Single-cell transcriptomic analysis in a mouse model deciphers cell transition states in the multistep development of esophageal cancer

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Esophageal squamous cell carcinoma (ESCC) is prevalent in some geographical regions of the world. ESCC development presents a multistep pathogenic process from inflammation to invasive cancer; however, what is critical in these processes and how they evolve is largely unknown, obstructing early diagnosis and effective treatment. Here, we create a mouse model mimicking human ESCC development and construct a single-cell ESCC developmental atlas. We identify a set of key transitional signatures associated with oncogenic evolution of epithelial cells and depict the landmark dynamic tumorigenic trajectories. An early down-regulation of CD8+ response against the initial tissue damage accompanied by the transition of immune response from type 1 to type 3 results in accumulation and activation of macrophages and neutrophils, which may create a chronic inflammatory environment that promotes carcinogen-transformed epithelial cell survival and proliferation. These findings shed light on how ESCC is initiated and developed.
Esophageal squamous cell carcinoma (ESCC) is a common gastrointestinal malignancy in some parts of the world like China, with a poor prognosis and high mortality mainly due to the lack of specific measures for early diagnosis and effective therapies. ESCC represents an extraordinary paradigm of carcinoma development, shaped in a sequential manner from inflammation (INF), hyperplasia (HYP), dysplasia (DYS), carcinoma in situ (CIS) to invasive carcinoma (ICA)\(^1\). However, up to date, how ESCC initiates and develops is largely unknown. This long-standing question is the major factor obstructing the early intervention and clinical care improvement of the disease. As such, exploring the mechanisms underlying ESCC formation and identifying biomarkers are the crucial tasks for early detection, diagnosis, and precision treatment of the cancer.

Recent genome studies, including The Cancer Genome Atlas (TCGA) project, have identified many genome variations in ESCC by using whole-exome or whole-genome sequencing on clinical tissue samples\(^2,3\). Although these studies have revealed an important role of the identified genome alterations in ESCC, it remains unresolved that how the normal epithelial cells may transit by the mutations through precancerous lesions to invasive carcinoma because all these previous studies were in cross-sectional design.

Another important issue in this regard is that somatic mutations solely might not be sufficient for ESCC initiation and development because such mutations also occur in pathologically normal human esophageal tissues\(^4\). These findings imply that other mechanisms such as transcriptome aberrance in ESCC tumorigenesis may merit further investigation. In addition, it has been demonstrated that the complex context of tumor microenvironment (TME) also has important roles in tumor initiation and development. Therefore, elucidating dynamic transcriptomic changes of TME cellular compositions during tumorigenesis are significant and inevitable in discovering how ESCC develops. Recently established single-cell transcriptomic analysis is a promising approach, which allows to analyze the complex cellular compositions and to decipher cell state transition in tissue samples\(^5\).

Capturing continuous tumorigenic lesions from sole patient over time to perform such study is impossible and, therefore, to address this important issue would be highly dependent on well-established animal models. Fortunately, it has been shown that chemical carcinogen 4-nitroquinoline 1-oxide (4NQO) can induce mouse ESCC development in a manner that mimics the tumorigenic processes of ESCC in humans\(^6,7\). The distinct multiple stages of ESCC tumorigenesis induced by the carcinogen provide an excellent opportunity to interrogate cell state transition by single-cell RNA sequencing (scRNA-seq), which would elucidate the dynamic ESCC tumorigenesis.

Here we report a single cell-based transcriptomic profiling study on various types of cells across every pathogenic stage during ESCC initiation and development in a mouse model induced by 4NQO. We have built a complete atlas and characterized the transcriptomic profiles for the transition of esophageal epithelial cells under the attack of carcinogen and elucidated how they evolve over time in a holistic approach. We have also depicted the transition landscapes of non-epithelial cells, i.e., fibroblasts and immune cells, in the esophageal microenvironments of different stages of tumorigenesis. Furthermore, we have found that some key changes in mice also occur in human esophageal tissue samples. These results shed light on the phenotypes and transition fates of different cell types across the tumorigenic processes of ESCC in animal model and may be implicated in human ESCC.

**Results**

**Bulk RNA-seq and scRNA-seq of mouse esophageal samples.** To explore the transcriptomic alterations at various pathological stages during ESCC tumorigenesis, 8-week-old female C57BL/6 mice were treated with 4NQO for 16 weeks, which resulted in five recognizable precancerous and cancerous lesions in the esophagus, i.e., INF, HYP, DYS, CIS, and ICA (Fig. 1a; Supplementary Fig. 1a). We examined mice receiving 4NQO and found they all developed the expected lesions in the esophagus at different time points of experiment. The ESCC number per animal (mean ± SD) at week 26 was 6.0 ± 3.6. We first performed conventional RNA-seq on mini-bulks of normal epithelial samples obtained by laser-capture microdissection (LCM) from control mice at different ages of 1, 2, 8, and 25 months and various precancerous or cancerous lesions from 4NQO-treated mice. Principal component analysis (PCA) of the differentially expressed genes showed that the expression programs in mice exposed to 4NQO were substantially different from that in control mice; however, there existed some overlaps in the expression profiles between 4NQO-exposed and non-exposed mice. For instance, the transcriptomic profile for stage INF in 4NQO-exposed mice was similar to that in control mice aged 25 months (Fig. 1b), indicating that conventional RNA-seq could not precisely clarify the path of malignant cell transition during the development and progression of ESCC due to high intra-tissue heterogeneity.

We therefore conducted a time-ordered single-cell transcriptomic profiling on various esophageal lesions in 4NQO-exposed mice. For stage NOR or stage INF, we used the whole esophagus from 30 or 20 mice. For other stages, we used the lesion foci and the sample numbers were 32 HYP from 25 mice, 24 DYS from 17 mice, 24 CIS from 20 mice and 25 ICA from 23 mice, respectively (Fig. 1c). A total of 66,089 cells including 29,975 CD45\(^+\) immune cells and 36,114 CD45\(^-\) non-immune cells were obtained across various pathological stages (Fig. 1c; Supplementary Fig. 1b). The median unique molecular identifier (UMI) per cell was 7748 in immune cells and 9370 in non-immune cells (Supplementary Fig. 1c); with a median signal detect ability of 1936 genes for immune cells and 2620 genes for non-immune cells (Supplementary Fig. 1c), respectively. Based on the expressions of canonical markers, we classified immune cells into T cells, B cells, myeloid cells and natural killer cells (Fig. 1c; Supplementary Fig. 1d, e) and identified four clusters of non-immune cells including epithelial cells, fibroblasts, endothelial cells and myocytes by using t-distributed stochastic neighbor embedding (tSNE) (Fig. 1c; Supplementary Fig. 1f, g).

**Identifying epithelial cell types during ESCC tumorigenesis.** To discover how normal esophageal epithelium develops into invasive carcinoma, we next examined the expression alterations and functional changes in epithelial cells during the transition from normal to precancer or cancer. We identified 1756 epithelial cells across all six stages that were classified into six subtypes designated as EpiC 1 to EpiC 6 (Fig. 2a; Supplementary Fig. 2a and Supplementary Table 1). Through the analysis of pathway activities (Fig. 2b; Supplementary Fig. 2b), we found that EpiC 1 (n = 339) had higher expression of genes (e.g., Birc5, Mki67, Top2a, and Ube2c) in mitosis and proliferation\(^6-11\) compared with other cell clusters, probably reflecting the ability of routine self-renewal of the esophageal epithelium. EpiC 2 (n = 463) had significantly higher expression of genes such as Gstm1, Gstm2, Adh7, and Aldh3a1, which were involved in the xenobiotic detoxification, likely due to the 4NQO exposure\(^12-14\). EpiC 3 (n = 421) had substantially higher expression of genes in response to extracellular carcinogen stimuli, pro-inflammation and Hif-1 signaling (e.g., Jun, Junb, Zfp36, and Atf3)\(^15-18\). EpiC 4 (n = 110) had a higher expression program for keratinization (e.g., Krt4, Krt13, S100a8, and S100a9)\(^19,20\). EpiC 5 (n = 213) featured by the upregulation of genes in the epithelial–mesenchymal transition (EMT) pathways (e.g., Mmp13
Fig. 1 Experimental design of RNA-seq on 4NQO-induced esophageal lesions in mice. a Induction of esophageal precancerous and cancerous lesions in mice. Mice were treated with 4NQO in drinking water (100 μg/ml) for 16 weeks and then kept without 4NQO treatment for another 10 weeks (upper panel). Mice were killed (week 0), during (week 12) and after treatment (weeks 20, 22, 24, or 26), respectively. Hematoxylin–eosin (H&E) staining and immunohistochemistry (IHC) analysis of Mki67 on esophageal epithelium slides clearly identified six different pathological lesions, i.e., normal (NOR), inflammation (INF), hyperplasia (HYP), dysplasia (DYS), carcinoma in situ (CIS), and invasive carcinoma (ICA) (lower panel). b Overview of the experimental design of single-cell diffusion (scRNA-seq). Pathological lesions of the esophagus were dissected and digested into single-cell suspensions for further separation using FITC-CD45 antibody via FACS (1–4). CD45+ and CD45− cells whose numbers in different lesions are shown on right panel were scRNA-sequenced, respectively.

and Itga6)21,22 and angiogenesis (e.g., Vegfa)23. EpiC 6 (n = 210) had significantly upregulated expression in the genes controlling cancer invasiveness (e.g., Mmp14 and Ecm1)24,25 and metastasis (e.g., Tm4sf1 and Vim)26,27. Besides, EpiC 5 cells also showed an expression pattern resembling the combination of EpiC 3, EpiC 4, and EpiC 6. Three subtypes, EpiC 1–3, were all present in normal esophageal epithelium and at the various stages of 4NQO-induced abnormal epithelia, although the proportions of cells at each stage were different (Fig. 2c), suggesting that these subtypes of epithelial cells were the background components of esophageal tissues. EpiC 4 and EpiC 5 appeared from INF and HYP, respectively, while EpiC 6 was present uniquely at ICA as the most malignant EpiC 6, processing through EpiC 2 to EpiC 5 cells (Fig. 3a; Supplementary Fig. 3a, b). The evolution of EpiC 1 to EpiC 6 was mainly along component 1. Gene set variation analysis (GSVA) of component 1 revealed a significant enrichment of genes related to cell invasiveness, EMT and angiogenesis (Fig. 3b, c; Supplementary Fig. 3c), which was concordant with the expression programs of EpiC 6 cells (Fig. 2b). As EpiC 6 cells appeared only at the ICA stage, these results implied that

Identifying cell fates of epithelial cell status transitions. We performed pseudotime and PCA analysis and found two evolution fates of esophageal epithelial cells during ESCC tumorigenesis both starting from EpiC 1 cells that had the lowest pseudotime value. Some cells transformed from proliferative EpiC 1 to normal differentiated EpiC 4 while other cells transformed to malignant EpiC 6, processing through EpiC 2 to EpiC 5 cells (Fig. 3a; Supplementary Fig. 3a, b). The evolution of EpiC 1 to EpiC 6 was mainly along component 1. Gene set variation analysis (GSVA) of component 1 revealed a significant enrichment of genes related to cell invasiveness, EMT and angiogenesis (Fig. 3b, c; Supplementary Fig. 3c), which was concordant with the expression programs of EpiC 6 cells (Fig. 2b). As EpiC 6 cells appeared only at the ICA stage, these results implied that
component 1 might be the underlying molecular mechanism for malignant transition of the esophageal tissues (Supplementary Fig. 3d).

We then examined whether the alterations of any transcription factors (TFs), well-documented ESCC-related mutation, or methylation dysregulation were included in the oncogenic evolution along component 1. We found that the expression levels of Pitx1, Trp53, and Bclaf1, which are known tumor suppressor TFs,28–30 were substantially decreased while TFs promoting EMT (e.g., Ets1 and Snai3)31,32, immunosuppression (e.g., Eomes33, cell migration and invasion (e.g., Creb3 and Elk3)34,35 were significantly upregulated. We also found two previously reported methylation-regulated genes in human ESCC, Rab2536, and Met37, were substantially down- or upregulated, respectively, along component 1. Furthermore, the expression levels of Notch1, a tumor suppressor frequently mutated in human ESCC38, displayed a gradual decrease over time along the evolution of component 1 (Fig. 3d; Supplementary Fig. 3e).

Fig. 2 Distinct epithelial cell populations and their expression signatures. a tSNE plot of 1756 epithelial cells based on their different expression. b Heatmap showing pathway activities scored per cluster by GSVA. c Stacked histogram showing epithelial cell composition across the six pathological stages. d Diffusion map of all epithelial cells using the first two diffusion components. Dot represents single cell and arrows indicate five branches starting from stage NOR to the other stages. e Histograms of scale normalized expression levels of six representative genes in each pathological stage. f Left: IHC staining of protein levels produced by the six genes in mouse esophageal tissues with different lesion. Scale bars, 100 μm. Right: stacked histograms showing quantification of IHC staining scores (0, negative; 1+, weak positive; 2+, median positive; 3+, strong positive). Each column was summarized from at least three visual fields.
Genes in component 2 were mainly related to cell proliferation (e.g., Birc5, Mki67, and Top2a). Cell cycle regression analysis and GSVA scoring revealed a correlation between proliferative ability and value of component 2 (Fig. 3e, f). We found that EpiC 6 cells had the highest expression of proliferation-related genes and the highest proportion of cells at G2/M status as compared with other clusters except EpiC 1 cells (Fig. 3e, f; Supplementary Fig. 3f–h), indicating increased proliferative ability of this cell cluster. On the other hand, EpiC 4 cells were at the G1 and S status (Supplementary Fig. 3g, h), showing reduced proliferative capacity. Together, these unsupervised analyses depicted two clear cell fates during ESCC carcinogenesis and indicated that attacked by the carcinogen, a portion of esophageal epithelial cells went into oncogenic route (from EpiC 1 and EpiC 2 to EpiC 6) but not into normal differentiation route (from EpiC 1 and EpiC 2 to EpiC 4). To exclude the impact of potential uncertainty of pathologically staging of early lesions such as hyperplasia and dysplasia, we also performed a sensitive analysis by excluding cells in week 20 or week 22. As a result, the cell state transition was similar to that without the exclusion (Supplementary Fig. 3i, j).

We further investigated the key pathways driving epithelial cells from stage INF to stage HYP and found that non-malignant EpiC 1–4 epithelial cells had significantly elevated expressions of the genes involving in carcinogen detoxification such as Nqo1, Aldh3a1, and Gstp1 (Fig. 3g), which reflected normal cellular response to the damage induced by 4NQO. The continuous damage might induce immune response via the stimulator of G2/M...
interferon genes (STING), AIM2 and NF-κB signaling because the expression levels of Tmem173, Aim2, Nfkbia, and Nfkbi2 were significantly elevated (Fig. 3g). In addition, we observed substantial differences in the expressions of NF-κB downstream genes in epithelial cell clusters (Fig. 3h). EpiC 3 cells had an increased expression of some transcription-related genes such as Junb and Fos, whereas EpiC 5 cells showed higher expression levels of inflammation genes (e.g., Cxcl1, Il6, Tnf, and Csf3) compared with other EpiCs, implying that they had crosstalks with immune cells. EpiC 5 cells also had high levels of Hif1α, which may lead to metabolism remodeling that promoted cell transformation. As malignant cells, EpiC 6 showed strong activation of survival-related genes (e.g., Bcl2 and Xiap) (Fig. 3g, b). These results indicated that under 4NQO attack, some epithelial cells might endure the damage and activate the inflammatory response, resulting in consequent cell survival and transformation.

Transcriptomic changes in fibroblasts promote tumorigenesis.

We next examined the transcriptomic alterations of cells in the tissue microenvironment and identified eight fibroblast clusters (FibCs) by different gene expression patterns (Fig. 4a; Supplementary Fig. 4a, b) that showed similar expression pathways and dynamics to epithelial cells (Fig. 4b–d). FibC 1 was the dominant composition in stage NOR and then markedly decreased with the processing of tumorigenesis (Fig. 4c, d). FibC 3 cells were the major fibroblasts in stage INF and had a strong interferon response and MHC-mediated antigen presentation activity (Fig. 4b), representing the initial immune response to tissue damage. FibC 4 and FibC 6, the major fibroblasts in stage DYS, had the elevated expression of genes involved in the TGF-β, STAT5 induced by IL-2 or STAT3 induced by IL-6 signaling pathways. FibC 8 cells increased along the tumorigenic stages with high expressions of genes in Myc and angiogenesis pathways. The replacement of FibC 3 by FibC 4 and FibC 6 during INF to HYP transition further confirmed the shift of immune response during early ESCC development. Specifically, beginning from stage HYP, fibroblasts actively recruited immune cells through increasing the expressions of complements and chemokines (Fig. 4e). For example, we found that FibC 6 and FibC 8 had significantly elevated expressions of various chemokine-related genes (e.g., Ccl7 in FibC6 and Cxcl12 in FibC 8, Supplementary Fig. 4c), which were well-known molecules that recruit immune cells. The dynamic changes of fibroblast clusters suggested that the immune response is modulated during ESCC tumorigenesis.

Turndown of adaptive anticancer immune during tumorigenesis.

We then explored the T cell status during ESCC tumorigenesis and identified 4 clusters designated as CD8+ T cell, CD4+ T cell, CD4−CD8− T cell 1 and CD4−CD8− T cell 2, respectively (Fig. 5a). We further classified CD8+ T cells into 7 clusters (Fig. 5b) and CD4+ T cells into 7 clusters (Fig. 5c), respectively. During 4NQO-induced tumorigenesis, all CD8+ T cells expressed naive and memory marker genes (e.g., Tcf7, Il7r, and Gcr7; Supplementary Fig. 5a) rather than cytotoxic markers. In stage NOR, the major type was naive cells (CD8-TN1) while in stage INF, the major cell type was replaced by memory T cells, mainly CD8+ tissue resident memory T (CD8-TRM) cells and effector memory T (CD8-TEM) cells (Fig. 5d),
suggesting that the immune responses were active in these two stages. Furthermore, we observed a high expression of the exhaustion markers (e.g., Pdcd1 and Lag3) in addition to the effective markers (e.g., Gzmk, Gzmb, and Prf1) in these memory T cells (Fig. 5c); but the proportion of these memory T cells were declined along with tumorigenic processing after stage INF, indicating a non-effective CD8+ T cell dominant microenvironment throughout precancerous stages.

Considering CD4+ T cells (Fig. 5f; Supplementary Fig. 5b), we noticed an imbalance among CD4-Th1-like, CD4-Th2, and CD4-Th17 cells, involving in three major kinds of cell-mediated effector immunity, respectively. Beginning at stage HYP, the proportion of CD4-Th1-like cells was decreased and replaced by CD4-Th2 and CD4-Th17 cells (Fig. 5f, g). CD4-Th1-like cells expressing high levels of Tbx21, Gzmb, and Ifng (Fig. 5f; Supplementary Fig. 5c) had been shown to have anti-tumor capability through activating monocytes and responding to interferon-γ. Differential gene expression analysis also revealed that some genes relative to immune activation (e.g., Ifitm1, Ifitm2, and Rora) were significantly higher in CD4-Th1-like cells than in CD4-Th2 cells (Supplementary Fig. 5d), a type of immune cells that their role in tumorigenesis was uncertain. The activation of CD4-Th17 produced high levels of Il22, Il17a, and Rorc at stage HYP (Fig. 5h) that may activate monocytes and recruit neutrophils to respond to epithelial injuries. The proportion of CD4-Th17 cells increased sharply after stage INF (Fig. 5f). The reduced anti-tumor role of CD4+ T cells and increased inflammatory response suggest an immune response Type 1 to Type 3 transition in the esophageal microenvironment during tumorigenesis.
Activated myeloid cells create inflammatory microenvironment. We found that, among the 11 clusters of myeloid cells (Fig. 6a; Supplementary Fig. 6a), the proportions of monocyte/macrophage (Mo/Mϕ)-C 1, Mo/Mϕ-C 3, cDC2-C 1, tolerogenic dendritic cell (tDC), and neutrophils changed substantially along the stages of tumorigenesis (Fig. 6b, c). After stage INF, Mo/Mϕ-C 1 cells were replaced by Mo/Mϕ-C 3 cells, featured by decreased expression levels of the genes (e.g., H2-Ab1 and C1qa) relative to antigen presentation and complement (Supplementary Fig. 6b)\(^{19,50}\). Meanwhile, the expression level of genes involved in immunosuppression (e.g., Il10, Arg1, and Adora2b, Fig. 6d)\(^{31-33}\) elevated along with the increase in Mo/Mϕ-C 3 proportion. Furthermore, the proportion of cDC2-C 1 sharply decreased since stage NOR with the downregulation of Icosl, a gene representing the immune stimulation activity\(^{13}\) (Fig. 6d). In contrast, the immune suppressive tDC cells with high expressions of Cd274, Pdcd1lg2, and Ido1 were persisted throughout all the tumorigenic stages (Fig. 6d). In addition, we found that the number of neutrophils continuously increased, and these cells expressed high levels of Mmp9, Prok2, Vegfa, and Nos2, but not tumor suppressors (Supplementary Fig. 6c). These results suggested that myeloid cells were activated during the processes of ESCC tumorigenesis, resulting in an inflammatory microenvironment and CD8\(^+\) T-associated adaptive immune suppression, which might allow initiated ESCC cells to survive and proliferate. Transcriptomic analysis also revealed that the immune suppression microenvironment might be created by the complex chemotaxis among various types of cells in this microenvironment.
For example, neutrophils recruitment could be mediated through its high expression of Cxcr1/2 receptors by their ligands Cxcl1/2/5 from other cell types or by their brother neutrophils through the simultaneous expression of Ccl3 and Ccr1. Similarly, tDC, Th17, and CD4+CD8+ T cells could be recruited by chemokines secreted by different cell types (Fig. 6e).

Besides the roles of chemokines, cells in the esophageal microenvironment were also stimulated by each other through various signaling pathways. We found that from stage HYP, the expression levels of Il17a in CD4+ T cells and Il17ra in myeloid cells were elevated (Fig. 6f), which were well-known to have ability to activate macrophages and neutrophils54,55. On the other hand, we found significant correlations between Il1b and Il23a levels in macrophages and neutrophils and their receptors Il1r1 and Il23r in CD4+ T cells, implying close-loop activation among these cells. The activated immune cells secreted high levels of cytokines (Il1b, Il6, Il18, Tnfr) and formed an inflammatory microenvironment starting from stage HYP (Fig. 6f). Analyses of interaction indicated increased interactions between malignant epithelial cells (Epice 5 and Epiec 6) and inflammatory immune cells, Th17 and neutrophils (Supplementary Fig. 6d, e). The inflammatory microenvironment might further promote carcinogen-initiated epithelial cells to transit along the carcinogenic roadmap as illustrated in Figs. 2b and 4b.

Validation of the expression profiles in human ESCC samples.

We last performed a validation examination of the identified expression profiles with mouse model in tissue samples obtained from four ESCC patients having both precancerous and cancerous lesions in the esophagus. RNA-seq was conducted with mini-bulk tissues of invasive carcinoma (ICA, n = 9), carcinoma in situ (CIS, n = 6), dysplasia (DYS, n = 17) and tumor-adjacent normal epithelia (taNOR, n = 8) obtained by LCM (Supplementary Fig. 7a). PCA showed a clearly different expression profile between cancer tissues (CIS and ICA) and non-cancer tissues (taNOR and DYS); however, an extensive overlap was present between taNOR and DYS (Fig. 7a; Supplementary Fig. 7b). In addition, we found that the six representative genes identified in mouse different epithelial cell clusters were also expressed in the precancerous lesions from ESCC patients (Fig. 7b) and the expression patterns were in line with those in mice. ALDH3A1 and S100A8 were highly expressed in the early precancerous lesions while MMP14 and ITGA6 were mainly present in invasive ESCC, which was concordant with their biological functions. As LCM captured mainly epithelial cells and the samples were not suitable for analyzing the status of other cell types, we performed bulk RNA-seq using biopsy samples. The results also showed a trend of increased neutrophils in the microenvironment, although this trend along the stage progress were marginally significant (trend test, P = 0.066; Fig. 7c). These results suggested that strongly supported our findings in mouse model and provided new clues in understanding ESCC initiation.

Discussion

In this study, we employed a high-throughput scRNA-seq analysis and have depicted a complete cell atlas of transition fates, characterized the transcriptomic features of cell clusters at each pathological stage, and elucidated the possible evolutionary trajectories of cells in the multistep processes of ESCC development. We majorly focused on the expression transitions of squamous epithelial and TME cells associated with tumorigenesis, because how they transform from normal to malignant phenotypes is a long-standing question. In this regard, our comprehensive findings shed light on the underlying molecular mechanism of carcinogen-induced esophageal squamous carcinoma.

Many previous studies have shown that human ESCC is a complex disease largely attributed to the exposure to carcinogens from the environment and lifestyles35. 4NQO represents a chemical carcinogen that can specifically induce ESCC in mice and this animal cancer model has been shown to mimic ESCC development in humans46,47. Thus, as it is impossible to obtain continuous tumorigenic lesions from the same human individuals over time, our results from this mouse model demonstrate its value for precisely understanding the gene expressions that may drive cell evolution from normal into malignancy in the multistep development of ESCC in humans. Indeed, we examine and validate the transcriptomic characters of epithelium and TME from patients bearing precancerous lesions.

Another interesting and important finding of this study is that the transcriptomic expression pattern of esophageal epithelial cells in 4NQO-induced early lesions is similar to that of esophageal normal epithelial cells in aged mice, while aging is a known risk factor for cancer development in humans. Previous genomic mutation studies in human esophagus have shown that although the exonic mutation burden in normal esophageal epithelium derived from ESCC-free human individuals was increased with age, no cancer-related lesions occurred in view of histopathology, suggesting that the transcriptional expression alterations and other factors such as immune suppression driven by carcinogen insults may show crucial effects in ESCC tumorigenesis.

Given that epithelial cells have two different fates, including oncogenic and differentiation, the deviation from the default fates during tumorigenesis implies a permissive or even supportive microenvironment for tumor development. Indeed, by analyze the dynamic changes of non-epithelial cell types in the microenvironment, we found decreased antigen presentation in both fibroblasts and myeloid cells reduced early downregulation of CD8+ T cell response during tumorigenesis. After inflammatory stage, no active cytotoxic responses by CD8+ T cells were detected indicating that neither epithelial cells nor fibroblasts in this pathogenic stage and hereafter had the activity tends to stimulate and activate cytotoxic T cells, whose activation has been considered to be a crucial mechanism against tumorigenesis. In addition, CD8+ T cell suppression may also result from a substantial change of myeloid cells because after pathogenic stage INF, MoMφ-C 3 and tDC cells, both of which are well-known as immune suppressors, became the main myeloid cell types in the tumor microenvironment. In addition, starting from hyperplasia stage, the macrophages and neutrophils activated by CD4-Th17 cells may form an inflammatory microenvironment during tumorigenesis. These findings strongly support our notion that the general immune suppression and chronic inflammatory microenvironment have crucial roles in promoting tumor development. Increased inflammatory responses and decreased cytotoxic T cell activities were also reported in the multistep process during cancer initiation for gastric cancer56 and pancreatic cancer57 depicted by single-cell analysis.

Although several important findings have been presented in this study, we acknowledge some limitations. First, the cells in this study were obtained from the mouse esophageal tissues induced by the carcinogen. Although the 4NQO-induced mouse ESCC model may mimic human ESCC development and we validated the expression of some genes and cells in human esophageal samples, caution should be taken in translating these results to humans. Second, although we have identified the carcinogen-induced epithelial cell and microenvironment transition fates, the underlying molecular mechanisms require further investigation. Furthermore, due to small size of esophageal epithelial tissues in mice and the vulnerable nature of esophagus epithelial cells in vitro, our data sets for early lesions were based...
Fig. 7 Validation of expression features in human epithelial tissues with different lesions. a PCA plot of mini-bulk tissue RNA-seq on human esophageal tissues with different lesions as indicated by different colors and each dot represents a sample. b Immunofluorescence image (upper panel; scale bar, 100 μm) and quantification of selected gene expression levels (lower panel) for human epithelia tissue samples with different lesions. Independent samples from stage INF to ICA are n = 5, 13, 8, 3, and 10, respectively. Data represent mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 for two-sided Wilcoxon rank-sum test, compared with stage INF. P-values for TOP2A: 0.0098 (HYP), 0.0295 (DYS), 0.0357 (CIS), 0.0007 (ICA); ALDH3A1: 0.0007 (ICA); ATF3: 0.0007 (ICA); S100A8: 0.0127 (ICA); MMP14: 0.0140 (HYP), 0.0451 (DYS), 0.0357 (CIS), 0.0007 (ICA); ITGA6: 0.0357 (CIS), 0.0007 (ICA). c Proportion of neutrophils (relative to stage INF) in different precancerous and cancerous lesions based on bulk tissue RNA-seq using CIBERSORT. Each dot represents a sample, data represent mean ± S.D. P = 0.066, determined by two-sided Mantel-Haenszel chi-square test for linear trend. Independent patient samples from stage INF to ICA are n = 6, 15, 11, 6 and 7, respectively.
on relatively small amount of epithelial cells. This limitation could not have biased our conclusion because the major results were derived from the comparison between different clusters of epithelial cells rather than setting the precursor stage data set as a benchmark for the interpretation. However, experimental methods such as stripping the epithelial layer, reducing sample processing time and raising sensitivity of vulnerable cell detection should be improved in the future studies.

In conclusion, we have conducted the single-cell transcriptomic analysis of carcinogen-induced ESCC in mice, which mimics human ESCC development. By analyzing epithelial cells in different pathogenic stages, we have depicted a roadmap in carcinogen-induced mouse ESCC development and identified some key expression signatures. Comprehensive analysis of non-epithelial cells in tumor microenvironment revealed the suppression of anticancer immune response and robust expression of chronic inflammation genes. These results shed light on the transcriptomic alterations and transition status of various cell types in the esophagus when ESCC develops. Because the mouse model used in this study mimics human esophageal tumorigenesis, the findings may also represent human ESCC development.

Methods

Human biopsy specimen collection. ESCC tumor, dysplasia lesions and tumor-adjacent (>5 cm) normal tissues of the same patients (n = 4) used for LCM and esophageal lesions of different pathological stages (n = 45) used for bulk RNA sequencing were collected during surgery or endoscopy in Linzhou Esophageal Cancer Hospital (Henan Province, China) from 2018 to 2019. The various lesions were diagnosed independently by at least two pathologists according to the American Joint Committee on Cancer Eighth edition. No patient had received chemotherapy or radiotherapy before biopsy or surgery. This study was approved by the Institutional Review Boards of Cancer Hospital, Chinese Academy of Medical Sciences and informed consent was obtained from each patient. Clinical information was collected from patients’ medical records.

Induction of multi-staged ESCC development and sample preparations. Animal experiments in this study were conducted in compliance with approved protocols and guidelines from the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences. Eight-week-old female C57BL/6 mice, purchased from the Beijing Huafulang bioscience company in China, were maintained in local housing facility of a controlled condition (23 ± 1 °C, 50 ± 10% humidity and 12–12 h light-dark cycle). Mice were treated with 4NQO (Sigma-Aldrich) in drinking water (100 μg/ml) for 16 weeks to induce multi-staged ESCC carcinogenesis. Drinking water containing the carcinogen was replaced once a week with freshly prepared one and mice were allowed to access drinking water ad libitum during treatment. After 16 weeks of carcinogen treatment, 4NQO drinking water was replaced by sterile water until the mice were killed.

The esophageal lesions were identified by two independent pathologists based on the histopathological criteria described previously29 (Fig. 1a). Briefly, stage NOR was well-oriented stratified epithelium consists of basal zone and superficial zone. Stage INF was normal epithelium with focal aggregates of epithelial lymphocytes. Stage DYS was defined as loss of polarity in the epithelial cells, nuclear pleomorphism, hyperchromatic, and increased or abnormal mitoses. In stage HYP, these abnormalities were confined to the lower third of the epithelium while in DYS they present in lower two thirds of the epithelium. Lesions with such abnormal changes involving the entire thickness of epithelium were considered as carcinoma in situ (stage CIS). Stage ICA was defined as a lesion with invasion into the sub-epithelial tissues. Esophageal samples of 4NQO-induced mice were subjected to single-cell RNA sequencing at six different time points and indicated different pathological stages (n = 4, 5, 4, 5, 4, and 2 mice for NOR, INF, HYP, DYS, CIS, and ICA, respectively). The esophagus was removed immediately when the animal was killed. Cross-sections of the esophagus were cut and stored at −80 °C and sections of the frozen tissues were stained with hematoxylin–eosin (H&E) for histopathological examination and microdissection for bulk RNA sequencing.

Single-cell RNA sequencing (scRNA-seq) and data analysis. For mice at stage NOR and INF, the whole esophagus were taken and for mice at other stages, the dysplastic/malignant lesions were taken immediately after killed. The tissue samples of ESCC and various precursor lesions were gently minced into small pieces and digested for with for RPMI-1640 medium (Invitrogen) containing collagenase IV (Gibco) and hyaluronidase (Sigma-Aldrich). CD45-FITC (553880, BD Biosciences, dilution 1:20) antibody staining was performed for fluorescence activated cell sorting (FACS) on a FACSAria sorter (BD Biosciences). Single cells with or without GFP signal, representing immune or non-immune cells, were sorted and captured respectively in nanoplotter droplets using Chromium (10X Genomics). scRNA-seq libraries were prepared using Chromium Single Cell 5′ Reagent Kits (10X Genomics) and sequencing was accomplished with an Illumina HiSeq x10 System.

Gene set variation analysis (GSVA). Pathway analyses were predominantly performed on the 50 hallmark pathways described in the molecular signature database, exported using the MsigDB database (version 6.2)50. We also assessed biological process activities using a described biological process of Gene Ontology (GO) dataset. To assign pathway activity estimates to individual cells, we applied the GSVA using standard settings, as implemented in the GSVA package (version 1.3.0)50. To assess differential activities of pathways between sub-cluster of cells, we contrasted the activity scores for each cell using Limma package (version 3.38.351). Differential activities of pathways were calculated for each identified cluster. T-values of the results of some significant differential pathways (P < 0.05) in top 10 were visualized using heatmaps with average pathway activity scores of each cluster.

Analysis of transcription factor expression. SCENIC (version 1.1.0)52 was used to assess the transcriptional activity of epithelial cells with high quality (UMI > 5500). The analysis used the motifs database for RcisTarget and GRNboost (corresponding to GENIES 1.4, AUCCell 1.4.1 and RcisTarget 1.2.1; with mm10_refseq_r80__10kb_up_and_down_tss.mc9nr). The input matrix was read.

Cell transition trajectory and diffusion map analysis. Monocle 2 (version 2.10.1)68 was used for the trajectory analysis on high quality epithelial cells (UMI > 5500). A total of 100 markers of each cell type were used for the cell comparison. Dimensionality reduction and trajectory construction were performed on the selected genes with default methods and parameters. We calculated diffusion components using the RunDiffusion function as implemented in Seurat package with default parameters. The first three dimensions were used to draw diffusion maps. Mean coordinates of each cluster were calculated at the center of the cluster. The farthest cell of stage NOR from the total distance of other stages was the start point.
Analysis of interaction between cell types. We used CellPhoneDB (version 2.0.6)\(^{79}\) with default arguments to reveal interaction between cell types. Each cluster analyzed was downsampled to 100 cells since the low cell number of some epithelial clusters. Interactions of epithelial cells with immune cells and immune cells with epithelial cells were demonstrated respectively.

Immunohistochemistry and immunofluorescent detection. Formalin-fixed paraffin-embedded (FFPE) sections of esophageal precursor lesions were collected from 30 patients between 2016 and 2018 in Linzhou Cancer Hospital, including INF (n = 5), HYP (n = 13), DYS (n = 8) and CIS (n = 3), to validate the results obtained in mice. The protein expression levels of the marker genes were detected by IHC staining for mice tissues and immunofluorescence for human specimens with antibodies (Abcam) shown in Supplementary Table 3. The samples were incubated with antibody against Ki67 (1:150 for IHC, ab16667), Top2a (1:8000 for IHC), 1:10000 for IF, ab221659), Si10a (1:500 for IHC, 1:1500 for IF, ab92331), Mmp14 (1:2000 for IHC, 1:6000 for IF, ab51074), or Itgα6 (1:250 for IHC, 1:750 for IF, ab18551). Opal multiplex staining was performed according to the Opal 5-Color Manual IHC Kit (Perkin Elmer). Opal DAPI, Opal 520, Opal 570, Opal 566, Opal 620, and Opal 690 were used to generate different signals. Slides were counterstained with DAPI (1:2000) for nuclei visualization, and subsequently coveredslipped using a VectaShield Hardset mounting media. The slides were imaged using Vectra Polaris Automated Quantitative Pathology Imaging System (Perkin Elmer). We used inform software (Perkin Elmer) to unmix and remove auto-

Statistical analysis. Statistical analyses were conducted by using R v3.5.1\(^{70}\) and Prism 7 (Graphpad Software). Pearson’s correlation was calculated with the R function cor() and the significance was determined using two-sided unpaired Wilcoxon rank-sum test. P < 0.05 was considered statistically significant.

Data availability
The raw sequencing data and processed gene expression matrix of mouse model have been deposited in GSA (Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, http://gsa.big.ac.cn) under the accession number CRA002118. The raw sequencing data of human esophageal tissues has been deposited in GSA-Human (https://bigd.big.ac.cn/gsa-human) under the accession number HR4000093. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. Source data are provided with this paper.

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Received: 13 January 2020; Accepted: 29 June 2020; Published: 24 July 2020
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Acknowledgements

This project was supported by the National Natural Science Foundation of China (81725015 to C. Wu, 21675098 to J.W., 21927802 to J.W.), Medical and Health Technology Innovation Project of Chinese Academy of Medical Sciences (2016-I2M-3-019 to D.L., 2016-I2M-4-002 to C. Wu, 2019-I2M-2-001 to D.L. and C. Wu), Beijing Outstanding Young Scientist Program (BJJWZYJH01201910023027 to C. Wu), 2018 Beijing Brain Initiative (Z181100001518004 to J.W.), Beijing Advanced Innovation Center for Genomics, and Beijing Advanced Innovation Center for Structural Biology.

Author contributions

J.W., C. Wu, and D.L. conceptualized and supervised this study. J.Y., Q.C., W.F., Y.C., and T.L. contributed to the study design and performed most experiments. W.G., Y.X., and L.L. were engaged in sample collection and preparation. Y.M., J.Y., X.Z., and Y. Lin contributed to bioinformatics and statistical analysis. C. Wang, L.P., Y. Luo, and A.L. were responsible for animal experiments. J.Y., Q.C., W.F., and Y.C. drafted the manuscript. W.T., C. Wu, J.W., and D.L. reviewed and prepared the final manuscript. All authors approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17492-y.

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Peer review information Nature Communications thanks Marnix Jansen, Shao Li and the other, anonymous, reviewer for their contribution to the peer review of this work. Peer reviewer reports are available.

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NATURE COMMUNICATIONS | (2020) 11:3715 | https://doi.org/10.1038/s41467-020-17492-y | www.nature.com/naturecommunications