

LETTER TO THE EDITOR

A new mouse esophageal cancer cell line (mEC25)-derived pre-clinical syngeneic tumor model for immunotherapy

Dear Editor,

Esophageal cancer (EC) is the sixth leading cause of death from cancer worldwide [1]. It has remained a large burden in the world, especially in China [2]. Esophageal squamous cell carcinoma (ESCC) is the major histological subtype of EC and is mostly regarded as a 'silent' tumor in its early stage. Most patients are diagnosed at a late stage, due to a lack of symptoms in the early stages of the disease [3]. Despite notable improvements in surgical techniques, adjuvant chemoradiotherapy, and screening with endoscopy using Lugol's iodine staining, and nutritional intervention, ESCC still has the poorest prognosis among gastrointestinal cancers. The overall 5-year survival rate of patients with ESCC after radical esophagectomy remains below 30% [4].

Recently, immunotherapy, especially programmed death-ligand 1/programmed cell death protein 1 (PD-L1/PD-1) blockade therapy, has proven its great potential in clinical trials as an EC treatment option [5, 6]. However, immunotherapies often display mixed success in many solid tumors, including ESCC [7]. These discrepancies might be partially explained by the intricate regulation of antitumor immune responses within the tumor microenvironment (TME) [8]. Thus, investigations into the immunosuppressive TME in solid tumors are needed to develop a novel immunotherapy strategy. Unlike other solid tumors, studies on the immunosuppressive TME are still scant in the context of EC [6]. One possible reason for this is the lack of a mouse-derived ESCC cell line, which could be used to establish a syngeneic mouse model of ESCC.

Cancer immunotherapies are designed to work in conjunction with a patient's immune system to increase native anti-tumor responses [9]. In this field of study, conventional xenograft models lack relevance due to the animals' immunocompromised status. Syngeneic mouse models, or allograft mouse tumor systems, consist of tumor tissues derived from the same genetic background as a given mouse strain. A syngeneic mouse model (e.g., 4T1 and MC38 cell lines), therefore, provides an effective approach for studying how cancer therapies perform in the presence of an intact and functional immune system. Previous reported mouse ESCC models with an intact immune system have generally been established by inducing primary mouse esophageal tumors with carcinogen 4-Nitroquinoline-1-Oxide (4-NQO) [10]. However, generating such a chemical-induced mouse ESCC model is not only time consuming but also inconvenient to investigate the immune-suppressive mechanisms in the TME or to explore new immunotherapeutic approaches. As such, we believe that generating a mouse-derived ESCC cell line to establish a syngeneic mouse model of esophageal cancer will provide an effective tool for research into antitumor immunity in ESCC.

To date, the most commonly used ESCC cell lines were derived from humans. There was one study which previously established a rat-derived esophageal cancer cell line, however, comprehensive analyses and applications of this rat-derived ESCC cell line were limited [11]. To develop a syngeneic mouse model for ESCC, we initially induced the primary mouse ESCC tumors with 4-NQO in C57BL/6 mice, according to a previously described protocol [10]. The materials and methods for this study are detailed in the Supplementary information. Massive tumors with histological characteristics of squamous cell carcinoma of the esophagus on 4-NQO treated mice were observed but no obvious lesion on the esophagus of control mice was found (Supplementary Figure S1). Next, we transplanted the primary ESCC tissue from the esophagus of 4-NQO-treated mouse into immune incomplete BALB/c nude mice to further enhance the

Abbreviations: EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1; TME, tumor microenvironment; 4-NQO, 4-Nitroquinoline-1-Oxide; EpiCM-2, Epithelial Cell Medium-2; CAF, cancer associate fibroblast; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PBS, Phosphate-buffered saline; PDT, population doubling time; SOX2, sex determining region Y-box 2; SD, standard deviation; i.t., intra-tumoral; H&E, hematoxylin and eosin

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tumorigenicity of cancer cells. The explants were successfully established *in vitro* and resected to isolate primary ESCC cells.

A previous study reported that the interaction between epithelial cells and fibroblasts could facilitate the growth of cancer cells and maintain their characteristics *in vitro* at the beginning of primary culture [12]. However, the rapid proliferation of fibroblasts would dilute the ratio of cancer cells and disturb the generation of cancer cell clones. To limit the proliferation of fibroblasts and promote the cancer cell growth, we used Epithelial Cell Medium-2 (EpiCM-2) medium, which selectively supports the growth of epithelial cells, to culture all the single cells isolated from ESCC tissues. With this approach, we found that the mouse ESCC cells grew well in the EpiCM-2 medium after five passages of primary culture (Supplementary Figure S2). In our method, the cancer-associated fibroblasts (CAFs) could exist as feeder cells to sustain the cancer cell growth at the beginning of *in vitro* culture, but their proliferation was limited by the EpiCM-2 medium without disturbing the generation of cancer cell colonies. After five passages of culture in EpiCM-2 medium, we changed this culture medium to a commonly used medium (Dulbecco's Modified Eagle Medium [DMEM] containing 10% fetal bovine serum [FBS]) for the primary mouse ESCC cells, which maintained the proliferative activity at a split ratio of 1:3 every 3 to 4 days.

A small proportion of CAFs may exist when the primary ESCC cells were cultured in complete DMEM medium. As such, we completely removed the CAFs by using the time difference of adhesion and detachability between the CAFs and ESCC cells [13]. In the first five passages, the cancer cells were detached by two-step Trypsin digestion. After 2-min digestion with 0.25% Trypsin, the CAFs were removed and the remaining attached ESCC cells were washed with phosphate-buffered saline (PBS), followed by a second digestion for 10 min. In the following 10 cell passages, the ESCC cells were detached by routine one-step Trypsin digestion.

Finally, after 15-passage culture in complete DMEM medium, we successfully established a stable mouse ESCC cell line and named it as mEC25. The mEC25 cells grew as an adherent monolayer with epithelial morphologic features (Figure 1a). Karyotypic analysis revealed that the chromosomes of mEC25 cells have both numerical and structural abnormalities (Figure 1b). The modal number of chromosomes ranged from 112 to 127, with a median of 118. These cells were successfully sub-cultured at a split ratio of 1:3 every 3 days. The population doubling time (PDT) of mEC25 at passage 20 and passage 50 was 26.8 hours and 25.8 hours respectively ($P = 0.610$), indicating that the *in vitro* growth ability of mEC25 cells could be consistently maintained (Figure 1c and d). We fur-

ther determined that mEC25 cells have enhanced migration and invasion abilities compared with primary mouse normal esophageal epithelial cells mNEEC (Figure 1e). Importantly, the mEC25 cell line formed solid tumors in all five BALB/c nude mice tested ($n = 100\%$), suggesting its potency of *in vivo* tumorigenicity (Figure 1f). We also detected molecular markers for epithelial or squamous cells in mEC25 cells. Specifically, the epithelial markers including cytokeratin, E-cadherin, and β -catenin were commonly expressed in mEC25 cells (Figure 1g). Moreover, most mEC25 cells exhibited high expression of the markers associated with squamous cell carcinoma, such as sex determining region Y-box 2 (SOX2) and p63 [14].

It is essential to establish reliable preclinical mouse models that could recapitulate the clinical features, molecular genetics, biological heterogeneity, and immune microenvironment of human cancers. Although fully murine models are the best approach for initial screening, however, despite chemically-induced primary mouse ESCC model has been reported, using them would be time-consuming [10]. A syngeneic mouse model induced by transplanting a cancer cell line would be more convenient for studies on ESCC immunotherapy. To determine if our C57BL/6 mouse-derived ESCC cell line could be used to establish a syngeneic mouse model of ESCC, we initially measured its *in vivo* tumorigenicity in C57BL/6 mice. We subcutaneously injected 4×10^6 mEC25 cells/mouse combined with matrigel (1:1) into syngeneic C57BL/6 mice. The injected mEC25 cells formed solid tumors in 90% (10/11) of the mice till day 10 (Figure 1h). Subsequent histological analysis of the mEC25-derived syngeneic tumor tissue displayed the characteristics of squamous cell carcinoma (Figure 1i), indicating that the mEC25 cells had a strong *in vivo* tumorigenicity in C57BL/6 mice with an intact immune system. This immunocompetent mouse tumor allograft model may provide a convenient way to investigate the regulation of anti-tumor immunity in the TME or to exploit the novel immunotherapy strategies in animals. Indeed, we further investigated the potential of this model for anti-PD-1 treatment (Figure 1j), a popular immunotherapy that has shown promising clinical outcomes in ESCC [15]. Compared with IgG control treatment, intra-tumoral injection of 200 $\mu\text{g}/\text{mouse}$ of PD-1 antibody therapy significantly inhibited the tumor growth (Figure 1k) and completely eliminated the ESCC tumors in 80% of the C57BL/6 mice after 16 days of treatment (Figure 1l and m). These findings indicated that this syngeneic mouse ESCC model could be useful not only for exploiting the mechanism of anti-PD-1 therapy but also for designing novel anti-PD-1 based therapies with enhanced anti-tumor efficiency.

In conclusion, we successfully developed a syngeneic tumor model by using a new mouse esophageal cancer cell

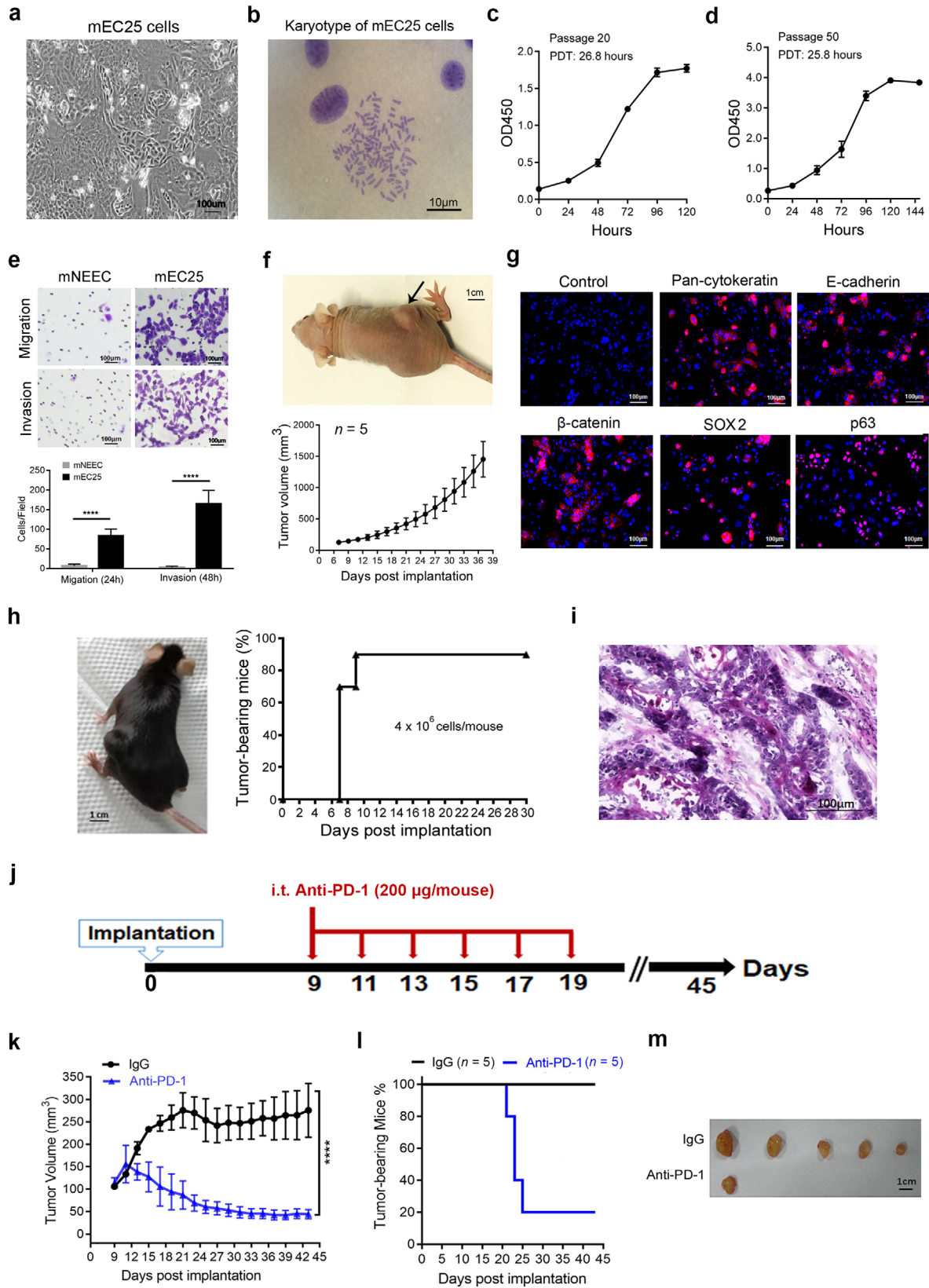


FIGURE 1 Establishment of a new syngeneic mouse model of ESCC which shows an immune response to anti-PD-1 treatment. **a**. Cell morphology of the mEC25 cell line. **b**. Karyotype analysis of the mEC25 cell line with hyperdiploidy chromosomes (1000 ×). **c-d**. Growth curves of mEC25 cells at passage 20 (**c**) and passage 50 (**d**). **e**. Representative images (upper panel) and quantification (lower panel) of mEC25 and primary mouse normal esophageal epithelial cells (mNEEC) that migrated at 24 h or invaded at 48 h. The data represent the means ± SD of three independent experiments. ****, $P < 0.0001$. **f**. mEC25 cells form solid tumors in BALB/c nude mice. Representative image of the

line (mEC25). Antitumor immune response observed with anti-PD-1 treatment further validated the applicability and reliability of this mouse model. Our study will provide an effective tool to investigate immune regulation in the initiation, progression, and treatment of ESCC.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animal study protocol was approved by the Committee on Experimental Animal Ethics at Shenzhen University School of Medicine.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Additional data is available online as an additional file at *Cancer Communications*. All materials and methods mentioned in the manuscript and additional file are available upon reasonable request from the corresponding author.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

TH and LF designed the experiments and wrote the manuscript. TH, JY and BL performed the experiments, analyzed the data. LF supervised the study. All authors read and approved the final manuscript.

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esophageal cancer, immunotherapy, syngeneic mouse model

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tumor-bearing mouse is shown in the upper panel. The growth curve of mEC25 xenografts is shown in the lower panel. g. Molecular markers were detected by immunofluorescence to characterize the mEC25 cells. h. Subcutaneous ESCC tumors were established by implanting 4×10^6 mEC25 cells with matrigel in C57BL/6 mice ($n = 11$; left panel). The percentage of tumor-bearing mice at indicated times after mEC25 cells injection is shown (right panel). i. Representative H&E staining of subcutaneous mEC25 tumor tissue. j. Schematic of the drug intervention protocol for anti-PD-1 in mEC25-implanted C57BL/6 mice. k-l. Growth curves of anti-PD-1 treated ESCC tumors (k) and the percentage of tumor-bearing mice at the indicated times by anti-PD-1 treatment (l) are shown. ****, $P < 0.0001$. m. Syngeneic mEC25 tumors from IgG ($n = 5$) and anti-PD-1-treated C57BL/6 mice ($n = 5$) sacrificed 43 days post-implantation. Abbreviations: ESCC, esophageal squamous cell carcinoma; PD-1, programmed cell death protein 1; SD, standard deviation; i.t., intra-tumoral; H&E, hematoxylin and eosin

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