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LncRNA ZEB2-AS1 promotes the proliferation, migration and invasion of esophageal squamous cell carcinoma cell through miR-574-3p/HMGA2 axis

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Abstract. – OBJECTIVE: Esophageal squamous cell carcinoma (ESCC) is a common malignant epithelial tumor in the elderly, and the cause is very complicated. Therefore, the study of the pathogenesis of ESCC is conducive to the effective treatment of ESCC. Many studies indicated that lncRNAs were important regulatory factors in tumor formation and disease development. However, the regulatory network of lncRNA in ESCC has not been fully explored.

MATERIALS AND METHODS: The expression of miR-574-3p, ZEB2-AS1, and HMGA2 was measured using qRT-PCR. The protein expression of PCNA, Cleaved-caspase-3, MMP9, and HMGA2 was detected through Western blot. Proliferation or apoptosis of transfected cells was calculated via CKK-8 assay and flow cytometry. Transwell assay was applied to detect cell migration and invasion of ESCC cells. Luciferase reporter assay and RNA pull-down were used to determine the relationship among miR-574-3p, ZEB2-AS1, and HMGA2 in ESCC. Moreover, the regulatory network of ZEB2-AS1 has been verified *in vivo* in this study.

RESULTS: We found that ZEB2-AS1 was up-regulated in ESCC tissues and cells. The knockdown of ZEB2-AS1 could inhibit cell proliferation, migration and migration, as well as promoted cell apoptosis in ESCC. Interestingly, miR-574-3p deficient or HMGA2 promotion could reverse the effect of si-ZEB2-AS1 on ESCC cell progression. Luciferase reporter assay indicated that miR-574-3p was a target miRNA of ZEB2-AS1, and HMGA2 was a target gene of miR-574-3p in ESCC.

CONCLUSIONS: In this paper, we first verified a novel regulatory mechanism of lncRNA ZEB2-AS1 in ESCC cellular process. LncRNA ZEB2-AS1 promoted the proliferation, migration, and invasion of ESCC by modulating miR-574-3p/HMGA2 axis, indicating that ZEB2-AS1 plays essential roles in cell progression in ESCC, providing a new therapeutic target of ESCC.

Key Words:
Cell progression, LncRNA, Regulatory mechanism.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a type of esophageal cancer, a malignant tumor with high invasion and poor prognosis[1-3]. The current treatment methods are very limited, mainly including surgery, radiotherapy, and chemotherapy[4]. Because of the inability to eliminate the lesions, ESCC has a poor prognosis and high recurrence rate. Therefore, studying the pathogenesis of ESCC is an important way to effectively treat ESCC.

LncRNA belongs to a non-coding RNA and is more than 200 nt in length[5]. LncRNA is abnormally expressed in a variety of cancers and is widely involved in important cellular physiological processes, such as cancer cell metabolism and tumor formation[6-8]. LncRNA, as a ceRNA, regulates mRNA expression by binding to miRNAs and is an important regulator of cell progression in cancer[9]. Multiple lncRNAs have been shown to be involved in the metabolic activities of ESCC cells, such as UCA1, HOTAIR, and GAS5[10-12]. Wang et al[10]

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reported that lncRNA UCA1 suppressed cell progression in ESCC by activating the Wnt signaling pathway. Moreover, lncRNA is also closely related to cancer diagnosis and treatment in ESCC[13-15]. ZEB2-AS1 has been shown to be highly expressed in a variety of cancers including bladder cancer, pancreatic cancer, lung cancer and gastric cancer[16-19]. However, the regulatory mechanism of ZEB2-AS1 in ESCC has not been fully elucidated.

Herein, we found that ZEB2-AS1 was highly expressed in ESCC; further, it was predicted by bioinformatic that miR-574-3p was a potential target miRNA of ZEB2-AS1, and HGMA2 was a candidate target gene of miR-574-3p. The purpose of this study was to elucidate the potential mechanism by which ZEB2-AS1 was involved in the regulation of ESCC cell growth.

Materials and Methods

Patients and Samples

ESCC tissues and adjacent tissues were collected from 30 patients who had been diagnose[d] with ESCC in Tangshan Gongren Hospital (Ta[ngshan], Hebei, China). This experiment was appro[ved] by the Ethics Review Board of Tangshan Go[ngren] Hospital. Written informed consent was obtai[ned] from all patients involved in the [study].

Cell Culture and Cell Tr[ans]fecti[on]

Normal human esop[hag]al squ[amous] cell (HET-1A) and esophagea[l] [carcino]ma (ESCC) cell lines [EC109], KYSE30, and KYSEE150) w[ere] purchase[d] American Type Culture Co[llection] (ATCC; M[anassas], VA, USA). All cell[s] cultured in Dulbecco's Modified Eag[le] medi[um]-MEM; Thermo Fisher Scientif[ic] Inc., Waltham[, MA], USA) with 10% fetal bo[vine] serum (FBS, HyC[lone], South-Logan, UT, U[SA]), 1% p[enicillin]-streptomycin at 37°C in a hu[midi]fied a[tmo]sphere of 5% CO_2.

T[he] cells [were] trans[fected] with si-ZEB2-AS1, anti-mi[R-574-3p], HM[GA]2 OE (HMGA2), and [the] negat[ive] [control] using Lipofectamine 2000 rea[gent] (Invitr[ogen], Carlsbad, CA, USA) according [to] the manufa[cturer]'s instructions.

Prolif[eration]

Cell proliferation was calculated using Cell [Count]ing Kit-8 (CCK-8; Dojindo Laboratories, Ku[mamo]to, Japan) according to the manufacturer's instructions. After transfection for 24 h, cells were seeded into 96-well plates and incubated at 37°C. Then 10 ul CCK-8 solutions were added to each well and incubated at 37°C. The absorbance was d[etect]ed using a spectrophotometric microplate reader (Beyotime Institute of Biotechnology, Shanghai, China) at 450 nm.

Cell Apoptosis

Flow cytometry was applied to detect cell apoptosis of transfected cells. Briefly, 2×10^5 transfected cells were seeded in 6-well plates and incubated at room temperature with 5% CO_2. After the cells were harvested and stained with Annexin V-FITC and PI for 30 min in the dark.

Cell [Migr]ation and [Inva]sion

Migration and invasion of transfected cells were measured using transwell chambers (Corning Inc., Corning, NY, USA). For cell invasion of transfected cells, the upper chamber with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used. For migration of transfected cells, the upper chamber without Matrigel was used. The cells were seeded into the upper chamber, and the lower chamber was filled with medium containing 10% FBS. Then, the plates were incubated at 37°C for 24 h. After incubation, the invasive and migrated cells were stained using 0.1% crystal violet and counted using an optical microscope.

QRT-PCR

The total RNA was isolated from tissues and cells with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. For miR-574-3p expression, the TaqMan® MicroRNA Real Time-PCR Assay reagents (Applied Biosystems; Foster City, CA, USA) and SYBR® Premix Ex Taq™ reagent (TaKaRa, Otsu, Shiga, Japan) were applied. For ZEB2-AS1 and HMGA2 expression, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and SYBR® Premix Ex Taq™ reagent (TaKaRa, Otsu, Shiga, Japan) were used to detect. GAPDH and snRNA U6 were used as endogenous controls of mRNA and miRNA, respectively. The relative mRNA and miRNA expression were calculated using $2^{-\Delta\Delta Ct}$ methods. The primers are as follows: lncRNA ZEB2-AS1 forward 5'-ATGAAGAACGCGCGAAGTGT-3' and reverse 5'-CACCCACATTATCACATGCCCT-3'; HMGA2 forward 5'-AGTCCCTCTA-

AAG-CAGCTCAAAAG-3' and reverse 5'-GCCATTTCC-TAGGTCTGCCTC-3' GAPDH forward 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse 5'-AGTCTTCTGGGTGGCAGTGAAT-3'. MiR-574-3p reverse 5'-CACGCTCATGCACACACCCCACA-3' U6 forward 5'-CGCTTCGGCAGCACATATACTA-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCA-3'.

Western Blot

Tissues and transfected cells were detached with trypsin and centrifuged. After washed with PBS, cell lysis buffer was added and incubated to extract protein. The BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) was used to measure protein concentration. The proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked in non-fat milk, the membrane was incubated with primary antibody (PCNA, Cleaved-caspase3, MMP9, GAPDH, and HMGA2, 1:2000, ProteinTech, Chicago, IL, USA). Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Finally, the blot was developed and analyzed using an enhanced chemiluminescence (ECL) Western blotting kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Luciferase Reporter Assay

The wild type of ZEB2-AS1 or mutated type of ZEB2-AS1 was inserted into Luciferase reporter plasmid (pGL3, Promega, Madison, WI, USA) to construct the vector of ZEB2-AS1-WT or ZEB2-AS1-MUT. Then, ZEB2-AS1-WT or ZEB2-AS1-MUT was cotransfected with miR-574-3p or miR-NC into KYSE30 and KYSEE150 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Similarly, the wild type of HMGA2 or the mutated type of HMGA2 was inserted into Luciferase reporter plasmid (pGL3, Promega, Madison, WI, USA) to construct the vector of HMGA2-WT or HMGA2-MUT. Then, HMGA2-WT or HMGA2-MUT was cotransfected with miR-574-3p or miR-NC into KYSE30 and KYSEE150 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA) was applied to detect Renilla and firefly Luciferase activities.

RNA Pull-Down

The cDNA sequence of ZEB2-AS was synthesized and inserted into pBluescript and transfected into KYSE30 and KYSEE150 cells. After Biotin-labeled miR-NC and miR-574-3p were transcribed and purified, 100 µl RIP buffer with cell lysate was added. Then, 50 µl of washed streptavidin agarose beads were added and the beads were washed with RIP buffer times. The extracted protein was detected by immunoblotting or mass spectrometry.

Xenograft Assay

Cells transfected were injected subcutaneously in the right regions of nude mice (4-week-old). Tumor volumes were monitored every 7 days. Tumor volume = width × length × (width + length)/2.5. Mice were euthanized at day 28 after injection and the tumor weight was measured. Animal studies were approved by the Ethics Review Board of the Zhongshan Gongren Hospital.

Statistical Analysis

All data were analyzed and performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Values were presented as mean ± standard deviation (SD).

Comparison groups were calculated using Student's *t*-test to analyze the difference between groups. Three or more sets of groups were calculated using One-way analysis of variance (ANOVA) followed by Tukey's test. $p<0.05$ was considered to be statistically significant.

Results

LncRNA ZEB2-AS1 was Upregulated in ESCC Tissues and Cell Lines

First, we measured the expression of ZEB2-AS1 from 30 ESCC tissues and normal tissues. As shown in Figure 1A, the expression of ZEB2-AS1 was significantly higher in ESCC tissues than that in normal tissues. The expression of ZEB2-AS1 in the ESCC cell lines (EC109, EC9706, KYSE30, and KYSEE150) was significantly higher than in the Normal human esophageal squamous cell (HET-1A) (Figure 1B). Notably, the survival analysis found that the high expression of ZEB2-AS1 of patients with ESCC had a poor prognosis (Figure 1C). These results indicated that ZEB2-AS1 played important roles in ESCC formation and treatment.

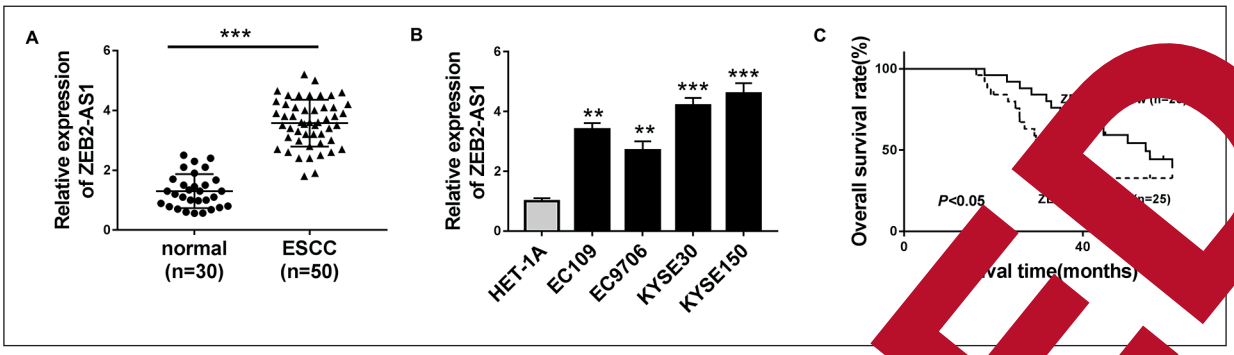

Figure 1. LncRNA ZEB2-AS1 was upregulated in ESCC tissues and cell lines. **A,** The ress -AS1 w easured in ESCC tissues and normal tissues. **B,** The expression of ZEB2-AS1 was measu ESCC es (E EC9706, KYSE30, and KYSEE150) and normal human esophageal squamous cell (HET-1A Kaplan-Mei he survival rates of patients with ESCC in relation to ZEB2-AS1. *p<0.05, **p<0.01.

LncRNA ZEB2-AS1 Promoted Proliferation, Migration and Invasion As Well As Inhibited Apoptosis in ESCC Cell Lines

Next, in order to verify the function of ZEB2-AS1 in ESCC, ZEB2-AS1 was knocked out for transfection into KYSE30 and KYSEE150 cell lines and stably expressed (Figure 2A). The l- ysis of CKK-8 assay showed that the kno of ZEB2-AS1 significantly inhibited the f- eration of KYSE30 and KYSEE150 cells (F 2B and 2C). Conversely, the apoptotic rate o ZEB2-AS1 transfection was signif tly elevate (Figure 2D and 2E). Meanw cing the expression of ZEB2-AS1 al greatly creased the migration and invasi ESCC (Fig- ure 2F-2I). The protein e ion MMP9 was signific while the protein expression cleaved- 3 was remarkably increa inhibition B2-AS1 in KYSE30 a 150 cell lin (Figure 2J-2L). There , inhi of ZEB2-AS1 could suppress proliferation ation, and invasion, an moted cell apopt n ESCC cells, imply that Z B2-AS1 promoted growth in ESC ell lin

LncRN -AS1 ectly Interacted Mi 3

-PCR a as used to detect the expression el of ZE -AS1 in the nucleus and cyto ults showed that the expression of ZE -AS1 in the cytoplasm was high ure 3A). Next, we predicted the presence of iR-574-3p binding site in ZEB2-AS1 by DI tool (Figure 3B). Therefore, we performed Luciferase reporter assay in KYSE30 and KYS cells, resp y, and showed that th pression of miR 4-3p combined with 2-AS1-WT significantly reduced Luciferase vity in cell ther than ZEB2-AS1-MUT ure 3C and). Moreover, the results of R ull-dow howed that the miR-574-3p pro ore ZEB2-AS1 expression compared to NC probe, indicating that miR-574-3p effectively bound ZEB2-AS1 in ESCC cells 3E). More than that, the knockdown of ZEB2 S1 significantly induced the expression of miR-574-3p in KYSE30 and KYSE150 cells (Figure 3F). Lowly expressed miR-574-3p in ESCC tissues was negatively correlated with ZEB2-AS1 expression (Figure 3G and 3H). All results determined that miR-574-3p was a target miRNA of ZEB2-AS1 in ESCC cells.

LncRNA ZEB2-AS1 Regulated ESCC Progression by Sponging MiR-574-3p

To further clarify the regulatory mechanism between ZEB2-AS1 and miR-574-3p, we co-transfected si-ZEB2-AS1 and anti-miR-574-3p into KYSE30 and KYSEE150 cells, respectively. The result showed that the induction of miR-574-3p by knockdown of ZEB2-AS1 was significantly decreased by downregulating miR-574-3p in KYSE30 and KYSEE150 cells (Figure 4A). Otherwise, cell proliferation, invasion, and migration were significantly decreased by si-ZEB2-AS1 transfection, which was induced by anti-mIR-574-3p transfection in KYSE30 and KYSEE150 cells (Figure 4B, 4E and 4F). As shown in Figure 4C, the promotion effect of si-ZEB2-AS1 on cell apoptosis was inhibited by the suppression of miR-574-3p in KYSE30 and KYSEE150 cells. Knocking out ZEB2-AS1 not only reduced PCNA

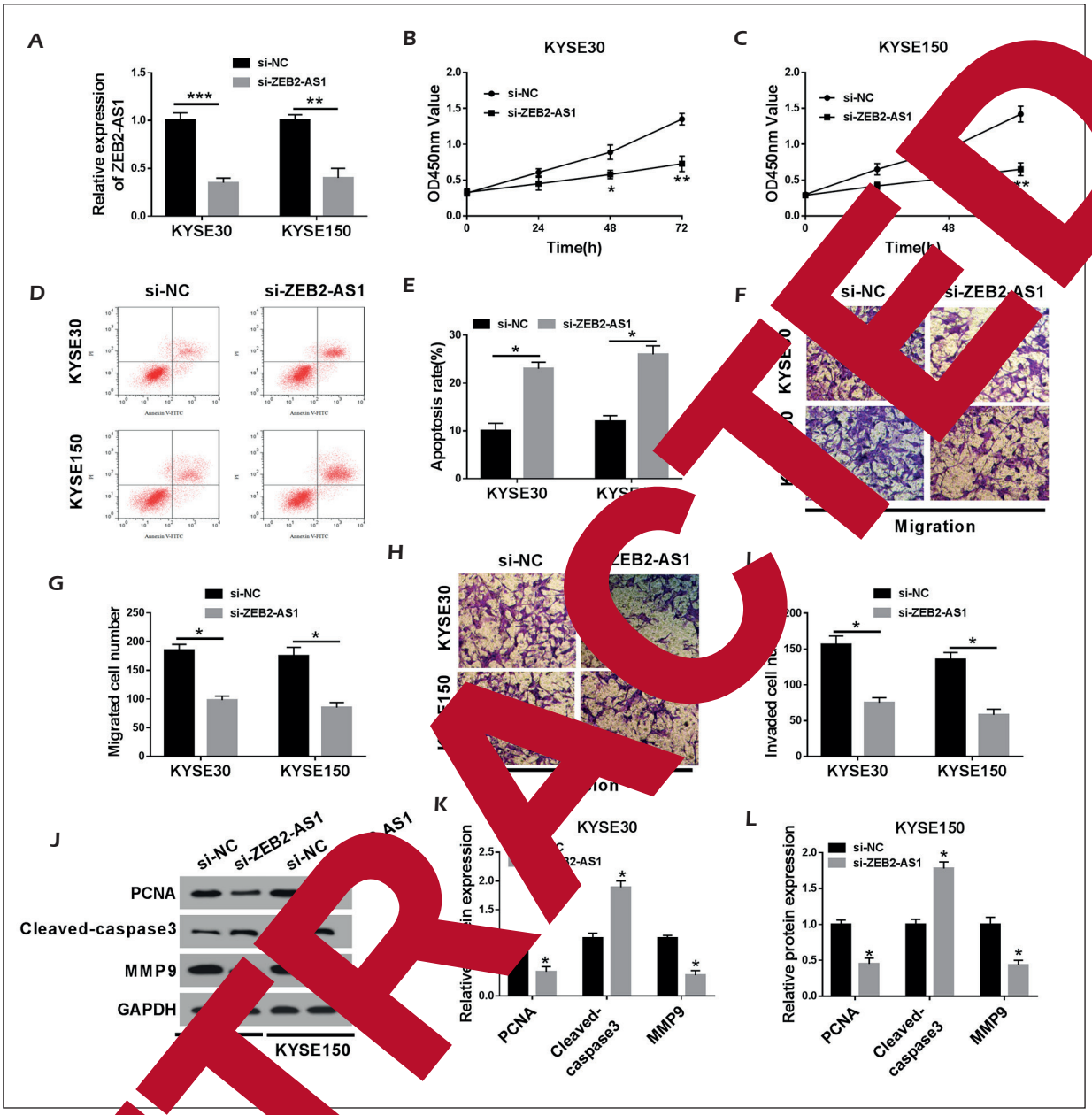

Figure 2. RNA ZEB2-AS1 p proliferation, migration and invasion as well as apoptosis in ESCC cell lines. **A,** The exp n of ZEB2-AS1 was de ted in KYSE30 and KYSE150 cells transfected with si-NC or si-ZEB2-AS1 *via* qRT-PCR. d **C,** Cel iferation was measured in KYSE30 and KYSE150 cells transfected with si-NC or si-ZEB2-AS1 using CCK say. **D.** , Cell a optosis rate was calculated in KYSE30 and KYSE150 cells transfected with si-NC or si-ZEB2-AS1 etry. **F-I** migration (**F** and **G**) and invasion (**H** and **I**) was detected in KYSE30 and KYSE150 cells transfec -NC or B2-AS1 using transwell assay (100×). **J-L,** The protein level of PCNA, Cleaved-caspase3 and 9 was ed i SE30 and KYSE150 cells transfected with si-NC or si-ZEB2-AS1 with Western blot. *p<0.05, l.

an in expression, but also promoted ved-cas se3 protein expression, which was ersed by underexpression of miR-574-3p in 30 and KYSEE150 cells (Figure 4G and 4H us, lncRNA ZEB2-AS1 regulated ESCC progression by sponging miR-574-3p.

LncRNA ZEB2-AS1 Promoted HMGA2 Expression by Sponging MiR-574-3p in ESCC Cells

The predicted results of starBase showed that there was a miR-574-3p binding site in the 3' non-coding region of HMGA2 (Figure 5A). To

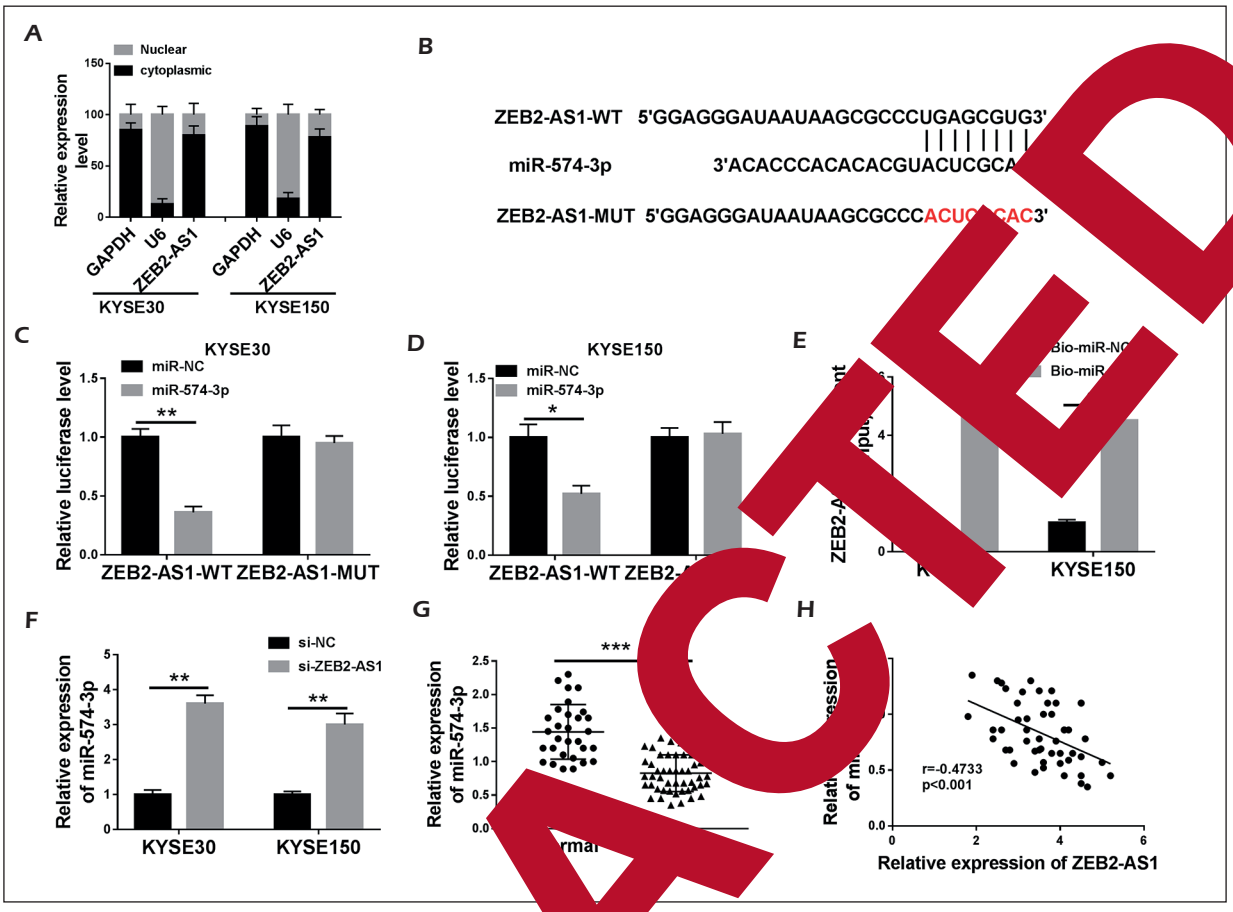

Figure 3. LncRNA ZEB2-AS1 directly interacted with miR-574-3p in ESCC cells. **A,** QRT-PCR assay was used to detect the expression level of ZEB2-AS1 in the nuclear and cytoplasmic fractions of KYSE30 and KYSE150 cells. **B,** The predicted sites of miR-574-3p binding to ZEB2-AS1 were predicted by DIANA tools, and **D,** Luciferase activity was measured in KYSE30 (**C**) and KYSE150 (**D**) cells cotransfected with ZEB2-AS1-WT or ZEB2-AS1-MUT and miR-574-3p or miR-NC using luciferase reporter assay. **E,** RNA pull-down was used to detect the enrichment level of ZEB2-AS1 in Bio-miR-NC and Bio-miR-574-3p groups. **F,** The expression of miR-574-3p in KYSE30 and KYSE150 cells transfected with si-NC or si-ZEB2-AS1. **G,** The expression of miR-574-3p detected in ESCC tissues and normal tissues. **H,** The expression of ZEB2-AS1 was significantly correlated with that of miR-574-3p in ESCC tissues through Pearson's correlated analysis.

further investigate the regulatory mechanism of ZEB2-AS1 in ESCC, the luciferase reporter assay was used to demonstrate the targeting relationship between miR-574-3p and HMGA2. The results showed that the overexpression of miR-574-3p inhibited the luciferase activity in the cells transfected with HMGA2 3'-UTR-WT KYSE30 and KYSEE150 cells (Figure 5B and 5C). The mRNA and protein expression of HMGA2 was sharply inhibited by the promotion of miR-574-3p in KYSE30 and KYSEE150 cells (Figure 5D and 5E). In addition, the knockdown of ZEB2-AS1 reduced the expression of HMGA2 mRNA and protein. When co-transfected with miR-574-3p inhibitor, the mRNA and protein expression of HMGA2 was partially increased (Figure 5F-5G). Of note, HMGA2, which was highly expressed in ESCC tissues, was negatively correlated with miR-574-3p expression but positively correlated with ZEB2-AS1 expression (Figure 5J-5L). All data determined that ZEB2-AS1 promoted HMGA2 expression by sponging miR-574-3p in ESCC cells.

Restoration of HMGA2 Reversed the Effects of LncRNA ZEB2-AS1 Knockdown in ESCC Cells

To investigate the effect of the ZEB2-AS1/miR-574-3p/HMGA2 regulatory network on ESCC cell progression, si-ZEB2-AS1 and HMGA2 overexpression (HMGA2) were co-transfected into KYSE30 and KYSEE150 cells. The results

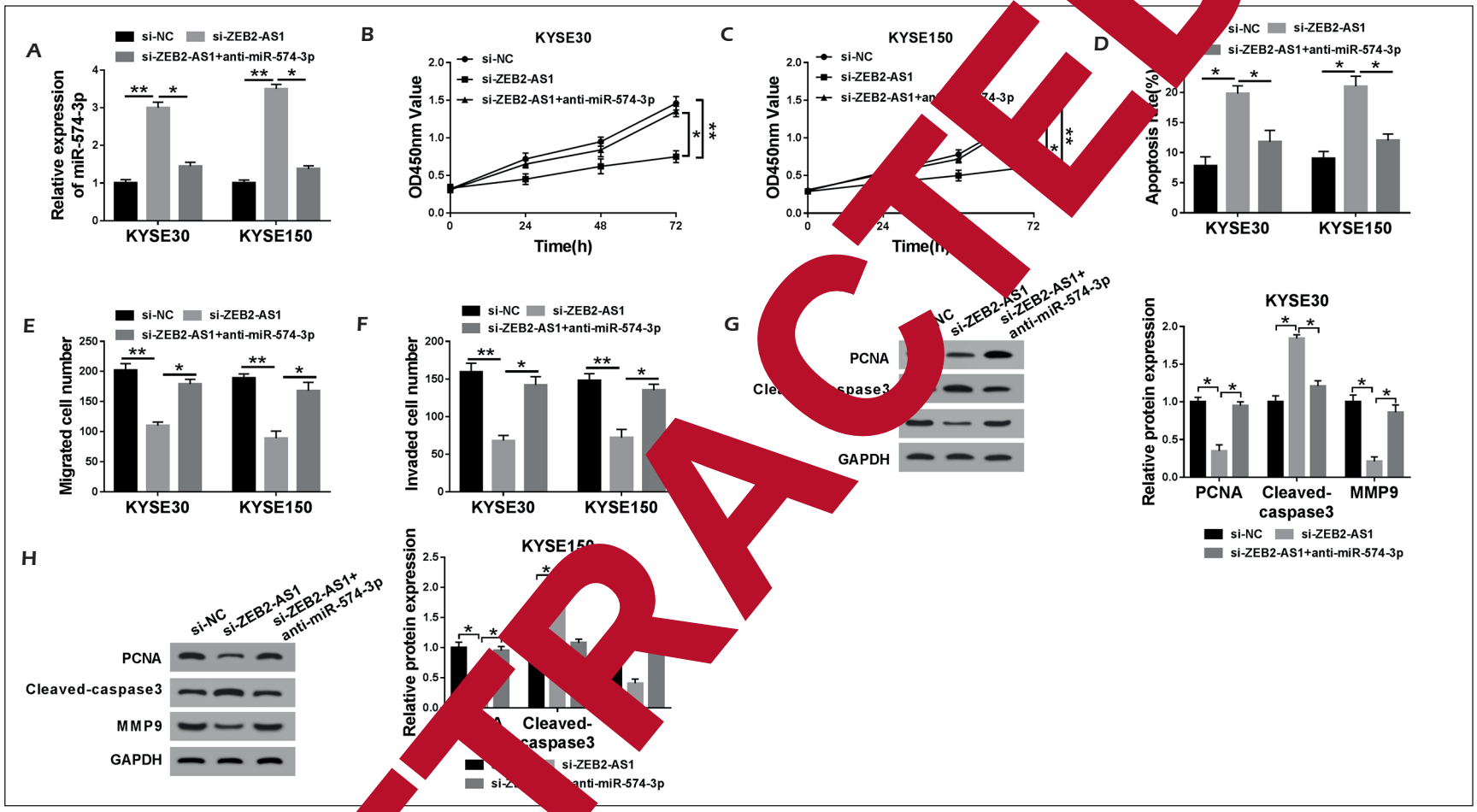

Figure 4. LncRNA ZEB2-AS1 regulat[ed] SCC pr[o]ssion by sponging miR-574-3p. **A,** The expression of miR-574-3p was detected in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS1 or si-ZEB2[-AS1+] anti-[miR-]574-3p *via* qRT-PCR. **B,** and **C,** Cell proliferation was measured in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS1 or si-ZEB2-AS1+ anti-miR-[574-3p] using CCK[-8] assay. **D,** Cell apoptosis rate was calculated in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS1 or si-ZEB2-AS1+ anti-miR-574-3p *via* flow cy[tometry] assay. **E,** a[nd] **F,** Cell migration (**E**) and invasion (**F**) was detected in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS1 or si-ZEB2-AS1+ anti-miR[-574-3p] using [Transwell] assay. **G,** and **H,** The protein level of PCNA, Cleaved-caspase3 and MMP9 was measured in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS1, si-ZEB2-AS1+anti-miR-574-3p with Western blot. *p<0.05, ****p<0.01.

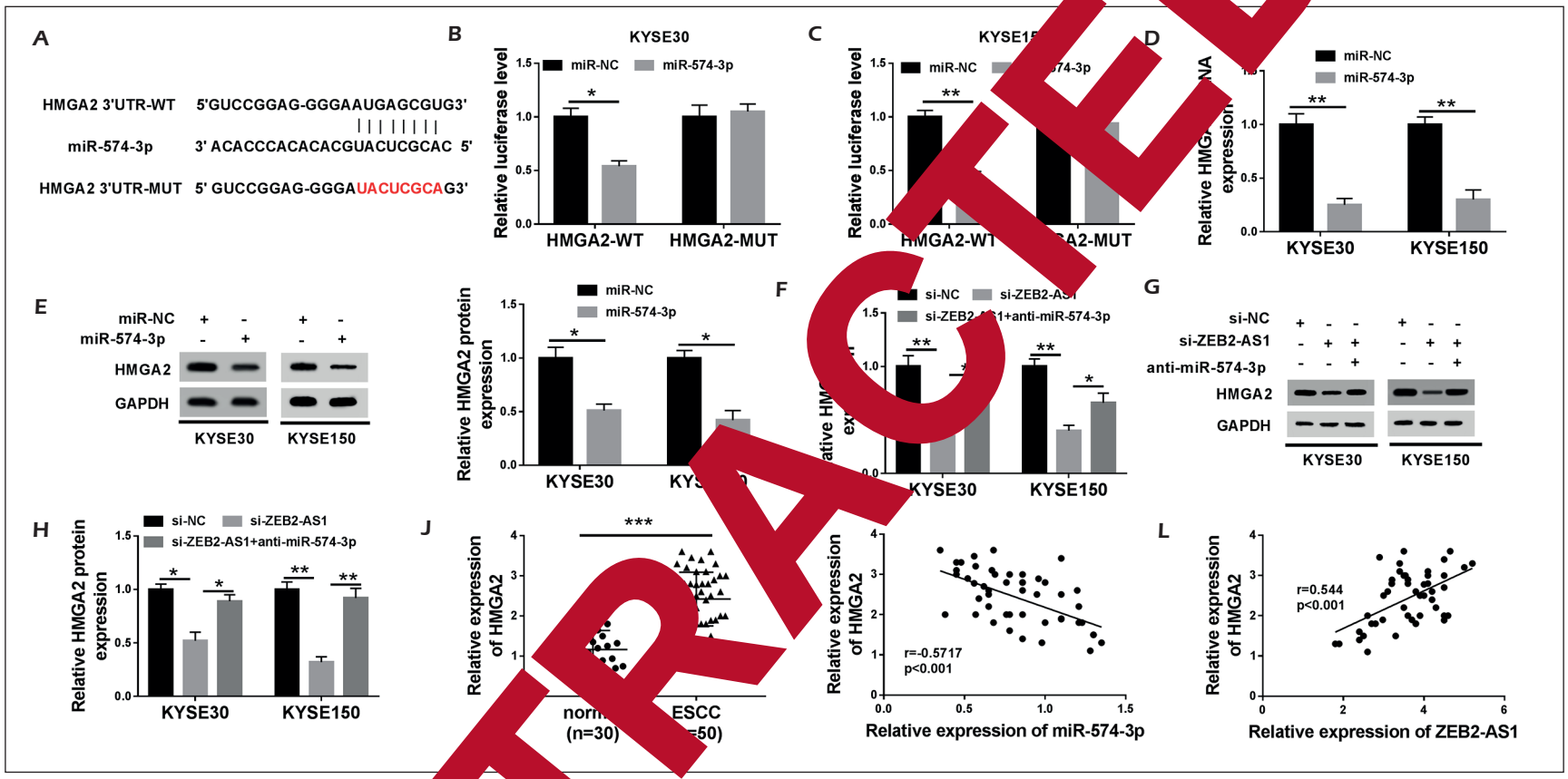

Figure 5. LncRNA ZEB2-AS1 promoted HMGA2 expression by sponging miR-574-3p in ESCC cells. **A,** The predicted sites of miR-574-3p binding to 3'UTR of HMGA2 were detected using starBase. **B,** and **C,** Luciferase activity was measured in KYSE30 (**B**) and KYSE150 (**C**) cells cotransfected with HMGA2 3'UTR-WT or HMGA2 3'UTR-MUT and miR-574-3p or miR-NC using luciferase reporter assay. **D,** and **E,** The mRNA (**D**) and protein (**E**) expression of HGM was detected in KYSE30 and KYSE150 cells transfected with si-NC or miR-574-3p. **F-H,** The mRNA (**F**) and protein (**G** and **H**) expression of HMGA2 was detected in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS1 or si-ZEB2-AS1+ anti-miR-574-3p *via* qRT-PCR and Western blot. **J,** The expression of HMGA2 was detected in ESCC tissues and normal tissues. **K,** The expression of HMGA2 was significantly negative correlated with miR-574-3p in ESCC tissues through Pearson's correlated analysis. **L,** The expression of HMGA2 was significantly positive correlated with that of ZEB2-AS1 in ESCC tissues through Pearson's correlated analysis.

indicated that overexpression of HMGA2 could weaken the suppression effect of si-ZEB2-AS1 on HMGA2 mRNA and protein expression in KYSE30 and KYSEE150 cells (Figure 6A and 6B). Knockdown of ZEB2-AS1 significantly inhibited cell proliferation, invasion and migration, and promoted apoptosis, which was rescued by overexpression of HMGA2 (Figure 6C-6G). Furthermore, PCNA and MMP9 protein expression were inhibited and Cleaved-caspase3 protein expression was induced by downregulation of ZEB2-AS1, which was reversed by the promotion of HMGA2 in KYSE30 and KYSEE150 cells (Figure 6H and 6I). Thus, the restoration of HMGA2 reversed the effects of lncRNA ZEB2-AS1 knockdown in ESCC cells.

Knockdown of ZEB2-AS1 Inhibited Tumor Growth In Vivo

To further investigate the effects of ZEB2-AS1 on the formation of ESCC, we performed experimental validation in mice. We injected KYSE30 cells transfected with sh-con and sh-ZEB2-AS1 subcutaneously into the right side of the nude mice (N=5). Tumor volum[illegible]s measured every 7 days, and tumors w[illegible] moved after transfection for 35 days. T[illegible]e-sults showed that ESCC tumor volume[illegible] weight were significantly inhibited after KE[illegible] AS1 was knocked out (Figur[illegible]7A and 7B). In ESCC tissues, Sh-ZEB2[illegible]ificantly increased the expression of[illegible]K-574[illegible] and decreased the expression of[illegible]B2-AS1[illegible]ure 7C and 7D). In addition, the[illegible]in e[illegible] HMGA2, PCNA, an[illegible]MP[illegible]ignificantly lower in Sh-ZEB2-[illegible] group t[illegible]the sh-con group, while the[illegible]in expressio[illegible]leaved-caspase3 was[illegible]Figure 7E). Therefore, the knockdo[illegible]of ZE[illegible]S1 inhibited tumor growth *in*[illegible].

Discussion

Due[illegible] comp[illegible]etiology of esophageal [illegible], th[illegible]e rate is high, and the pr[illegible]sis is p[illegible]. In order to further clarify the [illegible]ogenesis [illegible] ESCC and improve the therap[illegible]cRNAs, which act as important [illegible]ators, [illegible]eded to further investigation. In-[illegible]asing reports[21-25] showed that lncRNAs were [illegible]ed in cellular processes, such as cell proli[illegible]n and apoptosis in a variety of cancers. Moreover, lncRNAs were also associated with immune responses and drug sensitivity[26,27]. LncRNA PCAT1 was related to poor pr[illegible]sis and contributed cell proliferation, in[illegible] gration, and EMT in osteosarcom[illegible] LncRN[illegible] has also been proposed to play[illegible]s in ESCC cellular process and metasta[illegible] LncRNA AFAP1-AS1 was upregulated in ES[illegible]lls and promoted cell growth, a[illegible]l as re[illegible]cel[illegible] apoptosis in ESCC[32]. [illegible]is study, co[illegible] with previous researc[illegible]we fou[illegible]hat lncR[illegible]A ZEB2-AS1 expres[illegible]as i[illegible]sed in ESCC tissues and cells, [illegible]ndi[illegible] ZEB2[illegible] was associated with[illegible]C fo[illegible]n. Mo[illegible]er, Lan et al[34] report[illegible]hat ZEB2-[illegible]ipated in tumor gro[illegible] metastasis [illegible]patocellular carcinon[illegible]lea[illegible], the similar function of ZEB2-AS1 has bec[illegible]ified in colon cancer, pan[illegible]cancer, bla[illegible]ancer, and gastric ca[illegible][9,35]. However, t[illegible] function of ZEB2-[illegible] in ESCC has not been fully clarified. Our [illegible]dy is the fir[illegible] investigate that the knock-[illegible]n of ZEB2-[illegible]1 could inhibit ESCC cell p[illegible]ration, i[illegible]on, and migration while promot[illegible].

Furth[illegible]re, we also explored the regulatory [illegible]echanism of ZEB2-AS1 in ESCC cell pro-[illegible]. Gao et al[17] reported that ZEB2-AS1 [illegible]ec[illegible] pancreatic cancer formation and cell invasion by modulating miR-204/HMGB1 axis. In this study, Luciferase reporter assay determined that miR-574-3p was a target miRNA of ZEB2-AS1 and was negatively regulated in ZEB2-AS1 expression. MiR-574-3p is also abnormally expressed in many types of cancer[36-38]. Su et al[39] indicated that miR-574-3p had important biological significance in gastric cancer and was closely related to the early diagnosis of gastric cancer. Of note, in our study, we found that miR-574-3p was downregulated in ESCC tissues and reduction of miR-574-3p could reverse the suppressive effects of si-ZEB2-AS1 on ESCC cell growth, indicating that ZEB2-AS1 play roles in ESCC progression by targeting miR-574-3p.

In addition, we further explored the regulatory mechanism of ZEB2-AS1 and found that HMGA2 was a target mRNA of miR-574-3p. Functional experiments showed that HMGA2 overexpression could weaken the repressive effects of knockdown of ZEB2-AS1 on ESCC cell progression. Many studies[40-42] determined that HMGA2 was closely related to EMT in human pancreatic cancer cells and gastric cancer. Sun et al[43] reported that HMGA2/TET1/HOXA9 axis participated in the regulation of breast cancer growth and

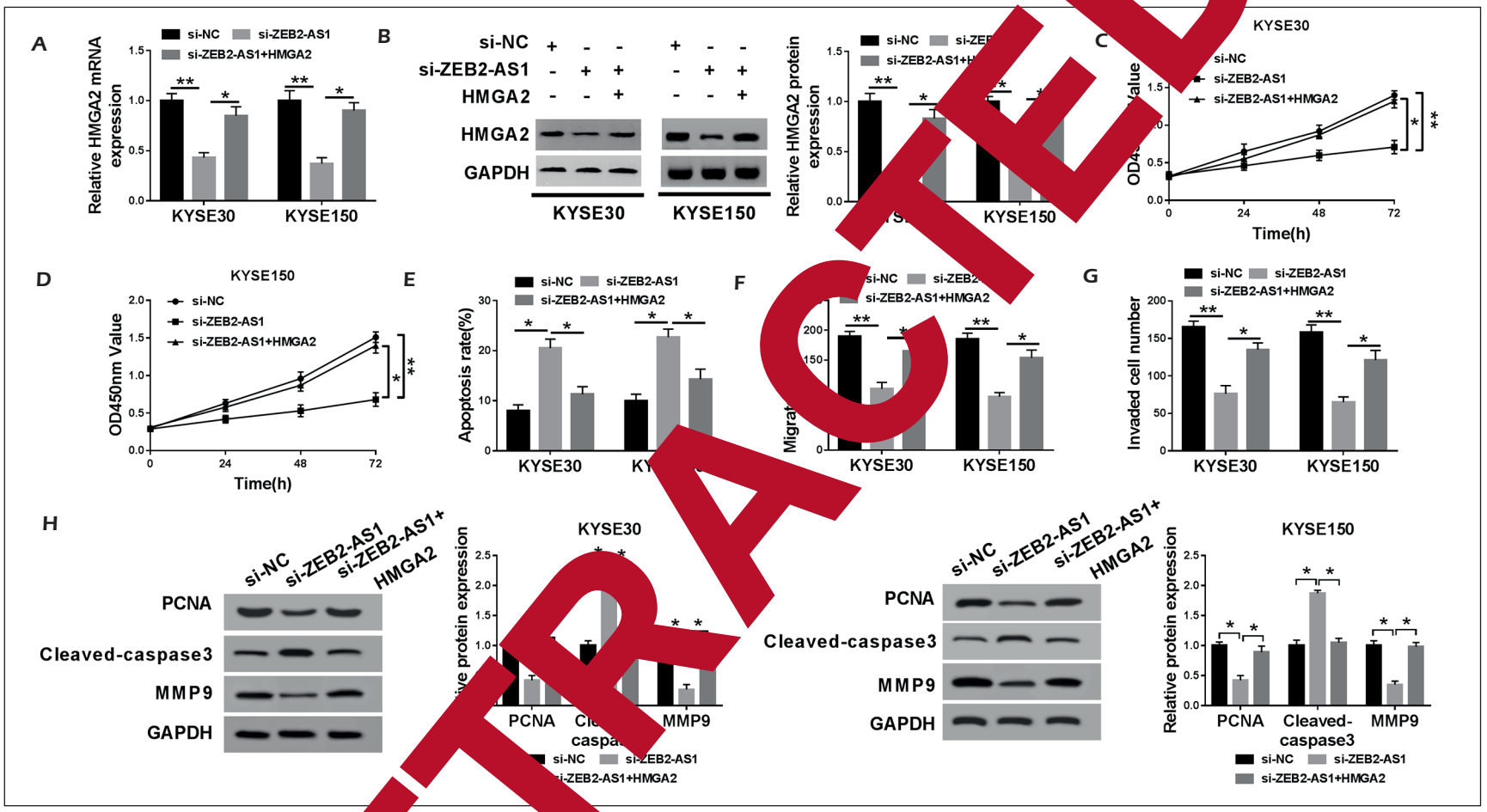

Figure 6. Restoration of HMGA2 reve[illegible]ne effe[illegible] of lncRNA ZEB2-AS1 knockdown in ESCC cells. **A,** and **B,** The mRNA (**A**) and protein (**B**) expression of HMGA2 was detected in KYSE30 and KYSE150 ce[illegible]ansfec[illegible]ith si-NC, si-ZEB2-AS1 or si-ZEB2-AS1+ HMGA2 *via* qRT-PCR. **C,** and **D,** Cell proliferation was measured in KYSE30 (**C**) and KYSE150 (**D**) cells transfected [illegible]i-ZEB[illegible]1 or si-ZEB2-AS1+ HMGA2 using CCK-8 assay. **E,** Cell apoptosis rate was calculated in KYSE30 and KYSE150 cells transfected with si-NC, si-ZEB2-AS[illegible]EB2-A[illegible]HMGA2 *via* flow cytometry. **F,** and **G,** Cell migration (**F**) and invasion (**G**) was detected in KYSE30 and KYSE150 cells transfected with si-NC, si-[illegible]S1 o[illegible]B2[illegible]+HMGA2 using transwell assay. **H,** and **I,** The protein level of PCNA, Cleaved-caspase3, and MMP9 was measured in KYSE30 (**H**) and KYSE15[illegible] ce[illegible]nsfecte[illegible]i-NC, si-ZEB2-AS1 or si-ZEB2-AS1+HMGA2 with Western blot. *p<0.05, **p<0.01.

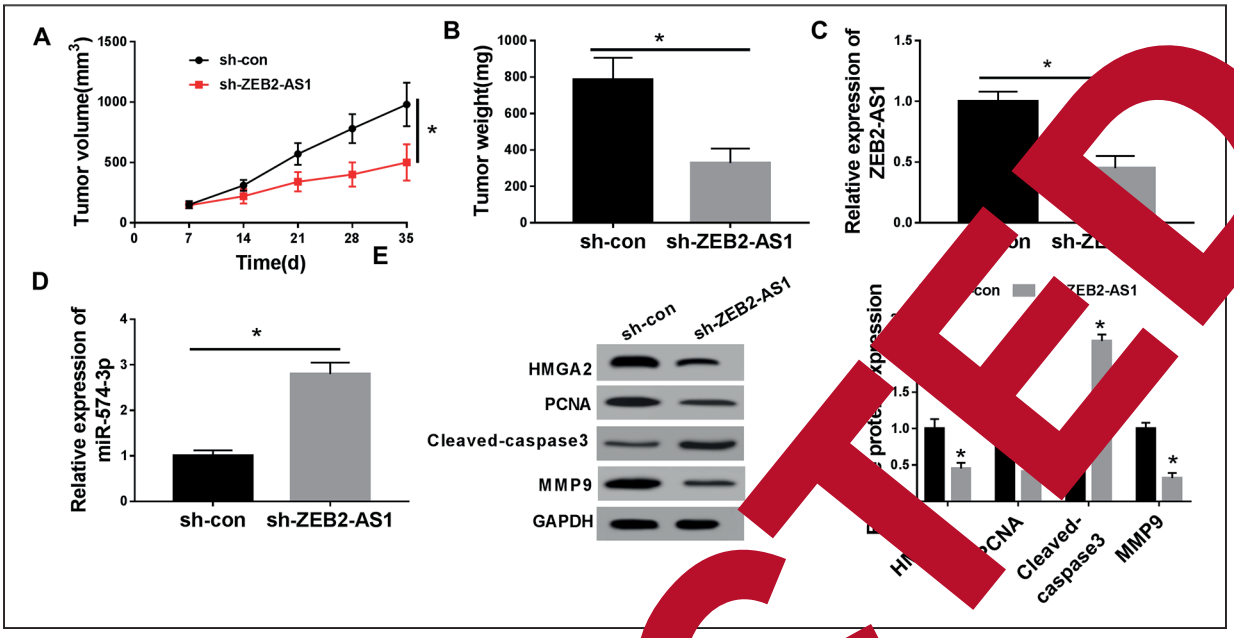

Figure 7. Knockdown of ZEB2-AS1 inhibits tumor growth *in vivo*. **A,** and **B,** Tumor volume (**A**) and weight (**B**) were calculated in sh-con and sh-ZEB2-AS1 groups in mice. **C,** and **D,** Relative expression of ZEB2-AS1 (**C**) and miR-574-3p (**D**) was measured in sh-con and sh-ZEB2-AS1 groups in mice *via* qRT-PCR. The protein levels of PCNA, Cleaved-caspase3, MMP9, and HMGA2 was measured in sh-con and sh-ZEB2-AS1 groups in mice *via* Western blot. $*p < 0.05$, $**p<0.01$.

metastasis. In ESCC, HMGA2 has been reported to be a target gene of miR-490-3p, miR-125b-5p and miR-195 and regulated cancer formation, cell proliferation, metastasis and apoptosis. In our study, we first verified the ZEB2-AS1/miR-574-3p/HMGA2 axis played an essential role in ESCC cell proliferation, migration, metastasis, and apoptosis. Taken together, our findings revealed the novel regulatory mechanism of lncRNA in ESCC progression.

Conclusions

In this paper, we first verified the novel regulatory mechanism of lncRNA ZEB2-AS1 in ESCC cells. LncRNA ZEB2-AS1 promoted the proliferation, migration, and invasion of ESCC by miR-574-3p/HMGA2 axis, including ZEB2-AS1 played essential roles in cell progression in ESCC and providing a new therapeutic of ESCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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