Reconstruction of single-cell lineage trajectories and identification of diversity in fates during the epithelial-to-mesenchymal transition

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Exploring the complexity of the epithelial-to-mesenchymal transition (EMT) unveils a diversity of potential cell fates; however, the exact timing and mechanisms by which early cell states diverge into distinct EMT trajectories remain unclear. Studying these EMT trajectories through single-cell RNA sequencing is challenging due to the necessity of sacrificing cells for each measurement. In this study, we employed optimal-transport analysis to reconstruct the past trajectories of different cell fates during TGF-beta-induced EMT in the MCF10A cell line. Our analysis revealed three distinct trajectories leading to low EMT, partial EMT, and high EMT states. Cells along the partial EMT trajectory showed substantial variations in the EMT signature and exhibited pronounced stemness. Throughout this EMT trajectory, we observed a consistent downregulation of the EED and EZH2 genes. This finding was validated by recent inhibitor screens of EMT regulators and CRISPR screen studies. Moreover, we applied our analysis of early-phase differential gene expression to gene sets associated with stemness and proliferation, pinpointing ITGB4, LAMA3, and LAMB3 as genes differentially expressed in the initial stages of the partial versus high EMT trajectories. We also found that CENPF, CKS1B, and MKI67 showed significant upregulation in the high EMT trajectory. While the first group of genes aligns with findings from previous studies, our work uniquely pinpoints the precise timing of these upregulations. Finally, the identification of the latter group of genes sheds light on potential cell cycle targets for modulating EMT trajectories.

EMT | cell fate | scRNA-seq

The epithelial–mesenchymal transition (EMT) is a pivotal process underpinning a range of biological phenomena from embryonic development and wound healing to tumor metastasis (1–5). During EMT, epithelial cells lose their apical–basal polarity and adhesion to other cells and acquire mesenchymal traits such as invasiveness and migratory capabilities (3–5). At the molecular level, this process is accompanied by the downregulation of epithelial markers such as E-cadherin (CDH1) and a concurrent upregulation of mesenchymal markers like N-cadherin (CDH2), vimentin (VIM), and fibronectin (FN) (6, 7). Importantly, EMT is not merely a binary transition from an epithelial (E) to a mesenchymal (M) state. Recent findings redefine EMT as a continuum, with cells capable of occupying intermediate states, often referred to as “partial” EMT (8, 9). Progression along this spectrum is tightly regulated by a set of key transcription factors, including members of the Snail, Zeb, and Twist families (10, 11). The expression and activities of these transcriptional factors are governed by a complex network of several epigenetic regulators and signaling pathways, encompassing TGF-beta, Wnt, EGF, FGF, PI3K/Akt/mTOR, IL-6/JAK/STAT3, and NOTCH (5, 12–16).

Cells in a syngeneic, phenotypically homogeneous population have been observed to adopt distinct fates upon treatment with an EMT inducer (17, 18). However, the intricate mechanisms that drive early cell states to branch into unique EMT trajectories are yet to be fully understood. The idea of divergent trajectories, through a developmental Waddington landscape (19), is well accepted in stem cell biology (20). Given the close association between EMT and stemness (21, 22), we aimed to investigate whether the heterogeneous response to EMT inducers extends beyond mere temporal variations and involves multiple distinct trajectories. To this end, we analyzed previously published time series scRNAseq data from MCF10A cells treated with TGF-beta (18) (Fig. 1A).

While scRNAseq data offer a wealth of insights into the heterogeneity of cellular states (23, 24), the inherent need to sacrifice cells at each time point precludes the ability...
to trace individual cell lineages over time. This restriction poses a challenge to reconstructing trajectories from time-series scRNA-seq data (25). To address this challenge, we employed a method based on OT analysis (26, 27), known as Waddington OT (WOT) (28). This method stands in contrast to other widely used trajectory tools such as pseudotime analysis, which infers a temporal sequence within a cell population but cannot deduce direct cell-to-cell transitions (29, 30). Another method, RNA velocity, utilizes additional information from unspliced and spliced RNA to predict the direction of movement across RNAseq space of individual cells (31, 32). This method deepens our insight into the velocity field and short-term cellular changes.

However, the applications of the RNA velocity method have sometimes been found to lack precision and can yield ambiguous results, particularly due to assumptions of constant kinetic rate parameters (33). To address these challenges, Qiu et al. developed,
a method that precisely infers the vector field from time-resolved, metabolically labeled scRNA-seq data (34). In contrast, our study utilizes conventional, daily-collected scRNA-seq data. We employ WOT specifically for its ability to analyze direct cell-to-cell transitions within scRNA-seq data at discrete, predetermined time points. This approach avoids the complexities and potential noise associated with velocity field inference, ensuring that our analysis remains precise and directly interpretable.

Utilizing the WOT technique, we reconstructed lineage trajectories at single-cell resolution using the time series scRNA-seq data from MCF10A cells undergoing EMT stimulated by TGF-beta (18), enabling identification of diverse trajectories leading to distinct EMT fates. In this study, we extend previous EMT research by not only examining state heterogeneity at various time points within a single EMT process but also by uncovering the diversity of EMT responses as unique, distinct processes under the same treatment. We delved into the roles of stemness, proliferation, and cellular hypoxic response signatures. While these signatures have known associations with EMT (5, 21, 35), their variations across different EMT trajectories have not been extensively explored.

Furthermore, our trajectory analysis at the single gene level enabled us to predict differentially expressed genes (DEGs) in the early phases of each fate. Early gene expression changes linked to a specific fate were then partially validated through methods such as inhibitor screens of EMT regulators and CRISPR-associated gene knockout screens (1, 15, 16, 36), highlighting the robustness of our predictions. We then included a wider array of genes implicated in EMT regulation but not yet fully examined. This approach led to several insights, notably that cell cycle-related genes are up-regulated in the ancestors of cells entering the high EMT state. Additionally, we found that genes linked to cell surface markers that play a critical role in cell–matrix and cell–cell adhesion are markedly up-regulated in the ancestors of cells transitioning to the partial EMT state. An overview of the general framework is provided in Fig. 1 B–F.

**Results**

**Uncovering Three Distinct EMT Trajectories via Optimal Transport Analysis.** Given that scRNA-seq data cannot be obtained from individual cells at multiple time points of lineage tracing experiments, due to the assay’s destructive nature, we set out to computationally infer likely ancestor cell states for different EMT fates. In the study by Deshmukh et al. (18), an immortalized human mammary epithelial cell line, MCF10A, was treated with TGF-beta for 1, 2, 3, 4, or 8 d (Fig. 1A), and scRNA-seq data were obtained from populations sacrificed at each time point. Through cluster analysis of the scRNAseq data at day 8, we identified three subpopulations representing three significantly different cell fates (Materials and Methods). These fates were categorized as low, medium, and high EMT by utilizing the 76GS and KS scoring metrics to compute the average EMT scores (37–40), for each subpopulation (Fig. 2A, day 8). For instance, using the 76GS method, we derived average EMT scores of −0.63, 0.23, and 0.32 for the low, medium, and high EMT categories, respectively, with

![Optimal transport recovers diverse trajectories of EMT.](https://www.pnas.org/content/121/32/e2406842121.full/fig2.png)

Fig. 2. Optimal transport recovers diverse trajectories of EMT. (A) The colormap presents the inferred ATF distributions, showcasing the probability of early cell states (from day 0 to day 4) serving as ancestors for the three fate subpopulations by day 8. (B) Barycentric coordinate projection visualizes ATF distributions. For each time point, every individual cell is associated with a three-dimensional probability vector, as determined by that specific time point’s ATF distributions of the three fates (each column in A). This vector is then mapped onto an equilateral triangle (SI Appendix, S3). A position at one of the triangle’s vertices indicates a 100% commitment of the cell state to the corresponding fate.
significant p-values (t-test, P < 0.005) for each pairwise comparison (SI Appendix, Table S1, day 8). Use of the KS method yielded consistent results (SI Appendix, Table S2, day 8).

To infer the trajectories of individual cell states across the sequential scRNAseq dataset, we utilized WOT (28). The dataset consists of six distinct batches, each sourced from a uniformly mixed single culture of around 10,000 cells. This setup provides a consistent starting point for each batch before the application of TGF-beta, allowing us to assume uniform initial conditions across the batches. Leveraging this baseline, the WOT method predicts a unique transition probability (i.e., the likelihood that one cellular state is the ancestor and the other the descendant) between two adjacent scRNAseq time points. The WOT approach assumes that cellular states navigate the gene expression space using the shortest overall distance (SI Appendix, Fig. S1 and Materials and Methods). By multiplying the inferred transition probabilities from initial to subsequent time points within our scRNAseq data series, we computed the probability of each early cell state, termed “ancestors”, transitioning into a final cell state at day 8 or “fate” (Fig. 2A). We refer to these transition probabilities as “ancestor-to-fate (ATF) distributions.” To validate our inferred distributions, we followed an approach of omitting data of a specific time point, designated as test data, and comparing our estimated cell state distribution to the actual data of this time point. The results showed minimal deviations between predictions and actual data, confirming our predictions’ reliability when contrasted with other intrinsic cellular variations and unbiased interpolations (SI Appendix, S1). Note that in the main text, both the inference and the validation of ATF distributions were confined to the first 30 PCA dimensions of the gene expression space, as validated in the original WOT paper to accurately predict cell states in the test data at held-out time points (37). Additionally, to broaden our analysis, we expanded the dimensional range up to 3,000 and repeated our analysis for comparative purposes. Our results demonstrated consistency in all main conclusions of the inferred ATF distributions across various dimensionalities (SI Appendix, S2).

To identify cellular origins leading to various fates, we categorized cells with over 75% probability of transitioning to specific fates as “top ancestors” (SI Appendix, S3). Notably, prior to treatment, the percentage of top ancestors for the low EMT fate constituted double the combined percentage of the other two fates (5.52% vs. 2.85% at day 0). By the second day of treatment, the proportions of top ancestors across all three fates converged, with values of 13.57%, 12.35%, and 13.31% for low, medium, and high EMT (Fig. 2B and SI Appendix, Fig. S2 and Table S3). This temporal shift in proportions indicates a delayed inclination toward the medium and high EMT fates, induced by TGF-beta. Additionally, cells falling below the probability threshold for any EMT fate were classified as “undetermined ancestors.” With ongoing TGF-beta treatment, the portion of the undetermined ancestors decreased sharply from 75% to 90% (Fig. 2B and SI Appendix, S5 and Fig. S5). This approach unveiled three distinct trajectories, each showing unique average EMT score trajectories with nonoverlapping 95% CI, throughout the course of TGF-beta treatment (Fig. 3B and SI Appendix, Fig. S6A). The clear separation into low, medium, and high EMT trajectories was consistently observed using both the 76GS and KS EMT scoring methods (SI Appendix, Fig. S6B and Materials and Methods).

Notably, the separation of trajectories was observed even before the initiation of TGF-beta treatment on day 0, implying that early EMT hallmarks could predetermine cellular EMT fates (Fig. 3B). In light of this finding, we limited our WOT analysis to the gene expression space encompassing genes associated with the EMT signaling pathway (42, 43), and repeated the computation of ATF distributions and gene signature dynamics across the three trajectories. We found that the three trajectories remained profoundly divergent, similar to the ATF computations using the full gene space. However, when examining early time point (before day 3), we observed that the ancestral cell populations were less separable when analyzed using the EMT gene set compared to the full gene set (SI Appendix, S6).

Furthermore, we analyzed stemness, hypoxia response, and proliferation signatures among cells belonging to the three fates. For each cell, we computed those signatures using single-sample gene set enrichment analysis, ssGSEA (Fig. 3A and Materials and Methods). All trajectories showed an over 1.9 z-score increase in both stemness and hypoxia response (Fig. 3B and SI Appendix, Fig. S6A). This trend aligns with prior research that links hypoxia to enhanced stemness in EMT (44, 45). Like the EMT signature, these three trajectories stood out with their nonoverlapping 95% CI when characterized by these two signatures (Fig. 3B and SI Appendix, Fig. S6A). Of particular interest was that by day 8, the medium EMT trajectory exhibited the highest levels of stemness and hypoxia response, with enrichment scores of 1.7 for both. In comparison, the low and high EMT trajectories displayed scores of 1.4 and 1.2, respectively (Fig. 3B and SI Appendix, Fig. S6A). These findings resonate with earlier studies identifying an intermediate EMT stage characterized by heightened stemness and a pronounced response to hypoxia (4, 35, 46).
Upon analyzing the proliferation signature trajectories, we noted enrichment z-score declines from day 0 to day 1 (low EMT: $-0.12$ to $-0.84$, medium EMT: $-0.26$ to $-1.04$, high EMT: $-0.11$ to $-0.80$, Fig. 3B). A similar trend was observed in the G2M checkpoint and mitotic spindle hallmarks (SI Appendix, Fig. S6A). This decrease reflects the known role of TGF-beta in inhibiting cell division (47–49).

From day 1 through day 8, cells regain their proliferative capacity, evidenced by enrichment score of proliferation rebounds of 2.0 for low, 1.8 for medium, and 2.1 for high EMT (Fig. 3B and SI Appendix, Fig. S6A). Based on these changes, we concluded that the medium EMT trajectory was distinctive, exhibiting the most pronounced decline and the least recovery in proliferation signatures. This unique trend in the medium EMT cells corresponds with their pronounced response to the TGF-beta inducer, evident by TGFBI (a TGF-beta-induced gene) showing more elevated expression in this trajectory than in the other two (SI Appendix, Fig. S7).

Fig. 3. OT analysis reveals unique cellular signatures across distinct EMT trajectories. (A) Color maps illustrate the EMT signature score (using the 76GS method), stemness signature score (via ssGSEA), and proliferation signature score (via ssGSEA) for all cellular states gathered from day 0 to day 8. (B) The panels depict the time progression of average cellular signature scores (left to right: EMT, stemness, and proliferation) across the three distinct EMT trajectories. Shaded regions denote the 95% CI. (C–E) Temporal evolution of mean gene expression across the three EMT trajectories. Shaded regions denote the 95% CI (C) for CDH1 and CDH2 genes, (D) for POSTN and KRT8 genes, and (E) for HIF1A and SNAI1 genes. (F) Two-dimensional plots illustrate the time-course progression of average cellular signature scores for paired signaling pathways. Lines connect daily average scores for each signature pair, with arrows highlighting the directional flow of time.
We then further analyzed the dynamics of individual genes pivotal to EMT, such as CDH1 and CDH2. We found that the medium EMT trajectory initially displays high CDH1 expression that diminished toward the end of treatment, shifting from a z-score of 0.09 on day 0 to −0.17 on day 8 (Fig. 3C). This downward trend aligns with previous findings indicating that CDH1 downregulation triggers partial EMT (50). Conversely, CDH2 expression notably increased in the medium EMT trajectory, diverging from the patterns seen in the high and low EMT trajectories (Fig. 3C). Furthermore, these gene expression patterns are markedly distinct, underscored by their nonoverlapping 95% CI. This finding aligns with a previous study showing elevated expression of CDH2 in partial EMT using the same cell line and treatment type (36). Beyond CDH2, Zhang et al. (36) highlighted elevated expression of POSTN and KRT8 expressions as indicators of the partial EMT phase. In our study, the medium EMT trajectory mirrored this finding, with POSTN and KRT8 expression levels surpassing those in the high and low trajectories (Fig. 3D). Additionally, we detected a pronounced rise in HIF-1α and Snail expression within the medium trajectory compared to the others (Fig. 3E). This finding further supports the classification of the medium EMT as partial EMT, given the known roles of these genes in hypoxia and partial EMT fates (35, 51).

To investigate whether TGF-beta treatment correlates with other essential EMT-related signaling pathways, we further conducted pairwise comparisons of various cellular signatures over time (SI Appendix, Fig. S8 and Materials and Methods). Across all trajectories, we found positive correlations between TGF-beta signaling and the IL6-JAK-STAT3, Wnt, and PI3K-AKT-mTOR pathways, with Pearson correlation coefficients ranging across trajectories from 0.95 to 0.97, 0.92 to 0.96, and 0.76 to 0.92, respectively (Fig. 3F). These observations are consistent with previous findings regarding the concurrent regulation of these pathways throughout the EMT process (9, 52, 53). Particularly, the intricate interplay between TGF-beta and PI3K signaling pathways, which includes both antagonistic and cooperative interactions, has been discussed previously (9). In our study, while the TGF-beta pathway activity increased from day 0 to day 8 across all three EMT trajectories, the PI3K pathway interestingly showed a decline in the partial EMT trajectory by the end of treatment.

In contrast, the enrichment scores for the other two trajectories remained relatively stable (Fig. 3F). With PI3K signaling recognized as a prominent driver of cell growth and proliferation (54), this observed decline aligns with the lower proliferation scores and G2M checkpoint pathway activity levels noted along the partial EMT trajectory (Fig. 3B and SI Appendix, Fig. S6A).

Note that the signature trajectories calculated in this section represent only the mean scores for cells on a specific path. The nonoverlapping CI clearly confirm the distinct separations of these mean dynamics. Indeed, variations in these signature scores exist within the entire cell population, as illustrated in SI Appendix, Fig. S6B. In the next section, we further expand our analysis to encompass the full distribution of scores. Furthermore, to explore the potential variations in lineage trajectories across different EMT models, we applied the WOT method to an additional dataset (17). Our findings confirm that variations in lineage trajectories indeed exist across different cell lines, even under the same EMT inducer, TGF-beta (SI Appendix, S7).

Unveiling Increased EMT Heterogeneity Within the Partial EMT Trajectory. To deepen our understanding of cellular heterogeneity across EMT trajectories, we studied the temporal evolution of EMT signature distributions along the three identified paths. We employed several methodologies to evaluate the within-trajectory distributions. First, we incorporated chronological sequences of triangle plots (Fig. 2B) with time-ordered individual cellular EMT signature scores (Fig. 4 A and B and SI Appendix, Fig. S9). This integration elucidated the relationship between ancestral cell EMT states and their potential to transition into a specific fate (Materials and Methods). The triangle plots demonstrate that the top ancestors, showing over 75% commitment to the high/lower EMT fate, consistently exhibited high/lower EMT signatures during the initial stages of the treatment process (Fig. 4B). Conversely, for the top ancestors of the partial EMT fate, EMT scores were notably heterogeneous, encompassing the full spectrum from low to high EMT cell types (Fig. 4B SI Appendix, Fig. S9). This pattern is discernible throughout days 0-3 (Fig. 4B SI Appendix, Fig. S9), suggesting that this early phase of the partial EMT trajectory displays a greater degree of variability in EMT expression scores compared to the early phases of the other EMT trajectories.

To explore the variability within the three EMT trajectories, we assessed the distributions of EMT, stemness, proliferation, and hypoxia scores among the top ancestors of the three identified EMT fates (SI Appendix, S8). We found that one distinguishing feature of the partial EMT trajectory was its broad variation in the EMT signature, paired with consistent stemness, proliferation, and hypoxia signatures (Fig. 4 C and D and SI Appendix, Fig. S10). We used Levene’s test for equality of variances to determine whether any population had a significantly different variance from the others. For instance, the top ancestors of the partial EMT trajectory exhibited a more pronounced variance in the EMT scores compared to those of the high EMT trajectory (Levene’s test, P-value < 1e-10 in days 1 to 8, Fig. 4C and SI Appendix, Table S1). In contrast, the partial EMT trajectory exhibited a stemness score variance similar to, or even less than, that of the high EMT trajectory (Levene’s test, P > 0.05 on days 1, 4, and 8). On days when significant differences did occur (Levene’s test, P < 1e-5 on days 2 and 3), the variances were more significant in the high than the partial EMT trajectory (Fig. 4D and SI Appendix, Table S4).

To further characterize the extent of heterogeneity within the partial EMT trajectory, we calculated pairwise cell state distances (55) (SI Appendix, S9), focusing on the differences between the partial and high EMT trajectories. The low EMT trajectory was excluded due to the high number of outliers (for details, see SI Appendix, Table S5). To determine whether the high variability was uniquely tied to the EMT signature, we computed cell state distances across three gene expression spaces: the full gene set, the EMT signature gene set, and genes differentially expressed between the partial and high EMT fates (SI Appendix, S10 and Table S6). Our findings reveal that, within the EMT gene expression space, variability in cell states was substantially greater in the partial EMT trajectory compared to the high EMT trajectory, as supported by statistically significant differences (\( t \text{-test}, P < 1e^{-9} \)) with fold changes of 1.11, 1.09, 1.10, and 1.09 for days 1 through 4, respectively (Fig. 4E and SI Appendix, Table S7). Conversely, during this period, these differences were not significant when cell state heterogeneity was analyzed using either the full gene set or the DEG set (Fig. 4E and SI Appendix, Table S7). This specific variability of the EMT score in the partial EMT trajectory aligns with prior research suggesting a lack of association between core EMT transcription factors and the partial EMT state (56).

To further explore the interplay between EMT and stemness signatures, we examined the joint distributions of these signatures at various time points (SI Appendix, S8). Our analysis revealed that the three trajectories during days 2 to 8 occupied different regions in the two-dimensional EMT and stemness score space. Specifically, the EMT signature predominantly distinguished...
Fig. 4. Tracing cellular signature variations across three EMT trajectories. (A) EMT signature scores for cell states from days 1 and 2 (for the complete time course see SI Appendix, Fig. S6). (B) EMT signature scores from (A) are paired with ATF distributions and plotted within a triangle using barycentric coordinates. As in Fig. 2C, a point’s location represents its ATF distribution. Concurrently, the color map showcases the EMT score. Dashed lines demarcate a 75% commitment to the fate linked to the corresponding triangle vertex. (C and D) Violin plots depict the distribution of each cellular signature score for the top ancestors of each fate: (C) for EMT score (via 76GS method) and (D) for stemness score (via ssGSEA). (E) The error bar plots depict the mean of weighted pairwise distances in cellular transcriptomics (indicated at the center of each bar), and the SD errors of these pairwise distances (symbolized by the length of the error bars). Significance levels are denoted by asterisks: one star for $\alpha = 1e^{-4}$, two stars for $\alpha = 1e^{-8}$, and three stars for $\alpha = 1e^{-12}$. (F) Scatter plots display paired cellular signature scores for days 2, 4, and 8. Color codes designate the top ancestors for each trajectory.
between the low and high EMT trajectories, whereas a pronounced stemness signature demarcated the partial EMT trajectory from the other two (Fig. 4F). Additionally, within the low EMT subset, a consistent positive correlation between EMT and stemness signatures was observed from days 1 to 8 (Pearson coefficients ranging from 0.22 to 0.44). In contrast, the high EMT subset presented a negative correlation between EMT and stemness signatures (Pearson coefficients ranging from −0.3 to −0.6) (SI Appendix, Table S8). This analysis reveals that cells with marked EMT signatures, whether extremely low or high, display reduced stemness. This trend is in line with earlier research suggesting that cells moving toward a distinctly differentiated state, whether closer to a pure E or M state along the EMT continuum, tend to exhibit less stemness (22).

Leveraging CRISPR Screening for Validation of Key Early Predicted Genes in EMT. To validate our identified trajectories, we compared our findings with a recent study that reported a substantial induction of the partial EMT fate following TGF-beta treatment in a background of PRC2 dysfunction, which was conducted across various epithelial cell lines including HMLER and MCF10A cells (36). The 2D gene expression maps showed that the levels of EED and EZH2—key constituents of PRC2—were notably diminished in areas aligning with the high-probability regions for the partial EMT trajectory (Fig. 5A and SI Appendix, Fig. S11A). To validate this observation, we quantified the expressions of these genes across the three trajectories. Both genes exhibited distinct average expression trends, each distinctly demarcated by nonoverlapping 95% CI. Importantly, there was a noticeable decline in EED and EZH2 expressions, predominantly within the partial EMT trajectory (Fig. 5B and SI Appendix, Fig. S11B). Concurrently, within the top ancestors of the partial EMT, there was a discernible contraction in the distribution of EED expression, marked by a decrease in the number of cells exhibiting high gene expression, which is evidenced by a shift in the mean of the distribution (SI Appendix, Fig. S11C and Table S9). Similar patterns were observed for the EZH2 gene (SI Appendix, Fig. S11C and Table S10).

In line with these findings, the CRISPR screen study revealed that knocking out EED and EZH2 promotes a partial EMT state with increased stemness (36). This study was performed using the HMLER cell line, which, like MCF10A, is an immortalized human mammary epithelial cell line and exhibits similar changes in gene expression during TGF-beta-induced EMT as the MCF10A cell line (36, 57). Therefore, we curated an EMT-related gene list from both the time course data (18) and the CRISPR screen study (36) (Materials and Methods). Two mesenchymal states were identified in the CRISPR study: C1-sgEED-Mes (partial EMT with EED gene knockout) retained some epithelial traits, while C1-sgKMT2D-Mes (high EMT with KMT2D knockout) lacked them. We then examined the differential expression of the curated gene set between the partial and high EMT fates in our dataset on day 8, and between the C1-sgEED-Mes and C1-sgKMT2D-Mes cells in the CRISPR screen data (SI Appendix, S10 and Fig. S12). Remarkably, three out of the top four ranked genes—TGFBRI, POSTN, and KRT8—were significantly up-regulated in the

**Fig. 5.** Early predictors of EMT fate through early DEG analysis and CRISPR knock-out screening. (A and B) EED gene expression analysis: (A) color maps display the ATF distributions for the partial EMT trajectory alongside the expression levels of the EED gene across all cellular states from day 0 to day 8. In the trajectory map, the color gradient signifies probability, while in the gene expression map, it indicates the level of gene expression. (B) Line plots illustrate the average dynamics of EED gene expression over. Shaded regions denote the 95% CI. (C and D) Early DEGs of proliferation-related genes (C) and stemness-related genes (D). Distinct color codes showcase the differential gene expressions in cell states from a specific trajectory when contrasted with the combined cell states of the remaining two trajectories.
partial EMT state in both datasets ($t$ test, $P < 1e-10$; fold changes in SI Appendix, Table S11). This analysis further supports our characterization of the partial and high EMT fates within our dataset.

We then evaluated early differential gene expression patterns between each pair of cellular states in our data, weighted according to their ancestral distributions on day 2. We compared our findings with a standard differential gene expression analysis conducted on two groups of CRISPR knockout epithelial cells, C1-sgEED-Epi and C1-sgKMT2D-Epi (SI Appendix, S10 and Fig. S12). These two groups of epithelial cells were the ancestral cells for their respective EMT fates: the C1-sgEED-Mes and C1-sgKMT2D-Mes cells, respectively (36). Notably, four of the top five ranked genes overlapped between our and the CRISPR study—TGFβ1, KRT8, and CDH1—were significantly up-regulated, while PHF19 was significantly down-regulated in the partial EMT trajectory ($t$ test, $P < 1e-9$; fold changes are in SI Appendix, Table S12). The concordance observed between our predictions and the results from the CRISPR screen study partially validates our inference approach of predicting early key genes in EMT.

Our methodology leverages the inherent heterogeneity of cellular states that culminate in diverse cell fates under a single EMT inducer. This approach enables the identification of crucial early-stage genes that govern specific cell destinies, effectively circumventing the necessity for extensive preexisting biological knowledge when selecting a specific EMT inducer or applying CRISPR to knock out a specific gene for a corresponding cell fate. We further applied our early DEG analysis to two comprehensive gene sets—the ones that we employed to delineate stenness and proliferation patterns (58, 59). Our results highlighted that in the early phase of TGF-beta-induced EMT, genes such as CENPF, CKSI1B, and MKI67 were significantly up-regulated in the ancestors of the high EMT state on day 2 ($t$ test, $P < 1e-10$ and fold changes 2.16, 1.80, and 1.76, respectively) (Fig. 5C). Similar patterns were observed on days 1 and 3 (SI Appendix, Fig. S13). In contrast, in the early cellular states of the partial EMT state, genes like LAMA3, LAMB3, and ITGB4 were prominently expressed on day 2 ($t$ test, $P < 1e-10$ and fold changes 1.66, 1.55, and 1.50, respectively) (Fig. 5D). Again, similar trends were observed on days 1 and 3 (SI Appendix, Fig. S13). Our findings concerning the increased expression of LAMA3, LAMB3, and ITGB4 align with prior research that identified their role in demarcating cancer stem cell-enriched populations in a partially mesenchymal state (60, 61). Our methodology provides insights by enabling the identification of DEGs across a temporal spectrum (SI Appendix, Fig. S7). For instance, our time-resolved analysis reveals that the differential expression of ITGB4 in the partial EMT state, when compared to the high EMT state, is more pronounced during the early stages than it is in the later phases of EMT. When comparing day 2 to day 8, the fold changes were 1.50 vs. 1.09, respectively. These findings potentially underscore crucial moments for timely interventions to influence the direction of EMT evolution.

**Discussion**

In this study, we utilized WOT to infer EMT trajectories as a data-driven model; however, the underlying mechanisms remain unidentified. Existing work in the field employs computational single-cell approaches to model EMT, utilizing mechanistic methods such as bifurcation and stability analysis from dynamical systems theory. These methods illustrate varying EMT responses to TGF-beta, corroborating our findings from data-driven models and further elucidating the underlying mechanisms of these diverse responses (62, 63). Moreover, another mechanistic approach involves constructing gene regulatory network circuits through a combination of transcriptomics data and network modeling. This approach helps identify the context-specific activity dynamics of common EMT transcription factors (64), and the activity dynamics of common EMT transcription factors in varying contexts (65). Therefore, future work should consider integrating these mechanistic models with OT analysis to enhance the predictions and uncover the underlying mechanisms driving these predictions.

Furthermore, WOT is based on optimal transport theory, which assumes that cells traverse the gene expression space via the shortest overall distance (26, 27). This foundational assumption serves as an unbiased starting point for cell state transitions (28). Future refinements could integrate prior knowledge of specific gene expression changes, adjusting gene distances based on this knowledge. This approach would allow us to leverage WOT more adaptively, inferring unknown system parts from existing biological understanding. Additionally, WOT employs an unbalanced optimal transport method, accommodating the effects of cell proliferation and death in the transport of cell states. However, the estimation of cell proliferation and death depends on our selection of gene sets from the literature. A recently published tool, TIGON (66), addresses this limitation by simultaneously reconstructing dynamic trajectories and population growth directly from the data.

Despite these caveats, the use of WOT has uncovered several insights into individual EMT trajectories. These insights, when integrated with existing EMT research, can offer a more comprehensive view of the EMT landscape. First, we found that the low EMT trajectory is determined early on, within a day of treatment. This result suggests that the initial state of these cells renders them resistant to TGF-beta, providing insights into two prior studies on EMT resistance: one study identified a subpopulation of epithelial cells with similar capabilities to receive and process TGF-beta signals but exhibited a notably weaker downstream response compared to more sensitive cell populations (36). Another study revealed that sustained EPCAM expression acts as a marker for epithelial clones in metastatic breast cancer that resist EMT induction, a trait shaped by the interplay between human ZEB1 and its target, GRHL2 (67).

Additionally, we observed that the expression of the EED and EZH2 genes was down-regulated from day 0 to day 1 following TGF-beta treatment in the MCF10A cell line (Fig. 5B and SI Appendix, Fig. S11B). Although there is no established mechanism for this effect, we hypothesize that the TGF-beta-induced cytostatic effect is associated with decreased expression of PRC2 components. PRC2 components, particularly EZH2, are well-documented targets of cell cycle transcriptional regulation, which is up-regulated in proliferating stem cells and cancer cells (68, 69). Consequently, from day 0 to day 1, MCF10A cells show sensitivity to the TGF-beta-induced reduction in cell proliferation (Fig. 3B and SI Appendix, Fig. S64), likely contributing to the reduced expression of EED and EZH2. Furthermore, after day 1, the dynamics of EED and EZH2 expression diverged across the three trajectories. As shown previously (36), either a stable or transient loss of PRC2 function is sufficient to activate an EMT trajectory and generate a partial mesenchymal cell state. As depicted in Fig. 5B and SI Appendix, Fig. S11B, after day 1, EED and EZH2 maintained a low expression level in the partial EMT trajectory, suggesting a functional reduction of PRC2, which aligns with the previous findings (36). In contrast, the low and high EMT trajectories showed a restoration of EED and EZH2 levels to pretreatment levels, indicating that PRC2 remains functional.

Last, leveraging the heterogeneity of cellular responses to TGF-beta-induced EMT, our method effectively pinpoints early differentially expressed genes across distinct EMT trajectories from a broad set of candidates. For instance, we distinguished ITGB4,
LAMA3, and LAMB3 due to their pronounced differential expres-
sions in the early stages of the partial versus high EMT trajectories. As previously highlighted, ITGB4 serves as an integrin subunit that
interacts with specific matrix proteins, while LAMB3 and LAMA3
engage with different integrin subunits than does ITGB4 (70, 71).
Future validation of our findings could employ cell surface markers
encoded by these genes to isolate early-phase cells and observe their
responses under a consistent TGF-β treatment timeline.

Materials and Methods

scRNA-seq Data Analysis. The single-cell RNA-seq datasets analyzed here
were obtained from published studies (18, 36). For the dataset from Deshmukh
et al., we used the processed sequencing data made available by the authors;
the raw sequencing reads are available from the NCBI Sequence Read Archive
(72) (BioProject Accession No. PRJNA698642). From the dataset from Zhang et al.,
processed single-cell RNA-seq profiles of HMLE cells subjected to EED/EZH2
knockout were downloaded from the Gene Expression Omnibus (GEO) database
(73) (GEO Accession No. GSE158115). The procedures for quality control, data
normalization, batch correction, and other steps for this dataset were performed
as in the original paper (36). Detailed descriptions of the scRNA-seq data dimen-
sionality reduction and clustering analysis are available in SI Appendix, S11.

Inferring Trajectories with WOT. We employed the WOT (28) method to ana-
lyze cell state transition probabilities over time in the scRNA-seq data, using
normalized expression matrices and day annotations. Cell growth rates were
determined using a logistic function based on cells’ proliferation and apoptosis
signatures from MsigDB gene sets (42, 43). These rates were then incorporated
into an unbalanced transport optimization to model transitions over consecutive
days, with parameters previously validated (28). This methodology enabled the
prediction of transition maps following TGF-β treatment and facilitated the
computation of AIF distributions, which quantify the likelihood of each cell differ-
entiating into specific fate subpopulations at early time points (SI Appendix, S12).

Assessing EMT Scores: The 76GS and KS Methods. EMT scores were calculated
using two distinct methodologies, each employing different gene sets and met-
rics. The consistency between these methods has been verified through a com-
parative study involving multiple individual samples (40). In the 76GS method
(37, 39), we computed the EMT score as a weighted sum of the expression levels
of 76 EMT-related genes. The weight assigned to each gene was determined
by its correlation with the CDH1 (E-cadherin) expression level. The scores
were subsequently adjusted such that the mean is 0. As a result, a negative score
signifies that a cell’s EMT state is closer to the epithelial (E) state than the mes-
enchymal (M) state. We then rescaled the scores by taking their negatives, thus
aligning the direction of the scores with the progression from the E to M state.
The second method, known as the KS method, was initially established based on
a comparison between the cumulative distribution functions (CDFs) of the E
and M signatures (38). According to this method, the EMT score is computed as
the maximum difference between the two CDFs, i.e., the CDF of the M signature
minus the CDF of the E signature. Therefore, a positive score for a sample indicates
its closeness to the M state, and vice versa.

Computation of Cellular Signature Scores by ssGSEA. For determining
the expression level of the stemness signature, we adopted gene sets from Lim
et al. (58), specifically designed to distinguish between stemness and mature cell
signatures by investigating mammary stem and luminal cells. For the hypoxia
response signature, we used gene sets from MSigDB (42, 43). The prolif-
eration signature was determined using a gene set from Ben-Porath et al. (59).
This set was compiled by merging three distinct gene groups: those that are
functionally involved in proliferation, those with cyclical expression within the
cell cycle, and those that were instrumental in the clustering of proliferative
subpopulations within human breast tumor expression data. Additionally, we
calculated the proliferation signature using two other gene sets associated with
specific proliferation signaling pathways, G2M and mitotic spindle, from MSigDB
(43). Details of the gene sets used for stemness, proliferation, hypoxia, and G2M
signatures are provided in SI Appendix, Tables S13–S17. For further signaling
pathway analysis, we investigated the gene sets of TGF-beta, PI3K-AKT-mTOR,
Wnt, and I6-JAK-STAT3 hallmarks from MSigDB (42, 43). We performed single-
sample Gene Set Enrichment Analysis (ssGSEA) on all gene sets using GSEA
(v1.0.4), a Python package (74). The enrichment scores for gene sets were
transformed into z-scores, with adjustments made by shifting the mean and
normalizing by the SD.

Data, Materials, and Software Availability. All code used to process data
and generate figures is available on a public GitHub repository at https://github.
com/Michorlab/OF-EMT (75). Previously published data were used for this work
(72, 73).

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