

# Aberrant methylation of EYA4 promotes epithelial-mesenchymal transition in esophageal squamous cell carcinoma

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EYA4, one of the four members of the EYA gene family, is associated with several human cancers. However, its biological functions and molecular mechanisms in the progression of cancer, particularly in esophageal squamous cell carcinoma (ESCC), remain unknown. In the present study, we found that EYA4 was underexpressed and hypermethylated in most of the ESCC cell lines tested (85.7%, 6/7). Treatment with 5-aza-dC and/or trichostatin A (TSA) restored EYA4 expression in ESCC cell lines, which indicates that EYA4 expression was epigenetically regulated. Similarly, EYA4 was aberrantly hypermethylated in ESCC tissues (78%, 39/50) and downregulation of EYA4 occurred in approximately 65% of primary ESCC at protein level where it was associated significantly with TNM stage and lymph node metastases. Knockdown of EYA4 in KYSE30 and KYSE70 ESCC cells using small hairpin RNA increased migration and invasive motility in vitro. Conversely, the overexpression of EYA4 in KYSE180 and KYSE450 promoted an epithelial phenotype, which consisted of decreased migration and invasion abilities and a decrease in TGF- $\beta$ 1-induced epithelial-mesenchymal transition. Mechanistically, EYA4 overexpression reduced the phosphorylation of Akt and glycogen synthase kinase (GSK) 3 $\beta$ , which led to the inactivation of slug. In addition, we found that TGF- $\beta$ 1 decreased EYA4 expression in both a dose-dependent and a time-dependent manner in KYSE30 cells, accompanied by an increase in the expression of DNA methyltransferases, especially DNMT3A. In summary, EYA4 is frequently hypermethylated in ESCC and may function as a tumor suppressor gene in the development of ESCC.

## KEYWORDS

DNA methylation, epithelial-mesenchymal transition, esophageal squamous cell carcinoma, EYA4, TGF- $\beta$ 1

## 1 | INTRODUCTION

Esophageal cancer is among the most common digestive malignancies worldwide and is the fourth most malignant disease in China.<sup>1</sup> An estimated 477 900 newly diagnosed esophageal cancer cases and approximately 375 000 deaths occurred in China in 2015.<sup>2</sup> Esophageal

squamous cell carcinoma is the predominant histological type of esophageal cancer, as it accounts for approximately 90% of esophageal cancer cases in northern and central China. Despite the development of multimodal therapies, the 5-year overall survival rate after surgery remains at approximately 20%.<sup>3</sup> Thus, to explore the underlying molecular mechanisms and identify potential therapeutic targets is critical.

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The eyes absent (*Eya*) gene family, which was initially identified in *Drosophila*, is a key regulator of eye development.<sup>4</sup> Four EYA proteins (EYA1-4) are encoded in the mammalian genome and are characterized by a highly conserved C-terminal domain (ED) and a poorly conserved N-terminal domain (NTD). The ED domain is essential for mediating the interaction with other proteins, such as Six and Dach family proteins.<sup>5,6</sup> The NTD domain contains a proline-serine-threonine-rich region and functions as a transcriptional activator.<sup>7,8</sup>

Recently, accumulating evidence has shown that EYA functions in the mediation of DNA repair, cell apoptosis, angiogenesis and tumor growth.<sup>9-13</sup> EYA4 was found to be epigenetically silenced and was reported to function as a potential tumor suppressor gene in various types of common human cancers such as esophageal adenocarcinoma,<sup>14</sup> breast cancer,<sup>15</sup> lung cancer<sup>16</sup> as well as in oral dysplasia.<sup>17</sup> In contrast, EYA4 has been reported to play an oncogenic role in peripheral nerve sheath tumors and breast cancer.<sup>13,18</sup> Thus, the functions of EYA4 might be context-dependent and might differ depending on the cell type. However, the biological functions and molecular mechanisms by which EYA4 affects tumor progression, particularly that of esophageal squamous cell carcinoma (ESCC), have not yet been fully explored.

In this study, we primarily revealed the epigenetic changes and the expression of EYA4 in ESCC. Furthermore, we examined the tumor-suppressor function of EYA4 in ESCC cells in vitro and in vivo and explored the possible pathway through which EYA4 might function.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and tissue samples

Seven human ESCC cell lines were maintained in RPMI-1640 and Ham's F12 (1:1) (HyClone, Utah, USA) media with 10% FBS (Gibco, NY, USA). An immortalized normal esophageal epithelial cell line (Het-1A) was cultured in BEBM (Lonza, Walkersville, MD, USA) along with all the additives except GA-1000. All the tissue samples were collected from patients with primary ESCC undergoing surgery at the National Cancer Center/Cancer Hospital between April 2008 and June 2009. No patient in the study had received preoperative radiation or chemotherapy before surgery. The adjacent non-tumor tissues were >5 cm away from the edge region of tumor and confirmed by microscopy. Written informed consent was obtained from all patients, and the study was approved by the institutional review boards.

### 2.2 | Drug treatment

Cells were seeded in 10-cm culture dishes for 24 hours and then treated with 10  $\mu\text{mol/L}$  5'-aza-2'-deoxycytidine (5-aza-dC, Sigma-Aldrich, St Louis, MO, USA) for 72 hours followed by treatment with 0.25  $\mu\text{mol/L}$  TSA (Sigma-Aldrich, St Louis, MO, USA) 12 hours. For the 5-aza-dC or TSA treatment, the cells were exposed to 10  $\mu\text{mol/L}$  5-aza-dC for 72 hours or DMSO for 72 hours followed by

incubation with 0.25  $\mu\text{mol/L}$  TSA for 12 hours. Culture medium with drugs were refreshed every 24 hours. TGF- $\beta$ 1 treatment was performed at the indicated concentration (Peprotech, Rocky Hill, NJ, USA).

### 2.3 | DNA extraction, bisulfite modification, methylation-specific PCR and bisulfite sequencing

Genomic DNA was isolated using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany), 500 ng of genomic DNA was then subjected to bisulfite conversion using EZ DNA Methylation Kits (Zymo Research, Orange, CA). MSP was performed with specific primers (listed in Table S1) for either methylated or unmethylated DNA, as previously described.<sup>19</sup> Bisulfite sequencing was performed by amplifying bisulfite-treated genomic DNA of EYA4 promoter region by using primers containing no CpG site (see Table S1). The PCR products were cloned into pGM-T cloning vector (Tiagen, Beijing, China) and sequenced.

### 2.4 | RNA isolation and quantitative reverse transcription-PCR

Total RNA was isolated from cell lines and tissues using TRIzol Reagent (ThermoFisher, Rockford, USA) according to the manufacturer's protocol. Reverse transcription was performed with 1  $\mu\text{g}$  of total RNA using RevertAid First Strand cDNA Synthesis Kits (ThermoFisher, Rockford, USA). EYA4 cDNA were quantified using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used for real-time PCR are shown in Table S1.

### 2.5 | Western blotting

All cells were harvested in RIPA lysis buffer (Cwbiochem, Beijing, China) and Halt protease inhibitor cocktail (ThermoFisher, Rockford, USA) on ice. The primary antibodies used included the following: EYA4 (1:250 dilution; Santa Cruz, USA), E-cadherin, vimentin, slug, MMP-2, MMP-13, Akt, p-Akt Ser473, GSK-3 $\beta$ , p-GSK3 $\beta$  Ser9, Histone H2A.X, p-Histone H2A.X Ser139, DNMT1, DNMT3A (1:1000 dilution; Cell Signaling Technology, USA) and  $\beta$ -actin (1:5000 dilution; Abgent, Suzhou, China).

### 2.6 | Plasmid construction and transfection

The sequences of 2 human shRNA used to repress EYA4 expression are shown in Table S2. All the shRNA were cloned into the pLKO.1 vector. The EYA4 expression vector was purchased from OriGene (MD, USA) and was transfected into KYSE180 and KYSE450 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells transfected with empty vector or scrambled shRNA vector were used as controls. Puromycin (final concentration: 1  $\mu\text{g/mL}$ ) was used to select stable cell lines. All constructs were confirmed by sequencing.

### 2.7 | Wound healing and Transwell invasion assays

Wound healing assay was performed with serum-starved cells, and images were captured at 24 and 48 hours after cell scratching. Measurements were quantified by ImagePro Plus.

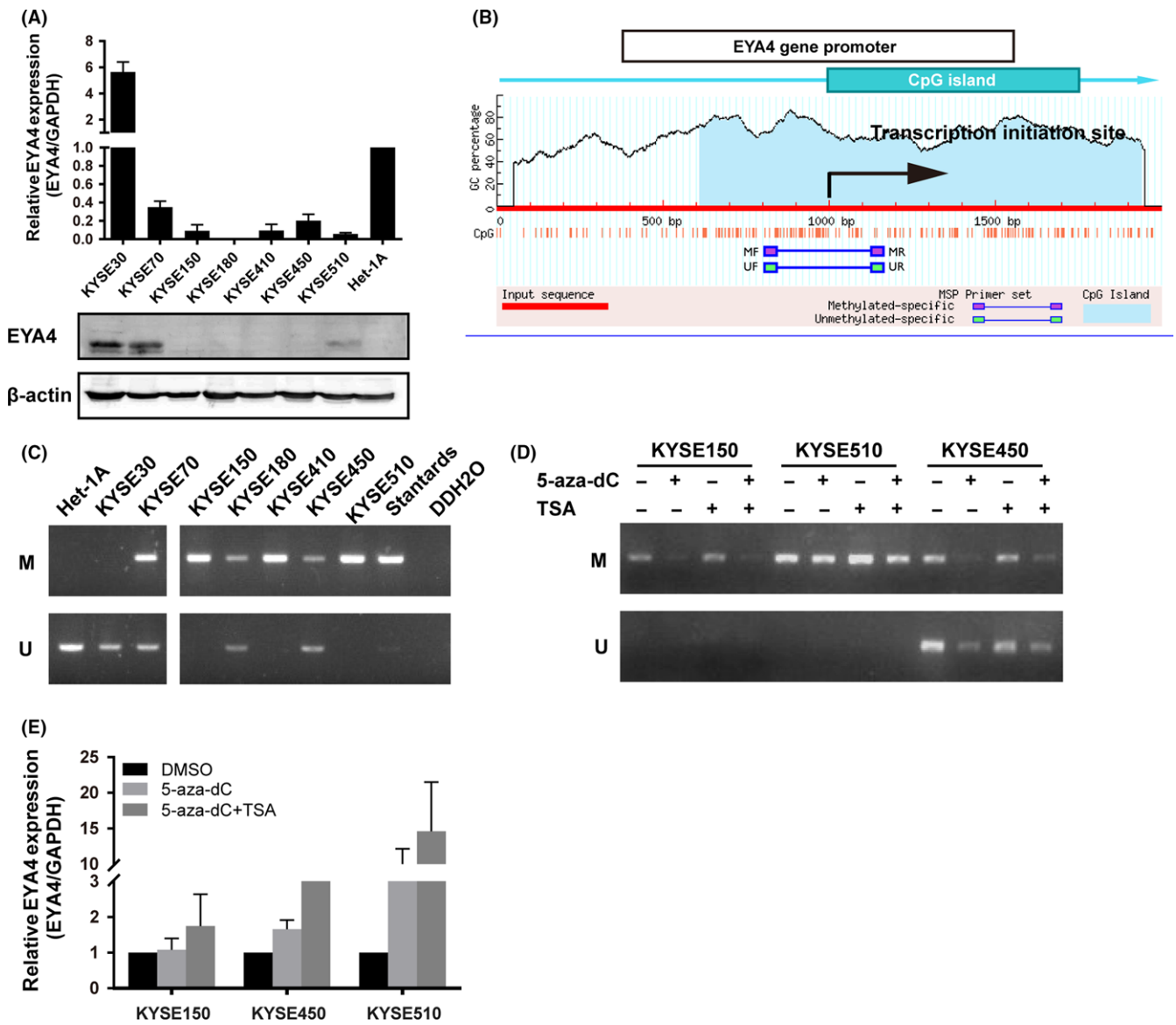
Serum-starved cells were suspended in 200  $\mu$ L of serum-free medium and loaded onto each 8.0- $\mu$ m pore membrane insert (Corning Costar, USA) in triplicate. After incubation for 24 or 48 hours, the cells that migrated to the lower surface were fixed in methanol and stained with Giemsa (Baso, Zhuhai, China). The number of cells in the membrane was counted in 9 different views under a microscope at 100 $\times$  magnification.

The invasion assay was performed in the same manner as the migration assay except Matrigel Transwells (Corning Costar, USA) were used.

For the drug treatment, cells were preincubated with TGF- $\beta$ 1 (5 ng/mL) or LY294002 (20  $\mu$ mol/L, Cell Signaling Technology, USA) for 24 hours in a CO<sub>2</sub> incubator. Then the Transwell assay was performed as described above.

### 2.8 | Immunofluorescence

Cells were cultured in a 96-well plate, fixed in formaldehyde, permeabilized with 0.25% Triton X-100, and incubated with an EYA4 antibody

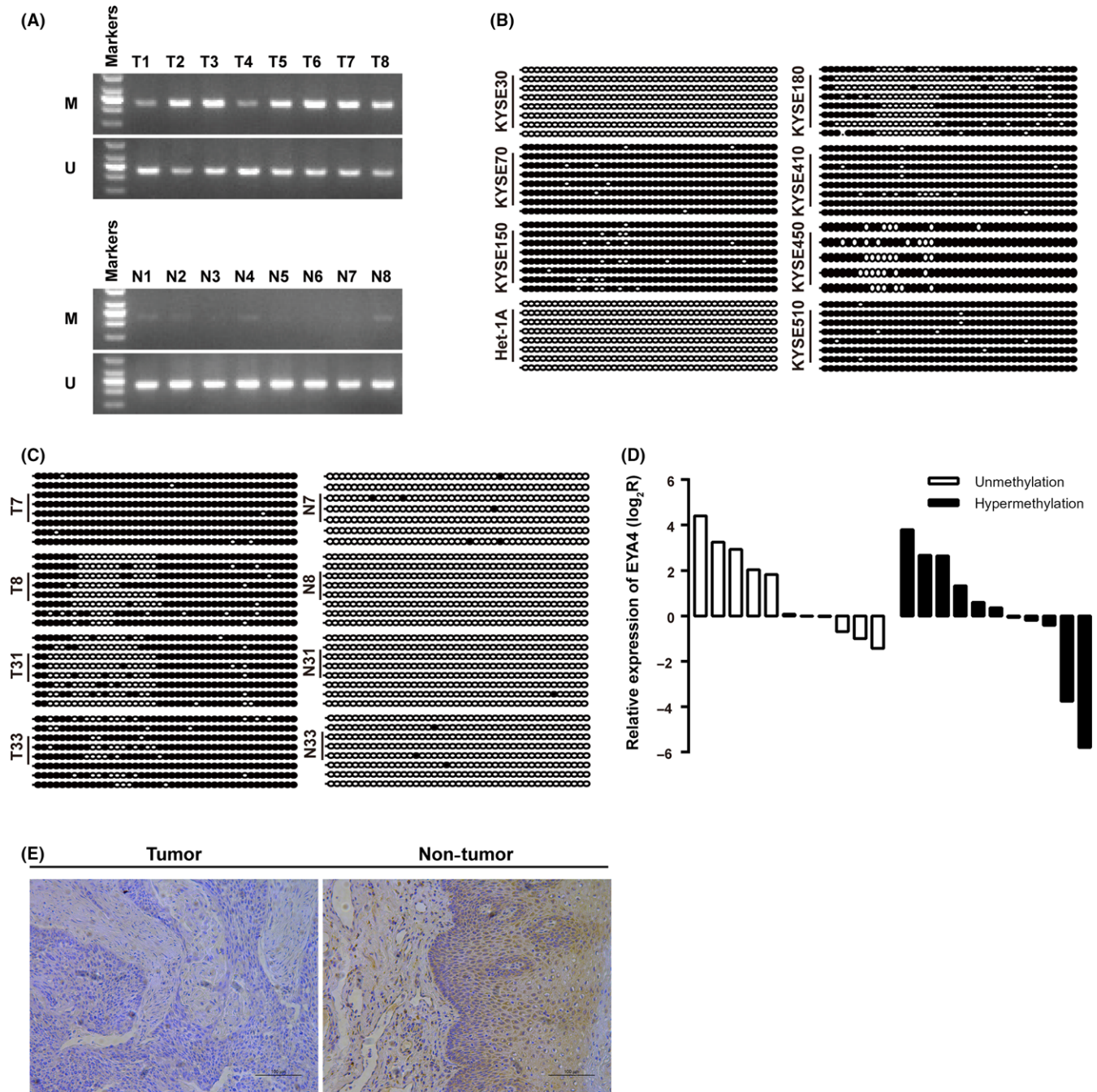


**FIGURE 1** Representative results of EYA4 expression and methylation in esophageal cancer cells. A, EYA4 mRNA expression detected by quantitative RT-PCR (qRT-PCR) and western blot in esophageal squamous cell carcinoma (ESCC) cell lines and a normal esophageal mucosa-derived cell line (Het-1A). B, Genomic map of the EYA4 promoter region. PCR regions for methylation-specific PCR (MSP) are shown. C, MSP results of EYA4 in human ESCC cell lines. M and U lanes indicate methylated and unmethylated alleles, respectively. Standards indicate methylated and unmethylated standard, respectively. D, ESCC cell lines were treated with 10  $\mu$ mol/L 5-aza-dC and/or trichostatin A (TSA) treatment and methylation status of EYA4 was detected by MSP. E, qRT-PCR analysis of EYA4 expression after 5-aza-dC and/or TSA treatment in ESCC cell lines

(1:50), and antibodies against either E-cadherin, vimentin or slug. Then, the cells were incubated with Texas Red-conjugated anti-rabbit and anti-goat IgG antibodies (1:500, Life Technologies, USA) and stained with 4,6-diamidino-2-phenylindole (Life Technologies). The images were viewed and captured using a microscope at a magnification of 200 $\times$ .

## 2.9 | In vivo tumorigenicity assay

First,  $2 \times 10^6$  cells were suspended in 200  $\mu$ L PBS and injected into the tail vein of nude mice. After 8 weeks, all mice were killed. Next, the lung tissues were dissected and fixed in



**FIGURE 2** Representative results of EYA4 expression and methylation in primary esophageal squamous cell carcinoma (ESCC). A, methylation-specific PCR (MSP) analyzed the EYA4 promoter methylation status in 50 esophageal squamous carcinoma tissues and adjacent normal tissues. M and U lanes indicate methylated and unmethylated alleles, respectively. Standards indicate methylated and unmethylated standard, respectively. Bisulfite-sequencing PCR demonstrated an aberrant hypermethylation status of the EYA4 promoter in ESCC cell lines (B) and ESCC specimens (C). Black and white circles represent methylated and unmethylated CpG, respectively. D, Correlation of EYA4 mRNA expression to EYA4 promoter methylation in ESCC specimens. E, The protein level of EYA4 in 30 primary ESCC and 10 non-tumor tissues as determined by immunohistochemistry. Representative images of EYA4 in tumor tissues and non-tumor tissues



**TABLE 1** Clinicopathological characteristics and EYA4 methylation status of 50 patients with esophageal squamous cell carcinoma

Characteristics	Total n = 50	EYA4 methylation status		P-value
		Methylated n = 40 (80%)	Unmethylated n = 10 (20%)	
Age				
≤59	24	19	5	.887
>60	26	21	5	
Gender				
Male	41	33	8	.854
Female	9	7	2	
Differentiation				
Poorly	14	9	5	.083
Moderately/ well	36	31	5	
Tumor stage				
I/II	5	5	0	.239
III/IV	45	35	10	
Metastasis				
Negative	19	15	4	.884
Positive	31	25	6	
Tumor size				
≥5 cm	11	10	1	.306
<5 cm	39	30	9	

Bouin's solution for at least 24 hours and were analyzed by H&E staining.

## 2.10 | Cell proliferation assay and determination of the IC50 by WST-8 assay

Cell proliferation was examined using a CCK8 assay (Dojindo, Kumamoto, Japan).

KYSE30 were cultured in 96-well (1000 cells/well) for 5 days. The absorbance at 450 nm was measured by a microplate reader.

KYSE30 were cultured in 96-well plates (1500 cells/well) overnight and then treated with serial dilutions of cisplatin (1-10 μmol/L). The cells were incubated for 72 hours, and CCK8 assays were performed as described above to determine the IC50. The IC50 was calculated by GraphPad Prism v5.0 (GraphPad Software). Experiments were repeated in triplicate.

## 2.11 | Cisplatin-induced apoptosis

Cells were treated with 10 μmol/L cisplatin for 24 hours and harvested. The cells were then digested with 0.25% trypsin without EDTA (Invitrogen, USA) and were washed twice in PBS. The double staining method with AnnexinV-FITC/propidium iodide (PI) (BD Bioscience, USA) was used to detect phosphatidylserine (PS) at the cell surface to calculate the number of apoptotic cells.

**TABLE 2** Clinicopathological correlation of EYA4 expression in ESCC

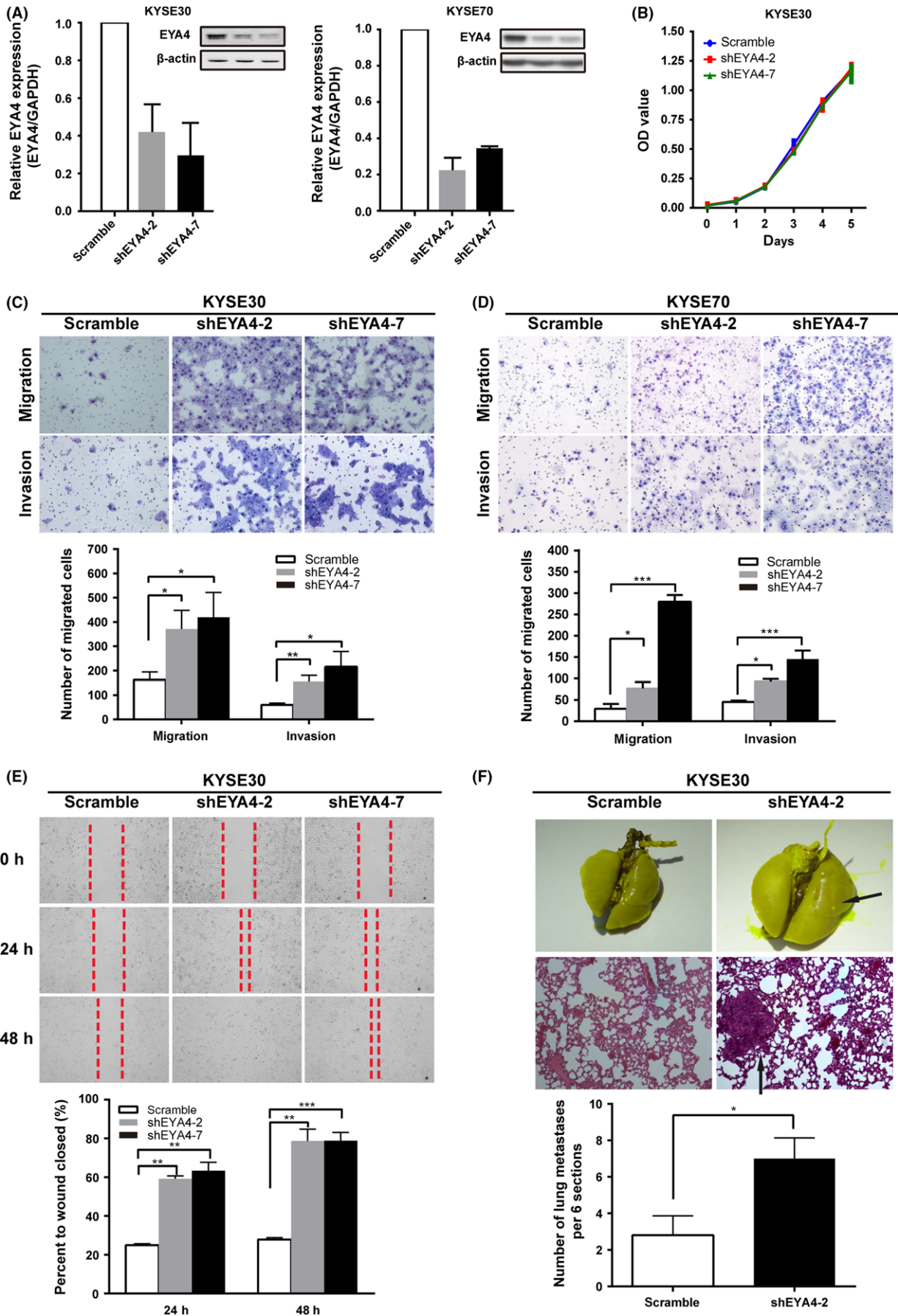
Characteristics	Total (n = 30)	EYA4 expression		P-value
		Low	High	
Age				
<60 y	17	10	7	.708
≥60 y	13	9	4	
Gender				
Female	6	3	3	.641
Male	24	16	8	
Smoking				
No	14	9	5	.919
Yes	16	10	6	
Drinking				
No	13	7	6	.346
Yes	17	12	5	
Family history				
No	23	14	9	1.000
Yes	7	5	2	
Differentiation				
High	5	4	1	.542
Middle	17	11	6	
Low	8	4	4	
T classification				
T1	4	1	3	.095
T2	5	3	2	
T3	16	9	7	
T4	5	5	0	
N classification				
N0	15	6	9	.021
N1	15	13	2	
TNM stage				
I	4	1	3	.007
II	14	6	8	
III	7	7	0	
IV	5	5	0	

## 2.12 | Immunohistochemistry

Briefly, tissue sections were de-waxed, rehydrated and incubated in retrieval buffer solution for antigen recovery. The DAB Kit (Zsgb-bio, Beijing, China) was used to visualize protein expression after staining with anti-EYA4 (1:50 dilution). The intensity of immunohistochemistry (IHC) staining in the tumor cells was analyzed independently by 2 pathologists.

## 2.13 | Statistical analysis

Data are presented as the means ± standard error of the mean (SEM). To evaluate significant differences between 2 matched pair



**FIGURE 3** Suppression of EYA4 promotes epithelial-mesenchymal transition (EMT) and invasion of human esophageal cancer cells. A, Quantitative RT-PCR and western blot analyses were used to detect the knockdown efficiency of EYA4 in KYSE30 and KYSE70 cells. B, The proliferation of KYSE30 cells treated with shRNA or scramble was measured by CCK8 assay. C,D, Transwell invasion assay showing that EYA4 inhibited cell migration and invasion. Representative images of invaded cells are shown in the upper panel and the results are summarized in the lower panel. The results are expressed as the mean  $\pm$  SEM of 3 independent experiments. E, Wound healing assay showing that EYA4 inhibited cell migration. Representative images were taken at 0, 24 and 48 h after scratching. The results are summarized in the lower panel. F, At 8 wk after tail vein injection of scramble or shEYA4-transfected KYSE30 cells, metastasis and/or tumor formation in lung was determined under a dissection microscope and by histological analysis using H&E staining. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , independent Student *t* test

groups or between 2 independent groups of samples, the paired *t* test and the Mann-Whitney *U* tests were used, respectively. Statistical analyses were performed using GraphPad Prism 5.0 or SPSS 20.0 (Chicago, IL, USA). Values for which  $P < .05$  were considered statistically significant.

### 3 | RESULTS

#### 3.1 | EYA4 is frequently methylated in human esophageal squamous carcinoma cell lines and esophageal squamous cell carcinoma tissues

EYA4 expression in 7 ESCC cell lines and a normal esophageal mucosa-derived cell line (Het-1A) were evaluated. Compared with that in Het-1A, EYA4 expression was decreased in most ESCC cell lines except KYSE30 cells (Figure 1A). To investigate the regulation of EYA4 in ESCC cells, the methylation status was detected by MSP in all ESCC and Het-1A cells (Figure 1B). We found methylation in all ESCC cell lines except KYSE30 cells and no methylation in Het-1A cells (Figure 1C). EYA4 mRNA expression were restored after treatment with 5-aza-dC and/or TSA in 3 ESCC cell lines including KYSE150, KYSE450 and KYSE510 (Figure 1D,E).

The methylation status of EYA4 in 50 primary ESCC tumor tissues and adjacent non-tumor tissues was analyzed by MSP. Methylation of EYA4 was detected in 39 of 50 (78%) primary ESCC and only 29 of 50 (58%) adjacent non-tumor tissues (Figure 2A). To further assess the methylation density, we performed clonal bisulfite sequencing of 43 CpG sites around the transcription initiation site (from  $-218$  bp to  $162$  bp) of the EYA4 promoter. EYA4 was downregulated in KYSE150 and KYSE510 with an average methylation density of approximately 93.5% and 97.4%, respectively. In contrast, methylation was rarely detected in Het-1A cells (Figure 2B). Similarly, we detected a significantly higher density of methylated CpG sites in primary ESCC tissues compared with adjacent non-tumor tissues (Figure 2C). Next, we integrated the methylation data with paired mRNA expression data from ESCC tissue samples and found that samples with EYA4 hypermethylation were more likely to have reduced EYA4 expression than samples without EYA4 methylation (Figure 2D). The association between EYA4 methylation and the clinicopathological features of ESCC was also analyzed. No significant association was found between the methylation of EYA4 and differentiation, tumor size or TNM stage (Table 1).

In addition, we detected the expression of EYA4 by IHC in 30 ESCC tumor tissues and 10 non-tumor tissues and found that 63%

(19/30) of tumor tissues showed low expression of EYA4 (negative staining), whereas only 30% (3/10) of non-tumor tissues had low expression of EYA4 (Figure 2E). This difference was marginally significant ( $P = .07$ , chi-square test). The results of IHC indicated that the expression of EYA4 was frequently reduced in ESCC, which is consistent with the results of methylation status of EYA4. Next, the relationship between EYA4 expression and clinicopathological characteristics were analyzed (Table 2), and we found that the expression of EYA4 was significantly associated with TNM stage and lymph node metastases, which suggests that the epigenetic silencing of EYA4 gene is correlated with the loss or reduction of EYA4 expression in ESCC and may play a critical role in epithelial-mesenchymal transition (EMT) during esophageal squamous cell carcinoma development and tumor aggressiveness.

#### 3.2 | EYA4 suppresses esophageal squamous cell carcinoma cell migration and invasion via the inhibition of epithelial-mesenchymal transition

Two shRNA were used to suppress endogenous EYA4 expression in KYSE30 and KYSE70 cells. EYA4 downregulation at the mRNA and protein levels was confirmed via quantitative RT-PCR (qRT-PCR) and western blotting, respectively (Figure 3A). The knockdown of EYA4 did not alter the cellular growth rate (Figure 3B). We next explored the effects of EYA4 on cell mobility. Transwell assays revealed that the knockdown of EYA4 in KYSE30 and KYSE70 cells led to a significant increase of cell migration and invasion ability (Figure 3C,D). Similarly, wound healing assays demonstrated that ESCC cells exhibited faster rates of wound healing when EYA4 was silenced (Figure 3E).

To further elucidate the inhibitory effects of EYA4 on tumor metastasis, experimental metastasis assays were performed. KYSE30-shEYA4 or control cells were intravenously injected into the lateral tail vein of nude mice. After 8 weeks, the mice were killed and the lungs were harvested. The number of metastatic nodules on the surface of the lungs was significantly higher in mice injected with KYSE30-shEYA4 cells than that injected with control cells (Figure 3F). H&E staining confirmed that the nodules on the surface of the lungs were metastatic tumors. Our data indicate that EYA4 is involved in the control of ESCC metastasis *in vivo*.

In contrast, KYSE180 and KYSE450 cells were stably transfected with the EYA4 construct, and ectopic expression of the EYA4 in these cells was determined (Figure 4A). Transwell assay showed that EYA4 overexpression in KYSE180 and KYSE450 cells was associated with decreased migratory ability (Figure 4B).

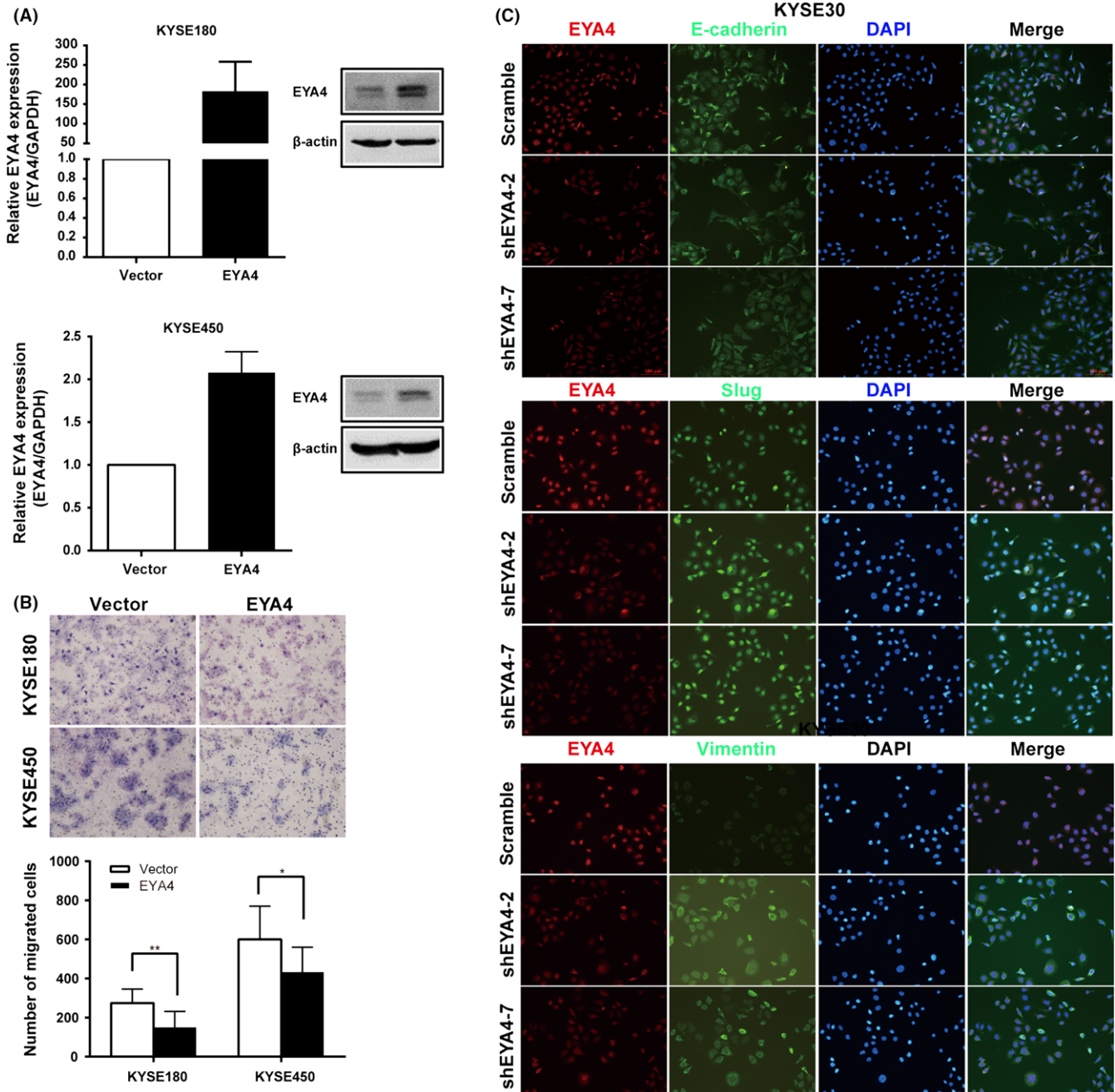


To explore the effect of EYA4 on EMT, IF was used to assess the epithelial and mesenchymal markers' expression. The results showed that E-cadherin expression was obviously decreased, while the expression of vimentin and slug was increased in the EYA4-knockdown group (Figure 4C). Furthermore, the staining of slug is predominantly nuclear in EYA4 knockdown cells. qRT-PCR and western blotting also demonstrated that the expression of vimentin, slug, MMP2 and MMP13 were elevated in EYA4-knockdown cells but were reduced in EYA4-overexpression cells (Figure 5A-C).

Taken together, these results suggest that EYA4 inhibits ESCC cell migration and invasion through the inhibition of EMT.

### 3.3 | EYA4 inhibits epithelial-mesenchymal transition via the PI3K/AKT/GSK3 $\beta$ /slug signaling pathway

To determine whether the Akt/GSK-3 $\beta$ /slug pathway is involved in EYA4-mediated EMT, we tested the expression of several proteins



**FIGURE 4** EYA4 inhibits the migration and epithelial-mesenchymal transition (EMT) of human esophageal cancer cells. A, Quantitative RT-PCR and western blot analyses were used to detect the ectopic expression efficiency of EYA4 in KYSE180 and KYSE450 cells. B, Decreased cell migration and invasion caused by ectopic expression of EYA4 was determined by Transwell assay (\* $P < .05$ , \*\* $P < .01$ , independent Student  $t$  test). C, Representative IF images showing increased expression of vimentin and slug and decreased expression of E-cadherin in shEYA4-transfected KYSE30 cells compared with shScramble-transfected cells. Nuclei were counterstained with DAPI



involved in this pathway. A significant increase in p-Akt, accompanied by an increase in p-GSK-3 $\beta$  and slug expression, was detected in EYA4-knockdown cells (Figure 5C). In contrast, we found a significant decrease in p-Akt and a slight decrease in slug expression in EYA4-overexpressing cells, while the change of p-GSK-3 $\beta$  expression was not obvious (Figure 5C).

To confirm whether Akt/GSK-3 $\beta$ /slug inactivation mediated the suppression of EYA4 on cell migration, we detected cell migration after LY294002 treatment in EYA4 knockdown cells and found that LY294002 significantly decreased KYSE30-shEYA4 cell migration (Figure 5D). Western blotting also showed that LY294002 effectively decreased the expression of p-Akt, p-GSK-3 $\beta$  and slug induced by the silencing of EYA4 in KYSE30 cells (Figure 5E).

### 3.4 | TGF- $\beta$ 1 induces an increase in DNMT and a decrease in EYA4 expression

To better characterize TGF- $\beta$ 1-induced EMT, the mRNA and protein expression of EYA4 and EMT-related genes were detected. We found that TGF- $\beta$ 1 decreased EYA4 expression in both a dose-dependent and a time-dependent manner in KYSE30 cells (Figure 6A). Furthermore, the expression of EYA4 and CDH1 was significantly decreased upon TGF- $\beta$ 1 treatment in KYSE30 cells, accompanied by an increase in the expression of vimentin and slug (Figure 6B). To elucidate the potential mechanisms that underlie the TGF- $\beta$ 1-induced changes in EYA4 expression and DNA methylation, we examined the expression of DNA methyltransferases including DNMT1 and DNMT3A, and found that TGF- $\beta$ 1 increased the expression of DNA methyltransferases, especially DNMT3A, in both a dose-dependent and a time-dependent manner (Figure 6B). TGF- $\beta$ 1, therefore, might decrease EYA4 expression through increasing the expression of DNA methyl-transferases.

### 3.5 | EYA4 inhibits TGF- $\beta$ 1-induced epithelial-mesenchymal transition

To investigate the role of EYA4 in TGF- $\beta$ 1-induced EMT in ESCC, KYSE180 and KYSE450 cells that overexpressed EYA4 were treated with TGF- $\beta$ 1. Overexpression of EYA4 significantly inhibited TGF- $\beta$ 1-induced EMT in KYSE180 and KYSE450 cells as indicated by migration and invasion assays (Figure 6C). Consistent with this, overexpression of EYA4 significantly reduced the expression of slug and upregulated the expression of E-cadherin after TGF- $\beta$ 1 treatment. Moreover, overexpression of EYA4 also downregulated the expression of MMP2 and MMP13 after TGF- $\beta$ 1 treatment in ESCC cells (Figure 6D,E).

### 3.6 | EYA4 plays a role in DNA damage repair in esophageal squamous cell carcinoma

Deregulation of EYA4 has previously been reported to have an impact on DNA damage repair, such as double-stranded breaks, in other types of cancer cells. We assessed the sensitivity of

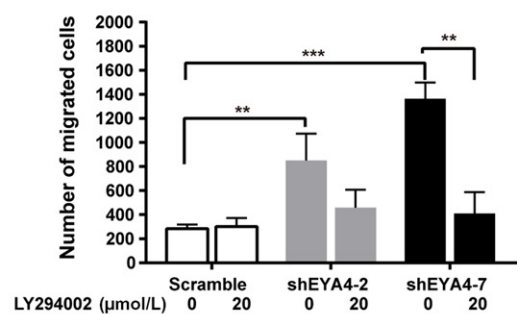
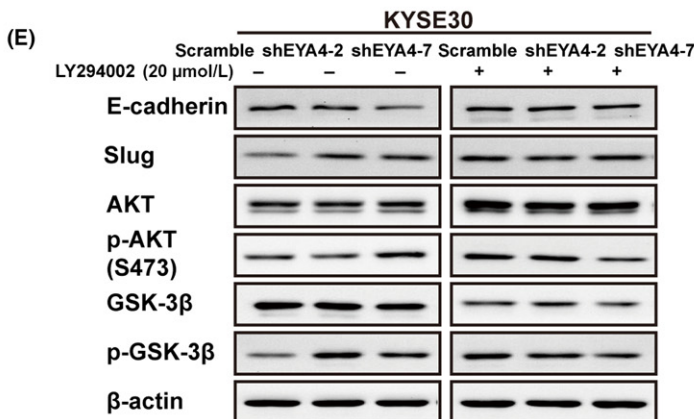
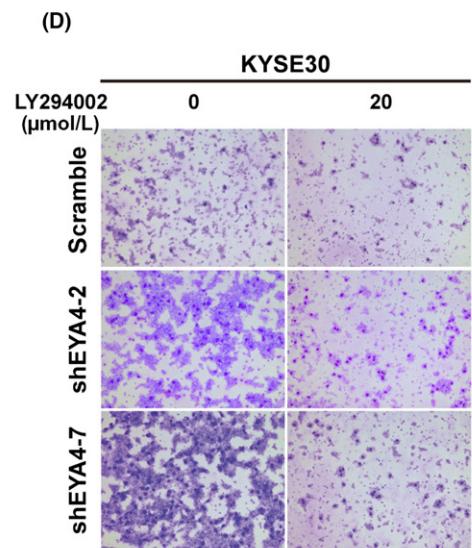
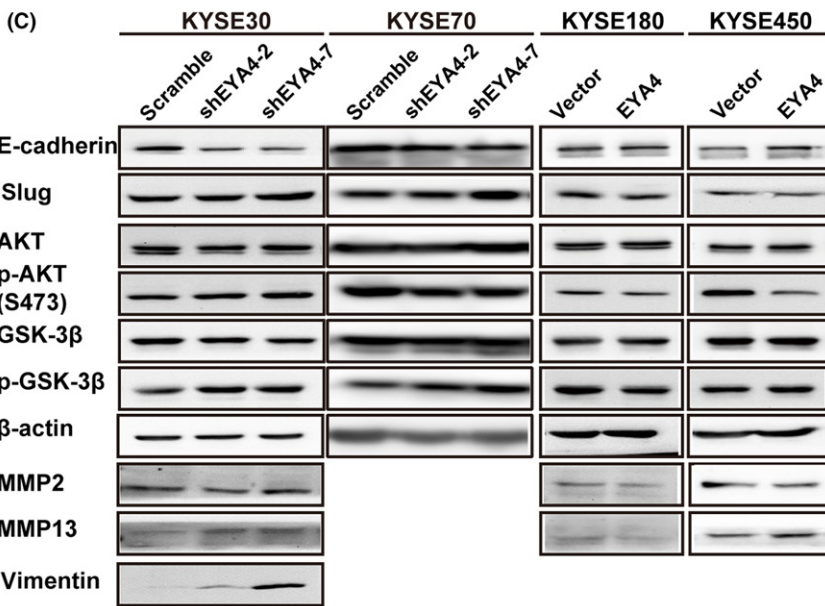
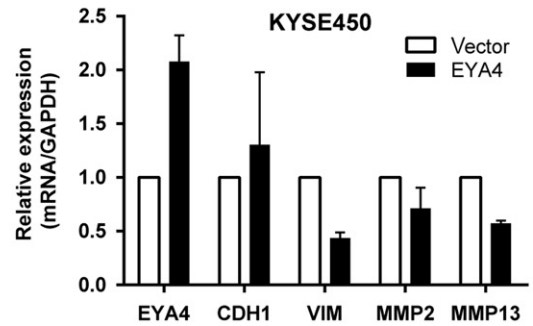
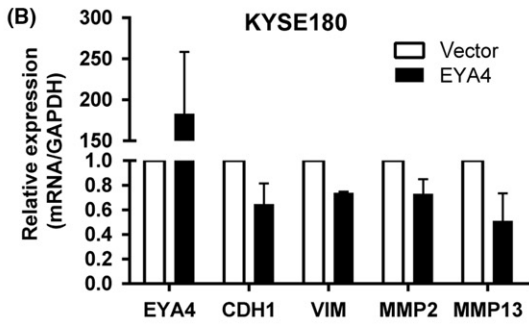
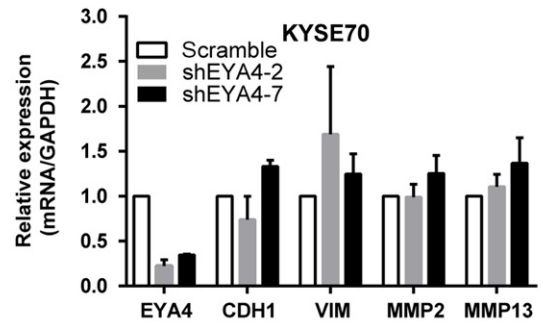
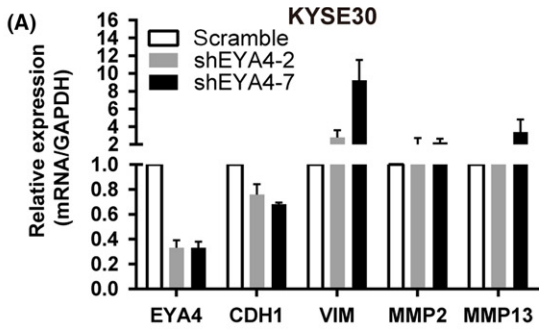
KYSE30-shEYA4 and control cells to cisplatin, and found that cisplatin inhibited cell viability in a concentration-dependent manner in both KYSE30-shEYA4 and control cells, but KYSE30-shEYA4 cells were more sensitive to cisplatin than the control cells (Figure 7A). Cisplatin-induced apoptosis assays showed that early apoptosis was significantly elevated in KYSE30-shEYA4 cells but was reduced in EYA4-overexpression cells (Figure 7B). Furthermore, we assessed the expression of  $\gamma$ H2AX and Ser-139 phosphorylation of H2AX in KYSE30-shEYA4 cells after cisplatin treatment, and found markedly higher expression of Ser-139-phosphorylated H2AX in KYSE30-shEYA4 cells in response to cisplatin (Figure 7C). These results suggest that EYA4 plays a role in DNA damage repair in ESCC.

## 4 | DISCUSSION

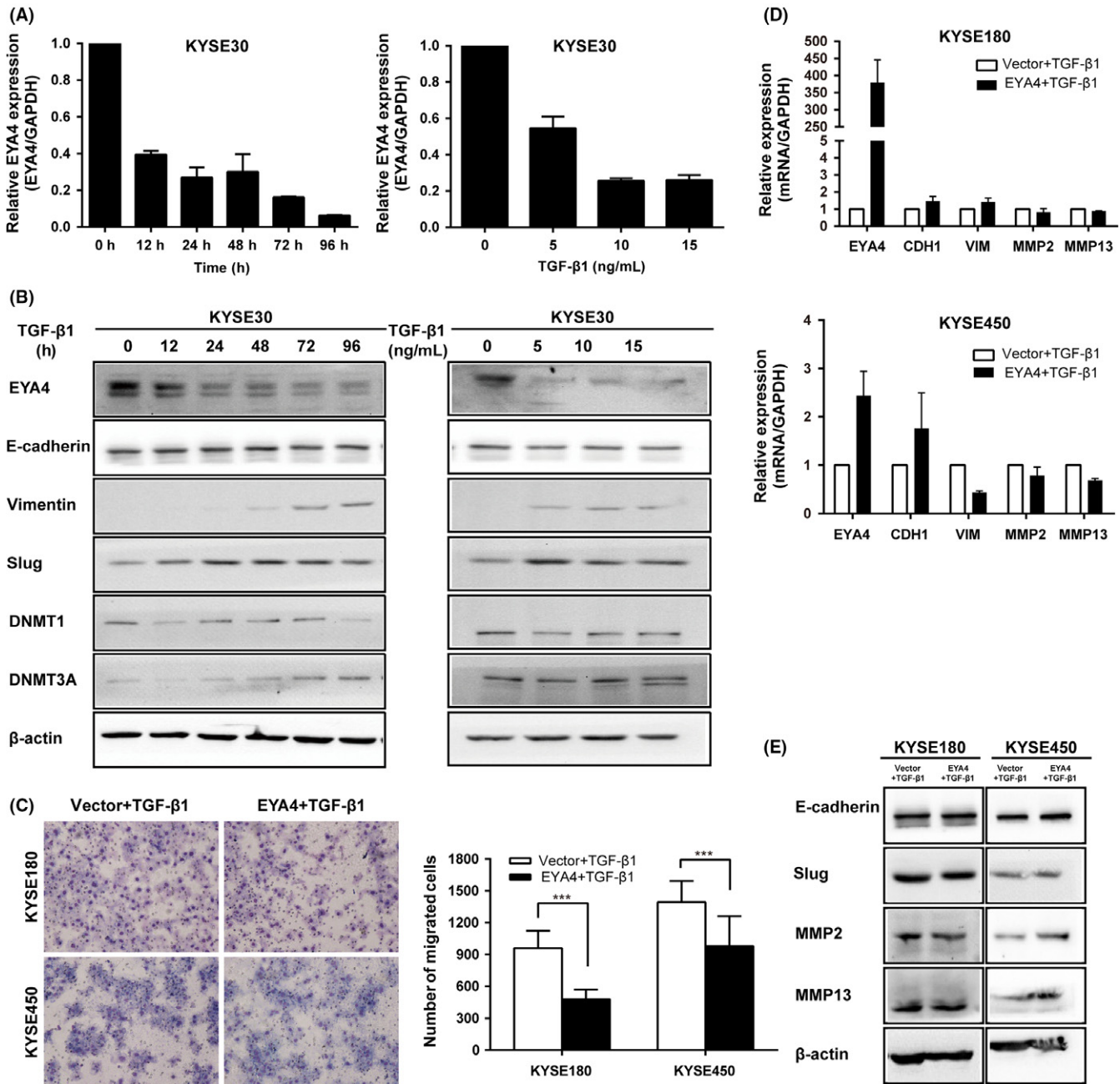
EYA4 is a key regulator of cell apoptosis and angiogenesis and has been shown to be associated with human cancers. Thus, we sought to study its role in ESCC. Numerous studies have indicated that EYA4 is aberrantly expressed in a variety of cancers, such as hepatocellular carcinoma, intrahepatic cholangio-carcinoma, colorectal cancer and acute lymphoblastic leukemia.<sup>16,19-22</sup> Consistent with these findings, in the present study, we found that EYA4 was aberrantly hypermethylated in both ESCC specimens and cell lines. Then, we assessed the expression of EYA4 in ESCC cell lines and found that reduced EYA4 expression in 6 of 7 ESCC cell lines was associated with methylation status. In vitro assays showed that EYA4 knockdown promoted tumor cell migration and invasiveness, whereas the overexpression of EYA4 resulted in inverse effects. Experimental metastasis assays confirmed that EYA4 knockdown promoted ESCC cell metastasis to the lung in vivo. These data imply that EYA4 functions as a tumor suppressor gene in ESCC.

The majority of previous studies have reported that EYA4 is frequently methylated and that it functions as a potential tumor suppressor gene in some types of cancer. In non-small-cell lung cancer, EYA4 was found to be frequently biallelically inactivated, and its low expression has been associated with poor survival of patients with sporadic lung cancers. Restoration of EYA4 expression inhibits the growth and colony formation ability of lung adenocarcinoma cells.<sup>16</sup> Using the Illumina Human Methylation 450K array, EYA4 was found to be methylated in more than 90% of CIMP-H colorectal cancers.<sup>23</sup> In addition, EYA4 functioned as a tumor suppressor gene in pancreatic ductal adenocarcinoma.<sup>24</sup> However, EYA4 has been reported to promote the adhesion and migration of malignant peripheral nerve sheath tumor cells and to play an oncogenic role in those tumors.<sup>18</sup> Thus, it seems likely that the role and function of EYA4 greatly depend on the context in which it is expressed.

Distant metastasis is one of the main reasons for treatment failure in ESCC. EMT is a critical event in the progression toward cancer metastasis and many signaling pathways are involved in the regulation of EMT in ESCC, such as Akt/GSK-3 $\beta$ /Snail, MAPK/ERK and TGF- $\beta$ 1/Smad.<sup>25-28</sup> Several studies have reported that the AKT/



**FIGURE 5** EYA4 inhibits the Akt/GSK-3 $\beta$ /Slug pathway to inhibit epithelial-mesenchymal transition (EMT). A, Relative expressions of E-cadherin, vimentin, slug, MMP2 and MMP13 were compared by quantitative RT-PCR between (A) EYA4-knockdown and control cells and (B) EYA4-overexpression and control cells. Western blots comparing EYA4-knockdown and EYA4-overexpression cells with their respective control cells are seen in relative expression of (C) Akt, p-Akt-S473, GSK-3 $\beta$ , p-GSK-3 $\beta$ .  $\beta$ -actin was used as a loading control. D, The representative figures and data of Transwell assay for shEYA4-transfected KYSE30 cells and shScramble-transfected cells after PI3K inhibitor LY294002 treatment (20  $\mu$ mol/L) (\*\* $P$  < .01, \*\*\* $P$  < .001, independent Student  $t$  test). E, Western blot analysis of the effects of LY294002 on the E-cadherin, slug, Akt, p-Akt-S473, GSK-3 $\beta$ , p-GSK-3 $\beta$  protein levels in EYA4-knockdown cells and control cells

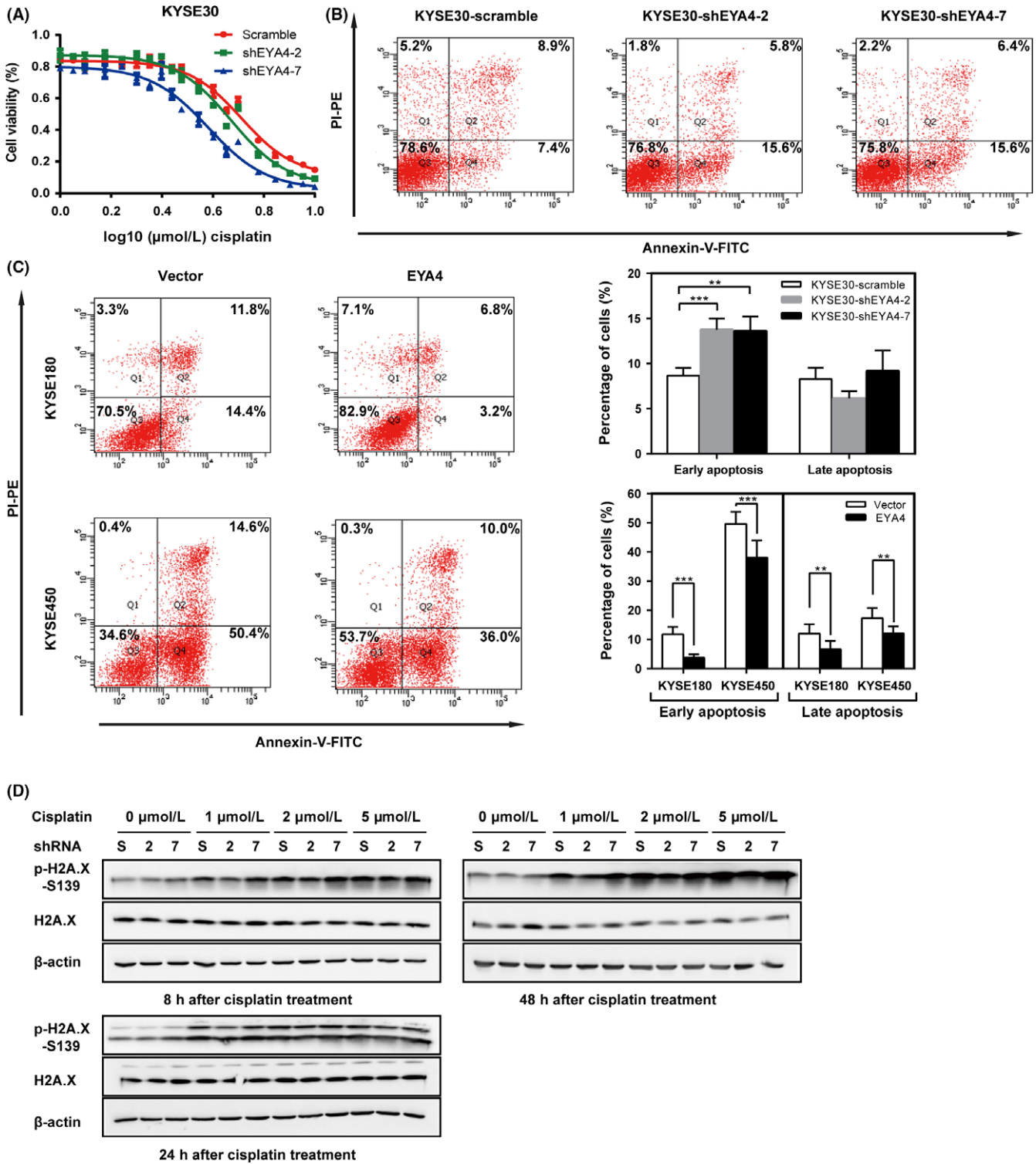


**FIGURE 6** Effect of TGF- $\beta$ 1 on the expression of EYA4 protein and cell migration in esophageal squamous cell carcinoma (ESCC) cell lines. A, Quantitative RT-PCR and (B) western blot analyses demonstrate that TGF- $\beta$ 1 induced a decreased EYA4 expression in a time and dose-dependent manner. KYSE30 cells were treated with TGF- $\beta$ 1 (5 ng/mL) for various periods of time as indicated or with various concentrations of TGF- $\beta$ 1 for 48 h. C, The representative figures and data of Transwell assay for EYA4-overexpression KYSE180 cells and KYSE450 cells with their respective control cells after TGF- $\beta$ 1 treatment (5 ng/mL) (\*\* $P$  < .001, independent Student  $t$  test). D,E, Quantitative RT-PCR and western blot analyses of the effects of TGF- $\beta$ 1 on the E-cadherin, slug, MMP2, MMP13 mRNA and protein expression in EYA4-overexpression KYSE180 and KYSE450 cells with their respective control cells



GSK-3 $\beta$  signaling pathway is involved in the progression of EMT in human malignancies, such as gastric cancer, hepatocellular carcinoma and prostate cancer.<sup>29-31</sup> In this study, we found that in EYA4-

knockdown cells, the expression of p-AKT, p-GSK-3 $\beta$  and slug were significantly increased. Moreover, an immunofluorescence assay detected the localization of slug in the EYA4-knockdown cells and



**FIGURE 7** EYA4 plays a role in DNA damage repair in esophageal squamous cell carcinoma (ESCC). A, Dose-response curves show that shEYA4-transfected KYSE30 cells exhibit decreased cell viability after treatment with cisplatin, compared to control cells. B, Representative flow cytometry diagram of cell apoptosis analysis in EYA4-knockdown and EYA4-overexpression cells after cisplatin treatment, compared to their respective control cells. The results are expressed as the mean  $\pm$  SEM (\*\* $P$  < .01, \*\*\* $P$  < .001, independent Student  $t$  test). C, KYSE30 cells in which EYA4 was knocked down show increased  $\gamma$ H2AX after cisplatin treatment in a time and dose-dependent manner. H2AX and  $\beta$ -actin serve as loading controls



found that slug was primarily expressed in the nucleus. Slug is an important transcription factor that regulates EMT, and its elevated expression is directly associated with tumor grade and distant metastasis in many kinds of human cancers, including ESCC.<sup>32-34</sup> Conversely, EYA4 overexpression reduced the expression of p-AKT, p-GSK-3 $\beta$  and slug. Therefore, we hypothesized that EYA4 could inhibit tumor cell invasiveness and metastasis in esophageal cancer, possibly through the inhibition of the activation of the AKT/GSK-3 $\beta$  signaling pathway. This would lead to instability in the expression of slug in the nucleus, the upregulation of E-cadherin expression and the downregulation of vimentin and MMP expression.

TGF- $\beta$ 1 is one of the important inducers of EMT in tumor cells. In the present study, we found that TGF- $\beta$ 1 could markedly induce the downregulation of EYA4 in both a dose-dependent and a time-dependent manner in ESCC cells, which was accompanied by remarkable changes in morphology and expression of EMT-related molecules. In addition, TGF- $\beta$ 1 promoted the expression of DNMT1 and DNMT3A, especially DNMT3A, which suggests that TGF- $\beta$ 1 plays a certain role in the regulation of EYA4 expression in ESCC. In other human malignancies, TGF- $\beta$ 1 has also been found to affect transcription and expression of downstream target genes via the regulation of DNMT expression.<sup>35-37</sup> In ovarian cancer cells, TGF- $\beta$ 1 induced the expression of DNMT and led to extensive genomic hypermethylation, which influenced the transcription of EMT-related genes and promoted the EMT process.<sup>38</sup> In hepatocellular carcinoma, TGF- $\beta$ 1 transformed HCC cells into cells with a spindle-shaped morphology, which was accompanied by hypermethylation of CDH1 and the methylation of its promoter.<sup>39</sup> However, several studies have found that TGF- $\beta$ 1 downregulated the expression of DNMT, which resulted in the demethylation of downstream genes.<sup>40</sup> Our results may provide new strategies for study on the regulation of EYA4 expression in other tumors.

DNA damage caused by various factors are the most harmful lesions, as they may lead to chromosomal aberrations and tumorigenesis if they are not repaired properly. Some studies have shown that after DNA damage, especially damage that consists of DNA double-strand breaks (DSB), phosphorylation of H2AX on serine 139 results in the formation of  $\gamma$ H2AX foci, which recruits other DNA damage response proteins and DNA repair-related proteins.<sup>41</sup> In our study, after cisplatin-induced DNA damage in ESCC cells, we found that EYA4 promoted the repair of DNA damage, inhibited apoptosis induced by cisplatin, and maintained genomic stability. Taken together, these data suggest that EYA4 not only regulates the invasion and metastasis abilities of ESCC cells, but it also plays a role in the process of carcinogenesis through the regulation of the stability of genomic DNA.

In summary, our study reveals that EYA4 is frequently methylated in ESCC tissues and cell lines and that EYA4 inhibits cell migration and invasion through the inactivation of the Akt/GSK-3 $\beta$ /slug pathway and the inhibition of EMT. Moreover, EYA4 plays an important role in DNA damage repair in ESCC cells. Therefore, this protein might be a potential target for anti-metastatic drugs in future clinical practice.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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