Maelstrom Directs Myeloid-Derived Suppressor Cells to Promote Esophageal Squamous Cell Carcinoma Progression via Activation of the Akt1/RelA/IL8 Signaling Pathway

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Abstract

Maelstrom (MAEL) is a novel cancer/testis-associated gene, which is not only expressed in the male testicular germ cells among human normal tissues, but is also aberrantly expressed in various cancer tissues. In our study, MAEL was characterized as a tumor-promoting gene and was significantly associated with esophageal squamous cell carcinoma (ESCC) recurrence and unfavorable prognosis. Kaplan–Meier analysis showed that patients with high MAEL expression had a shorter survival time. Functional experiments showed that MAEL promoted tumor cell growth and inhibited cell apoptosis. These results prompted us to investigate the factors affecting the tumorigenicity of MAEL. Further experimentation demonstrated that MAEL enhanced the expression of phosphorylated Akt1, with subsequent phosphorylation of nuclear factor kappa B (NF-κB) subunit RelA in tumor cells, and chemotracted myeloid-derived suppressor cells (MDSCs) by upregulating interleukin-8 (IL8) to accelerate tumor progression in the tumor microenvironment. We also found that TGF-β secreted by MDSCs could upregulate MAEL by inducing Smad2/Smad3 phosphorylation. In summary, this study revealed a mechanism by which MAEL could upregulate IL8 through Akt1/RelA to direct MDSCs homing into the tumor, suggesting that MAEL could be an attractive therapeutic target and a prognostic marker against ESCC. Cancer Immunol Res; 6(10); 1246–59. ©2018 AACR.

Introduction

Maelstrom (MAEL) is a novel cancer/testis-associated gene, which is not only expressed in male testicular germ cells among human normal tissues, but is also aberrantly expressed in various cancer tissues (1–3). MAEL was initially identified in Drosophila and linked to spermatogenesis and meiosis (4). MAEL, comprising a MAEL-specific domain and a high-mobility group domain, was considered to be indispensable for Piwi-mediated silencing (5, 6). Researchers identified MAEL from 1q24, one of the most frequent chromosomal alterations in hepatocellular carcinoma (HCC; ref. 1), urothelial carcinoma of the bladder (UCB; ref. 7), and colorectal cancer (8). A growing body of evidence has shed considerable light on the important roles of MAEL in cancer progression (9, 10). A previous study demonstrates that MAEL is an important player in the progression of HCC by activating Akt/GSK3β to direct MDSCs homing into the tumor (10). MAEL, regulated by miR-186, can promote tumor aggressiveness by epigenetically downregulating the metastasis suppressor 1 (MTSS1) gene through DNA methyltransferase (DNMT)3B in UCB (7). In colorectal cancer, MAEL can induce epithelial–mesenchymal transition (EMT) and stem cell properties to promote the progression of patients with colorectal cancer (8).

The tumor microenvironment (TME) plays a key role in tumor progression. The underlying mechanism by which MAEL regulates tumor progression in TME has not been elucidated. Myeloid-derived suppressor cells (MDSCs) have been extensively studied as one of the most important immunosuppressive cells in the TME (11, 12). Previous studies reveal that interleukin-8 (IL8; CXCL8), a chemokine with tumor-promoting effects in the TME (13, 14) and is functional on CXCR1 and CXCR2, can chemottract MDSCs to the tumor site (15). MDSCs may drive tumor progression through secretion of cytokines and chemokines with promtumorigenic functions, depending on the TME (16–19). Given the immunosuppressive
function of MDSCs, the IL8–MDSC axis offers an attractive immunotherapeutic target of cancer.

Esophageal squamous cell carcinoma (ESCC), the uppermost histologic subtype of esophageal cancer, is common in China (20). Despite improvements in treatment, the 5-year overall survival (OS) rate of patients is only 17% (20, 21). Therefore, search for immunotherapy targets is of great importance. Nonetheless, potential oncogenic function of MAEL and corresponding molecular mechanisms in ESCC have not yet been addressed. In the present study, we found that MAEL was overexpressed in esophageal tumor tissues and was positively correlated with worse OS and progression-free survival (PFS). The molecular mechanisms underlying the important role that MAEL plays in regulating the TME of ESCC were elucidated. Overall, this study provides an explanation for ESCC tumorigenesis and progression with MAEL high expression.

Materials and Methods

Patients and tumor samples

A total of 120 paired tumor (typically 1 cm³) and corresponding nontumor tissues (located more than 3 cm away from tumor tissues) were freshly obtained from ESCC patients (diagnosed in nontumor tissues (located more than 3 cm away from tumor tissues) were freshly obtained from ESCC patients (diagnosed in 2012–2014) at the department of Thoracic Surgery of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The tissues were washed with PBS and cut into small pieces. Some tissues were used for RNA extraction using TRIzol, according to the standard protocol, and some tissues were processed by paraffin embedding and sectioned for IHC staining studies treated with 4% paraformaldehyde. No chemotherapy, radiotherapy, or other therapy was performed prior to entry into the study. Samples used in this study were approved by Zhengzhou University Medical Center Institutional Review Board protocols in accordance with Declaration of Helsinki. Written informed consent was obtained from each patient with available follow-up information prior to participation. All specimens were deidentified prior to analysis. The clinicopathologic features were analyzed according to age, gender, stage, differentiation, and lymph node metastasis, and patients were staged according to the tumor–node–metastasis staging system of the American Joint Committee on Cancer (AJCC, 6th edition).

ESCC cell lines and cell culture conditions

Seven ESCC cell lines (EC109, EC1, EC9706, TE1, TE7, KYSE450, and KYSE70) and one immortalized cell line (Het-1a) were purchased from Cellbank Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences, preserved in our laboratory, and frozen in liquid nitrogen until further use in our experiments. All ESCC cell lines were passed to the fifth or sixth generation and cultured in RPMI 1640 (SH30809.01, HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin (100 U/mL), and streptomycin (100 μg/mL) for 48 hours before used in experiments. Cells were maintained in a humidified incubator at 37 °C in 5% CO₂. All cell lines were tested for Mycoplasma. Cell lines were authenticated by STR profiling and stored in liquid nitrogen container when not in use.

RNA extraction and qRT-PCR

Detailed experiments were performed as described previously (21). Total RNA was extracted from ESCC cell lines and tissue specimens by TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa) in Agilent Mx3005P. Primer sequences (Sangon Biotech) for qRT-PCR were listed in Supplementary Table S1. GAPDH was used for normalization of data. The data were analyzed by 2⁻ΔΔCt.

Plasmid construction, transfection, and cell sorting

The stable overexpression of MAEL in TE7 cells and detailed experiments were performed as described previously (20). Briefly, full-length MAEL cDNA (Supplementary Table S2) was amplified by PCR using PrimeSTAR HS DNA polymerase (TaKaRa), and cloned into XbaI and salI sites of pEGFP-N1 vector (Invitrogen), and then transfected into TE7 cells (MAEL-TE7) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells transfected with empty vector were used as controls (Vector-TE7). After 48 hours of transfection, the transfectants with mock vector and MAEL expression vector were sorted by MoFlo XDP (Beckman) based on the expression of green fluorescent protein (GFP). After several rounds of sorting, the purity of MAEL expression cells was over 95%.

Stable knockdown of MAEL in TE1 by short hairpin RNAs (shRNA) was achieved using the hI6-MCS-ubiquitin-EGFP-ires-puro vector plasmid purchased from GENE (GeneChem) and transfected into TE1 cells according to the manufacturer's instructions. Cells transfected with empty vector were used as controls. Stable knockdown of MAEL in TE1 cells and detailed experiments were performed as described previously (21). The purity was over 90%.

Western blotting

Total cell proteins of Vector-TE7 (Vec), MAEL-TE7 (MAEL), shControl-TE1 (shC), shMAEL-TE1 (shM), TE7, and TE1 cells were extracted using RIPA lysis buffer (Biyunian). Protein concentration was detected and modulated using Coomassie blue staining (G250). Equal amounts of proteins (20–30 μg) were loaded onto a 10% SDS–polyacrylamide gel. Following electrophoresis, the proteins were blotted onto polyvinylidene fluoride membrane. The membrane was blocked in 5% nonfat milk and incubated with primary antibodies overnight at 4 °C, followed by treatment with secondary antibody for 2 hours at 37 °C. Primary antibodies used were as follows: anti-MAEL (1:2,000), anti-Smad2 (1:5,000), anti-Smad3 (1:5,000), anti-pSmad2 (1:1,000), anti-pSmad3 (1:1,000), anti-Akt1 (1:5,000), anti-pAkt1 (1:5,000), anti-pRelA (1:5,000), anti-pRelA (1:5,000), and anti-IL8 (1:1,000) were purchased from Abcam, and anti-β-actin antibody (Cell Signaling Technology; 1:10,000). For inhibitor treatment, cells were treated with PI3K inhibitor (LY294002, LY; HY-10108), Akt1 inhibitor (AZD5363, AZD; HY-15431), RelA inhibitor (QNZ (EVP); HY-13812), TGFβR1 inhibitor (SB431542, SB; HY-10431), Selleck Chemicals), and CXCR1/CXCR2 inhibitor (repaxtinix; HY-15251; MCE).

IHC and evaluation of IHC staining

Detailed IHC and evaluation of IHC staining experiments were performed as described previously (22). The paraffin-embedded ESCC tissues and their paired marginal tissues were detected for the expression of IL8 (1:1,000; Abcam) and MAEL (1:800; Abcam) by IHC. Sections were treated with 3%
hydrogen peroxide (H₂O₂) and 5% bovine serum albumin and incubated overnight with primary antibodies at 4°C. After incubation with horseradish peroxidase–conjugated secondary antibody for 1 hour at 37°C, sections were washed and counterstained with hematoxylin.

All sections were assessed at 20× magnification by one pathologist and two experienced observers and visualized under a microscope (Olympus, IX73). Staining was evaluated based on intensity (negative = 0; weak = 1; moderate = 2; and high = 3) of immunostaining and density (0% = 0; 1%–40% = 1; 41%–75% = 2; >76% = 3) of positive tumor cells. The final immunoreactivity score of each sample was acquired by multiplying the intensity and density scores.

Cell proliferation assay

The cell growth rate was assessed by the CCK-8 (Dojindo) kit at a density of 1 × 10⁶ cells per well in 100 µL RPMI-1640 (SH30809.01, HyClone). Cells were seeded in 96-well plates. After every 24 hours for 3 days, cells were stained with 10 µL of CCK-8 reagent at 37°C for 1 hour, and the absorbance was measured at 450 nm (MULTISKANMK3, Thermo Scientific). The number of viable cells was determined by OD value. The results are expressed as the mean ± SEM of 3 independent experiments.

Apoptosis assay

After 48 hours of transfection with Vector-TE7 (Vec) and MAEL-TE7 (MAEL), shControl-TE1 (shC) and shMAEL-TE1 (shM) cells were harvested and washed with ice-cold PBS twice. Next, the cells were collected and suspended in the 200 µL Annexin V-binding buffer (BioLegend) to a final concentration of 10⁶ cells/mL. Cells were incubated with 1 µL AlexaFluor647 Annexin V (BioLegend) for 15 minutes at 4°C in the dark, and then propidium iodide (PI; Sigma) was added. Samples were immediately analyzed by flow cytometry (BD FACSCTanto II).

Sphere formation assay

Five hundred shControl-TE1 (shC) and shMAEL-TE1 (shM) cells were grown in the DMEM/F12 medium (Invitrogen LifeTechnologies) supplemented with heparin (4 µg/mL; Sigma), 2% B27 (Gibco, Life Technologies), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF; 20 ng/mL; both from PeproTech), penicillin (100 IU/mL), and streptomycin (100 µg/mL). They were then seeded in 24-well ultralow cluster plates (Corning Costar) and cultured for 2 weeks. The number of spheres was quantified by microscope (Olympus, IX73).

Three-dimensional (3D) culture

Detailed 3D culture experiments were performed as described previously (23). Briefly, before seeding into 3D fibrin gels, Vector-TE7 (Vec) and MAEL-TE7 (MAEL) were maintained in RPMI-1640 culture medium containing 10% FBS and antibiotics, and cell density was adjusted to 10⁶ cells/mL. Fibrinogen was diluted into 2 mg/mL with T7 buffer (pH 7.4, 50 mmol/L Tris, 150 mmol/L NaCl). Fibrinogen and the cell solution were mixed at a ratio of 1:1, resulting in 1 mg/mL fibrinogen and 5,000 cells/mL in the mixture. Cell/fibrinogen mixture (250 µL) was seeded into each well of a 24-well plate and mixed well with preadded 5 µL thrombin (0.1 U/µL). The plate was then moved into a 37°C cell culture incubator and incubated for 10 minutes. Finally, 1 mL RPMI-1640 medium was added.

Enzyme-linked immunosorbent assay (ELISA)

One hundred milliliters of supernatant from each well of cultured Vector-TE7 (Vec), MAEL-TE7 (MAEL), shControl-TE1 (shC), shMAEL-TE1 (shM) cells, and PMN-MDSCs were collected at 48 hours for measurement of IL8 protein (without dilution) using a Human CXCL8/IL8 Quantikine ELISA kit (R&D Systems Inc.). IL6, IL10, TGFβ, and TNFα proteins were, respectively, detected by human IL6, IL10, TGFβ1, and TNFα Quantikine ELISA kits (R&D Systems). Each assay had duplicated wells for the standard curve and samples. Concentration was calculated from the standard curve using the recombinant cytokines, according to the manufacturer’s instructions.

Multiplex bead-based assay

The Human Chemokine and Cytokine detection kit uses multiplex bead-based assay panel provided by BioLegend LEGENDplex (#740001). Supernatants were collected from the same quantity of Vector-TE7 (Vec), MAEL-TE7 (MAEL), shControl-TE1 (shC), and shMAEL-TE1 (shM) cells after culturing for 48 hours and were centrifuged prior to analysis. Results were normalized to standards provided by the BioLegend LEGENDplex. Detailed experiments were performed according to the standard protocol.

FACS analyses and sorting of PMN-MDSCs and CD8⁺ T cells

Peripheral blood from 20 ESCC patients was collected in 20 mL heparin-containing tubes. Peripheral blood mononuclear cells were isolated by Ficoll density gradient (Tianjin HY) centrifugation. Then, anti-CD14 and anti-CD11b MACs magnetic sorting system including QuadroMACS Starting Kit (#130-050-201 and 130-049-601), MACS LS columns (130-042-401), and MACS Running Buffer (130-091-221, Miltenyi Biotec) was used to isolate CD14⁺CD11b⁺ PMN-MDSCs according to the manufacturer’s instructions. CD14⁺ cells were collected and prepared for CD11b⁻ cells isolation. APC-Cy7–CD14 (clone 63D3), PE-Cy7–CD11b (clone ICRF44), PE-CD33 (clone P67.6), FITC–HLA-DR (clone L243), APC–Lin (clones UCH11, HCD14, 3G8, HB19, 2H7, HCD56), and PerCP-CCD5 (clone W6D3; BioLegend) were used to mark PMN-MDSCs. PE-cy7–CXCR1 (8F1/CXCR1) was purchased from BioLegend. Eighty percent of sorted CD14⁺CD11b⁺ cells were Lin⁻HLA-DR⁻CD33⁻CD15⁻CD14⁻CD11b⁺. The remainder of the cell suspension from the same patients was used to purify CD8⁺ T cells by the MACS magnetic sorting system including the QuadroMACS Starting Kit (#130-096-495), MACS LS columns, and MACS Running Buffer (Miltenyi Biotec). FITC–CD3 (clone HIT3a) and PE-Cy7–CD8 (clone SK1; BioLegend) were used to mark CD8⁺ T cells.

Transwell assay

The chemotactic migration of PMN-MDSCs was evaluated in 24-well plates with 5-µm pore size polycarbonate filters (Corning Inc.). Freshly isolated PMN-MDSCs from patient peripheral blood were seeded onto the upper chamber. Tumor cells or
anti-IL8 neutralizing antibodies (50 μg/mL, Abcam, #ab18672) were added to the lower chamber. PMN-MDSCs that had migrated into the lower chamber were counted after 24 hours.

Animal model
All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University. To generate a xenograft mouse model, female BALB/c nude mice (Vital River Laboratory Animal Technology Co. Ltd.) ages 5 weeks were randomly divided into groups. The flank of 6 groups of nude mice received subcutaneous injections of shControl-TE1 (shC), shMAEL-TE1 (shM), shC + PMN-MDSCs, shM + PMN-MDSCs, shC + reparixin, or shC + reparixin + PMN-MDSCs (10 mice/group) near the right thigh. The flank of 8 groups of nude mice received subcutaneous injections of Vector-TE7 (Vec), MAEL-TE7 (MAEL), Vec + PMN-MDSCs, MAEL + PMN-MDSCs, Vec + reparixin, MAEL + reparixin, Vec + PMN-MDSCs + reparixin, or MAEL + PMN-MDSCs + reparixin (8 mice/group; 5 × 10⁶ tumor cells in 100 μL of PBS), respectively, at day 0. Half of the mice in each group were treated as follows: mice were inspected every other 2 days, and tumor growth was evaluated by measuring length and width of tumor mass with calipers. At day 6, mice with detectable tumors were injected 2.0 × 10⁷ PMN-MDSCs (i.v.). Reparixin (20 μg) was administered subcutaneously daily from days 6 to 24. At day 24, mice were sacrificed to obtain tumor tissues used for detection of the tumor weight. Tumor volumes were calculated using the formula: (length × width)²/2. The remaining half of the mice in each group were handled as follows: reparixin (20 μg) was administrated subcutaneously daily from days 18 to 24. At day 23, mice were injected 2.0 × 10⁷ PMN-MDSCs (i.v.), and at day 24, mice were sacrificed to obtain tumor tissues that were used to assess the percentage of PMN-MDSCs recruited to tumor site by flow cytometry.

Immunosuppressive assay
T-cell proliferation experiments were performed as described previously (24). CD8⁺ T cells (1 × 10⁵) were labeled with CFSE and washed. Control or prestimulated PBS, CFSE-labeled CD8⁺ T cells were stimulated with 5 × 10⁴ CD3/CD28 beads (Miltenyi Biotech) and IL2 (100 U/mL), and cocultured with purified PMN-MDSCs for 48 hours at ratios of 1:0 and 1:2, respectively. Analysis was performed by flow cytometry. In functional experiments, 5 × 10⁵ CD8⁺ T cells were incubated with 1 × 10⁶ PMN-MDSCs or not for 48 hours, and granzyme B and perforin production was detected by flow cytometry assay. The analysis was gated on CD8⁺ T cells (25). Flow cytometry antibodies used were as follows: PE-cy7–CD8 (SK1), FITC-granzyme B (GB11), and APC-perforin (B-D48; BioLegend).

Human recombinant proteins
Recombinant human IL6 was purchased from PeproTech (#200-06), recombinant human IL10 was purchased from PeproTech (#200-10), recombinant human TGFβ was purchased from PeproTech (#100-21). TE7 cells were respectively treated with IL6 (20 ng/mL), IL10 (20 ng/mL), and TGFβ (20 ng/mL) for 48 hours. For inhibitor treatment, TE7 and TE1 cells were pretreated with TGFβR1 inhibitor (SR; 10 μmol/L) for 1 hour and incubated with TGFβ for 48 hours.

TCGA database analysis
The expression of MAEL and IL8 of 190 esophageal cancer cases were obtained from The Cancer Genome Atlas in 2016 (TCGA; https://cancergenome.nih.gov/). All the cases were esophageal cancer patients without any treatment.

Statistical analysis
A paired t test was used to compare MAEL and IL8 expression between ESCC tissues and nontumor tissues, using GraphPad Prism 5 software (GraphPad Software). The Pearson χ² test analyzed the association between MAEL expression and clinicopathologic features, and MAEL and IL8. Photoshop software was used to detect the gray value. OS and disease-free survival (DFS) curves were evaluated by the Kaplan–Meier method and log-rank tests. Results were considered statistically significant when P < 0.05.

Results
MAEL was upregulated and associated with survival of patients in ESCC
MAEL expression was previously studied in HCC (1), UCB (7), and colorectal cancer (8). In our study, we found that MAEL expression was higher in 120 esophageal tumor tissues compared with nontumor tissues by qRT-PCR (Fig. 1A). In human esophageal cancer cell lines (EC109, TE1, TE7, KYSE450, KYSE70, and EC9706), MAEL expression was highest in TE1 and lowest in TE7 (Fig. 1B).

To study the potential clinical relevance of MAEL expression in patients with ESCC, we performed IHC on tissue sections, and selected representative IHC images are shown in Fig. 1C. We found that MAEL was expressed in tumor tissues and was absent or weakly stained in marginal tissues. Kaplan–Meier analysis revealed that higher MAEL expression was associated with lower OS and poorer disease-free survival (DFS) rates of patients with ESCC (Fig. 1D). Our results also revealed that MAEL expression was upregulated with increased tumor stage (P = 0.0398), lymph node metastasis (P = 0.0076), differentiation (P = 0.0378), and tumor size (P = 0.0338), whereas MAEL expression had no significant correlation with patient age or gender (Supplementary Table S3). Collectively, MAEL was considered as an independent prognostic marker for ESCC.

Overexpression or knockdown of MAEL influences the tumorigenesis of ESCC cell lines
To evaluate the oncogenic function of MAEL, we transfected a gene construct expressing GFP-MAEL into TE7. After several rounds of sorting based on GFP expression, stable MAEL-overexpressing TE7 cells were obtained, and the purity was over 97% (Supplementary Fig. S1A). The efficiency of MAEL upregulation at the mRNA and protein levels was respectively detected by qRT-PCR (Fig. 2A) and Western blot (Fig. 2B), and the gray value was summarized by histogram (Supplementary Fig. S1B). Ectopic expression of MAEL was shown to promote cell growth (Fig. 2C) by CCK-8 assay and inhibit cell apoptosis (Supplementary Fig. S1C) by flow cytometry assay. The colony formation assay yielded a higher number and larger colonies in MAEL-transfected cells than in control cells (Fig. 2D). We also found that sphere-forming ability of MAEL-overexpressing cells was significantly increased compared with control cells (Fig. 2E). We further investigated whether MAEL could increase the stemness of ESCC cells and found that MAEL could
upregulate many stemness-associated genes (Oct4, NANOG, and NOTCH1) and surface antigen CD133, which is associated with cancer stem cells (Fig. 2F).

To validate the effect of MAEL on tumor growth in vivo, a xenograft tumor mouse model was established by subcutaneously injecting MAEL-overexpressing and control cells into the left dorsal flank of mice. We found that volumes and weights of tumors were significantly greater in MAEL-overexpressing cells than in controls (Fig. 2G). IHC staining also confirmed that expression of MAEL in MAEL-transfected cells was higher compared with control in xenograft tumors (Fig. 2H). Lump images of xenograft tumors formed in nude mice injected with MAEL- and vector-transfected cells are shown in Supplementary Fig. S1D.

In order to demonstrate tumorigenic function, MAEL was silenced by shRNA. The sorting purity of transfected cells was over 90% (Supplementary Fig. S1E). The efficiency of MAEL downregulation at the mRNA and protein levels was respectively detected by qRT-PCR (Fig. 3A) and Western blot (Fig. 3B), and the gray value was calculated by histogram (Supplementary Fig. S1F). MAEL knockdown could inhibit cell growth (Fig. 3C) by CCK-8 assay and promote cell apoptosis (Supplementary Fig. S1G) by flow cytometry assay. A colony formation assay yielded a lower number and smaller colonies in MAEL-silenced cells than in control cells (Fig. 3D). The sphere-forming ability of MAEL-silenced cells was significantly decreased compared with control cells (Fig. 3E). MAEL knockdown also downregulated stemness-associated genes (Oct4, NANOG, and NOTCH1) and surface antigen CD133 (Fig. 3F).

We found that volumes and weights of tumors were significantly smaller and lighter in MAEL-silenced cells than in control cells in vivo (Fig. 3G). Representative images from IHC staining also confirmed that MAEL expression was significantly decreased in MAEL-silenced cells compared with control cells (Fig. 3H). Images of the xenograft tumors formed in nude mice injected with MAEL-silenced and control cells are displayed in Supplementary Fig. S1H. Overall, MAEL overexpression or knockdown could promote or inhibit cell growth, self-renewal ability, and stemness...
Figure 2.
Overexpression of MAEL played an important oncogenic role in ESCC cells. A, qRT-PCR analysis of MAEL expression in sorted TE7 cells expressing MAEL-TE7 (MAEL) and Vector-TE7 (Vec). B, The expression of MAEL detected by Western blot in MAEL and Vec cells. C, Rate of cell growth of MAEL and Vec by CCK-8 assay. D, 500 cells were seeded in 6-well plates, and after 2 weeks of culture, representative images of foci formation in monolayer cultures of MAEL and Vec cells, and the number of colonies detected. E, Representative images of spheroid formation of MAEL and Vec cells after 5 days in culture within a 90-Pa 3D fibrin gel. Data are expressed as mean ± SEM of 3 independent experiments. F, The expression of stemness-associated genes (Oct4, NANOG, and NOTCH1) and surface antigen CD133 (associated with cancer stem cells) was detected in MAEL and Vec cells. G, Volumes and weights of xenograft tumors from nude mice injected with either MAEL or Vec cells (5 mice per group) were detected at 6, 9, 12, 15, 18, 21, and 24 days. H, MAEL expression in xenograft tumors from nude mice was analyzed by using IHC (original magnification: 400×). Mice were injected with $5 \times 10^5$ either MAEL or Vec cells, and sacrificed after 24 days. Data are shown as mean ± SEM of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student t test).
of ESCC cells, and tumor formation and development in vivo, indicating that MAEL had a strong oncogenic function in the progression of ESCC.

IL8, regulated by MAEL, positively correlated with patient prognosis

Accumulating evidence suggests that TME plays a crucial role in promoting the development of solid cancers (11, 26). In TME, cytokines and chemokines relevant to tumor progression are thought to be key tumor-promoting factors. In the present study, we detected 10 cytokines and 13 chemokines using a human multiplex bead-based kit and found that only IL8 had a significant and contrary change in MAEL-overexpressing and MAEL-silenced cells at the protein level (Fig. 4A). The expression of 14 chemokines was detected by qRT-PCR, represented as heat map (Fig. 4B). The results demonstrated that expression of MAEL decreased its effect on tumorigenicity.

Figure 3.

Silencing MAEL decreased its effect on tumorigenicity. A, The relative expression by qRT-PCR of MAEL in shMAEL-TE1 (shM) and shControl-TE1 (shC) cells sorted from TE1 cells. B, Expression of the MAEL between shM and shC cells by Western blot. C, Rate of cell growth between shC and shM detected by CCK-8 assay. D, 500 cells were seeded in 6-well plates, and after 2 weeks of culture, representative images of foci formation in monolayer culture between shC and shM cells, and the number of colonies detected. E, Representative images showed sphere-forming ability between shM and shC cells at 14 days. F, The expression of stemness-associated genes (Oct4, NANOG, and NOTCH1) and surface antigen CD133 (associated with cancer stem cells) was detected in shC cells and shM cells. G, Volumes and weights of xenograft tumors from nude mice injected with either shC or shM cells (5 mice per group) were detected at 6, 9, 12, 15, 18, 21, and 24 days. H, MAEL expression in xenograft tumors from nude mice was analyzed by using IHC (original magnification: 400×). Mice were injected with 5 × 10⁶ either shC or shM cells, and sacrificed after 24 days. *P < 0.05; **P < 0.01; ***P < 0.001 (Student t test). Results are expressed as mean ± SEM of 3 independent experiments.
of CCL5, CCL22, CXCL5, and IL8 was significantly upregulated in MAEL-transfected cells compared with control cells (Fig. 4C), whereas the expression of CCL5, CXCL5, CXCL6, and IL8 was downregulated in MAEL-silenced cells (Fig. 4D). Only IL8 was significantly changed among different groups by various methods (Fig. 4E). In concordance with multicytokine and qRT-PCR analysis, ELISAs also demonstrated that MAEL-overexpressed cells induced a significant upregulation of IL8 in parallel with a significant downregulation of IL8 in MAEL-silenced cells (Fig. 4F). Thus, IL8 could be a downstream target of MAEL.

The expression of IL8 was positively correlated with MAEL expression and ESCC patient prognosis. A, Detection of cytokine and chemokine expression in the supernatant of cell lines cultured for 48 hours by a human multiplex bead-based kit at the protein level. Heat map shows differential downregulation and upregulation of genes between MAEL and Vec cells or between shM and shC cells. B, Heat map of the expression of cytokines and chemokines detected by qRT-PCR between MAEL and Vec cells or between shM and shC cells. Gene expression is represented as relative values normalized to GAPDH mRNA. C and D, Expression of chemokines was detected between MAEL and Vec cells or between shM and shC cells by qRT-PCR. E, Venn diagram of chemokines that changed congruously and significantly (detected by human multicytokine assay and qRT-PCR) between MAEL and Vec cells and between shM and shC cells. 1 (yellow circle): chemokines that changed significantly and congruously between MAEL and Vec cells and between shM and shC cells; 2 (blue circle): chemokines that changed significantly by qRT-PCR between MAEL and Vec cells; 3 (purple circle): chemokines that changed significantly by qRT-PCR between shM and shC cells. F, The amount of IL8 secreted into culture media was detected by ELISA between MAEL and Vec cells and between shM and shC cells cultured for 48 hours. G, IL8 protein expression in tumor and nontumor tissue was analyzed by using IHC (original magnification: 200×). H, Kaplan–Meier survival analysis of OS and DFS based on high (n = 30) and low (n = 34) MAEL expression (by IHC). Results are summarized as mean ± SEM of 3 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student t test).

PMN-MDSCs were chemotactically recruited by IL8
Multiple mechanisms of immune suppression can arise in the disease state to facilitate the progression of cancer (15, 27). Among them, tumor recruitment of MDSCs represents one major impediment to effective antitumor immune responses. According to previous studies, MDSCs can be recruited by IL8,
and cell supernatants of advanced cancer patients are usually rich in IL8 commonly produced by malignant cells (28). Therefore, we performed a classic transwell migration assays to verify the recruitment of PMN-MDSCs in patient PBMCs by IL8. Representative flow cytometry gating of PMN-MDSCs (Lin<sup>-</sup>HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup>CD14<sup>+</sup>) is shown in Figure 5A. We found that the numbers of MDSCs chemotacted by cell supernatant were higher in shControl (shC) than sh-MAEL (shM), and that in MAEL-overexpressed than in control cells (Fig. 5B). Migration of PMN-MDSCs could be blocked by IL8-neutralizing antibody (Fig. 5B). A positive correlation between the percentage of PMN-MDSCs and IL8 mRNA in ESCC tissues (N = 11) was found (Supplementary Fig. S2D). PMN-MDSCs were subjected to a suppression assay. These cells inhibited T-cell proliferation, shown by a reduced proliferation index in T cells (Supplementary Fig. S2E) and decreased expression of perforin and granzyme B secreted by effector T cells (Supplementary Fig. S2F–S2G). We then detected whether CXC1R1, the chemokine receptor for IL8, was expressed on esophageal cancer cells and PMN-MDSCs and found that the expression of CXC1R1 was respectively 16.20% ± 2.889% (N = 6) on esophageal cancer cells (Supplementary Fig. S2H) and 70.17% ± 4.540% (N = 10) on PMN-MDSCs (Supplementary Fig. S2I), indicating that the expression of CXC1R1 on PMN-MDSCs was significantly higher compared with that on esophageal cancer cells.

Reparixin, a described pharmacologic inhibitor of CXC1R1 and CXC2R2, has been shown to decrease the recruitment of MDSCs in vivo (15, 29, 30). To further investigate the effect of IL8 on PMN-MDSCs in vivo, a further study was designed as described in Fig. 5C. We found that volumes and weights of tumors were significantly greater in the shC + PMN-MDSCs group (compared with the shC group) and in the MAEL + PMN-MDSCs group (compared with the MAEL group), which could be counteracted by downregulating MAEL or using reparixin (Fig. 5D and E). Images of the xenograft tumors formed in nude mice are shown in Supplementary Fig. S3A and S3B. As shown in Fig. 5F and G, we found that the percentage of PMN-MDSCs chemotacted to the tumor site was significantly upregulated in the shC + PMN-MDSCs group compared with the shC group and also in the MAEL + PMN-MDSCs group compared with the MAEL group, and the effect could be counteracted by downregulating MAEL or using reparixin. From these results, we could infer that IL8, regulated by MAEL, chemotacted PMN-MDSCs to tumor site to promote cell growth, which could be blocked by downregulating MAEL or IL8-neutralizing antibody in vitro or reparixin in vivo.

MAEL upregulated IL8 by activating the Akt1/RelA signaling pathway

Li et al. demonstrate that MAEL can activate the Akt/GSK-3β/Snail signaling pathway in HCC (1), which prompted us to investigate whether MAEL-regulated IL8 expression is mediated by Akt1 protein in ESCC cells. As anticipated, Western blot results demonstrated that expression of phosphorylated Akt1 was substantially enhanced in MAEL-overexpressed compared with control cells (Fig. 6A), and reduced expression of phosphorylated Akt1 was observed in MAEL-silenced cells (Fig. 6B). The most common upstream target of chemokines is the nuclear transcription factor RelA, and it has been reported that RelA can upregulate IL8 (31–33).

As we expected, expression of phosphorylated RelA and IL8 was upregulated in MAEL-overexpressed compared with control cells (Fig. 6A), and opposite results were observed in MAEL-silenced cells (Fig. 6B). To further demonstrate that MAEL-regulated IL8 was through activation of the Akt1/RelA signaling pathway, tumor cells were treated with protein (PI3K, Akt1, and RelA) inhibitors. In line with previous reports, we revealed that PI3K inhibitor (LY) could downregulate phosphorylated Akt1, phosphorylated RelA, and IL8 expression. An Akt1 inhibitor (AZD) could also downregulate phosphorylated Akt1, phosphorylated RelA, and IL8 expression. The RelA inhibitor (QNZ (EVP)) could downregulate phosphorylated RelA and IL8 expression (Fig. 6A and B). ELISAs also demonstrated that expression of IL8 in the supernatants from tumor cells was significantly downregulated when cells were treated with either LY, AZD, or QNZ (EVP; Fig. 6A and B). By gray value analysis, we found that these results were statistically significant (Supplementary Fig. S4A and S4B).

PMN-MDSCs secreted TGFβ to upregulate MAEL through activating Smad2/Smad3

It has been reported that MDSCs supernatant could upregulate another cancer–testis antigen (CTA), MAGE-A4, in lung cancer (34). In accordance with previous studies, we found that MDSC supernatants could significantly upregulate MAEL, activate EMT, and upregulate stemness-associated genes in esophageal cancer cell lines (Fig. 7A). To demonstrate which cytokines in MDSC supernatants exerted this effect, we detected expression of several more common factors secreted by PMN-MDSCs, such as IL10, IL6, IL8, TGFβ, and TNFα by ELISA (35, 36), and we found that TGFβ, IL10, and IL6 was high (Supplementary Fig. S4E). It has been reported that TGFβ (37), IL10 (38), and IL6 (39) can activate EMT. In order to further confirm the cytokines in PMN-MDSCs supernatants that could upregulate MAEL, we treated TE7 cells with TGFβ, IL10, and IL6 and detected the expression of MAEL, EMT markers, and stemness-associated genes. We found that only TGFβ could upregulate MAEL (Fig. 7B) and that the expression of TGFβ was positively correlated with MAEL at the mRNA level (Supplementary Fig. S4F). These results demonstrated that TGFβ secreted by PMN-MDSCs could upregulate MAEL. Regarding the TGFβ/Smad signaling pathway, studies have revealed that TGFβ and TGFβR1 binding complexes can promote translocation of Smad complexes to the nucleus (40). To further verify whether TGFβ-modulated MAEL was also mediated by Smad proteins, we tested expression of several proteins involved in the TGFβ/Smad pathway by Western blot. In our results, when TE7 and TE1 cells were treated with TGFβ, the expression of pSmad2, pSmad3, and MAEL was significantly upregulated (Fig. 7C and D). As expected, opposite expression of these proteins was detected when TE7 and TE1 were treated with TGFβR1 inhibitor (SB; Fig. 7C and D). By gray value analysis, the results were statistically significant (Supplementary Fig. S4G–S4H). These results demonstrated...
Figure 5.
Human PMN-MDSCs could be recruited by IL8. A, Representative flow cytometry plots of gating strategy for PMN-MDSCs (Lin− HLA-DR− CD15+ CD14− CD11b+).
B, Numbers of PMN-MDSCs recruited to indicated stimuli in migration assays, including IL8-containing cell culture supernatants, between shM and shC cells, and MAEL and Vec cells. When indicated, IL8-neutralizing monoclonal antibody (mAb) was added to lower transmigration chamber.
C, Flow chart of in vivo mouse experiments: female BALB/c nude SPF mice were subcutaneously inoculated with tumor cells (5 × 10⁶) at day 0. Half of the mice in each group were treated as follows: at day 6, mice were injected 2.0 × 10⁷ PMN-MDSCs (i.v.), and then reparixin (20 µg) was administered subcutaneously daily from days 6 to 24. Mice were sacrificed at day 24. The remaining half of the mice were handled as follows: reparixin (20 µg) was administered subcutaneously daily from days 18 to 24. At day 23, mice were injected 2.0 × 10⁷ PMN-MDSCs (i.v.), and mice were then sacrificed at day 24. Repa: Reparixin; sc: subcutaneously; iv: intravenously.
D, Weights and volumes of xenograft tumors from nude mice injected with shC, shM, shC + PMN-MDSCs, shM + PMN-MDSCs, shC + reparixin, or shM + PMN-MDSCs + reparixin. Weights and volumes of xenograft tumors expressed as mean ± SEM.
E, Weights and volumes of xenograft tumors from nude mice injected with Vec, MAEL, Vec + PMN-MDSCs, MAEL + PMN-MDSCs, Vec + reparixin, MAEL + reparixin, Vec + reparixin + PMN-MDSCs or MAEL + reparixin + PMN-MDSCs. Weights and volumes of xenograft tumors expressed as mean ± SEM.
F and G, Percentage of PMN-MDSCs recruited to mouse tumor tissues by flow cytometry. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student t-test).
Three independent experiments were performed, and results are summarized as mean ± SEM.
that TGFβ secreted by PMN-MDSCs could upregulate MAEL by inducing Smad2/Smad3 phosphorylation. A schematic diagram of MAEL affecting the progression of ESCC is summarized in Fig. 7E.

**Discussion**

Esophageal cancer ranks as the sixth leading cause of cancer-related death and the eighth most commonly diagnosed malignant disease in China (41, 42). Although profited from the development of diagnostic techniques and therapeutic modalities, the prognosis of ESCC has been poor. Up to now, the exact cellular and molecular mechanisms leading to ESCC have not been systematically evaluated. Thus, there is urgency to find new treatment targets for ESCC, especially molecularly targeted therapies.

*MAEL*, as a novel CTA, was identified as a potential therapeutic target of human HCC (1), UCB (7), and colorectal cancer (8). However, tumorigenic function and the underlying mechanisms of MAEL in ESCC have remained unclear thus far. In our study, we found that expression of MAEL, correlating with tumor stage, differentiation, and lymph node metastasis, was higher in tumor tissues compared with non-tumor tissues. MAEL could promote cell growth, colony formation, and stemness in vitro and led to a significant increase in tumor volumes and weights of mice in vivo. Taken together, MAEL plays an important role in esophageal cancer progression and could be used as an effective therapeutic target.

Some studies have shown that some oncogenes, such as IL33, can target cancer stem cell growth and facilitate macrophage recruitment to indirectly promote the progression of colon cancer (43). Twist1’s role in directly promoting the development of gastric cancer and indirectly driving the transdifferentiation of quiescent fibroblasts into CAFs in the TME (44, 45) has also been demonstrated. Studies investigating MAEL have focused on studying its tumorigenicity (1, 7), with little attention paid to its possible role in the TME. In order to investigate whether MAEL also plays a certain role in regulating the TME in ESCC, the potential mechanisms of MAEL need to be further explored. An increasing number of studies have shown that chemokines (46, 47) or cytokines (48) can promote tumor development. Based on these results, we wanted to explore whether MAEL was also associated with chemokines or cytokines in the TME. Using multicytokine, qRT-PCR, and ELISA, we detected the expression of chemokines and cytokines and found that only IL8 was significantly changed. We further observed a significant positive correlation between the expression of MAEL and IL8 and that IL8 was a downstream target of MAEL.

Some researchers indicate that IL8 can promote tumor cell invasion and migration by directly interacting with CXCR1/2 of ESCC cells (49). Other researchers have found that IL8 can recruit MDSCs through their expression of CXCR1/2 to further facilitate the growth of tumor cells in the TME (15, 50, 51). In our study, we found that the expression of CXCR1 on PMN-MDSCs was significantly higher compared with that on esophageal cancer cells, indicating that IL8 mainly played a role in recruiting PMN-MDSCs, which was significantly attenuated by IL8-neutralizing antibody in vitro and reparixin in vivo. These results demonstrated that MAEL regulated the development of ESCC via regulation of IL8, recruiting MDSCs to tumor sites.

Nonetheless, the molecular mechanisms of MAEL-regulated IL8 have not been elucidated. In HCC (1), MAEL can promote tumor cell invasion and migration via activation of Akt/GSK3β/Snail signaling. Therefore, we hypothesized that MAEL could regulate IL8 via Akt1 in ESCC as well. In this study, we found that expression of phosphorylated Akt1 increased when MAEL was overexpressed, and opposite results were observed when MAEL was silenced. The expression of IL8 was decreased when cells were treated with LY or AZD. Many studies have demonstrated that the most common upstream target of chemokines is RelA (13, 52, 53), and RelA can regulate IL8 (13, 32). These results prompted us to investigate whether RelA could regulate IL8 in ESCC. Our data showed that IL8 was significantly decreased after tumor cell treatment with QNZ (EVP), and phosphorylated RelA increased or decreased accordingly when MAEL was overexpressed or silenced. We further explored whether Akt1 could regulate RelA in ESCC. In line with previous studies (54, 55), we found that inhibiting Akt1 in transfected cells by LY or AZD suppressed activation of the RelA signaling pathway, indicating that MAEL could regulate IL8, which recruited PMN-MDSCs to the tumor site to promote tumor progression via activation of Akt1/RelA signaling.

Shi and colleagues demonstrated that MAGE-A4, a CTA, could be regulated by MDSC supernatants (34), and Sampson and colleagues demonstrated TGFβ could upregulate another...
CTA, GAGEC1 (3). In our study, we found that MDSC supernatants also upregulated MAEL. These results prompted us to further investigate which cytokines in MDSC supernatants, such as TGFβ, IL6, IL10 or IL8, might influence expression of MAEL. Cytokines found to be produced were added to tumor cells to detect the expression of MAEL. Our data indicated that only TGFβ could upregulate MAEL. Accumulating evidence has revealed that blocking TGFβ/Smad signaling is a promising cancer therapy (56, 57). These results led us to investigate whether TGFβ-triggered MAEL upregulation could be Smad dependent. Our results demonstrated that the expression of MAEL and phosphorylated Smad2/Smad3 were considerably upregulated in TGFβ-treated cells, and when TGFβ inhibitor was added, the effects were reversed. These results indicated that TGFβ induced upregulation of MAEL via activation of Smad2/Smad3. In summary, MAEL played an important role in the progression of ESCC via activation of Akt1/RelA/IL8 signaling to recruit PMN-MDSCs to tumor sites, and TGFβ secreted by PMN-MDSCs could induce Smad2/Smad3 phosphorylation to upregulate MAEL to further promote tumor progression in patients with ESCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
Pupu Li, Xinfeng Chen, Guohui Qin, and Yi Zhang had full access to all data and take responsibility for its integrity and accuracy of the data analysis.

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