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ARTICLE

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Identification of the tumour transition states occurring during EMT

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In cancer, the epithelial-to-mesenchymal transition (EMT) is associated with tumour stemness, metastasis and resistance to therapy. It has recently been proposed that, rather than being a binary process, EMT occurs through distinct intermediate states. However, there is no direct in vivo evidence for this idea. Here we screen a large panel of cell surface markers in skin and mammary primary tumours, and identify the existence of multiple tumour subpopulations associated with different EMT stages: from epithelial to completely mesenchymal states, passing through intermediate hybrid states. Although all EMT subpopulations presented similar tumour-propagating cell capacity, they displayed differences in cellular plasticity, invasiveness and metastatic potential. Their transcriptional and epigenetic landscapes identify the underlying gene regulatory networks, transcription factors and signalling pathways that control these different EMT transition states. Finally, these tumour subpopulations are localized in different niches that differentially regulate EMT transition states.

EMT is a cellular process in which cells lose their epithelial characteristics and acquire mesenchymal features, which enable them to migrate more efficiently and invade the underlying mesenchyme. EMT is essential for gastrulation, somitogenesis and neural crest delamination during embryonic development and has been associated with various diseases. In cancer, EMT is associated with tumorigenesis, invasion, metastasis, tumour stemness and resistance to therapy[1,2]. Although EMT has traditionally been viewed as a binary switch, some in vitro data (mainly co-expression of epithelial and mesenchymal markers within the same cells) have indicated that EMT may proceed in a stepwise manner through the generation of subpopulations that represent different intermediate states between the epithelial and mesenchymal states[3–7]. However, it remains unclear whether EMT proceeds through these intermediate states in vivo, and if so how many intermediate steps exist, how plastic and reversible these intermediate states are, which mechanisms regulate the transition from one state to another and what the implications of these different EMT states are for tumour progression, stemness and metastasis[1,2].

Different tumour EMT transition states

To determine whether EMT in vivo occurs through a succession of different intermediate states, we used a genetic mouse model of skin squamous cell carcinoma (SCC) mediated by the conditional expression of $KRas^{G12D}$ and *p53* deletion ($p53^{KO}$) in hair follicles. This model generates skin tumours that undergo spontaneous EMT, containing epithelial YFP^+Epcam^+ and mesenchymal-like YFP^+Epcam^- tumour cells (TCs)[8]. Using flow cytometry (fluorescence-activated cell sorting, FACS), we screened cells from these tumours for a large panel of cell surface markers and assessed whether these markers were heterogeneously expressed in YFP^+Epcam^+ or YFP^+Epcam^- populations (Fig. 1a). The YFP^+Epcam^+ TCs were relatively homogenous, with only four markers being heterogeneously expressed (Fig. 1b). By contrast, half of the markers were heterogeneously expressed in YFP^+Epcam^- TCs (Fig. 1b, Extended Data Fig. 1a, b), suggesting that EMT is associated with important cellular heterogeneity. The markers that were most frequently heterogeneously expressed during EMT included CD61 (also known as Itgb3), CD51 (also known as Itgav) and CD106 (also known as Vcam1; Extended Data Fig. 1c), which mark subpopulations of TCs associated with tumour stemness, EMT or metastasis initiation in other tumour models[7,9–12]. Other markers were not as frequently heterogeneously expressed when analysed in a larger cohort of tumours (Extended Data Fig. 1c). Combinatorial multicolour FACS analysis revealed that CD106, CD61, and CD51 discriminated six distinct populations within YFP^+Epcam^- TCs in most (75%) mixed tumours (Fig. 1c, e, h, Extended Data Fig. 2). About 10% of mixed tumours with a high proportion of $Epcam^+$ cells presented only triple-negative ($Epcam^-$ $CD51^-CD61^-CD106^-$) and $Epcam^-$ $CD51^+CD61^+$ populations, whereas highly mesenchymal tumours with only $Epcam^-$ TCs contained $CD51^+$, $CD51^+CD61^+$, $CD106^+CD51^+$ and $CD106^+CD51^+CD61^+$ triple-positive tumour subpopulations with almost no triple-negative and $CD106^+$ subpopulations (Fig. 1c–g).

To define whether these different tumour populations correspond to distinct EMT transition states, we isolated the subpopulations by FACS and performed immunostaining on cytospin with epithelial (keratin 14, K14) and mesenchymal (vimentin) markers. Notably, loss of Epcam expression coincided with a gain in vimentin expression in all TCs, consistent with the first switch to the mesenchymal state (Fig. 1i, j). However,

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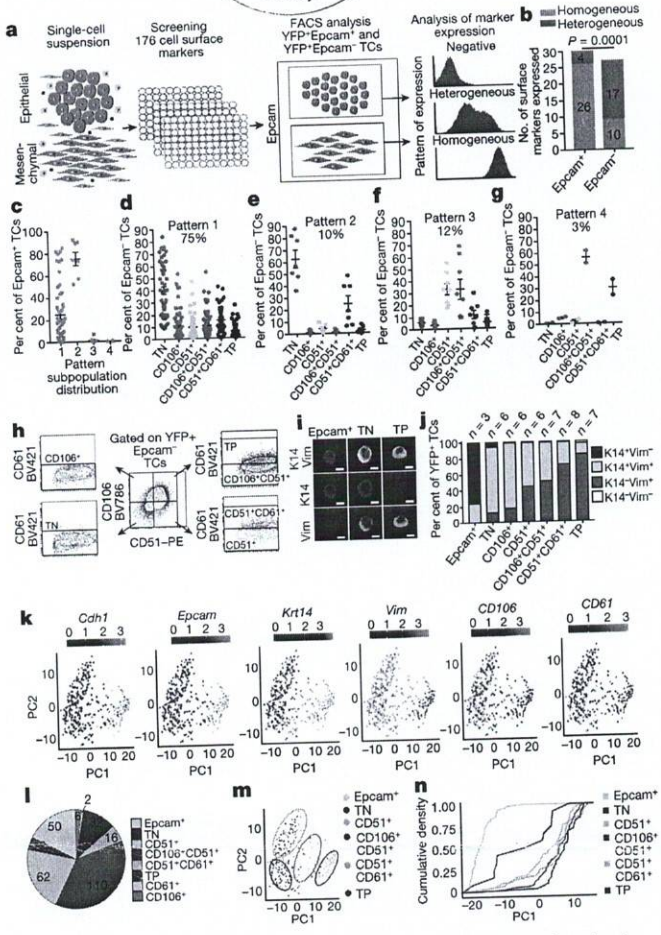

Fig. 1 | Identification of the tumour transition states occurring during EMT in vivo. a, Strategy for marker screening in hair follicle-derived skin SCC model presenting spontaneous EMT. **b**, Percentage of homogeneously and heterogeneously expressed markers in YFP$^+$Epcam$^+$ and YFP$^+$Epcam$^-$ TCs. Two-tailed Fisher's exact test. **c**, Percentage of Epcam$^+$ cells in the four groups of tumours that differ by the frequencies of the different subpopulations. **d**–**g**, Distributions of subpopulations in tumours with pattern 1 (**d**; mixed tumours containing Epcam$^+$ and Epcam$^-$ TCs), pattern 2 (**e**; differentiated tumours with a high percentage of Epcam$^+$ TCs), pattern 3 (**f**) and pattern 4 (**g**; mesenchymal Epcam$^-$ TCs) ($n = 12$ mice, mean ± s.e.m.). TN, triple-negative; TP, triple-positive. **h**, FACS profile showing the six CD106/CD51/CD61 subpopulations in the most frequent pattern (pattern 1). **i**, Co-immunostaining of keratin 14 (K14) and vimentin (Vim) in cytospin of FACS-isolated tumour cells. Scale bars, 20 μm; $n = 6$. **j**, Cytospin counts of TCs based on K14 and vimentin expression (average of 90 cells per condition and tumour). **k**, PCA of scRNA-seq of Epcam$^+$ and Epcam$^-$ TCs. Dots represent single cells; colour scale represents the normalized expression of each gene ($n = 66$ Epcam$^+$ and $n = 277$ Epcam$^-$ cells from one tumour). **l**, Proportion of single cells expressing Epcam, CD51, CD61 and CD106 markers. **m**, PCA plot coloured by expression of the markers used to define EMT subpopulations by FACS. Grey circle, epithelial tumour cells; blue, hybrid cells; yellow, early EMT cells; red, late EMT cells. **n**, Cumulative density of each marker combination across PC1, which correspond to the degree of EMT (**j**).

some Epcam$^-$ tumour subpopulations (triple-negative, CD106$^+$ and CD51$^+$) continued to express K14 and vimentin, whereas other Epcam$^-$ subpopulations (CD51$^+$CD61$^+$ and triple-positive) were essentially K14$^-$vimentin$^+$, with rare TCs expressing low levels of K14 (Fig. 1i, j). These data indicate that the subpopulations identified during spontaneous EMT of primary skin tumours correspond to different tumour populations with different degrees of EMT, with some subpopulations corresponding to the hybrid tumour phenotypes with epithelial and mesenchymal features that have been described in cancer cell lines in vitro[3–7].

To further assess cellular heterogeneity during spontaneous EMT, we performed single cell RNA sequencing (scRNA-seq) of FACS-isolated YFP$^+$Epcam$^+$ and YFP$^+$Epcam$^-$ TCs. Dimensionality reduction using principal component analysis (PCA) revealed that the first principal component (PC1), which explained 21% of the variability, could be attributed to EMT state (Fig. 1k). Our scRNA-seq data confirmed that Epcam$^-$ subpopulations showed greater transcriptional heterogeneity than Epcam$^+$ subpopulations (Fig. 1k). The expression of epithelial and mesenchymal markers at the single-cell level confirmed the progressive acquisition of EMT features with epithelial, mesenchymal and hybrid states (Extended Data Fig. 3) and the distribution of CD51, CD61 and CD106 markers correlated with the degree of EMT along PC1, confirming the EMT gradient found across the different tumour subpopulations (Fig. 1l–n).

To assess whether these different subpopulations of EMT TCs reflect a more general mechanism occurring during EMT, we assessed the expression of these cell surface markers in metaplastic-like mammary tumours arising from oncogenic *Pik3ca* expression and *p53* deletion and in *MMTV-PyMT* mammary luminal tumours, which have been reported to present EMT features[13–16]. Notably, a subset of mammary metaplastic-like and *MMTV-PyMT* luminal tumours also contained Epcam$^+$ and Epcam$^-$ TCs that could be subdivided into the same six subpopulations as found in *KRas^{G12D}/p53^{KO}* skin tumours (Extended Data Figs. 4a–d, 5a–c). Immunostaining on cytospin and real-time PCR with reverse transcriptase (RT–PCR) showed that the subpopulations isolated from the mammary tumours presented different degrees of EMT, similar to those identified in skin SCCs (Extended Data Figs. 4e, f, h, 5d, e, g), demonstrating that the different EMT transition states identified here represent a conserved mechanism during EMT.

To investigate whether these EMT transition states exist in human cancers, we assessed the expression of epithelial and mesenchymal markers in tumours derived from xenotransplantation (PDX) of poorly differentiated human breast cancers and SCCs. After several passages in immunodeficient mice, human stroma is entirely replaced by mouse cells[17], making it possible to differentiate human TCs that underwent EMT and lost the expression of epithelial markers from mouse stroma using an antibody against human antigen (Ku-80). We detected areas expressing only epithelial markers, areas co-expressing epithelial and mesenchymal markers and areas expressing exclusively mesenchymal markers in poorly differentiated breast cancer, lung and oesophageal SCCs (Extended Data Fig. 6). These data demonstrate that EMT in human cancers is associated with different transition states including hybrid states, as was suggested by scRNA-seq of human SCCs[18].

Stemness and plasticity of EMT states

EMT has been associated with cancer stemness, characterized by an increase in tumour-propagating cell (TPC) frequency[1,8,19,20]. As previously described[8], Epcam$^-$ TCs contained five times as many TPCs as did Epcam$^+$ TCs (Fig. 2a). Notably, all EMT subpopulations presented similar TPC frequencies (Fig. 2a). These data show that the earliest EMT state already exhibits increased TPC frequency, and tumour stemness does not increase further in later transition states. Whereas Epcam$^+$ TCs showed higher proliferation than Epcam$^-$ TCs, there was no difference in proliferation rate between the different EMT subpopulations (Fig. 2b). Thus, TPC frequency is inversely correlated to in vivo proliferation.

Cancer cells have been shown to be plastic in transplantation assays, with different tumour subpopulations able to transit back and forth between different states and to recapitulate primary tumour heterogeneity[21]. To determine whether the different subpopulations identified during EMT are similarly plastic, we analysed the tumour phenotypes of secondary tumours. As previously described[8], secondary tumours arising from the transplantation of Epcam$^-$ TCs comprise only Epcam$^-$ TCs (Fig. 2c). Although all EMT subpopulations presented a certain degree of plasticity, at early time points following transplantation (3–4 weeks), the triple-negative subpopulation was relatively primed towards the epithelial phenotype and preferentially gave rise to