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MTA2 promotes the metastasis of esophageal squamous cell carcinoma via EIF4E-Twist feedback loop

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Abstract

Metastasis-associated protein 2 (MTA2) is frequently amplified in many types of cancers; however, the role and underlying molecular mechanism of MTA2 in esophageal squamous cell carcinoma (ESCC) remain unknown. Here, we reported that MTA2 is highly expressed in ESCC tissue and cells, and is closely related to the malignant characteristics and poor prognosis of patients with ESCC. Through in vitro and in vivo experiments, we demonstrated that MTA2 significantly promoted ESCC growth, metastasis, and epithelial-mesenchymal transition (EMT) progression. This integrative analysis combined with expression microarray showed that MTA2 could interact with eukaryotic initiation factor 4E (EIF4E), which positively regulates the expression of Twist, known as a master regulator of EMT. Moreover, the results of chromatin immunoprecipitation revealed that MTA2 was recruited to the E-cadherin promoter by Twist, which reduced the acetylation level of the promoter region and thus inhibited expression of E-cadherin, and subsequently promoted the aggressive progression of ESCC. Collectively, our study provided novel evidence that MTA2 plays an aggressive role in ESCC metastasis by a novel EIF4E-Twist positive feedback loop, which may provide a potential therapeutic target for the management of ESCC.

KEYWORDS
EIF4E, ESCC, metastasis, MTA2, Twist

1 | INTRODUCTION

Esophageal cancer is one of the least studied and most aggressive malignancies worldwide.[1] The most prevalent histologic type of esophageal carcinoma is esophageal squamous cell carcinoma (ESCC).[2] The latest data showed that ESCC accounts for 90% of the newly diagnosed esophageal cancers each year in the world, with incidence and mortality varying across countries and world regions.[3] Despite the development of tumor diagnosis and therapy, the survival of esophageal cancer has still not significantly improved, and the overall 5-y survival rates range from 15% to 25%.[4] Squamous cell carcinoma is a histologic category of epithelial malignancies involving a high degree of metastatic outgrowth and invasion,[5] which accounts for poor outcomes for ESCC. However, the molecular mechanisms of tumorigenesis and metastasis of ESCC are still unclear.

Su-Li Dai1 and Si-Si Wei contributed equally to this work.

The metastasis-associated (MTA) family was first identified by Toh and colleagues in 1994 using a differential cDNA screening method.[6] Subsequently, the MTA protein family was discovered to play an indispensable role in the formation and progression of a wide variety of cancers, serving as a promising candidate cancer biomarker.[7-12] The MTA family mainly consists of 3 members: MTA1, MTA2, and MTA3. It has been reported that MTA2 regulates histone deacetylase activity to control downstream gene transcription, resulting in epithelial cell transformations and cancer cell invasion and metastasis.[13] MTA2 overexpression has been found in gastric cancer [14] colorectal cancer,[15] non–small-cell lung cancer,[16] and nasopharyngeal carcinoma.[17] Moreover, MTA2 has been shown to be associated with motile and anchorage-independent growth phenotypes of estrogen receptor α (ERα)-negative breast cancer cells, and higher levels of MTA2 have been associated with increased risk of early recurrence.[18] Chen and colleagues also revealed that high MTA2 expression in pancreatic ductal adenocarcinoma serves as an independent biomarker for poor survival.[19] In hepatocellular carcinoma, overexpression of MTA2 was associated with larger tumor size and poor differentiation.[20] Our previous study reported that MTA2 protein was highly expressed in ESCC and was positively associated with poor prognosis of patients with ESCC.[21] However, the detailed mechanism of MTA2 in ESCC has not been revealed until now.

Epithelial-mesenchymal transition (EMT) is one of the vital characteristics of squamous carcinoma, through which epithelial cell-derived malignant tumor cells obtain metastatic abilities. During the EMT progress, many genes involved in cell adhesion, mesenchymal differentiation, cell migration, and invasion are transcriptionally altered.[22,23] The best studied transcriptional modulation during EMT is repression of the E-cadherin gene *CDH1*, which is one of the critical regulators of the epithelial phenotype.[24] Accumulating evidence has shown that E-cadherin is regulated by specific transcriptional repressors, including the Twist/Mi2/NuRD complex, in which both MTA2 and Twist are all important components.[25]

In this study, we examined the *MTA2* gene and protein expression in ESCC tissues and cells and analyzed the correlation of *MTA2* expression with clinicopathological parameters and survival rate of the patients with ESCC. Furthermore, gain- and loss-of-function experiments were used to demonstrate that downregulation of MTA2 impeded the proliferation and metastasis of ESCC cells in vitro and in vivo. Additionally, we found that EMT mediated by EIF4E and Twist is the critical downstream biomarker of MTA2 function. Therefore, our data provide novel and powerful evidence between MTA2 and ESCC progression and suggest a new therapeutic target for ESCC.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens and cell culture

Tissue specimens were collected immediately after surgical resection from patients with ESCC (n = 98) at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) from January 2013 to December 2013. None of the patients underwent chemotherapy and/or radiotherapy before surgery. Our study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (approval no. EC112), and written prior informed consent and approval were obtained from all patients.

The esophageal cancer cell lines Eca-109 and TE-1 were obtained from the Shanghai Institute for Biological Sciences. YES-2, KYSE-30, KYSE-410, and KYSE-510 cell lines were kindly provided by Dr. Masatoshi Tagawa (Chiba University, Chiba-ken, Japan). KYSE-150, KYSE-180, and KYSE-450 and normal esophageal squamous epithelial cell line NE1 were donated by Dr. Qimin Zhan's laboratory from the Cancer Hospital of Chinese Academy of Medical Sciences (Beijing, China). All cells were maintained in RPMI 1640 medium (Gibco) containing 10% FBS (Biological Industries), 50 μg/mL streptomycin, and 50 U/mL penicillin (Invitrogen) in an humidified atmosphere of 95% air/5% CO_2 at 37°C.

2.2 | qRT-PCR

Total RNA was extracted using the TRIzol reagent (Thermo Fisher) method. In accordance with the manufacturer's protocol, cDNA was synthesized from total RNA using the GoScript™ Reverse Transcription System (Promega). A SYBR Green PCR kit (Promega) was used in the amplification process with a 7500 Real-time PCR system. Primers used for amplification are listed in the Appendix S1. qRT-PCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control.

2.3 | Immunohistochemistry

Immunohistochemistry (IHC) was performed, and intensity of the positive staining was measured as described in our previous study.[21] The antibodies used in this study were MTA2 (Abcam), Ki-67 and CD31 (ZSBG-BIO), E-cadherin (CST), N-cadherin (CST), vimentin (CST), and EIF4E (CST).

2.4 | Knockdown and ectopic expression of MTA2 in ESCC cell lines

MTA2 and EIF4E small interfering RNA (siRNAs) were purchased from GenePharma. After sequencing, the MTA2 siRNA and the negative control sequence were inserted into the lentiviral vector hU6-MCS-Ubiquitin-EGFP-IRES, named shNC and shMTA2. MTA2 cDNA was inserted into the eukaryotic lentiviral expression vector pCDH-CMV-MCS-EF1-GFP-CD511B. The CD511B plasmid encoding full-length MTA2 cDNA sequence and empty vector were transiently transfected into KYSE30 and KYSE510 cell lines using Lipofectamine 2000 reagent (Thermo Fisher). The expression of MTA2 in KYSE30 and KYSE510 cell lines was silenced using lentivirus-mediated short hairpin RNA (shRNA) and siRNA. The efficiency of transfection was verified by western blotting.

2.5 | Cell proliferation, migration, and invasion assays

Cells were seeded into 96-well plates (2000 cells per well) and grown in complete medium for 24, 48, 72, or 96 h in an humidified atmosphere at 37°C with 5% CO_2 in air. For analysis, 20 μL of MTS substrate (Promega) was added to each well. After culturing for 2 h, the absorbance of the wells was measured on a microplate reader (BioTek) at 492 nm. For the colony formation assay, cells were seeded at a density of 1000 cells per well in 6-well plates. After culturing for 12 d in an incubator, cells were fixed with paraformaldehyde (Solarbio) and stained with crystal violet (Solarbio). The number of colonies was counted under an inverse microscope (Nikon). For the wound healing assay, 24 h after transfection, scratch wounds were made using 100 μL sterile pipette tips. To remove the disrupted cells, plates were washed with PBS twice, and photographs were taken at 0 and 36 h, respectively. For the transwell assay, 80 000 cells in 200 μL serum-free RPMI1640 were seeded into the upper chamber of transwell cell culture inserts (Corning Incorporated) with or without Matrigel (BD Biosciences). Complete medium was added to the bottom chamber. After incubation for 20 h, the membranes were fixed with paraformaldehyde and stained with crystal violet. Cells were observed under a microscope and counted in 5 fields.

2.6 | Western blotting

Cells or tumor tissues were lysed with RIPA (Solarbio) reagent containing a protease inhibitor. Next 60 μg of protein were fractionated on a 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore), which were then blocked with 5% skimmed milk for 1 h at room temperature. Then, the membranes were incubated overnight at 4°C with primary antibodies. Fluorescent secondary antibodies (Rockland) and an infrared imaging system (LI-COR) were used to visualize the protein bands. Protein levels were normalized to those of GAPDH (Abcam).

2.7 | Microarray for the detection of MTA2-associated signaling

Total RNA from human KYSE30 ESCC cells, in which MTA2 was stably knocked down, and control KYSE30 cells were isolated and quantified. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Expression profiles were determined using a RiboArray™ Custom Array (12 × 90K A10000-1-90) and with an Axon GenePix 4000B scanner. Quantile normalization and the subsequent data processing were performed using the robust multi-array average (RMA) method. The transcript profiling data were deposited in the NCBI Gene Expression Omnibus and are accessible through the GEO series accession number GSE112495.

2.8 | Coimmunoprecipitation

Cells were harvested 72 h after transfection, and nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher) following the manufacturer's protocol. A small amount of nuclear protein lysate was prepared for western blotting, and the remaining lysate was incubated overnight at 4°C with MTA2 antibody (Proteintech) and acetylated-lysine antibody (CST), and rabbit IgG antibody as a negative control. Next, the lysates were pre-cleared with prepared protein A-agarose beads (Thermo Fisher) for 2 h at 4°C with gentle agitation. After high-speed centrifugation to remove the supernatant, the beads were washed 3 times with immunoprecipitation washing buffer and then boiled for 5 min in 2× SDS loading buffer (Solarbio). After centrifugation, the supernatant was used for western blot analysis. Membranes were incubated with Twist (Abcam) and E-cadherin antibody.

2.9 | Chromatin immunoprecipitation PCR

Chromatin immunoprecipitation (ChIP) experiments were conducted using an EZ-Magna ChIP A/G Kit (Millipore) in accordance with the manufacturer's protocol. The DNA-bound proteins from KYSE30 cells were cross-linked in 1% formaldehyde for 10 min at room temperature and quenched in glycine. Rabbit anti-Twist or acetylated-lysine antibody or normal rabbit IgG were used for immunoprecipitation. PCR was performed to detect the binding to the promoter of the E-cadherin gene. The eluted materials from the first immunoprecipitation (IP) were used in the Re-ChIP and then incubated with an MTA2 antibody (Proteintech). The binding sites of Twist in the E-cadherin promoter region were predicted with Jaspar (http://jaspar.genereg.net/). The primers for the E-cadherin promoter were F, 5′-GCAGGTCCATAACCCACCTA-3′ and R, 5′-CATAGACGCGGTGACCCTCTA-3′.

2.10 | Immunofluorescence

KYSE30 cells were treated with si-MTA2 for 24 h, fixed with 4% paraformaldehyde (Solarbio) for 30 min, permeabilized with 0.1% Triton X-100 (Solarbio) for 15 min, and blocked with 5% BSA for 30 min. The cells were then incubated with antibodies, including MTA2, E-cadherin, N-cadherin, and vimentin (CST) for 2 h at room temperature, followed by further incubation at room temperature for 1 h with rabbit IgG (Alexa Fluor 546, green). Nuclear DNA was labeled in blue with DAPI (Beyotime). Images were captured by confocal microscopy on a Zeiss LSM Image Examiner (Carl Zeiss).

2.11 | Nude mouse xenograft

KYSE30 cells were used in this study to establish stable cell lines via virus transfection that constitutively overexpresses the MTA2 protein or MTA2 shRNA. Four-wk-old male athymic BALB/c mice (Charles River)

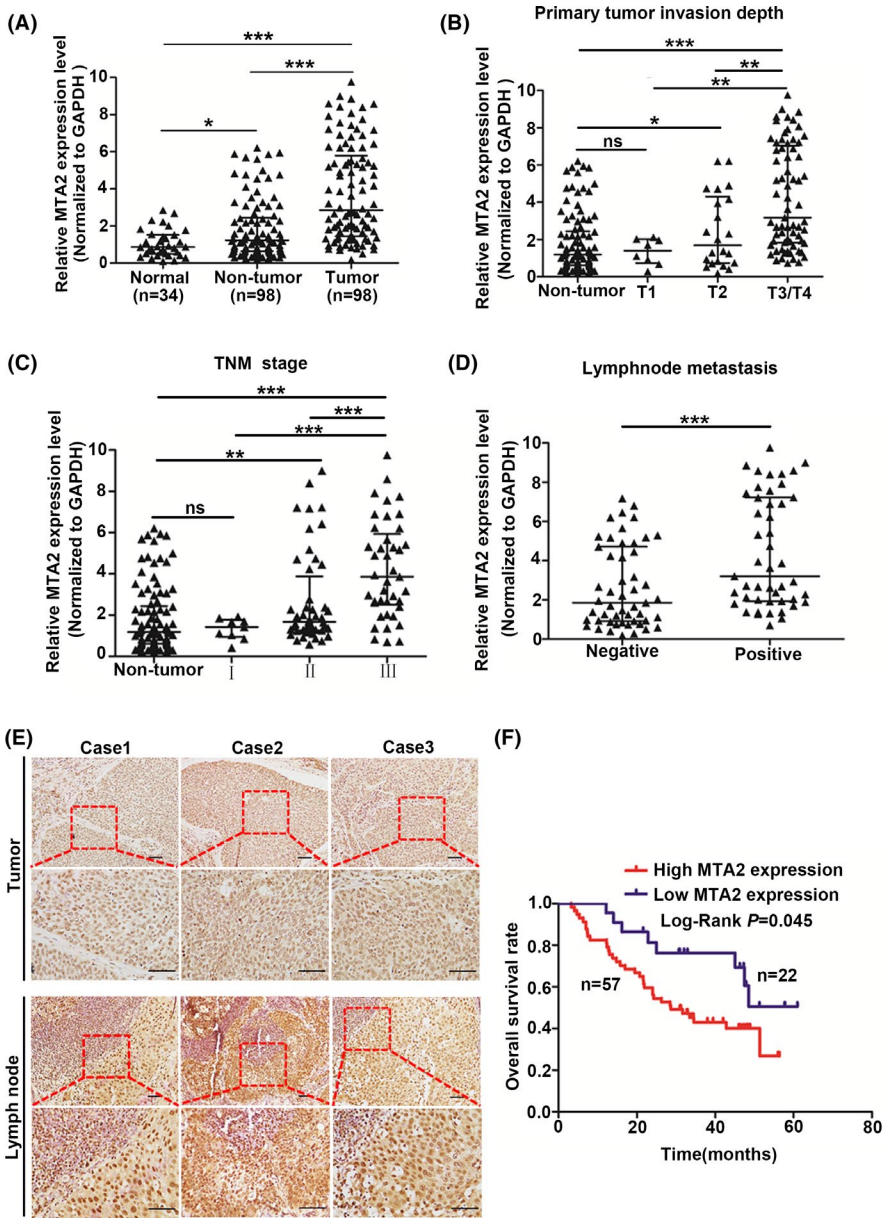


FIGURE 1 MTA2 overexpression in ESCC tissues correlates with ESCC aggressiveness. A, Expression of MTA2 was analyzed by qRT-PCR in ESCC tissues (n = 98), adjacent non-tumor tissues (n = 98), and normal esophageal epithelial tissues (n = 34). B-D, The correlation between MTA2 expression and primary tumor invasion depth (B), TNM stage (C), lymph node metastasis (D). E, Expression of MTA2 was analyzed by IHC in primary tumor tissues and the corresponding lymph node metastatic tumors tissues from 3 patients. F, Effect of the MTA2 expression level on clinical prognosis was analyzed by Kaplan-Meier survival analysis. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

were randomly divided into 2 groups. For the tumor formation assay, 1×10^6 KYSE30/shNC or KYSE30/shMTA2 and KYSE30/CD511B or KYSE30/MTA2 cells in 100 μL PBS were injected subcutaneously into the right flank of each mouse. Tumor nodules were measured every 7 d after their length exceeded 4 mm, and the volume was calculated using the following formula: $V = (\text{width}^2 \times \text{length})/2$. Xenografts were collected at the 6th wk for immunohistochemical staining and protein extraction.

For pulmonary metastasis models, 5×10^6 KYSE30/shNC or KYSE30/shMTA2 cells in 200 μL PBS were injected via the tail vein. All mice were sacrificed 2 mo later. Pulmonary metastases were examined in the gross specimens and with H&E staining of lung tissues.

2.12 | Statistical analysis

All observations were confirmed by at least 3 independent experiments. Statistical analyses were performed using SPSS version 13.0 software (IBM Corporation). We conducted independent sample *t* tests, Mann-Whitney *U* tests and one-way ANOVA (least-squares difference [LSD] post hoc) for the continuous variables, and chi-square tests for categorical variables. The correlation between MTA2 and EIF4E expression was analyzed with nonparametric Spearman test. Log-rank test using the Kaplan-Meier method was performed to provide survival analysis. A *P*-value < .05 was considered as statistically significant.

3 | RESULTS

3.1 | MTA2 overexpression in ESCC tissues correlates with ESCC aggressiveness

We firstly performed an IHC and western blotting analysis to investigate the MTA2 protein expression in normal esophageal squamous

epithelium, precancerous lesion tissues, and ESCC tissues. The result showed that expression of MTA2 was increased in ESCC, compared with precancerous lesion tissue or the corresponding non-tumor tissues (Figure S1a,b), suggesting that MTA2 was increasing following the malignancy of cells.

To illustrate the mechanism of MTA2 expression, we next investigated expression levels of the *MTA2* gene in ESCC malignancy. *MTA2* mRNA expression in 98 human ESCC tissues was performed using reverse transcription and quantitative PCR (qRT-PCR). *MTA2* was highly expressed in ESCC tissues compared with that in the corresponding non-tumor tissues from the same donor or that in normal esophageal epithelial tissue samples (Figures 1A and S1c). This result showed that the *MTA2* gene expression increased before its transcription. Further analysis revealed that the level of the *MTA2* gene was positively correlated with primary tumor invasion depth (Figure 1B), advanced TNM stage (Figure 1C), and lymph node metastasis (Figure 1D,E). Conversely, we divided the samples into an *MTA2* low-expression group and an *MTA2* high-expression group in accordance with median *MTA2* expression in the ESCC tissues to detect the correlation of *MTA2* with clinical pathological features of patients with ESCC. As expected, *MTA2* expression levels was positively correlated with primary tumor invasion depth, lymph node metastasis, distant metastasis, and TNM stage, although it did not correlate with tumor differentiation grade and tumor size (Table. 1).

Additionally, predictive analysis using 79 ESCC tissues with complete follow-up information revealed that high *MTA2* expression in ESCC tissues was associated with reduced overall survival (Figure 1F), supplementing GEPIA data (http://gepia.cancer-pku.cn/index.html), in which high *MTA2* expression was correlated with poor survival in mesothelioma (Figure S1d), adrenocortical carcinoma (Figure S1e), and hepatocellular liver carcinoma (Figure S1f), But it lacked survival data for ESCC. Taken together, we concluded that MTA2 overexpression was associated with ESCC malignancy and poor prognosis.

TABLE 1 Correlation between MTA2 expression and clinicopathological features in 98 patients with ESCC

Parameters	Total	Expression level of MTA2		*P*-value[a]
		Low	High	
Age (y)				
<60	41	19	22	.539
≥60	57	30	27	
Gender				
Male	76	36	40	.333
Female	22	13	9	
Differentiation				
Well	37	15	22	.145
Poor	61	34	27	
TNM				
T				
T1	14	11	3	.006**
T2	21	11	10	
T3	52	23	29	
T4	11	4	7	
N				
N0	57	34	23	.024*
N1	41	15	26	
M				
M0	93	49	44	.022*
M1	5	0	5	
Stage				
I	9	5	4	.001**
II	48	34	14	
III	36	10	26	
IV	5	0	5	
Lymphatic invasion				
Negative	51	31	20	.026*
Positive	47	18	29	
Tumor size (cm^3)				
≤5	16	8	8	.075
>5 and ≤10	21	15	6	
>10	61	26	35	

[a]Chi-squared test results.
*$P < .05$; **$P < .01$.

3.2 | MTA2 is required for ESCC cell proliferation, migration, and invasion in vitro

To clarify the function of MTA2 in ESCC cells, we first analyzed MTA2 expression in 8 ESCC cell lines and a normal esophageal epithelial cell line. Compared with the normal esophageal epithelial cell line, MTA2 expression was significantly higher in 8 ESCC cell lines (Figure S2a). MTA2 expression was knocked down in KYSE30 and KYSE510 cells through the transfection with siRNA or shRNA, and was overexpressed through the transfection with the pCDH-MTA2 plasmid (Figure S2b). As shown in Figure 2A, after transfection with siRNA to knockdown the expression of MTA2, the wound healing process was delayed. Moreover, the number of cells penetrating the membrane of the chambers in both the migration and invasion assays was significantly lower in the MTA2 knockdown group compared with that in the control group (Figure 2B,C). In contrast, exogenous overexpression of MTA2 facilitated wound closure of both the KYSE30 and KYSE510 cell lines (Figure 2D). Transwell migration and Matrigel invasion assays also implied that overexpression of MTA2 prominently enhanced the migratory and invasive capabilities of both ESCC cell lines compared with those of the controls (Figure 2E,F).

Moreover, we also found that compared with the controls, MTA2 depletion impeded KYSE30 and KYSE510 cell proliferation, based on an MTS assay (Figure S2c), while MTA2 overexpression promoted the viability of both cell lines (Figure S2d). Colony

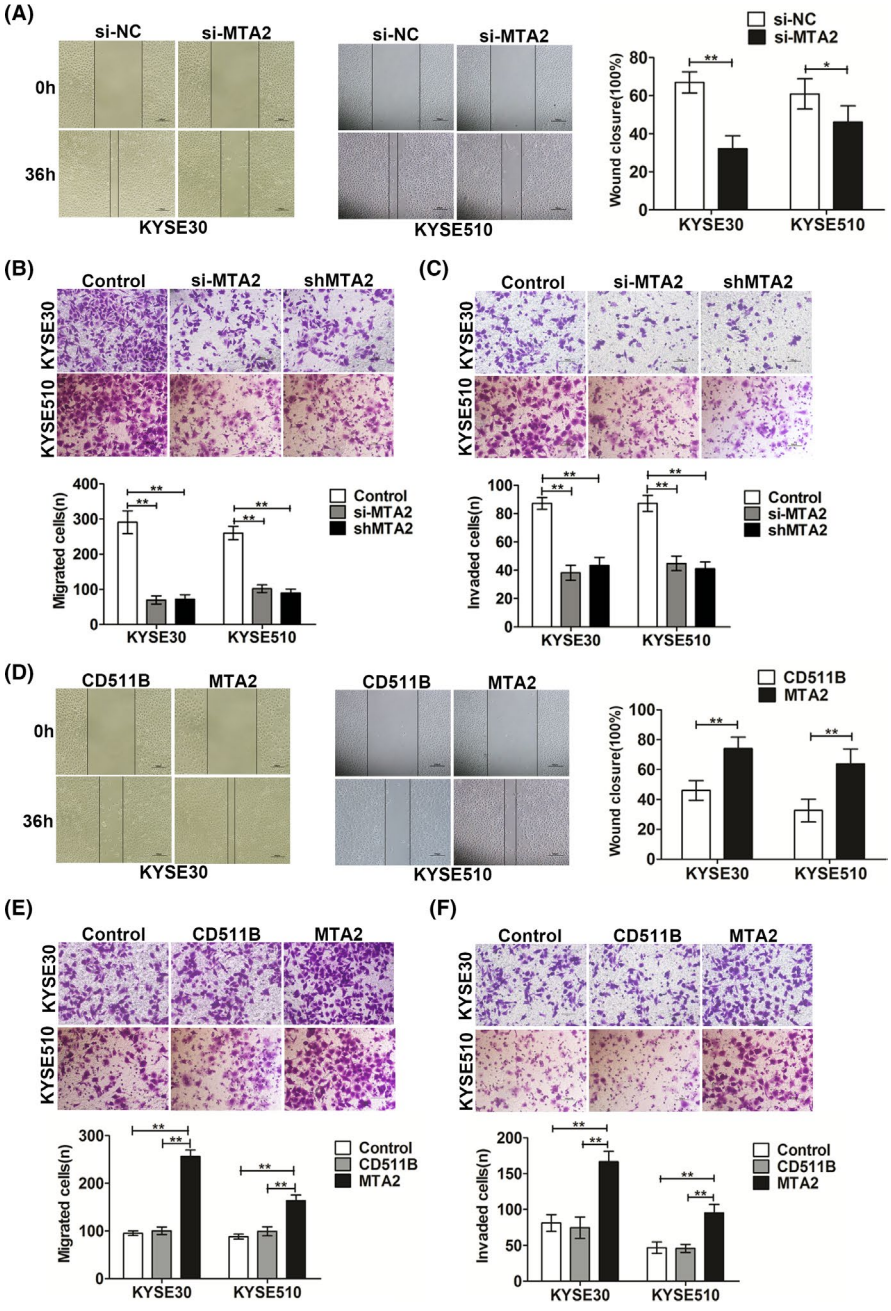


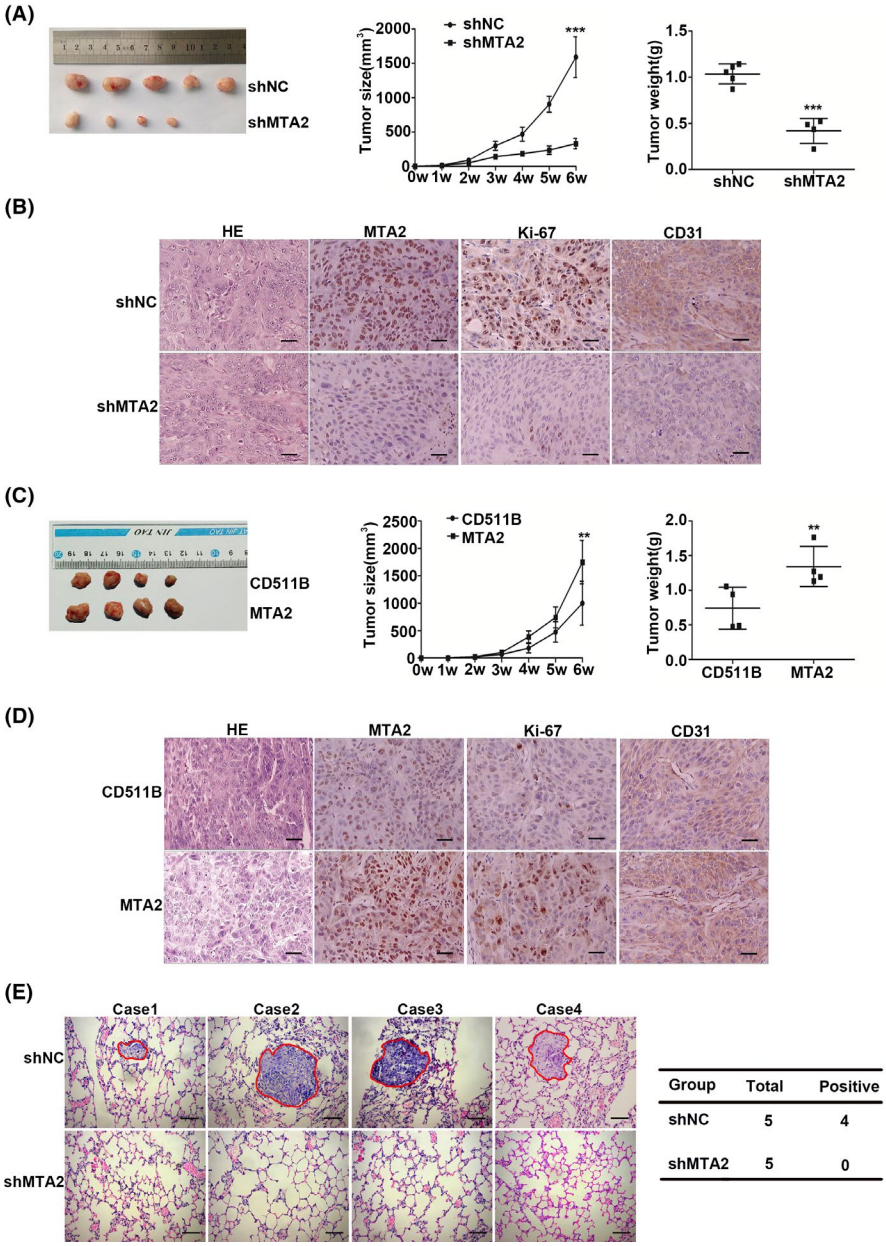
FIGURE 2 MTA2 is required for ESCC cell proliferation, migration and invasion in vitro. A, Wound healing assay were performed in KYSE30 and KYSE510 cells transfected with siRNA. B and C, Transwell and Matrigel assays were performed in control and MTA2-depleted KYSE30 and KYSE510 cells. D, Wound healing assays were performed in KYSE30 and KYSE510 cells transfected with different vectors. E and F, Transwell and Matrigel assays were performed in control and MTA2-overexpressed KYSE30 and KYSE510 cells. $^{*}P < .05$, $^{**}P < .01$

formation assay also demonstrated a similar function of MTA2 (Figure S2e).

Taken together, these data demonstrated that MTA2 played a carcinogenic role in ESCC by promoting viability, migration, and invasion of ESCC cell lines.

3.3 | MTA2 promotes the growth and metastasis of transplanted tumors in vivo

To further examine the oncogenic activity of MTA2 in tumor progression in vivo, we generated animal models by subcutaneously injecting KYSE30/shMTA2 or KYSE30/MTA2 cells into nude mice. Both the control and KYSE30/MTA2 groups formed tumors after injection, and the tumor formation rate in the KYSE30/shMTA2 group was 80% (4/5). Xenograft growth rate and average tumor weight were both lower in the MTA2 knockdown group compared with in the control group (Figure 3A). Moreover, expression levels of both the cell proliferation marker Ki-67 and the tumor angiogenesis marker CD31 were significantly reduced in MTA2 knockdown tumors (Figure 3B). In contrast, tumors formed by MTA2-overexpressed cells were markedly larger than those in the control group (Figure 3C). Correspondingly, MTA2 overexpression promoted the expression of Ki-67 and CD31 in tumors in the xenografts (Figure 3D). Conversely, the influence of MTA2 expression on ESCC cell metastasis was evaluated in vivo. KYSE30/shNC and KYSE30/shMTA2 cells were injected into the tail vein of nude mice. Histologic analysis showed that pulmonary metastatic



Group	Total	Positive
shNC	5	4
shMTA2	5	0

FIGURE 3 MTA2 promotes the growth and metastasis of transplanted tumors in vivo. A, C, KYSE30 cells stably downregulated (A) or overexpressed (C) MTA2 were subcutaneously injected into BALB/c nude mice. Representative images of mice from different treatment groups 6 wk after injections are shown. Tumor growth and tumor weight were analyzed. B and D, Representative immunohistochemical images showing the intensity of Ki-67 and CD31 expression (brown) in xenografted tumors from different groups. E, KYSE30 cells stably downregulated MTA2 were slowly injected into BALB/c nude mice via the tail vein. Representative images of HE staining of lung metastasis. $^{**}P < .01$, $^{***}P < .001$

foci were formed in the mouse lungs in the control group, while no pulmonary tumor nodules were found in mice in the MTA2 knockdown group (Figure 3E). These results strongly suggested that knockdown of MTA2 impeded the growth and metastasis of esophageal carcinoma cells in vivo.

3.4 | MTA2 is required for epithelial-mesenchymal transition in ESCC cells

EMT is involved in the different stages of tumor metastasis and promotes a malignant phenotype in tumors.[22] To further investigate the mechanism behind MTA2-mediated promotion of metastasis in ESCC cells, expression of molecules that are associated with transition from an epithelial to a mesenchymal phenotype was examined. The results indicated that compared with the controls, expression of N-cadherin, vimentin, MMP2, MMP9, and ZEB1 was suppressed, while expression of E-cadherin and ZO-1 was dramatically increased when the expression of MTA2 was silenced in both KYSE30 and KYSE510 cells (Figure 4A). Correspondingly, compared with the control cells, expression levels of N-cadherin, vimentin, MMP2, MMP9, and ZEB1 were significantly increased in MTA2-overexpressing cells, whereas those of E-cadherin and ZO-1 were decreased (Figure 4B). Furthermore, we used an IF staining assay to confirm this result. As shown in Figure 4C, compared with the control cells, MTA2 deficiency repressed the expression of N-cadherin and vimentin, while the MTA2 knockdown group exhibited increased E-cadherin staining at the cell membrane.

To verify this result in vivo, we also analyzed E-cadherin, N-cadherin, and vimentin expression in xenograft tumor tissues. The result was consistent with findings in vitro (Figure 4D). Collectively, all these data confirmed the hypothesis that the MTA2-mediated metastasis of ESCC was associated with promotion of EMT.

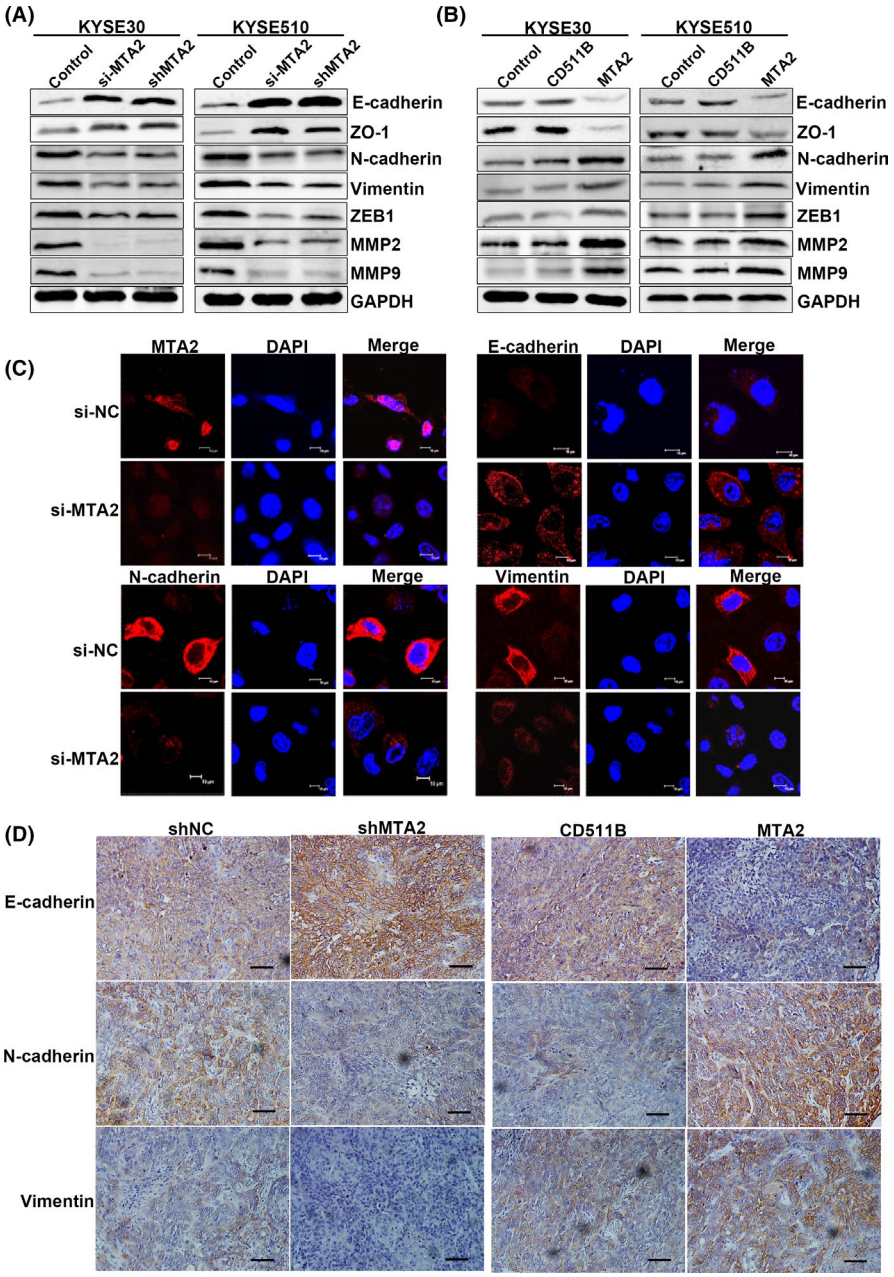


FIGURE 4 MTA2 is required for epithelial-mesenchymal transition in ESCC cells. A and B, Western blot analysis of the expression of EMT markers in MTA2-depleted or MTA2-overexpressed KYSE30 and KYSE510 cells. C, Immunofluorescence staining for EMT markers in KYSE30 cells. transfected with siRNAs. D, Expression of epithelial marker E-cadherin and the mesenchymal markers N-cadherin and vimentin in mice tumor tissues was examined by IHC

3.5 | EIF4E is a function target of MTA2

To further examine the molecular mechanism that underlay the protumorigenic role of MTA2, we conducted a gene expression microarray analysis to discover which molecules were altered coincident with MTA2 downregulation. We identified 93 genes with significant changes in expression (fold change > 2, $P < .05$, GSE112495) (Figure 5A). Further Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that some genes involved in TGF-β, acting cytoskeleton-related pathway and vascular endothelial growth factor (VEGF) signaling pathway were changed (Figure 5B), suggesting that MTA2 may contribute to EMT, cell motility, and angiogenesis progression. Next, mRNA levels of 6 representative genes (3 upregulated and 3 downregulated) were further confirmed by qRT-PCR, as shown in Figure 5C. Among these genes, we found that EIF4E expression was consistently and significantly decreased in ESCC cells with depletion of MTA2, compared with that in the controls. Then we detected the expression of *EIF4E* in ESCC tissues from 60 patients in our study, and the results showed that *EIF4E* expression was significantly increased in ESCC tissues compared with that in the matched para-cancerous tissues (Figure 5D). Further analysis revealed that *EIF4E* expression was positively correlated with primary tumor invasion depth (Figure 5E), lymph node metastasis (Figure 5F), and TNM stage (Figure 5G). Moreover, the Kaplan-Meier survival analysis demonstrated that higher *EIF4E* levels in patients were correlated with shorter overall survival compared with that in patients with low *EIF4E* levels (Figure 5H). Additionally, high *EIF4E* expression was correlated with low survival probability in patients with glioma (Figure S3a), patients with liver hepatocellular carcinoma (Figure S3b), and patients with lung adenocarcinoma (Figure S3c)

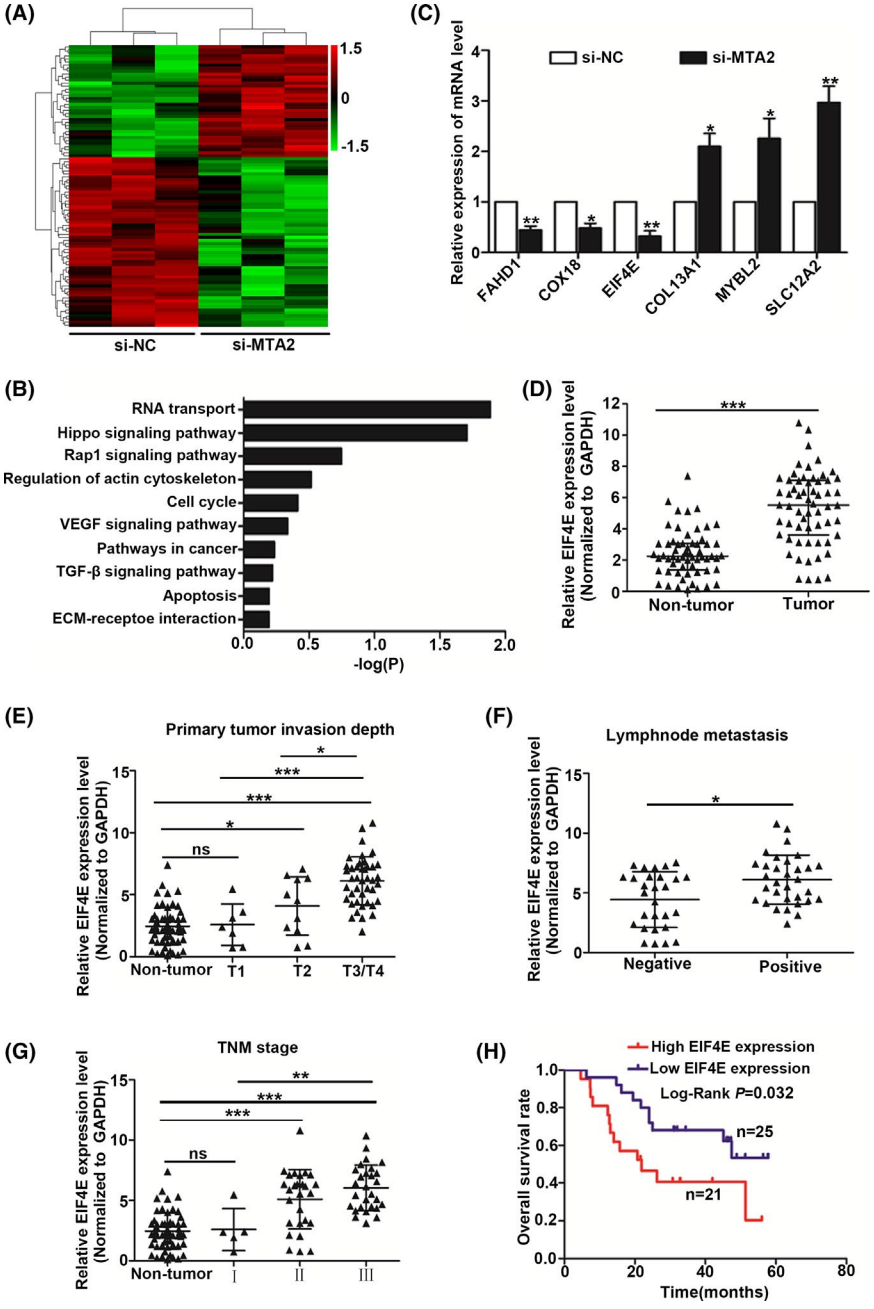


FIGURE 5 EIF4E is a function target of MTA2. A, Heatmap representation of the fold change in gene expression as determined by transcriptome analysis in MTA2-silenced cells and control cells (KYSE30). B, KEGG pathway analysis of MTA2-regulated genes. Pathways showing enrichment are presented. C, Expression of cancer-related genes after MTA2 knockdown was detected by qRT-PCR. D, Expression of EIF4E was analyzed by qRT-PCR in esophageal squamous cell carcinoma tissues (n = 60) and adjacent non-tumor tissues (n = 60). E-G, The correlation between EIF4E expression and primary tumor invasion depth (E), lymph node metastasis (F), and TNM stage (G). H, Effect of EIF4E expression level on clinical prognosis was analyzed by Kaplan-Meier survival analysis. *$P < .05$, **$P < .01$, ***$P < .001$

in GEPIA analysis, indicating a similar function with MTA2. These results suggested that EIF4E might play a vital role in the MTA2-mediated malignant phenotype of ESCC.

3.6 | MTA2-mediated promotion of malignancy in ESCC is mediated by EIF4E

Next, we will evaluate the detailed relationship of EIF4E with MTA2 function. Firstly, knockdown of MTA2 decreased the expression of EIF4E, while overexpression of MTA2 exerts contrast function in level of EIF4E (Figure 6A). Consistently, the immunohistochemistry results showed that the expression of EIF4E in transplanted tumors in the MTA2 knockdown group was decreased, while the expression of EIF4E in the MTA2 overexpression group was reversed (Figure 6B). Furthermore, a positive correlation between the expression of *EIF4E* and *MTA2* was found in 60 human ESCC tissues (Figure 6C), which was consistent with the data from the GEPIA (Figure S3d). These results demonstrated that *EIF4E* was regulated by *MTA2* in transcription and protein levels. Next, we investigated the effect of EIF4E on the migration and invasion of ESCC cells. First, we knocked down or overexpressed EIF4E in ESCC cells (Figure S4a,b), and then transwell assays were performed. The downregulation of EIF4E with siRNAs inhibited both the migration and invasion capacities of the cell lines (Figure 6D), while the migration and invasion ability of both cell lines was dramatically increased after EIF4E overexpression (Figure 6E). We next conducted a rescue experiment by reintroducing EIF4E expression in stable MTA2-silenced KYSE30 cells. Accordingly, the

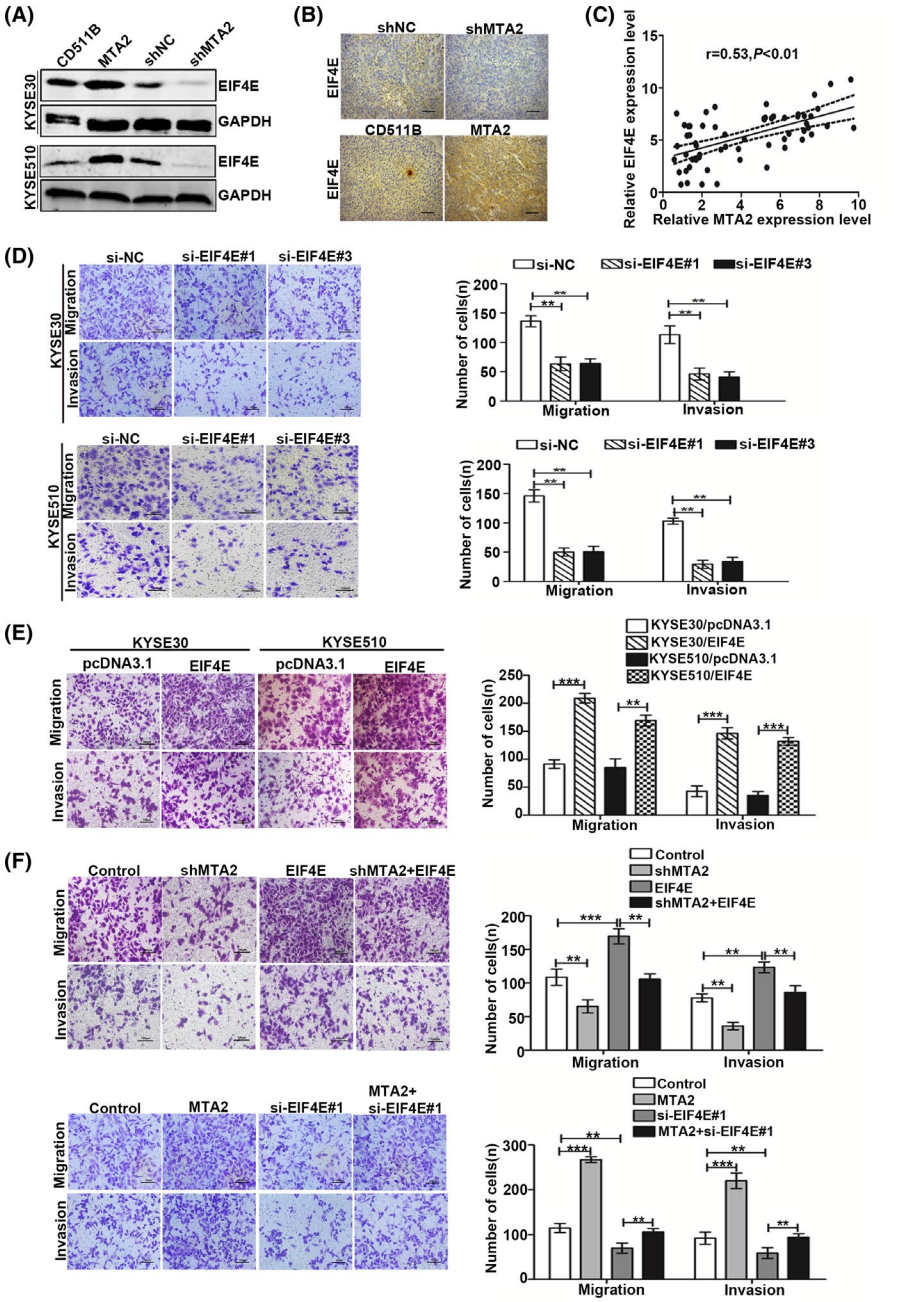


FIGURE 6 MTA2-mediated promotion of malignancy in ESCC is mediated by EIF4E. A, Western blot assay was performed to examine the expression of EIF4E after MTA2 knockdown or overexpressed in KYSE30 and KYSE510 cells. B, Expression of EIF4E in mice tumor tissues from different treat groups was examined by IHC. C, Correlation between MTA2 and EIF4E expression in ESCC tissues was analyzed by Spearman test. D, Transwell assays were performed in control and EIF4E-knockdown KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in 5 different fields. E, Transwell assays were performed in control and EIF4E-overexpressed KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in 5 different fields. F, Rescue experiments with transwell assays were performed in KYSE30 cells co-transfected with shMTA2 and EIF4E or MTA2 and si-EIF4E#1. The numbers of cells that migrated or invaded were counted in 5 different fields. $^{**}P < .01$, $^{***}P < .001$

inhibition of the migration and invasion that was induced by MTA2 knockdown was abrogated as a result of the forced expression of EIF4E. However, knockdown of EIF4E in MTA2 overexpression cells could partially inhibit the increase in migration or invasion caused by MTA2 overexpression (Figure 6F). Therefore, our findings first revealed that EIF4E was a major downstream mediator of MTA2-induced metastatic activity in ESCC.

3.7 | MTA2 promotes epithelial-mesenchymal transition in ESCC via the regulation of EIF4E

To underline the important role of EIF4E in MTA2-mediated promotion of ESCC, we were interested in determining whether EIF4E was involved in the MTA2-mediated regulation of EMT in ESCC. Our results showed that expression of E-cadherin and ZO-1 evaluated by western blotting was significantly reduced, while expression levels of N-cadherin, vimentin, MMP2, and MMP9 were increased after EIF4E overexpression in both KYSE30 and KYSE510 cell lines (Figure 7A). However, knocking down EIF4E with siRNAs demonstrated the opposite result (Figure 7B), and was in agreement with the pro-metastatic role of EIF4E. Then, rescue experiments showed that ectopic overexpression of EIF4E reversed the decrease in N-cadherin and vimentin levels caused by MTA2 depletion, while the MTA2-induced increase in N-cadherin and vimentin expression was inhibited by EIF4E absence. Downregulation of MTA2 led to a promotion of E-cadherin and ZO-1 expression, but this promotion was greatly attenuated by EIF4E overexpression (Figure 7C,D). These

data indicated that the inducible expression of EIF4E at least in part mediated the promotion of EMT by MTA2 in ESCC.

3.8 | MTA2 and EIF4E-twist formed a positive feedback to repress *E-cadherin* expression

Twist, a basic helix-loop-helix (bHLH) transcription factor, which recognizes the canonical E-box (CANNTG) to regulate gene transcription, plays a critical role in tumor metastasis. MTA2 is the vital component of protein complex Twist/Mi2/NuRD, in which Twist is the most important transcription factor that regulates EMT, as previously known.[25] In our study, we also found that the forced expression of Twist prominently enhanced the migratory and invasive capabilities of KYSE30 and KYSE510 cells (Figure 8A). Furthermore, compared with those of the controls, cells with enhanced expression of Twist suppressed E-cadherin and ZO-1 levels significantly, whereas N-cadherin and vimentin exhibited the opposite trend (Figure 8B). Therefore, we were interested in determining whether Twist participated in the MTA2-mediated regulation of EMT in ESCC. First, coimmunoprecipitation (Co-IP) with the extraction from MTA2-overexpressing KYSE30 cells, followed by immunoblotting with Twist antibody, verified that Twist was co-precipitated with MTA2 (Figure 8C). Additionally, this interaction was confirmed with endogenously expressed proteins in KYSE30 cells (Figure 8D). To investigate whether E-cadherin is a transcriptional target of Twist, we analyzed the promoter of the E-cadherin gene using bioinformatics and found several putative Twist consensus binding sites (Figure 8E). Furthermore, ChIP assay with anti-Twist followed by RT-PCR and qPCR validated the occupancy of Twist at the *E-cadherin* promoter. Furthermore, the immunoprecipitated materials associated with the anti-Twist complex were subjected to Re-ChIP using an antibody against MTA2 and also showed that MTA2 was enriched at the promoter of the E-cadherin gene (Figure 8F). These results collectively showed that MTA2 was recruited by Twist to the E-cadherin promoter. As a part of the

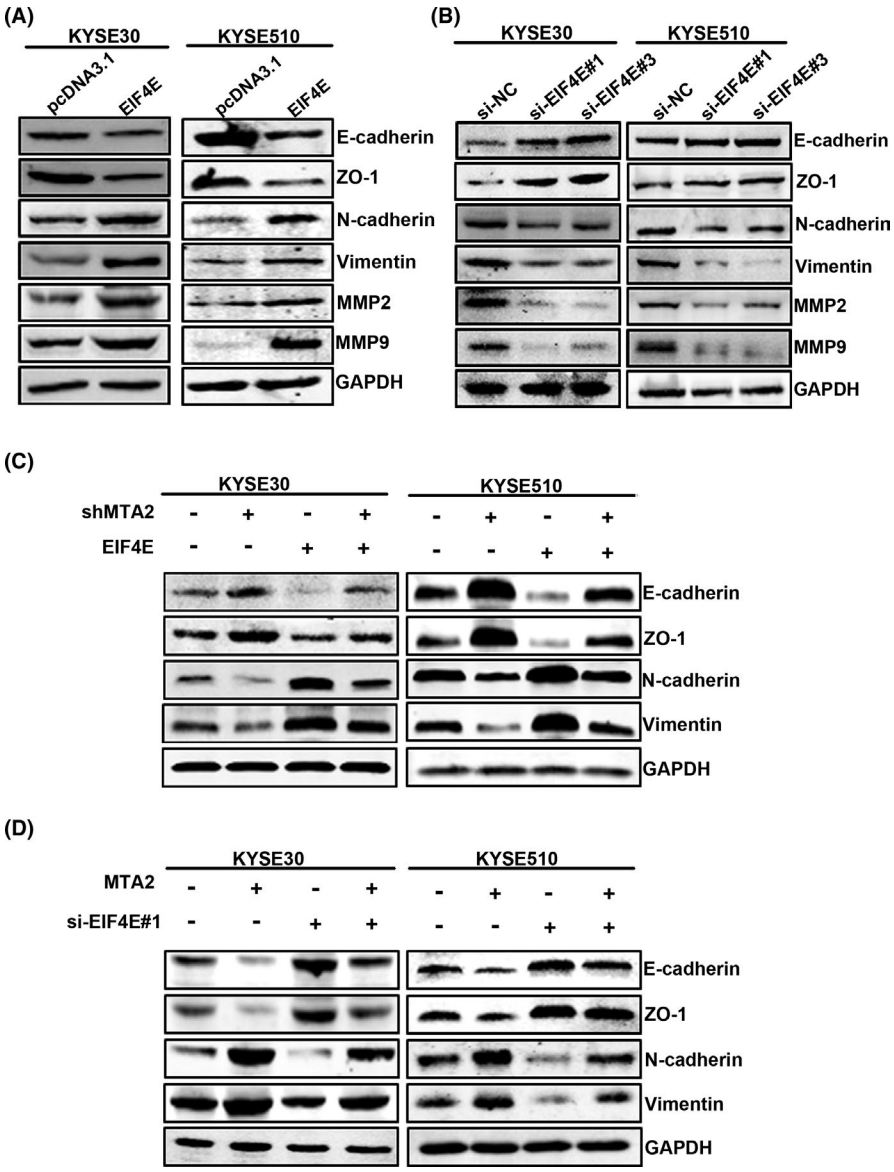
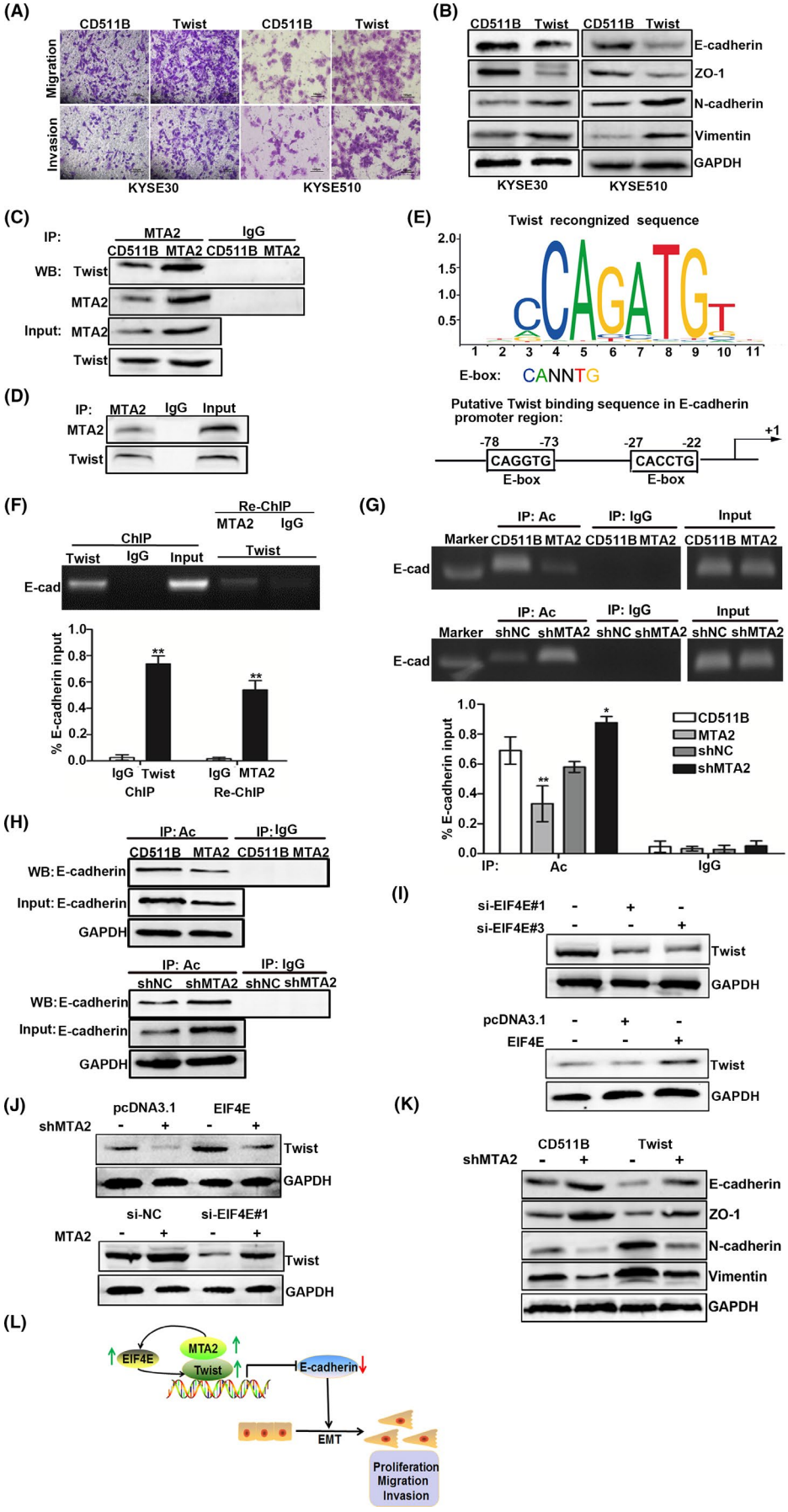


FIGURE 7 MTA2 promotes epithelial-mesenchymal transition in ESCC via regulation of EIF4E. A, Western blot analysis of the expression of EMT markers in EIF4E-overexpressed KYSE30 and KYSE510 cells. B, Western blot analysis of the expression of EMT markers in EIF4E-depleted KYSE30 and KYSE510 cells. C, Overexpression of EIF4E reversed the effect of MTA2 downregulation on EMT transformation. D, Knocking down EIF4E expression reversed the effect of MTA2 upregulation on EMT transformation


(A)
CD511B
Twist
CD511B
Twist
Migration
Invasion
KYSE30
KYSE510
(B)
CD511B Twist CD511B Twist
E-cadherin
ZO-1
N-cadherin
Vimentin
GAPDH
KYSE30
KYSE510
(C)
IP:
MTA2
IgG
CD511B MTA2 CD511B MTA2
WB: Twist
MTA2
Input: MTA2
Twist
(D)
IP: MTA2 IgG Input
MTA2
Twist
(E)
Twist recongnized sequence
E-box: CANNTG
Putative Twist binding sequence in E-cadherin promoter region:
-78 -73 -27 -22 +1
CAGGTG
CACCTG
E-box
E-box
(F)
Re-ChIP
MTA2 IgG
ChIP
Twist IgG Input Twist
E-cad
% E-cadherin input
IgG Twist
ChIP
IgG MTA2
Re-ChIP
(G)
IP: Ac
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WB: E-cadherin
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(I)
si-EIF4E#1
si-EIF4E#3
Twist
GAPDH
pcDNA3.1
EIF4E
Twist
GAPDH
(J)
pcDNA3.1
EIF4E
shMTA2
Twist
GAPDH
si-NC
si-EIF4E#1
MTA2
Twist
GAPDH
(K)
CD511B
Twist
shMTA2
E-cadherin
ZO-1
N-cadherin
Vimentin
GAPDH
(L)
EIF4E
MTA2
Twist
E-cadherin
EMT
Proliferation
Migration
Invasion

FIGURE 8 MTA2 and EIF4E-Twist formed a positive feedback to repress E-cadherin expression. A, Transwell assays were performed in control and Twist-overexpressed KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in 5 different fields. B, Western blot analysis of the expression of EMT markers in Twist-overexpressed KYSE30 and KYSE510 cells. C, Coimmunoprecipitation assays were performed in control and MTA2-overexpressed KYSE30 cells. IgG was used as a negative control. D, Endogenous MTA2 and Twist were coimmunoprecipitated from KYSE30 cells and the bound endogenous MTA2 and Twist were examined by immunoblotting. E, Twist recognized consensus site was identified in the promoter region of E-cadherin using a bioinformatics website (http://jaspar.genereg.net/). The number represents the nucleotide position relative to the transcription start site (+1). F, ChIP Re-ChIP assays were performed in KYSE30 cells to examine the recruitment of Twist and MTA2 onto E-cadherin promoter. G, ChIP assays were performed in KYSE30 cells to examine the promoter acetylation level of E-cadherin with or without MTA2. Ac, acetylated lysine. H, Coimmunoprecipitation assays were performed to detect the acetylation of E-cadherin in MTA2 overexpression or knockdown KYSE30 cells. Ac, acetylated lysine. I, Effect of EIF4E on the expression of Twist was analyzed by western blot. J, The influence of MTA2 and EIF4E on the expression of Twist was examined by western blot. K, Rescue experiment with western blot analysis on the influence of MTA2 and Twist on the expression of EMT markers in KYSE30 cells. L, Schematic model of MTA2 interaction with EIF4E and Twist involved in pro-metastatic function. $^{*}P < .05$, $^{**}P < .01$

NuRD complex, MTA2 can bind directly to HDAC2. This situation suggests that MTA2 may be capable of repressing E-cadherin expression through deacetylation. To verify this hypothesis, we performed the ChIP experiment again with acetylated-lysine antibody. The result suggested that, after MTA2 knockdown, the promoter acetylation level of E-cadherin was increased, while overexpression of MTA2 could decrease the level of acetylation (Figure 8G). To further test whether E-cadherin underwent regulation through acetylation modification by MTA2, we carried out a Co-IP experiment and acetylated lysine was immunoprecipitated and detected by western blotting using an anti-E-cadherin antibody. We found that the acetylation of E-cadherin was markedly increased upon MTA2 knockdown. However, overexpression of MTA2 produced the opposite result (Figure 8H). These results indicated that the Twist-dependent recruitment of MTA2 to the promoter repressed E-cadherin expression.

We confirmed that EIF4E can regulate EMT progression in ESCC cells. Next, we wanted to know whether expression of Twist was regulated by EIF4E. Interestingly, we also found that, compared with the controls, Twist expression was suppressed when EIF4E expression was silenced. In contrast, the expression levels of Twist were significantly increased in EIF4E-overexpressing cells (Figure 8I), which was consistent with a previous report.[26] Furthermore, we also detected that MTA2 could affect Twist expression through EIF4E (Figure 8J).

To further investigate the effect of the association between MTA2 and Twist on EMT in ESCC, we reintroduced Twist to stable MTA2-silenced KYSE30 cells. The rescue experiment showed that ectopic overexpression of Twist reversed the increase in E-cadherin and ZO-1 levels caused by MTA2 depletion, while expression of N-cadherin and vimentin was elevated after Twist reintroduction compared with that in the MTA2 knockdown group (Figure 8K). Collectively, these results indicated that MTA2 and EIF4E-Twist formed a positive feedback to regulate development of ESCC.

4 | DISCUSSION

In the present study, we found that the *MTA2* gene and protein levels were frequently overexpressed in ESCC and were correlated with the advanced TNM stage. Moreover, high MTA2 expression in ESCC tissues was associated with a poor survival prognosis. Functionally, MTA2 markedly promoted cell viability and metastasis via enhanced EMT progression.

Increasing evidence suggested that MTA2 plays an oncogenic role in the development of malignant carcinoma. Our present data demonstrated that MTA2 had lower basal expression in normal esophageal epithelial tissues (Figure S1a). This result further pointed out an important role of MTA2 in carcinogenesis of ESCC. Our functional data for MTA2 suggested that downregulation of MTA2 decreased the viability of ESCC cells in vitro and in vivo, demonstrating the requirement for MTA2 in ESCC metastasis, consistent with MTA2 function in many other cancers. Furthermore, results from our study also showed that CD31 was markedly decreased in MTA2-knockdown tumors in mice (Figure 3D), and that the VEGF signaling pathway was involved in the MTA2 affected gene dataset (Figure 5B), firstly revealing the promotion of angiogenesis by MTA2 in cancer, and further showing the pivotal role of MTA2 in progression of ESCC.

In the present study, expression analysis of a set of EMT markers was conducted to explore whether MTA2 was involved in EMT progression. Results showed that, compared with controls, MTA2 depletion suppressed the expression of mesenchymal markers, and upregulated the expression of epithelial markers. In MTA2-overexpressing cells, we observed the loss of epithelial markers, while expression of mesenchymal markers was strongly induced. These data indicated that the MTA2-mediated promotion of metastasis in ESCC is in an EMT-dependent manner. To further examine the molecular mechanism that underlay the pro-EMT role of MTA2 in ESCC, we performed transcriptome analysis in MTA2 knockdown cells. We found that the 93 differentially expressed genes were mostly involved in cancer-associated signaling pathways, such as the Rap1 and TGF-β signaling pathways, which are especially associated with metastasis and EMT process. Among these genes, we found that eukaryotic initiation factor 4E (EIF4E) expression was consistently and significantly decreased in ESCC cells with depletion of MTA2 compared with that in controls. EIF4E has been shown to be elevated in esophageal cancer[27] and plays a crucial role in EMT and tumor metastasis in breast cancer and NSCLC.[28,29] Although this protein is needed to

translate all cap-dependent mRNAs, elevated EIF4E expression selectively and preferentially enhances the translation of mRNAs linked to malignant transformation and metastasis.[30] According to Pettersson and colleagues, inhibition of EIF4E reduced breast cancer cell metastasis by suppressing TGFβ-induced EMT.[31] The phosphorylation of EIF4E promoted EMT and prostate tumor cell metastasis via translational control of SNAIL and MMP-3.[28] In our present study, the introduction of exogenous EIF4E promoted ESCC cell migration and invasion, as well as the EMT phenotype compared with those of the controls. Furthermore, the rescue experiment indicated that EIF4E was involved in MTA2-mediated regulation of EMT in ESCC. However, further research should be conducted to investigate the precise regulatory mechanism of MTA2 on EIF4E in ESCC.

Twist is overexpressed in various types of human cancers, including ESCC.[32,33] In the present study, our data also indicated that expression of Twist was suppressed when EIF4E was silenced, while EIF4E overexpression increased the Twist level. As shown in a previous report,[26] activation of EIF4E promotes metastatic progression via translation of several EMT-associated mRNAs such as Twist. EIF4E binds to the 5′-end 7-methylguanosine cap of mRNAs and recruits to the EIF4F complex (EIF4G , EIF4A and EIF4E), expanding mRNA secondary structure to expose the translation initiation codon and enable translation.[34] Some studies have shown that Twist plays an essential role in tumor metastasis by inducing EMT.[35,36] Although we know that silencing of E-cadherin expression is a characteristic of EMT[37,38] and Twist has been shown to silence E-cadherin transcription by binding to the E-box motifs in its promoter, the precise mechanisms were unknown. In addition to transcriptional inhibition, downregulation of E-cadherin during EMT can also be medicated at the epigenetic and post–translational levels, such as acetylation.[39-41] MTA2 was identified in the NuRD complex, which contains HDAC1/2 and deacetylates lysine. MTA2 was described to be recruited by Twist for inhibition of E-cadherin expression in HEK-293 cells.[25] The results of ChIP followed by PCR in our study demonstrated that MTA2 was recruited by Twist to bind to the E-cadherin promoter. Further studies indicated that MTA2 could reduce the acetylation level of E-cadherin, thus inhibiting expression of E-cadherin. The rescue experiment further confirmed that the promotion of EMT progression of ESCC by MTA2 was attributed to the interaction with Twist and the subsequent E-cadherin suppression.

Collectively, our findings illustrated that the aberrant expression of MTA2 promotes proliferation, invasion, and metastasis of human ESCC cells through regulation of EMT, which is mainly dependent on the EIF4E-Twist positive feedback loop (Figure 8L). These discoveries reinforce the hypothesis that MTA2 plays an essential and aggressive role in ESCC metastasis by promoting EMT progression, highlighting MTA2 as one of putative targets for treatment of ESCC metastasis.

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CONFLICT OF INTEREST

The authors have viewed the manuscript and declared that no conflict of interest exists.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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