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# Biophysical and biochemical attributes of hybrid epithelial/mesenchymal phenotypes

Running title: Characterizing hybrid epithelial/mesenchymal phenotypes

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## Abstract

The Epithelial- Mesenchymal Transition (EMT) is a biological phenomenon associated with explicit phenotypic and molecular changes in cellular traits. Unlike the earlier-held popular belief of it being a binary process, EMT is now thought of as a landscape including diverse hybrid E/M phenotypes manifested by varying degrees of the transition. These hybrid cells can co-express both epithelial and mesenchymal markers and/or functional traits, and can possess the property of collective cell migration, enhanced tumor-initiating ability, and immune/targeted therapy-evasive features, all of which are often associated with worse patient outcomes. These characteristics of the hybrid E/M cells have led to a surge in studies that map their biophysical and biochemical hallmarks that can be helpful in exploiting their therapeutic vulnerabilities. This review discusses recent advances made in investigating hybrid E/M phenotype(s) from diverse biophysical and biochemical aspects by integrating live cell-imaging, cellular morphology quantification and mathematical modeling, and highlights a set of questions that remain unanswered about the dynamics of hybrid E/M states.

## Keywords

Hybrid epithelial/mesenchymal phenotypes; Collective cell migration; epithelial-mesenchymal heterogeneity; mathematical modeling; cell-state transition; live-cell imaging

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## 1. Introduction

Epithelial Mesenchymal Transition (EMT) is a cellular process well-known for its involvement in embryogenesis, wound healing in adults, tissue fibrosis and cancer progression [1]. EMT is typically associated with one or more changes in the following cellular traits: change in morphology from a cobblestone-like to a fibroblast-like one, weakened cell-cell adhesion, loss of apico-basal polarity, and acquiring migration and invasion capabilities, conferred by molecular changes at transcriptional and/or post- transcriptional frontiers. Investigation into EMT by cell biologists, molecular biologists, cancer biologists and more recently, by biophysicists and systems biologists has exploded in the past 5-10 years (**Fig 1A**) [1].

A salient feature that has emerged for EMT from *in silico*, *in vitro*, *in vivo* (during development as well as in pathological contexts), and clinical observations reveals that EMT is not a binary process; instead, cells can display one or more hybrid epithelial/mesenchymal (E/M) phenotypes [2]. These phenotypes are not always merely transitory or intermediate states for cells *en route* EMT or its reverse – Mesenchymal Epithelial Transition (MET), but also can be stable states (i.e. end points of a transition). Nevertheless, these hybrid E/M phenotypes can be more plastic compared to the 'pure' epithelial and mesenchymal ones, a property which may contribute to their enhanced metastatic ability. The discovery of diverse hybrid E/M phenotypes has simultaneously triggered a fast adaptation in our vocabulary – from EMT to EMP (Epithelial-Mesenchymal Plasticity).

Hybrid E/M cells have been postulated to co-express epithelial and mesenchymal markers such as E-cadherin (CDH1) and Vimentin, and/or exhibit features such as collective cell migration [2]. Their enhanced ability to form tumors and metastases *in vitro* and *in vivo*, their enrichment in circulating tumor cells (CTCs), and their association with worse patient outcomes in multiple cancer types has driven a strong interest towards characterizing their biophysical and biochemical hallmarks and exploiting their therapeutic vulnerabilities (**Fig 1B**) [3]. Here, we review recent progress in identifying diverse hybrid E/M phenotypes across different cancer types, and highlight the open questions related to their complexity and plasticity and their functional consequences in cancer progression.

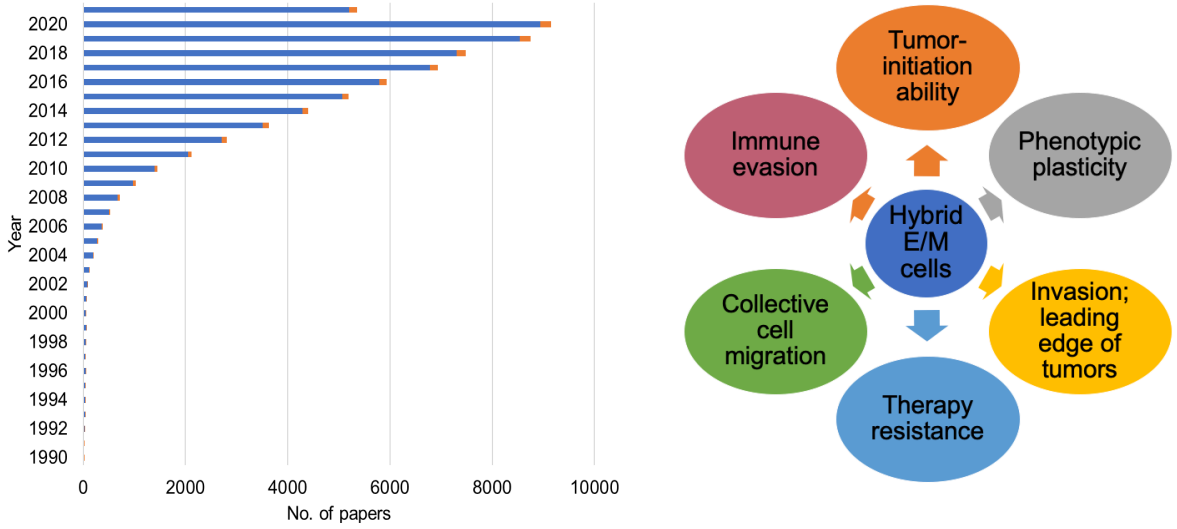


***Figure 1: Hybrid E/M phenotypes.*** *(Left) Numberof PubMed entries over the years containing the terms "hybrid E/M", "partial EMT", "incomplete EMT", "intermediate EMT" (Orange) relative to the total number of PubMed entries for "EMT" (Blue). (Right) Biophysical, biochemical and functional traits often associated with hybrid E/M cells.*

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## 2. Biochemical signatures of hybrid E/M phenotypes

### *2.1 Signaling pathways associated with hybrid E/M phenotypes*

Recent profiling at multiple time points during EMT induction (and/or reversal), often at single-cell level, have enabled identification of signaling pathways associated with one or more hybrid E/M phenotypes. Single-cell RNA-seq data for MCF10A cells treated with TGFβ1 revealed a gradual decrease in epithelial markers (CDH1, EpCAM) and increase in mesenchymal markers (CDH2, FN1, FAP) starting at 2 days of induction, indicating a spectrum of cell-states [4]. The distribution of EMT scores suggested heterogeneity along the epithelial-mesenchymal axis at all time-points, including the untreated MCF10A cell population. Cells treated with TGFβ1 for longer duration had a higher mean EMT score, suggesting that a higher percentage of those cells had a more mesenchymal signature [4]. Another multi-tiered (transcriptomic, proteomic, metabolomic) analysis of EMT in the MCF10A treated with TGFβ supported the observation of these pre-existing (i.e. prior to induction) mesenchymal cells. This analysis identified 20 cell clusters and suggested a more discrete transition from an epithelial phenotype to a hybrid E/M one. Both these studies identified many pathways (Notch, cell cycle, adherens junctions etc.) altered during early or late stages of EMT, many of which have been earlier well-reported to be implicated in EMT; however, fell short of identifying pathways specific to hybrid E/M states. TGFβ-driven EMT in MCF10A at least partly depends on ZEB1, as TGFβ failed to down-regulate CDH1 in ZEB1-KO cells, but upregulated mesenchymal genes such as Vimentin [5].

When comparing TGFβ-driven EMT to spontaneous EMT (where cells from a confluent colony migrate into unoccupied regions of the plate, similar to that seen in scratch-wound healing assay) in MCF10A cells, it was observed that the latter setup had a higher percentage of cells co-expressing E and M markers. KRAS signaling was sustained thoughout both scenarios, but the differentially expressed genes in both scenarios only showed a partial overlap. Further, inhibiting the RAF/MEK/ERK pathway did not prevent the activation of mesenchymal program but obstructed downregulation of epithelial program, thus locking cells in a hybrid E/M state showing co-expression of CDH1 with mesenchymal markers [6]. This analysis indicated that different pathways may be more effective “epithelial inhibitors” vs. “mesenchymal inducers”, thus introducing the context-specific dynamics of EMT driven by these signaling pathways. Such context-specificity in EMT was also endorsed by another study comparing 12 time-course EMT experiments: 4 different cell lines (A549 – lung, DU145 – prostate, MCF7 – breast and OVCA420 – ovarian) treated with 3 EMT-inducing factors: EGF, ENF and TGFβ1 [7]. A few canonical EMT genes such as CD44 and TGFB1 showed differential expression across all 12 conditions, but most others, including SNAI1, CDH1 (E-cadherin) and CDH2 (N-cadherin) did not exhibit this universal pattern. Interestingly, the responses of individual cell lines to different stimuli were noted to be more similar to one another, instead of that of diverse cell lines to the same stimuli. Thus, the manifestation of hybrid E/M phenotypes and pathways associated with them may be quite cell line and/or cancer subtype-specific.

In a mouse model of pancreatic ductal adenocarcinoma (PDAC), hybrid E/M phenotype was seen not at a transcriptional level, but via protein internalization [8]. In these *in vivo* tumors, two distinct types of EMT were observed: a “complete EMT” (C-EMT) and a “partial EMT” (P-EMT). While both of them had overlapping mesenchymal programs, P-EMT tumors retained expression of epithelial transcripts. Tumor spheres obtained from C- and P-EMT cell lines had diverse invasion patterns; C-EMT cells, having lost E-cadherin, exhibited predominantly single-cell invasion at edges of primary cell mass (typical of mesenchymal program), but P-EMT cells with remnant E-cadherin and cell-cell adhesion invaded through multicellular clusters as well.

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P-EMT program was found to be associated with high levels of late recycling endosome protein Rab11, which is responsible for recycling of epithelial proteins back to the cell surface [8]. Thus, the loss of epithelial program need not always be achieved at a transcriptional level as tacitly assumed often, but also can have a predominant post-transcriptional mode. In this study, the co-existence of C-EMT and P-EMT was not seen in an individual tumor, indicating that P-EMT is a stable end-point of transition for many PDAC tumors.

TGFβ treatment was capable of pushing P-EMT cells to C-EMT state [8]; thereby suggesting that the extent to which carcinoma cells undergo EMT can depend on TGFβ concentration in the stroma [9]. Further analysis revealed that a prolonged activation of calcium signaling can induce a P-EMT in multiple murine and human cell lines [10], showing the internalization of membrane-associated E-cadherin and other epithelial proteins and consequently increased cellular migration and invasion. Such prolonged activation of $Ca^{2+}$ can also activate NFATc signaling, which has been reported to stabilize hybrid E/M phenotype in non-small cell lung cancer (NSCLC) H1975 cells [11]. Other manifestations of partial EMT have been reported in head and neck squamos cell carcinoma (HNSCC) from an analysis of ~6,000 single-cells taken from 18 patients' primary tumors and metastases. The P-EMT cells were found to be at leading edge of primary tumors, indicative of their role in collective cell invasion in concert with neighboring cancer-associated fibroblasts (CAFs) [12]. Similar to differences seen in C-EMT and P-EMT in PDAC, the P-EMT HNSCC cells also retained expression of various epithelial markers and showed upregulation of only one EMT-transcription factor (EMT-TF) : SNAI2 (SLUG). SNAI2 often induces a weaker EMT as compared to its family member SNAI1 (SNAIL) and has been associated with a partial EMT state and/or collective cell migration across diverse biological contexts where EMT is involved – branching morphogenesis, wound healing and breast cancer [13,14].

Put together, these studies have highlighted individual pathways in context-specific manners which may stabilize cells in hybrid E/M phenotype(s). However, arriving at a more generic or universal partial EMT signature seems distant, if at all possible, given widely different flavours (transcriptional, post-transcriptional etc.) of hybrid E/M phenotypes reported across cancers.

### *2.2 Transcriptional factors associated with hybrid E/M phenotypes*

Besides SLUG [12–14], another transcription factor that can be associated with hybrid E/M phenotype(s) is ΔNP63α. In basal-like breast cancer, ΔNP63α can stabilize a hybrid E/M state by elevating the expression of pro-migratory components SLUG and AXL, as well as increase miR-205 which can silence ZEB1/2, thus promoting retention of epithelial traits [15]. ΔNP63α and SLUG expression can promote collective cell invasion *in vivo* of ductal carcinoma *in situ* (DCIS) tumors. They are also expressed in mammary stem cells and myoepithelial cells during mammary development, thus underscoring that hybrid E/M cell-state identity can be stably acquired during normal (i.e. non-cancerous) scenarios as well and that the reactivation of these pathways in cancer cells can mirror those traits of collective migration and enhanced stemness.

A partnership between SLUG and ΔNP63α can be seen beyond breast cancer as well. Their expression is correlated in lung squamous cell carcinoma (SCC), bladder and prostate cancer. ΔNP63α induces the expression of SLUG and atypical protocadherin FAT2 to promote lung SCC cell migration that prevent the establishment of mature cell-cell adhesions formed by E-cadherin, but does not abrogate E-cadherin fully. Live imaging of organotypic culture of DCIS cells revealed that ΔNP63α expressing cells invaded the ECM similar to that seen in canonical leader-follower relationship, but cells depleted of ΔNP63α could not invade into the ECM [16].

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Elevated expression of ΔNP63α, FAT2 and SLUG is associated with worse clinical outcomes in NSCLC cells as well as HER2-/ER- breast cancer patients [16], highlighting a poor prognosis for hybrid E/M phenotype.

A family member of FAT2 – FAT1 – is associated with hybrid E/M phenotype in SCCs. Deletion of FAT1 in mouse models of skin SCCs and lung tumors can promote hybrid E/M phenotype and consequent higher stemness and spontaneous metastasis [17]. Loss of function of FAT1 activates both the epithelial and mesenchymal branches. First, through the CD44-SRC axis, YAP1 nuclear localization is increased that upregulates ZEB1 expression, stimulating EMT. Second, EZH2 is inactivated which promotes SOX2 that can sustain an epithelial state. A phospho-proteomic analysis identified a decrease in EGFR-RAS-RAF-MEK-MAPK signaling upon Fat1 deletion, reminiscent of RAF/MEK/ERK pathway inhibition locking human mammary epithelial cells in hybrid E/M state as seen *in vitro* [6]. High expression of Fat1-deleted signature was associated with worse survival in lung SCC patients [17]. Interestingly, in this SCC mouse model, ΔNP63 was found to regulate early hybrid E/M states and prevented the transition from EpCAM (epithelial cell adhesion molecule)-positive cells to EpCAM-negative ones [18], while TGFβ, a common EMT-inducer, promoted the transition to more mesenchymal states. Thus, ΔNP63 seems to maintain hybrid E/M state across different cancers *in vitro* and *in vivo*.

The p63 gene can be activated transcriptionally by GRHL2 [19], a well-studied MET inducer [20]. GRHL2 and ZEB1 inhibit each other and can self-activate; different proportions of these molecules in a cell can allow for epithelial, mesenchymal and hybrid E/M phenotypes [21,22]. In addition to SLUG and ΔNP63α, NRF2 (encoded by NFE2L2 gene) has been proposed as a stabilizer of hybrid E/M phenotype [23]. Overexpression of NRF2 increases the frequency of cells co-expressing ZEB1 and E-cadherin in bladder cancer RT4 cells, while its downregulation can drive hybrid E/M H1975 cells to being more mesenchymal. In collectively migrating RT4 cells during scratch-wound healing assay, NRF2 can stabilize hybrid E/M (cells co-expressing ZEB1 and E-cadherin) phenotype close to the leading edge [24]. Expression levels of NRF2 and its downstream targets also associate with worse clinical outcome across cancers [23].

Alongside the characterization of transcription factors (TFs) specific to hybrid E/M phenotypes in diverse cancers, specific cancer types have been proposed to be enriched in hybrid E/M phenotypes, for instance, the highly aggressive Inflammatory Breast Cancer (IBC) which metastasizes primarily via clusters of circulating tumor cells (CTCs) [25,26]. Compared to non-IBC patients, IBC patients have a higher frequency of clusters which show accumulation of E-cadherin due to altered trafficking. Many cell line and mouse models of IBC possess cells co-expressing E-cadherin with one or more mesenchymal markers (Vimentin and/or ZEB1) [25]. Single-cell clones derived from SUM149 IBC cells can display a range of behaviors – epithelial-like (E), mesenchymal (M1, M2) and three distinct hybrid states (EM1, EM2 and EM3) [27]. Consistent with previous reports [28], the hybrid E/M cells had the maximum tumor-initiating potential *in vivo.* RNA-seq and ATAC-seq of these clones identified clusters of transcription factos associated with these cell-states. The RUNX TF family was specifically asscociated with hybrid E/M states, while KLF TF family activity was identified to decrease consistently as cells progressed through EMT. Knockdown of RUNX2 led to upregulation of CDH1 in EM2, EM3 and M1 clones, decrease in Snai1 and upregulation of OVOL1 in all three hybrid E/M clones, thus pinpointing the role of RUNX2 in enabling hybrid E/M state in IBC. RUNX2 has been shown to stimulate SNAI2 (SLUG) – a TF typically seen to be upregulated in hybrid E/M cells – in MCF7 cells [29]. However, what EMT and/or MET inducing TFs RUNX2 interfaces with in IBC still remains to be unearthed.

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Overall, while many EMT-TFs have been identified over the last two decades (ZEB1/2, SNAI 1/2, TWIST, GSC, FOXC2), the identification of hybrid E/M-inducing or stabilizing TFs (SLUG, NRF2, ΔNP63, RUNX2) (**Fig 2**) is relatively recent, and much work is needed to delineate their impact on various biochemical and biophysical aspects of EMT in a context-specific mode.

| Cancer | TF | Effect on E | Effect on M |
|---|---|---|---|
| Basal breast cancer | ΔNP63 | Retained (due to miR-205) | Increased SLUG, AXL |
| Lung, Squamous SCC | FAT1 (deletion) | Retained (through SOX2 upregulation) | Nuclear accumulation of YAP1; higher ZEB1 |
| Bladder cancer | NRF2 | Stabilized co-expression of E-cadherin and ZEB1 | |

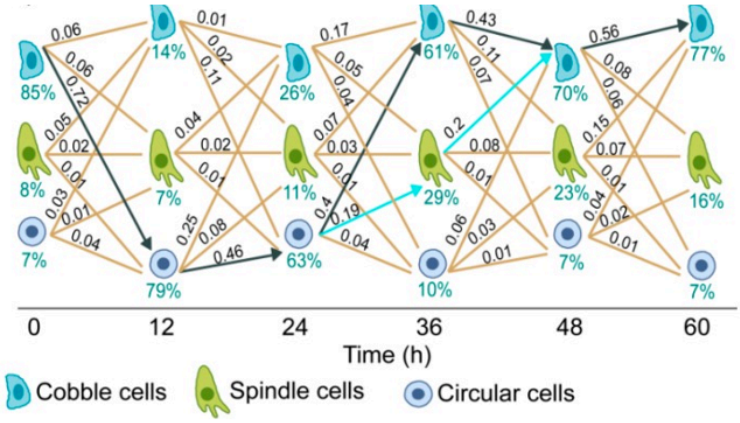


***Figure 2: Biochemical and biophysical aspects of hybrid E/M phenotypes.*** *(Top) A list of transcription factors so far associated with hybrid E/M phenotypes in different cancers, and their impact on epithelial and mesenchymal markers/traits. (Bottom) Cell-state transition diagram for the MDA-MB-468 cells treated with EGF; numerical values denote cell-state transition probabilitites or percentages of cells of a particular shape (cobble, spinder, circular) at a given induction timepoint. Image taken from Devaraj & Bose, J Clin Med 2019* [39].

*2.3 Epigenetic mechanisms associated with hybrid E/M phenotypes*

Among the TFs involved in EMT/MET, GRHL2 can behave as a pioneer factor, i.e., it can bind to enhancers and regulate chromatin accessibility, thereby influencing the binding of other TFs [20]. GRHL2 employs DNA methylation and histone modifications to regulate the transcription of epithelial genes; likely making them transcriptionally more accessible,, which helps stably maintain an epithelial and/or hybrid E/M phenotype. GRHL2 knockdown, therefore, can facilitate epithelial cells to switch to hybrid E/M and/or mesenchymal states, depending on chromatin remodeling driven by EMT-TFs such as ZEB1. Thus, the epigenetic status of various epithelial and mesenchymal genes (for instance; poised, active, repressive chromatin marks) can influence the ability of various EMT and MET inducers and TFs to drive cellular plasticity [20]. For instance, ZEB1-mediated chromatin remodeling can act as a barrier to GRHL2-driven MET, and conversely, GRHL2-mediated epigenetic changes can allow for EMT-resistant cells in a population [30,31]. Therefore, in addition to transcriptionally repressing each other, ZEB1 and GRHL2 may be engaged in epigenetic ‘tug of war’ as well, as often seen in developmental cell-fate commitment scenarios [20]. This functional antagonism can act as ‘checkpoints’ for

cellular transitions in and out of hybrid E/M phenotype(s). Similar antagonism was witnessed for BRD2 (pro-EMT) and ZMYND8 (anti-EMT), both of which can occupy largely overlapping cistromic regions including enhancers, acting as a fulcrum of cellular identity and plasticity [32].

To delineate specific epigenetic regulators of hybrid E/M phenotype(s), ChIP-Seq and ChIP-qPCR analysis was conducted for ETS2, HNF4A and JUNB whose expression transiently peaked (12-36 hours) during treatment of NSCLC A549 cells with TGFβ [33]. The maximal peak intensities for HNF4A and JUNB were found to be within 50 bp of each other and close to ETS2 binding sites. These master regulators also physically interact with one another and synergistically drive the formation of super-enhancers for many EMT-associated genes. Their knockdown abolishes TGFβ-driven EMT, pinpointing to their crucial role in EMT dynamics. Another factor involved in chromatin reorganization that has been implicated in EMT is CTCF [34,35]; however, how CTCF impacts the topological domains and 3D chromatin structures as cells progress through EMT remains to be identified in a context-specifc manner. Integrating multi-modal high-throughput data (ATAC-seq, ChIP-seq, RNA-seq), preferably at a single-cell level, will be instrumental in decoding the epigenetic hallmarks of hybrid E/M states.

### *2.4 Proteomic signatures for hybrid E/M phenotypes*

One of the first studies investigating the E-M spectrum in 38 NSCLC cell lines identified a proteomic signature of hybrid E/M cells [36]. These cell lines were categorized into epithelial, mesenchymal and hybrid E/M based on cell morphology and the ratio of cell-surface localized E-cadherin and Vimentin, as obtained from whole cell lysates. Many of the hybrid E/M cell lines exhibited aggressive behavior – collective migration and enhanced invasion, coupled with high aggregation – a combination of typical epithelial and mesenchymal traits. In these hybrid E/M cell lines, mRNA and protein levels of many cytoskeletal and actin-binding proteins were elevated, and a signature of 135 proteins was identified. Proteomic analysis of epithelial cells, H1437, after 8 days of TGFβ treatment identified that reorganization of cytoskeletal changes preceded the loss of CDH1 (noticed at protein levels after 14 days of TGFβ treatment) [36], supporting that mRNA or protein levels of CDH1 alone is not necessary to mark the acquisition of a hybrid E/M phenotype.

In a mass cytometry based single-cell proteomic analysis in NSCLC cells, eight distinct states were seen in NSCLC, out of which three were specificially mapped as partial EMT ones [37]. The partial EMT states co-expressed Vimentin and E-cadherin, and included a subset of Twist+ cells. This study also compared the trajectories followed by cells during EMT (upon treatment of TGFβ) with those during MET (upon withdrawal of TGFβ) and demonstrated hysteresis, as predicted earlier by mathematical models for EMT signaling [38].

Put together, these studies endorsed that co-expression of E-cadherin and Vimentin can be employed at a proteomic level too to identify hybrid E/M cells. However, a specific proteome and/or secretome signature of hybrid E/M cells has not yet been unequivocally identified.

## 3. Biophysical signatures of hybrid E/M phenotypes

### *3.1 Cell shapes associated with hybrid E/M phenotypes*

Similar to transcriptomic profiling at multiple time points during EMT, characterizing cell-shape dynamics as cells progress through the EMT has piqued recent interest. Observing TGFβ-treated HaCaT and AW13516 epithelial cells using phase-contrast microscopy has revealed

changes in cell shape. Five distinct phenotypes were identified based on cell shape, inter-cellular space and migratory structures (lamellipodia, filopodia): epithelial (polygonal-shaped, closely packed), epithelial-like (polygonal-shaped, bearing lamellipodia-like structures on their free surface), progessive mesenchymal (elongated dumbbell shapes but with cortical F-actin), semi-mesenchymal (stellate-shaped with distinct filopodia) and terminal mesenchymal (fusiform in shape with prominent lamellipodia and filopodia; vimetin and fibronectin present) [40]. With increasing time intervals of TGFβ exposure , the percentage of epithelial cells decreased while that of terminal mesenchymal ones increased, quantified by a Markov model that estimates the most likely trajectories of cells as they change their shapes. In a similar study conducted in MDA-MB-468 cells that were induced to undergo EMT through EGF, three distinct morphological states – cobble, spindle and circular – were observed; the latter two of which are migratory (**Fig 2**). This cell-shape heterogeneity was witnessed in untreated cells too (similar to biochemical heterogeneity in untreated MCF10A cells [4]), but the relative abundance of these phenotypes depended on dosage and exposure duration of EGF [39]. A population dynamics model fitted on the dynamic data collected here revealed that these cell-shape transitions were reversible and that spindle cells predominantly formed from circular ones. These investigations that integrate quantitative image analysis with population-level mathematical models offer not only a way to augment the molecular marker- based investigations of EMT, but also a pipeline to understanding the dynamical principles that govern the underlying cellular plasticity landscape. For instance, in A549 cells with endogenous RFP (red fluorescent protein) label for Vimentin [41], live-cell imaging- based analysis of individual cell trajectories (in a high-dimensional morphological feature space) identified two distinct cell-transition paths with varying Vimentin dynamics, thereby amplifying heterogeneity in hybrid E/M states.

Another study highlighting the quantitative analysis of cell-shape metrics involves digital holographic cytometry (DHC) – a non-invasive label-free imaging technique for 3D morphological data of cells in a monolayer. Imaging NMuMG cells by TGFβ for 96 hours using DHC led to capturing 27 different features grouped into four categories; one metric from each of these categories was used to arrive at an EMP score, upon which a linear classifier was trained [42]. For categorizing the TGFβ- treated and TGFβ inhibitor-treated NMuMg cells (both at varying concentrations), this classifier worked accurately well; however, its aaccuracy fell short with other cell lines. Therefore, while a DHC-derived classifier allows efficient monitoring of rate, degree and heterogeneity of cell-state transitions in a given cell system [42], its application can be limited by a possible lack of universality in quantitative morphological changes seen upon EMT induction, similar to observations for biochemical heterogeneity [7].

### *3.2 Cytoskeletal hallmarks of hybrid E/M phenotypes*

Image quantification can also reveal specific cytoskeletal attributes of hybrid E/M phenotypes. On treatment of NSCLC cells A549, H460 and H1299 with TGFβ, stress fibers increasingly aligned well among themselves, as reflected by a gradual change in the orientational order parameter [43]. Cells in partial or early EMT (14 hours post-treatment) stage had fewer spots for focal adhesion kinases (FAKs) but stress fibers in late EMT (48 hours post-treatment, i.e. towards a more mesenchymal) were capped on both ends with FAK. Atomic Force Microscopy (AFM) measurements revealed that the Young's modulus of the 'nest-like' partial EMT cells was intermediate to that of untreated and late stage EMT. Although the time-period for which cells were treated with TGFβ may seem insufficient to induce a complete EMT, but E-cadherin was drastically reduced both at mRNA and protein levels [43]. However, how loss of E-cadherin and the cytoskeletal changes can impact each other, needs further analysis.

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Another study conducted in human mammary epithelial cells (HMECs) using an inducible over-expression of ZEB1 (24 hour exposure: partial EMT; 5 day exposure: complete EMT) [44] demonstrates that not all biophysical features of hybrid E/M states are intermediate between those of epithelial and mesenchymal states. The nucleus-centrosome axis is oriented towards the inter-cellular junction in epithelial state and towards extracellular matrix in mesenchymal state, but random in the hybrid E/M state. Consequently, the hybrid E/M cells are poorly contractile and exert less cell traction forces; thus, migrating faster than both the epithelial and mesenchymal ones [44]. This enhanced migratory ability of hybrid E/M cells is reminiscent of their preferred localization at the invasive front of primary tumors as seen across clinical specimens [12,45]. Given relatively few studies on deciphering cytoskeletal hallmarks of hybrid E/M phenotypes, their generality still needs to be tested in other EMT model systems.

### *3.3 Cell-cell adhesion and collective cell migration in hybrid E/M phenotypes*

Computational modeling of invasion of a heterogeneous cell aggregate predicts that invasive potential is the maximum for intermediate values of cell-cell adhesion, which may correspond to hybrid E/M phenotypes [46]. Recent experimental observations showing an enrichment of hybrid E/M cells at the leading edge in luminal breast cancers [47] supports these model predictions as well as presents an opportunity to decode mechanistic underpinnings for similar clinical observations [12,45]. The frequency of hybrid E/M cells in clusters of circulating tumor cells (CTCs) as well as size distribution of those clusters depends on both the rate of EMT and the cell-cell adhesion parameters [48].

However, the association of hybrid E/M phenotypes with collective cell migration should not be assumed exclusive for various reaons. First, during collective migration, cells can often dynamically change their position from being a ‘leader’ to a ‘follower’ and *vice versa* involving metabolic reprogramming [49], but whether EMT-associated changes are witnessed concomitantly, and whether and how they contribute functionally to this dynamic phenotypic switching, requires further rigorous analysis. Second, epithelial cells undergoing unjamming transition (UJT) may also display collective migration without showing considerable changes in cell-cell junctions and apico-basal polarity [50]. Hence, understanding the dynamic inter-relationships among hybrid E/M phenotypes, cell-cell adhesion and collective cell migration is crucial for a rigorous definition of unique biophysical traits associated with hybrid E/M states.

## 4. Conclusion

Recent advancements in live-cell imaging, cell morphology quantification, lineage tracing and development of mathematical models to capture cell-state transition trajectories have fuelled our progress in understanding the biophysical and biochemical repertoire of properties that hybrid E/M phenotypes posses, and their functional implications in cancer progression. This deluge of high-throughput and dynamic data being collected can help make substantial leaps in answering some of the open questions: a) how do the different biophysical and biochemical changes catalogued so far for hybrid E/M phenotypes coincide with and influence one another? b) how the manifestation of hybrid E/M phenotypes in one model system differs from another? and c) do hybrid E/M cells occupy a discrete state-space in the composite feature space or is it a continuum of states; or can the concept of “macro-states” and “micro-states” be invoked to resolve seemingly conflicting data about cells traversing a multi-stable phenotypic landscape?

**Conflict of Interest** The authors declare no conflict of interest

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