMT and non-EMT cells were subcutaneously injected, intravasation of both cell types was observed, but lung metastases were formed only by non-EMT cells (Tsuji et al., 2008). These observations suggest that both non-EMT and EMT cells faced roadblocks in successful metastasis, their co-injection led to an emergent cooperative behavior. Thus, given their ability to self-renew and differentiate into potentially cooperating epithelial and mesenchymal subpopulations,

the hybrid E/M cells would be highly capable of repopulating tumors and forming metastatic colonies. In other words, these multipotent hybrid E/M display conceptual parallels with stem cells during development (O'Brien-Ball & Biddle, 2017).

Switching to a hybrid E/M state can also be an adaptive cellular stress response, as witnessed by the transition of both epithelial and mesenchymal sub-clones of HMLER cell line towards a hybrid E/M signature, when cultured in suspension (Grosse-Wilde et al., 2015). This notion is strengthened by a recent study that identified an interplay between TWIST1 and the oxidative stress-sensing molecular AMP-activated protein kinase (AMPK) (Saxena et al., 2018b). AMPK can modulate cancer cell survival response by switching the adaptive response between matrix-attached and matrix-detached states (Saha et al., 2018). Matrix deprival triggers AMPK activity, which can, in turn, upregulate TWIST and elicit a partial EMT response (Saha et al., 2018; Saxena, Balaji, et al., 2018b). Consistently, an AMPK gene signature is elevated in CTCs as compared to both primary tumors and metastases (Saha et al., 2018). Another study demonstrating that the exposure of both basal-like and luminal breast cancer cell lines to taxanes induced an adaptive transition to a hybrid E/M ($CD44^{hi}/CD24^{hi}$) phenotype, which formed aggressive tumors *in vivo* (Goldman et al., 2015). These drug-tolerant cells exhibited amplified Notch-Jagged1 signaling as compared to the parental population (Boareto et al., 2016), reminiscent of the role of Notch signaling and Jagged1 in mediating stemness in breast cancer (Sansone et al., 2007; Sharma, Paranjape, Rangarajan, & Dighe, 2012), and potentially indicating that tumor cells may amplify biochemical communication and coordination among themselves as a community, especially under stressed conditions Ben-Jacob et al., 2012.

### 4.2. Prostate cancer

In the CPKV mouse model discussed above, Ruscetti *et al.* found that hybrid E/M cells formed significantly more spheres *in vitro* and contained a higher proportion of $Lin^-/Sca1^+/CD49f^{hi}$ stem/progenitor cells, as compared to fully epithelial or mesenchymal cells (Ruscetti et al., 2015). The study further demonstrated that mesenchymal and hybrid E/M cells both formed more tumors *in vivo* relative to epithelial cells, and that both cell types were able to regenerate areas with both mesenchymal-like and epithelial-like glandular structures. Thus, hybrid E/M cells displayed inherent plasticity to give rise to both epithelial and mesenchymal phenotypes. Moreover, the hybrid E/M cells possessed enhanced spheroid forming potential *in vitro*, and could revert to an epithelial state, start proliferating, and form lung macrometastases (Ruscetti et al., 2015). This plasticity was consistently demonstrated in hybrid E/M cells from the PKV cell line: when cultured separately for 2 weeks, 80% of hybrid E/M cells reverted to either an epithelial or mesenchymal phenotype, while only 20-30% of epithelial or mesenchymal cells transitioned to any of the other phenotypes (Ruscetti et al., 2016).

Similarly, among phenotypically distinct clones generated from OPCT-1 cells that span the entire EMT spectrum, the hybrid E/M clone P4B6 possessed the highest clonogenicity, as assessed by sphere-forming assays (Harner-Foreman et al., 2017). This clone also expressed multiple stem cell markers such as SOX2, CD44, and NANOG, and formed faster and larger tumors *in vivo* as compared to parental or other sister clones. Likewise, an isogenic subline derived from DU145 concurrently co-expressed ZEB1 and E-cadherin, and displayed significant *in vitro* colony formation capacity and aggressive growth in mouse xenografts (Putzke et al., 2011). This study also showed co-expression of epithelial and mesenchymal markers in specific human tumor metastatic sites. Another study comparing the tumor-initiation frequency in two subpopulations of PC-3 cells demonstrated that the post-EMT subpopulation PC-3/S lacked tumor-initiating cells and had lower metastatic potential than the hybrid E/M PC-3/Mc cells. Consistently, induction of a full-blown EMT in hybrid E/M PC-3/Mc cells enhanced their invasiveness, but suppressed anchorage-independent growth and stemness, blunting their metastatic colonization potential (Celià-Terrassa et al., 2012), emphasizing that hybrid E/M cells may occupy a 'metastatic sweet spot' (Jolly, Mani, & Levine, 2018d; Shibue & Weinberg, 2017). Together, these studies underscore the remarkable capacity for hybrid E/M cells to generate heterogeneous subpopulations and form macrometastases through increased tumor-initiating capacity.

### 4.3. Lung cancer

The cell-surface marker CD133 was identified as a marker of CSCs in both SCLC and NSCLC (Bertolini et al., 2009; Eramo et al., 2008). Treatment of NSCLC cell lines LC31 and A549 with TGFβ1 induced a loss of epithelial morphology and acquisition of mesenchymal spindle-shaped morphology, upregulation of EMT-TFs TWIST and SLUG, downregulation of E-cadherin, and an increase in levels of stem cell markers SOX2, OCT4, and NANOG (Pirozzi et al., 2011). CD133 levels and consequent pneumosphere-forming and colony-forming ability of LC31 cells treated with TGFβ1 was also enriched, indicating that EMT may promote tumorigenicity *in vivo* in NSCLC (Pirozzi et al., 2011). However, recent studies have challenged this one-to-one correspondence between a full-blown EMT and stemness in lung cancer. CSCs in NSCLC expressing ITGB4 (Zheng et al., 2013) – a marker of hybrid E/M CSCs in breast cancer (Bierie et al., 2017) – tends to argue that the overlap between hybrid E/M phenotype and stem-like behavior can be extended beyond breast cancer. Moreover, Tirino *et al.* identified multiple distinct subpopulations of tumor-initiating cells in A549 cells with varied extents of EMT (Tirino et al., 2013). The subpopulations identified were $CD133^+$, $CD133^-$, side population (SP+), SP-. SP+ cells show a distinctly low Hoescht 33342 dye staining pattern in FACS and exhibits several CSC-like cell properties, including regeneration of both SP+ and SP- population cells, multidrug resistance, and elevation of telomerase activity (Ho, Ng, Lam, & Hung, 2007). TGFβ1 treatment promoted migration in $CD133^+$, but not in $CD133^-$ or SP+ cells and induced colony formation in the SP- cells. Thus, $CD133^+$ was proposed to be a migrating cancer stem-like subpopulation, while the SP+ cells were proposed to be stationary cancer stem cells (Tirino et al., 2013), reminiscent of the multiple subpopulations of cancer stem cells reported for breast cancer (Liu et al., 2014) and ovarian cancer (Biddle et al., 2011) with varying EMT phenotypes. A recent study in NSCLC indicated that TGFβ1-driven increase in $CD133^+$ is enriched in cells co-expressing E-cadherin and SLUG (Andriani et al., 2016), thus indicating that a hybrid E/M phenotype may facilitate plasticity of lung cancer cells from a non-stem to a stem-like state.

In addition to stem-like properties, hybrid E/M phenotypes in NSCLC have been implicated in resistance to multiple therapies. NSCLC patients whose tumors exhibit driving mutations in epidermal growth factor receptor (EGFR) typically benefit from EGFR tyrosine kinase inhibitor (TKI) treatments, such as erlotinib and gefitinib. However, resistance eventually develops, at least partially through EMT (Byers et al., 2013; Li et al., 2014a; Shien et al., 2013; Thomson et al., 2005; Thomson, Petti, Sujka-Kwok, Epstein, & Haley, 2008; Yauch et al., 2005). Interestingly, erlotinib- resistant 'mesenchymal-like' cells were found to be already present in cell lines and tumors before drug treatment, further highlighting the pre-existing intra-tumor heterogeneity and inherent phenotypic plasticity in populations of cancer cells (Yao et al., 2010). The existence of this therapy-resistant cell state may contribute to the observation of 'cellular memory' of EMT observed in H358 cells (Thomson et al., 2008). Removal of TGFβ from cultures of H358 pre-treated with TGFβ induced H358 cells led to their reversion to an epithelial state; however, the sensitivity to EGFR inhibitors was not immediately restored, indicating some 'cellular memory' associated with EMT (Thomson et al., 2008). Such 'memory' effects are commonly observed during cellular transitions, and are a hallmark of multi-stable systems (Wang et al., 2009a; Xiong & Ferrell, 2003), where cells in one state have the potential to spontaneously and reversibly switch to another state.

Recently, a study focusing on erlotinib-resistant HCC827 cell lines showed an increased frequency of cells co-expressing E-cadherin and vimentin as compared to parental cells, indicating a potential selection of hybrid E/M cells (Fustaino et al., 2017). These cells expressed higher levels of ZEB1, increased sphere-forming ability, and migrated collectively led by cells stained positive both for E-cadherin and vimentin, pointing towards enhanced resistance to EGFR TKI and CSC-like traits in hybrid E/M cells. Another study showing an abatement of tumor-initiating ability *in vivo* in patient-derived NSCLC cells belonging to full-blown mesenchymal phenotype (Tiran et al., 2017) further consolidates the emerging notion that 'stemness window' most likely resides within one or more transitional states along the epithelial-mesenchymal spectrum (Bocci, Jolly, George, Levine, & Onuchic, 2018a; Jolly et al., 2015b; Ombrato & Malanchi, 2014).

### 4.4. Ovarian cancer

In ovarian cancer, stem-like cells were first isolated as single-cell clones derived from the ascites of a patient with advanced ovarian cancer, and identified as being tumorigenic (Bapat, Mali, Koppikar, & Kurrey, 2005). These clones were anchorage-independent, formed spheroids in suspension and multicellular colonies in soft agar, and initiated tumors *in vivo* with histopathological similarity to human tumors. This 'stemness' program – together with the expression of stem cell markers such as OCT4, BMI1, and nestin – was later shown to be driven by SLUG and SNAIL (Kurrey, Ghanate, Chaskar, Doiphode, & Bapat, 2009), reinforcing the link between EMT and stemness reported across multiple cancers (Scheel & Weinberg, 2012). Later, primary ovarian cancer cultures co-expressing E-cadherin and laminin were observed to stain for multiple stem cell markers. Importantly, only cultures containing these co-expressing hybrid E/M cells, but not the ones enriched in mesenchymal cells, were able to form tumors *in vivo*, suggesting enhanced tumor-initiating capacity in the cells with a hybrid E/M phenotype (Strauss et al., 2011). A subset of hybrid E/M cells expressing stem cell markers was multipotent, adaptable and transdifferentiated to both epithelial and mesenchymal cells through partial MET and partial EMT, respectively (Strauss et al., 2011).

Besides enhanced tumor-initiating abilities, other traits of a hybrid E/M phenotype may fuel aggressive progression in ovarian carcinoma. First, their plasticity and adaptability may offer survival advantages. For instance, SKOV3 cells – characterized as intermediate mesenchymal – exhibited high anoikis resistance and spheroid-formation ability *in vitro* (Huang et al., 2013). Treatment with saracatinib (AZD0530; Src-kinase inhibitor) restored E-cadherin expression in SKOV3 and abrogated their spheroid formation ability (Huang et al., 2013), but not in purely mesenchymal OVCAR10 cells, again underscoring the enhanced plasticity in hybrid E/M phenotype(s) observed in multiple cases (Ruscetti et al., 2016; Varankar et al., 2018). Ovarian carcinoma cells may activate an endogenous EMT response upon spheroid formation involving signaling through the TGF-β pathway (Peart, Correa, Valdes, DiMattia, & Shepherd, 2012; Rafehi et al., 2016). In this context, saracatinib treatment may push cells towards a more 'locked' or 'rigid' epithelial state, thus compromising their tumor-initiation ability. Second, the enrichment of cells with hybrid E/M stem-like traits upon starvation indicates a better survival/fitness of hybrid E/M state (Strauss et al., 2011).

Third, ovarian cancer disseminates predominantly by direct extension of cancer cells from primary tumor cells into the intra-abdominal cavity. There they travel both as individual cells and as multicellular aggregates that subsequently adhere to peritoneal tissues, migrate into the sub-mesothelial matrix, and form secondary lesions (Klymenko, Kim, & Stack, 2017a). In other cancer types such as IBC, migration via multicellular aggregates have been linked to a hybrid E/M phenotype (Jolly, Boareto, et al., 2017c). Along these lines, a recent study showed that cells expressing both E-cadherin and N-cadherin form multicellular aggregates made up of both epithelial and mesenchymal cells (Klymenko et al., 2017b). These results suggest that the hybrid multicellular aggregates may contain both the multipotent hybrid E/M cells and more differentiated subpopulations. These aggregates containing cells across the EMT spectrum may indeed be the most aggressive, since they provide greater diversity of paracrine and autocrine signaling, cellular and extracellular attachments, and proliferative capacity. These potential advantages may underlie a more efficient formation of macrometastases by multicellular aggregates with multipotent hybrid E/M cells, as compared to homogeneous aggregates containing cells only E- or N- cadherin (Giuliano et al., 2018).

### 4.5. Squamous cell cancer

Cancer stem cells (CSCs) were first identified in SCC as a single CD44 + subpopulation (Prince et al., 2007). A high frequency of CD44+ cells correlates with a poor prognosis in SCC (Joshua et al., 2012). It is now also recognized that CSCs in SCC can adapt heterogeneous phenotypes (Biddle et al., 2011). Epithelial-like CSCs (identified by $CD44^{hi}$/$EpCAM^{hi}$) were shown to be able to give rise to both epithelial and mesenchymal populations and bi-lineage colonies *in vitro* (Biddle et al., 2011) – a trait that could be regulated by Wnt signaling mediated by GSK3β (Shigeishi et al., 2013). However, this trait was significantly impaired in the mesenchymal-like CSCs (identified by $CD44^{hi}$/ $EpCAM^{lo}$) (Biddle et al., 2011), indicating that cells locked in a post-EMT state may lose their plasticity. Interestingly, both the epithelial-like CSCs and a subset of the $CD44^{hi}$/$EpCAM^{lo}$ cells that could reconstitute tumor heterogeneity were both $ALDH1^+$ and EpCAM+. Single $ALDH1^+$ cells have been shown to be enriched in spheroid-derived cells and possess higher invasive and colony-formation ability than $ALDH1^-$ cells in multiple other SCC cell lines (Chen et al., 2011). This association between EpCAM expression and plasticity in mesenchymal-like CSCs further suggests that this ALDH+ subset may be a hybrid E/M CSC (Colacino et al., 2018; Jolly et al., 2014). Further credence to this hypothesis is gained by observations that NRF2, an oxidative stress response transcription factor that upregulates ALDH (Luo et al., 2018), is maximally expressed in a hybrid E/M phenotype (Bocci et al., 2018b).

ALDH1+ cells were recently shown to co-express various epithelial and mesenchymal markers at a single-cell level in breast cancer (Colacino et al., 2018) and are capable of giving rise to both classes of CSCs ($ALDH1^+$ and $CD44^+$/$CD24^-$) as well as differentiated cancer cells (Liu et al., 2014), supporting the suggestion that the hybrid E/M phenotype may be enriched for stemness in SCC (Biddle et al., 2011). Consistent with this notion, analysis of the *in vivo* identified hybrid E/M phenotypes highlighted that tumor-initiating capacity was increased in the earlier transitional states of EMT, and did not increase further as EMT progressed. In contrast, metastatic potential peaked in the earlier transitional states, but was reduced significantly with the progression of EMT, reinforcing the notion that a hybrid E/M state is associated with increased metastasis (Pastushenko et al., 2018). It is possible that the hybrid E/M state contains the truly 'bad actors' of carcinomas in which E-cadherin and other epithelial players provide proliferative signals through cell-cell junctions, the mesenchymal traits promote migration, invasion, and a pro-survival phenotype, and the stem-like capacity enables the generation of specialized subpopulations that can cooperate.

Mechanistic studies have identified some potential molecular drivers of a hybrid E/M state in SCC. Transcriptional and chromatin analysis identified that ΔNP63 promotes the existence of aggressive hybrid E/M states in SCC (Pastushenko et al., 2018). In addition, in oral SCC, while Notch1 and Notch3 cooperate to drive epithelial differentiation, the combination of Notch1 with TGFβ leads to an alternative outcome in which TGFβ-driven ZEB1 expression represses Notch3, which prevents cell differentiation, and permits the progression of a partial EMT (Natsuizaka et al., 2017). Given that TGFβ-induced EMT can activate Notch-Jagged1 signaling (Brabletz et al., 2011; Zavadil, Cermak, Soto-Nieves, & Böttinger, 2004), it is likely that cell-cell communication

mediated via Notch-Jagged1 signaling can laterally induce and/or stabilize a hybrid E/M phenotype in SCC (Boareto et al., 2016).

### 4.6. Pancreatic cancer

Pancreatic CSCs were identified as a CD24+/CD44+/EpCAM+ population capable of self-renewal, producing differentiated progeny, and with increased expression of developmental signaling pathways such as Hedgehog (Li et al., 2007). CSCs in PANC-1 cell line did not exhibit a full EMT, instead retained E-cadherin levels (Yin et al., 2011), indicating a hybrid E/M phenotype. Next, similar to the case of lung cancer (Ho et al., 2007), side population (SP) cells have been isolated in pancreatic cancer cell lines as well. Relative to the non-SP cells, SP cells in both PANC-1 and KP-1NL have enhanced tumor-formation potential, express higher levels of E-cadherin when cultured, and are more responsive to TGFβ treatment and removal (Kabashima et al., 2009), suggesting they display a more poised or plastic behavior, typical of hybrid E/M phenotype(s) (Ruscetti et al., 2016). A subset of CSCs, identified by CD133+/CXCR4+, was found at the invasive edge of the primary tumor, possessing migratory potential (Hermann et al., 2007). This heterogeneity in spatial localization and migration traits of subsets of CSCs is reminiscent of observations in breast cancer, where mesenchymal-like CSCs identified by CD44+/CD24- are found at the invasive edge, and hybrid E/M-like CSCs identified by ALDH+ are found in the tumor interior (Colacino et al., 2018; Liu et al., 2014). A gradient of EMT-inducing signals such as TGFβ, coupled with non-cell autonomous control of EMT and CSCs, may underlie this spatial pattern formation in pancreatic cancer (Bocci et al., 2018c). Future studies should further characterize molecular similarities and differences between the CSC subsets.

### 4.7. Other cancer types

Besides breast and pancreatic cancer, CSCs in colorectal cancer cell lines were identified by CD24+/CD44+ (Yeung, Gandhi, Wilding, Muschel, & Bodmer, 2010), suggesting a hybrid E/M phenotype. Colorectal carcinoma cells grown in 3D exhibit upregulated many CSC markers, including ALDH1A1, as well as increased levels of EMT markers. Intriguingly, the levels of E-cadherin also increased concomitantly in both HT29 and DLD1 cells, indicating a convergence towards hybrid E/M phenotype (Stankevicius et al., 2017), reinforcing the observations made in the case of breast cancer (Grosse-Wilde et al., 2015).

## 5. Therapeutic and clinical implications of hybrid E/M phenotype(s)

### 5.1. Breast cancer

Initial studies that viewed EMT as a binary process showed enhanced drug-resistance traits in mesenchymal phenotypes relative to epithelial ones (Singh & Settleman, 2011). Consistently, high levels of EMT-TFs and/or their targets were shown to associate with aggressive subtypes, such as basal-like breast cancer and with poor patient prognosis (Lehmann et al., 2016; Mani et al., 2007; Taube et al., 2010; Van Nes et al., 2012). However, recent reports have argued that co-expression of various epithelial and mesenchymal markers can also associate with both *de novo* and adaptive drug resistance, and patient survival across all subtypes of breast cancer. For instance, development of insensitivity to tamoxifen in MCF7 cells, that to trastuzumab in HER2-overexpressing cells, and that to taxanes in both luminal and basal-like breast cancer cells is usually accompanied by a partial EMT (Goldman et al., 2015; Hiscox et al., 2006; Vadgama et al., 2012).

Much before the association of EMT-TFs with stemness or survival was elucidated, co-expression of mesenchymal marker vimentin and epithelial/luminal markers cytokeratins 8 and 18, rather than the expression of vimentin alone, was shown to correlate with increased invasive and metastatic potential and poor survival (Hendrix, Seftor, Seftor, & Trevor, 1997; Ramaekers et al., 1983; Thomas et al., 1999). More recently, Grosse-wilde et al. showed that a 60 gene signature – a combination of 30 genes most differently expressed in epithelial cells and 30 genes most differently expressed in mesenchymal cells – predicted poor outcome across all subtypes of breast cancer; however, neither the mammaprint 70-gene signature nor the proliferation-associated PCNA metagene signature was able to predict poor survival in all subtypes (Grosse-Wilde et al., 2015). This 60 gene signature includes epithelial markers such as E-cadherin, EpCAM, and many keratins, and EMT inducers such as Wnt5a (Grosse-Wilde et al., 2015; Wang, Tang, Gong, Zhu, & Liu, 2017a). Consistently, levels of various other markers and stabilizers of hybrid E/M phenotype(s) can also associate with poor survival. First, among TNBC patients who received chemotherapy, enhanced levels of ITGB4 – a marker for a hybrid E/M state – associate with worse 5-year probability of relapse-free survival (Bierie et al., 2017). Moreover, primary tumor biopsies of TNBC patients have a much higher proportion of cells co-expressing epithelial and mesenchymal markers, as compared to other subtypes (Yu et al., 2013). Second, high levels of GRHL2 – a PSF for a hybrid E/M phenotype (Jolly et al., 2016) – correlates with poor relapse-free survival across multiple breast cancer subtypes – luminal A, luminal B, HER2+, and basal-like (Mooney et al., 2017). Third, elevated levels of P-cadherin – a proposed marker of a hybrid E/M state – correlate with shorter overall survival, disease-free survival, and locoregional relapse-free survival in breast cancer (Ribeiro & Paredes, 2015). Fourth, ΔNp63α – an inducer of a hybrid E/M state – associates with shorter overall survival in ER-/HER2- patients (Dang et al., 2015). Fifth, bi-phenotypic CTCs have been shown to initiate metastasis in mice: EpCAM+ CD44+ CTCs directly transplanted into the femoral medulla cavity of highly immunodeficient NSG mice formed bone metastases in eight months (Baccelli et al., 2013). Consistently, the abundance of these hybrid E/M CTCs can increase with disease progression and can correlate with the patient's metastatic burden and overall survival (Baccelli et al., 2013). Sixth, a co-expression of E-cadherin and vimentin associate with the worst disease-free and overall survival among all cases (E-cadherin or vimentin expression alone, or absence of both markers) (Yamashita et al., 2018). Therefore, while the evidence for clinical implications of a hybrid E/M signature in breast cancer has been long standing, recent studies have elucidated the molecular signatures underlying this behavior.

A notable example of aggressiveness of hybrid E/M phenotype in breast cancer is IBC. It constitutes only 2-4% of breast cancer cases, but IBC patients accounts for 10% of breast cancer related mortality annually (Jolly, Boareto, et al., 2017c). As compared to 5% of patients in non-IBC cases showing distant metastasis at the time of diagnosis, over 30% of IBC patients have metastases (Warren et al., 2015), indicating its aggressiveness. A salient feature of IBC biology is that unlike non-IBC cases, IBC primary tumors have ubiquitous presence of E-cadherin, and the lymphatic system and metastases contain frequent tumor emboli or clusters of CTCs (Colpaert et al., 2003; Kleer, van Golen, Braun, & Merajver, 2001). The presence of clusters of CTCs associates with poor survival across multiple cancer types (Fabisiewicz & Grybowska, 2017), including a prominent association in the case of IBC (Mu et al., 2015). Moreover, the network of genes usually involved in collective dissemination through clusters of CTCs is more hierarchically organized for IBC samples relative to non-IBC ones, suggesting a higher adaptability of IBC cells (Tripathi, Jolly, Woodward, Levine, & Deem, 2018). Due to the inherently high plasticity of a hybrid E/M phenotype prevalent in IBC (Jolly, Boareto, et al., 2017c), tumor emboli in IBC may contain cells in varying phenotypes; this heterogeneity and potential cooperation possible among heterogeneous subpopulations may endow cells with fitness advantages to drive metastatic dissemination and therapy resistance. For instance, the two subpopulations of CSCs identified in breast cancer (Liu et al., 2014) have markedly different metabolic vulnerabilities, thus, both glycolytic and redox metabolism pathways must be targeted to avoid tumor relapse (Luo et al., 2018). Moreover, in heterotypic clusters or emboli, potential metabolic symbiosis between tumor and stromal cells (Wang, Liu, & Wang, 2014) may offer

additional routes to escape therapy. Therefore, non-cell autonomous behavior such as cell-cell cooperation and coordination – as illustrated in multiple *in vitro* and *in vivo* cases in breast cancer (Grosse-Wilde et al., 2018; Neelakantan et al., 2017; Tsuji et al., 2008) – can be another significant contributor to aggressive behavior underlying the hybrid E/M phenotypes.

### 5.2. Prostate cancer

While early studies pinpointed EMT as a driver of castration resistant disease (Byrne et al., 2016; Kong et al., 2015; Sun et al., 2012), there is growing evidence for a provocative connection between therapy resistance and the hybrid E/M phenotype in prostate cancer For example, chronic exposure of LNCaP95 cells to increasing doses of the androgen receptor targeting agent, enzalutamide, resulted in enzalutamide-resistant cells with increased levels of SNAIL and vimentin and a spindle-like morphology, but no loss of E-cadherin (Ware et al., 2016). Analysis of metastatic biopsies from castration-resistant prostate cancer patients showed a similar partial EMT or hybrid E/M phenotype, with co-expression of SNAIL and cytokeratin (Ware et al., 2016). Similarly, in the 22Rv1 cell line, long-term exposure to TGFβ induced a hybrid E/M phenotype, with cells co-expressing E-cadherin and mesenchymal markers, such as N-cadherin, vimentin, and fibronectin (Shiota et al., 2015). These hybrid cells also displayed increased resistance to androgen deprivation. Furthermore, a docetaxel-resistant subline of DU145 showed phenotypic signs of partial EMT: relative to parental DU145 cells, docetaxel-resistant cells exhibited increased migration and colony formation and increased levels of ZEB1, but did not display a single-cell scattering phenotype typical of post-EMT cells (Hanrahan et al., 2017). Elevated resistance to docetaxel was also reported in a hybrid E/M sub-clone, P4B6, relative to the parental OPCT-1 cells (Harner-Foreman et al., 2017).

An additional connection between the hybrid E/M state(s) and therapy resistance in prostate cancer comes from studies with metformin. Metformin was identified as a potent drug against enzalutamide-resistant tumors, by targeting the TGF-β1/STAT3 pathway (Liu et al., 2017). Interestingly, metformin can also reduce the frequency of hybrid E/M cells (Bocci, Jolly, et al., 2018a). Metformin-mediated inhibition of CSCs has been reported earlier in other cancers (Whitburn, Edwards, & Sooriakumaran, 2017). Given the emerging notion across cancer subtypes about an enrichment of stemness traits in hybrid E/M phenotype (Bierie et al., 2017; Jolly, Boareto, Huang, et al., 2015a; Pastushenko et al., 2018), it is attractive to speculate that metformin inhibits enzalutamide-resistant tumors by reducing the numbers of hybrid E/M cells and thus restricting the resulting overall tumor-initiating capacity of the population.

Clinical data provides further significance to the overlap between castration-resistant prostate cancer and the hybrid E/M phenotype(s). First, over 80% of CTCs in patients with CRPC co-expressed various epithelial (EpCAM, cytokeratin), mesenchymal (vimentin, N-cadherin) and stemness (CD133) markers (Armstrong et al., 2011). Second, a comparison of tissue specimens from transurethral resection of the prostate between castration-resistant and hormone-naïve prostate cancer patients showed that the former had higher levels of N-cadherin than the latter. Furthermore, within the castration-resistant specimens, specimens from patients with metastases had higher N-cadherin levels than those without metastases, while there was no significant difference in E-cadherin levels (Jennbacken et al., 2010). However, current clinical validation of these EMT biomarkers is lacking incorporation into treatment algorithms, illustrating the complexity and challenges of EMT biomarker development. For example, a recent study found a panel of EMT factors was not prognostic of outcomes in localized prostate cancer (Armstrong et al., 2016), despite early evidence that an E- to N-cadherin switch in localized prostate cancer was associated with metastasis and death (Gravdal, Halvorsen, Haukaas, & Akslen, 2007). Many confounding factors, such as cohort size or the frequency of deaths and metastatic events (Armstrong et al., 2016), may influence this connection. Thus, a detailed investigation of EMT markers in localized vs. metastatic prostate cancer is warranted (Li, Yang, & Gao, 2014b).

### 5.3. Lung cancer

The ZEB/miR-200 feedback loop has been implicated in mediating therapy resistance in NSCLC. ZEB1 is upregulated in erlotinib-resistant HCC827 and HCC4006 cells, and inhibiting ZEB1 can restore erlotinib sensitivity in HCC4006ER NSCLC cells (Fustaino et al., 2017; Yoshida et al., 2016). Both ZEB1 and ZEB2 are upregulated in gefitinib-resistant HCC827 and PC9 cells, and cells undergo MET and regain drug sensitivity upon long withdrawal of the drug (Lee et al., 2017). Moreover, miR-200c can also mediate immune evasion in NSCLC through inhibiting the immune checkpoint molecule PD-L1 (Chen et al., 2014) and through loss of antigen (Tripathi et al., 2016). miR-200c dysregulates the levels of immunoproteasome subunits PSMB8, PSMB9, and PSMB10 through regulating STAT3 activity (Tripathi et al., 2016). The immunoproteasome generates peptides that are suitable for binding to HLA molecules, enabling antigen presentation to elicit CD8+ T-cell responses. Decreased levels of immunoproteasome can, thus, drive immune evasion. Altering STAT3 activity either via mTOR inhibitor rapamycin or by treating with a demethylating agent that can increase STAT1 phosphorylation led to recovery of the immunoproteasome subunit expression Tripathi et al., 2016. Reduced expression of these units both at mRNA and protein levels was observed in mesenchymal cell lines relative to the epithelial ones. A significant reduction in expression of these subunits with the acquisition of resistance to EGFR TKI further consolidates the role of EMT in driving resistance against multiple therapies in NSCLC (Tripathi et al., 2016). Furthermore, in NSCLC, EMT was found to be associated with significantly lower infiltration of CD4+ T cells and with lower ratio of CD4/CD8 T-cells, and over-expression of immune checkpoints such as CTLA-4 (Chae et al., 2018) and PD-L1 (Chen et al., 2014), driving immunosuppression against CD8+ T-cells.

Platinum-based chemotherapy is the first-line treatment for NSCLC, but recurrence happens in most patients (Liu et al., 2013). Cisplatin treatment of NSCLC cell lines H460 and H661 increased the frequency of $CD133^+$ cells through activating Notch signaling pathway; these enriched cells also exhibited cross-resistance to paclitaxel and doxorubicin, through elevated levels of ATP-binding cassette transporters ABCB1 and ABCG2 (Liu et al., 2013). Moreover, JAG1, a ligand of Notch signaling, can promote survival of HCC827 cells (Choi et al., 2009), thus suggesting a potential involvement of Notch-JAG1 signaling in enabling survival of NSCLC cells. Notch-JAG1 signaling has been suggested to be implicated in adaptive drug resistance, formation of clusters of CTCs in breast cancer, and possibly stabilizing a hybrid E/M phenotype (Boareto et al., 2016; Cheung et al., 2016; Jolly, Boareto, et al., 2017c). Thus, enhanced Notch-JAG1 signaling may mediate multidrug resistance by maintaining a hybrid E/M phenotype in NSCLC. This hypothesis is strengthened by observations that a) JAG1 represses the levels of JAG2 (Choi et al., 2009) – a potential attenuator of Notch-JAG1 signaling (Jolly et al., 2015c), b) JAG1 promotes metastasis *in vivo* in NSCLC (Chang et al., 2016a), and c) increased JAG1 levels associate with poor overall survival in NSCLC (Chang, Ho, et al., 2016a; Pancewicz-Wojtkiewicz et al., 2016). JAG1 is among the top surface membrane receptors in multiple chemo-resistant SCLC cell lines (Guo et al., 2010; Tripathi et al., 2017).

In SCLC, loss of E-cadherin associates with changes of genes at both mRNA and protein levels that are well-known to regulating EMT and stemness such as ALDH1 and ZEB1/2, supporting the presence of a mesenchymal subset of SCLC that exhibits cross-resistance also to BCL2, HDAC1 and TOP2A/B inhibitors (Stewart et al., 2017). Thus, a partial or full EMT drives resistance to multiple targeted therapies in SCLC (Stewart et al., 2017). Upregulation of various mesenchymal markers such as N-cadherin, FSP1, and ZEB1 upon irradiation, without any consistent changes in epithelial markers, indicate the potential adaptation

of SCLC cells towards a hybrid E/M phenotype (Meredith et al., 2016). This observation is reminiscent of a switch to a hybrid E/M phenotype seen in multiple breast cancer cell lines as an adaptive response to treatment with taxanes (Goldman et al., 2015). Furthermore, in NSCLC, a recent study demonstrated that a hybrid E/M phenotype – identified via co-expression of E-cadherin and SLUG – has significantly poorer survival as compared to those displaying largely epithelial features, or even epithelial and mesenchymal tumors combined (Andriani et al., 2016), endorsing the emerging notion across other cancer types that the hybrid E/M phenotype is perhaps the most aggressive one.

### 5.4. Ovarian cancer

The association between EMT and therapy resistance in ovarian cancer remains unclear. Consistent with the traditional view of EMT as a driver of chemoresistance, increased levels of EMT-TFs SNAIL and SLUG has been reported to be associated with resistance to radiotherapy and paclitaxel (chemotherapy) (Kurrey et al., 2009). Similarly, upregulation of the stem cell marker, NANOG, was found to drive EMT, the expression of the multidrug resistance gene MDR-1, and resistance to cisplatin (Liu et al., 2016; Qin, Li, Cao, Du, & Huang, 2017). Cisplatin resistance can also be driven by upregulation of EMT-promoting hematopoietic PBX interacting protein (HPIP) (Bugide et al., 2017) or EMT-TF TWIST1 (Roberts et al., 2016; Zhu et al., 2016), reinforcing the idea of the well-established correlation between EMT and chemoresistance noted across multiple cancer types (Singh & Settleman, 2011). Immunohistochemistry-based evaluation of ovarian tumors associated the expression of EMT-TFs and ECM components that cross-talk with EMT factors, with poor prognosis (Prislei et al., 2015; Takai et al., 2014; Yuzhalin, Urbonas, Silva, Muschel, & Gordon-Weeks, 2018). Several of these biomarkers are being considered as effective prognostic markers in ovarian cancer. EMT can also be accompanied by a dedifferentiated, pro-inflammatory, and immunomodulatory phenotype that can drive chemoresistance (Rohnalter et al., 2015; Taki et al., 2018). Thus, either targeting EMT-associated signaling pathways or inhibiting EMT via reversing aberrant epigenetic changes have been successful in restoring chemosensitivity both *in vitro* and *in vivo* (Deng et al., 2016).

Contrary to these observations, however, multiple reports have suggested that enrichment of an epithelial signature is associated with chemoresistance. For example, analysis of 46 ovarian cancer cell lines reveal higher resistance to cisplatin in epithelial cell lines, which is mediated by NF-kB pathway activation (Miow et al., 2015). Furthermore, cells from ascites of chemoresistant patients were found to be largely epithelial; conversely, cells and multicellular aggregates with mesenchymal markers were enriched in the chemo-naïve patients (Latifi et al., 2012). Similarly, overexpression of the epithelial regulatory microRNAs miR-200c/miR-141 led to an unexpected 6-8 fold increase in resistance to carboplatin (Brozovic, Duran, Wang, Francisco, & Sikic, 2015). This dichotomy in the association of epithelial vs. mesenchymal phenotypes with chemo-resistance can be highly cell-context dependent. For instance, the effect of miR-200c on expression of TUBB3, a class III β-tubulin that contributes to chemotherapy resistance in ovarian carcinoma, depends on the nuclear vs. cytoplasmic localization of the RNA binding protein, Hu-antigen R (ELAV1) (Sulaiman, Ab Mutalib, & Jamal, 2016). It is also worth noting that overexpression of miR-200c/miR-141 drove only a partial MET in OVCAR-3 and MES-OV cell lines (Brozovic et al., 2015). Therefore, a potential hybrid E/M state mediated by ectopic expression of miR-200 family members in post-EMT cell lines may explain the increased chemoresistance in this case.

To address the contradictory findings related to chemoresistance and EMT/MET in ovarian cancer, ovarian tumors were stratified based on miRNA and mRNA expression profiles into three sub-groups: epithelial, mesenchymal or mixed (Gardi et al., 2014; Yang et al., 2013). This stratification indicated the likely presence of hybrid E/M phenotypes within ovarian carcinoma tumors. Similarly, a 33-gene EMT signature was used to classify tumors into four states – epithelial, mesenchymal, and two intermediate E/M states, one of which was associated with a worse prognosis in patients (Huang et al., 2013). Consistently, in a recent study dissecting single-cell heterogeneity in HGSOC, cells co-expressing E-cadherin and vimentin expressed multiple stem cell markers and positively correlated with a metastatic trajectory (Gonzalez et al., 2018). CTCs expressing various epithelial, mesenchymal, and stem cell markers have been observed in ovarian cancer patients (Blassl et al., 2016); they may be selected for platinum-based chemotherapy, reflecting drug refractory outcomes (Chebouti et al., 2017).

Recently, it was argued that the efficacy of Simvastatin in reducing ovarian cancer metastasis was mediated via targeting of cellular plasticity through inactivation of the Hippo/YAP/ RhoA pathway and impairing stemness during spheroid growth (Kato et al., 2018) – a trait associated with a hybrid E/M phenotype (Grosse-Wilde et al., 2015). Thus, it appears that the hybrid E/M state that is demonstrated to retain maximal 'stemness' features is likely to be associated with poor prognosis for ovarian cancer patients. Although the mechanisms of therapy resistance are likely to be diverse and multifactorial, it is possible that the presence of a hybrid E/M phenotype as a driver of chemoresistance can explain some of the contradictions in ovarian cancer literature regarding the role of epithelial and mesenchymal cells in promoting chemotherapy resistance.

### 5.5. Squamous cell cancer

A detailed investigation in oral SCC has emphasized that a hybrid E/M phenotype may impart unique broad resistance to multiple cancer therapies (Biddle et al., 2016). In CA1 and LM OSCC cell lines, the epithelial subpopulation ($CD44^{hi}/EpCAM^{hi}/CD24^{-}$) was sensitive to paclitaxel, cisplatin, and salinomycin; the mesenchymal subpopulation ($CD44^{hi}$ / $EpCAM^{lo}$ /$CD24^{-}$) was sensitive to cisplatin and salinomycin; and a plastic hybrid E/M subpopulation ($CD44^{hi}$/ $EpCAM^{lo}/CD24^{hi}$ was resistant to all three drugs (Biddle et al., 2016). This study also identified that this drug-resistant sub-population can be enriched *in vitro* by simultaneous treatment with TGFβ and retinoic acid - an MET inducer (Woo & Jang, 2012; Wu, Kim, Chen, Yang, & Chang, 2017). Treatment with RA extends the time cells spend transitioning to EMT, and hence the time for which an undifferentiated population of hybrid E/M cells that can be cultured *in vitro*. While these studies illustrate the potential of external stimuli to promote a hybrid E/M phenotype, other clinically-relevant factors such as PSFs, as discussed earlier, may also promote the hybrid E/M state(s). The timescale over which this hybrid E/M state can be maintained remains to be identified in SCC; such a hybrid E/M state was shown to break down largely to epithelial or mesenchymal phenotypes in PKV prostate cancer cells within two weeks (Ruscetti et al., 2016), however, was relatively more stable over multiple passages over two months in H1975 NSCLC cells (Jolly et al., 2016). Given the high inherent plasticity of this drug-resistant state, this simultaneous treatment with TGFβ and retinoic acid may also generate various subpopulations with varying EMT phenotypes that may cooperate to drive tumor aggressiveness, as observed in breast cancer (Grosse-Wilde et al., 2018; Neelakantan et al., 2017; Tsuji et al., 2008). Therefore, in prostate cancer, targeting both epithelial and mesenchymal states may be promising (given that hybrid E/M largely breaks down to either of these subpopulations relatively quickly), while in cases with an enhanced stability of hybrid E/M, destabilizing that state through targeting PSFs followed by a combinatorial treatment targeting epithelial and mesenchymal subpopulations is likely to be more effective.

Numerous other reports have also associated therapeutic resistance with EMT in SCC, including resistance to cisplatin in TGFβ-responsive cells (Oshimori, Oristian, & Fuchs, 2015), and resistance to radiation treatment in invading cells that have undergone EMT (Gemenetzidis, Gammon, Biddle, Emich, & Mackenzie, 2015). Mechanistically, reduced levels of reaction oxygen species and increased levels of their scavenger

SOD2 in mesenchymal-like CSCs (Gammon, Biddle, Heywood, Johannessen, & Mackenzie, 2013) can also promote survival by combating the high oxidative stress experienced during metastasis (Piskounova et al., 2015). Yet, more detailed investigations to determine the contribution of post-EMT cells and/or hybrid E/M cells in therapy resistance need to be done. To do this, the accurate identification of hybrid E/M phenotypes, becomes an important task. A recent study tracing the expression of fibroblast specific protein 1 (FSP1) claimed that EMT was dispensable for metastatic dissemination (Fischer et al., 2015). However, gene expression analysis of subsets of CSCs in SCC (Biddle et al., 2016) identified that FSP1 was specifically upregulated only in full EMT phenotypes, thus, the use of FSP1 as a marker of EMT may have missed the hybrid E/M CSCs that are truly responsible for metastasis (Li & Kang, 2016).

Therapies designed to target hybrid E/M CSCs may hold promise for preventing metastases and acquisition of therapeutic resistance. For instance, thapsigargin, a drug that inhibits both lysosomal function and induces an unfolded protein response in the endoplasmic reticulum, was shown to target the hybrid E/M CSCs identified by $CD44^{hi}$ $EpCAM^{lo}$ $CD24^{hi}$ in both CA1 and LM cell lines (Biddle et al., 2016). While these drugs may be promising, the mutational landscape of the target tumor also needs to be taken into account. For example, in SCC, mutations inactivating the Notch1 and TGFβ signaling pathways are common, as these pathways have a tumor-suppressive effect in early stage lesions (Cammareri et al., 2016; Pickering et al., 2013). However, these pathways may cooperate later to drive or maintain a hybrid E/M phenotype (Natsuizaka et al., 2017), raising the intriguing question of whether these common tumor-promoting mutations may actually be inhibitory to metastasis. It is unlikely that monotherapies targeting hybrid E/M state will be of significant impact, given the high plasticity of both mesenchymal-like and epithelial-like CSCs in SCC.

Besides governing drug resistance, cells in a hybrid E/M phenotype at tumor invasive edge may also be highly aggressive, and thus, serve as indicator of poor prognosis in SCC. These cells – identified as tumor buds (Grigore, Jolly, Jia, Farach-Carson, & Levine, 2016) – expressed the EMT-TF ZEB1 but at levels weaker than that in adjacent stroma; they also expressed fibronectin (FN1) and exhibited decreased levels of members of miR-200 family (Jensen et al., 2015). Similarly, in head and neck SCCs, a partial EMT signature identified at a single-cell level was predictive of nodal metastasis and adverse pathological features such as lymphovascular invasion and extranodal extension (Puram et al., 2017). Intriguingly, in lung SCCs, both complete EMT and incomplete EMT – defined by co-expression of E-cadherin and vimentin – were associated with poor prognosis (Aruga, Kijima, Masuda, Onozawa, & Yoshizawa, 2018). Thus, future studies should investigate the tissue-specific roles of hybrid E/M phenotypes in SCC.

### 5.6. Pancreatic cancer

Various subsets of pancreatic CSCs – CD133+, CD24+/CD44+ – exhibit resistance against gemcitabine (Hermann et al., 2007; Yin et al., 2011), the standard chemotherapy for PDAC. Initial insights into mechanisms for resistance of CD24+/CD44+ CSCs against gemcitabine and radiation implicate reactive oxygen species and senescence regulator p16 (Wang et al., 2017b; Yin et al., 2011). Further characterization of underlying mechanisms would be crucial for any attempts towards significant progress in therapeutics, given its abysmally low 5-year survival rate and lack of specific druggable targets in PDAC (Gaianigo et al., 2017).

EMT has been implicated in drug resistance in PDAC both *in vitro* and *in vivo*. Across a panel of nine cell lines (PANC-1, Hs766T, AsPC-1, MIAPaCa-2, MPanc96, L3.6pl, BxPC-3, CFPAC-1, and SU86.86) treated with three conventional chemotherapeutic agents (gemcitabine, 5-flourouracil, and cisplatin), the resistant cell lines showed an upregulation of EMT-associated genes, including ZEB1 (Arumugam et al., 2009). Consistently, upregulation of miR-200 or let-7 – both of which can inhibit ZEB1 – can lead to reversal of EMT and increased sensitivity of gemcitabine-resistant pancreatic cancer cells (Wang et al., 2009b). Further, *in vivo* experiments in KPC mouse models show that knockdown of Twist or Snail led to enhanced sensitivity to gemcitabine and consequent prolonged survival in mice (Zheng et al., 2015), suggesting some common minimum program(s) triggered by various EMT-TFs in pancreatic cancer that can elevate resistance against various therapies. Although co-expression of ZEB1 and E-cadherin in the gemcitabine resistant subline of BxPC-3 indicates a correlation of hybrid E/M state with drug resistance (Gao et al., 2017), future studies are required for a more quantitative correlation between $IC_{50}$ of various drugs and the extent of EMT in PDAC cell lines.

### 5.7. Other cancer types

EMT has been associated with acquired resistance in colorectal cancer (CRC) cells too: 5-flourocuracil resistant HT-29 cells show enhanced migration, and upregulation of Twist, Zeb1, and Zeb2, but not a complete loss of E-cadherin at either mRNA or protein levels (Kim, Kwak, Je, Lee, & Jung, 2015), leaving open the possibility that they underwent a partial rather than a full EMT. Although a correlation between EMT markers and poor patient prognosis has been seen in colorectal carcinomas, similar to many other carcinomas (Shibue & Weinberg, 2017), tumor budding – the presence of small clusters of malignant cells in the tumor stroma – is considered as an additional prognostic factor for CRC (Grigore et al., 2016). Tumor buds have been proposed to mirror a partial EMT, with co-expression of nuclear Zeb1 and membranous E-cadherin, thus the jury is still out on whether a partial or a full EMT is more representative of aggressiveness in CRC. Markers specifically upregulated in hybrid E/M phenotype(s) may contribute in resolving this conundrum (Bocci, Tripathi, et al., 2018b; Chang et al., 2016b).

## 6. Conclusion

The existence and aggressive behavior of hybrid E/M phenotypes is an emerging notion across many tumor types. As discussed above, cells co-expressing different combinations of epithelial and mesenchymal markers have been identified in cancer cell lines, primary tumors, animal models of cancer metastasis, CTCs, and metastases. These hybrid E/M phenotype(s) may occupy – perhaps even stably – different spaces on the multi-dimensional and highly non-linear landscape of the EMT spectrum. EMT involves significant changes in a compendium of molecular and morphological traits, thus a rigorous characterization of these distinct hybrid E/M phenotypes is necessary to understand the dynamics of EMT in a context-specific manner. Recent studies that have identified multiple hybrid E/M phenotypes have challenged the dogmatic binary view of EMT, and demonstrated the aggressiveness of hybrid E/M states and emphasized that collective cell migration through clusters of CTCs can be a predominant route to metastasis (Cheung & Ewald, 2016; Fabisiewicz & Grybowska, 2017; Kulasinghe et al., 2018). Yet, a detailed molecular signature and morphological features that can be considered as robust hallmarks of hybrid E/M phenotype remain to be identified and clinically validated in most human cancer subtypes. Manifestations of EMT, and hence hybrid E/M, can be tissue-specific. For instance, K14-positive cells that led collective invasion in breast cancer lacked any canonical EMT markers and hence may help organize purely epithelial motion (Cheung et al., 2016), but a significant proportion of K14-positive cells expressed vimentin in SCC tumors (Pastushenko et al., 2018). Further, EMT and/or MET need not be a necessary or a sufficient condition for metastasis (Cheung & Ewald, 2016; Somarelli et al., 2016b). While recent data support the relevance of EMT and stemness factors in aggressive human carcinoma behavior (Ben-Porath et al., 2008; Taube et al., 2010), biomarker development remains a critical unmet need to translate these preclinical findings to the clinic for diagnostic and therapeutic purposes.

Cells in hybrid E/M phenotype(s) have been shown to be highly plastic and adaptable, and capable of generating epithelial, mesenchymal and hybrid E/M subpopulations, suggesting their enhanced stem-like properties. Indeed, *in vitro* and *in vivo* evidence has demonstrated hybrid E/M cells to be more tumor-initiating as compared to epithelial or mesenchymal ones. These de-differentiated properties associated with a hybrid E/M phenotype are reminiscent of a stem cell that is activated upon tissue injury to proliferate, differentiate, and restore tissue integrity (Jordan, Johnson, & Abell, 2011; O'Brien-Ball & Biddle, 2017). For instance, glomerular epithelial cells in the kidney can attain a hybrid E/M state that behave as bipotent kidney progenitors (Swetha, Chandra, Phadnis, & Bhonde, 2011). Similarly, upon rat liver injury, hybrid E/M cells function as bipotent adult hepatic progenitors (Yovchev et al., 2008). Consistently, hepatic stellate cells – liver resident mesenchymal stem cells – may maintain a hybrid E/M phenotype during quiescence and can transdifferentiate to myofibroblasts when needed (Cicchini et al., 2015; Wang et al., 2018). These properties – phenotypic plasticity and subsequent multi-lineage differentiation potential – can endow hybrid E/M cells with fitness advantage to drive metastatic dissemination (Jolly, Mani, & Levine, 2018d). Consistently, hybrid E/M phenotype has been associated with worse patient survival and more aggressive behavior across cancer types. While hybrid E/M cells may be much more likely to be stem-like relative to the 'extreme' epithelial or mesenchymal phenotypes (Bocci, Jolly, et al., 2018a; Jolly, Jia, Boareto, et al., 2015b; Ombrato & Malanchi, 2014), there may be hybrid E/M cancer cells across multiple carcinoma types that are not stem-like and/or stem-like cancer cells that are not hybrid E/M (Beerling et al., 2016; Bocci, Jolly, et al., 2018a; Jolly, Jia, Boareto, et al., 2015b; Sikandar et al., 2017).

Despite the significance of the hybrid E/M phenotype(s) as poor prognostic biomarker(s) across many carcinomas, a detailed identification of genetic and epigenetic factors that can stabilize one or more hybrid E/M phenotype(s), and that can impart them their aggravated tumor-initiation potential, therapy resistance, immune evasion, and metastatic propensity remains incomplete. Thus, future research directions should aim to decipher the fundamental design principles of cell-autonomous and non-cell autonomous effects – both genetic and non-genetic levels – that can promote the hybrid E/M phenotype(s). These principles can boost the efficiency of ongoing and planned therapeutic attempts to target EMT (Davis, 2014; Malek, Wang, Taparra, & Tran, 2017; Pattabiraman & Weinberg, 2017).

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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