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The mechanism of high mobility group box-1 in the proliferation and macrophage polarization in esophageal squamous cell carcinoma cells

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Abstract

Background Previous studies showed that high mobility group box-1 (*HMGB1*) facilitates the initiation and progression of esophageal squamous cell carcinoma (ESCC), and the current research investigated the detailed mechanisms implicated.

Methods The impact of *HMGB1* and *IGFBP3* levels on the survival of ESCC was examined by plotting Kaplan–Meier (KM) curves based on the data collected from The Cancer Genome Atlas (TCGA). Quantitative real-time PCR (qRT-PCR) was performed to detect the expressions of *HMGB1* in both human esophageal epithelial cells (HEEC) and ESCC cells. After cell transfection, the proliferation of ESCC cells was measured, and the cell metastasis was determined based on the levels of cadherins (CDHs) and Vimentin (VIM). Macrophage polarization was determined by calculating the mean fluorescence intensity (MFI) of *CD206* and *CD86*. In addition, co-immunoprecipitation and immunoblotting were applied to evaluate the interaction between insulin-like growth factor binding protein 3 (*IGFBP3*)/DNA-dependent protein kinase catalytic subunit (*DNA–PKcs*) and *HMGB1*.

Results A high level of *HMGB1* was predictive of an unfavorable prognosis of ESCC ($p < 0.05$). *HMGB1* showed a higher expression in ESCC cells ($p < 0.05$), while knockdown of *HMGB1* inhibited ESCC cell proliferation, downregulated the levels of *CDH2* and *VIM* and upregulated the level of *CDH1* ($p < 0.05$). In contrast, overexpressed *HMGB1* showed the opposite effects ($p < 0.05$), suggesting the role of *HMGB1* in the epithelial–mesenchymal transition (EMT) of ESCC. After the knockout of *HMGB1*, the MFI of *CD86* was increased but that of CD206 was reduced, indicating the polarization towards M1 macrophages ($p < 0.05$). However, the results were reversed when *HMGB1* was overexpressed ($p < 0.05$). Meanwhile, *HMGB1* could interact with the *IGFBP3*/*DNA–PKcs* complex ($p < 0.05$). Low-expressed *IGFBP3* was predictive of an unfavorable prognosis of ESCC, and *IGFBP3* silencing promoted the proliferation of ESCC cells ($p < 0.05$). Besides, *HMGB1* and *IGFBP3* could act antagonistically in influencing the proliferation of ESCC cells and macrophage polarization.

Conclusions Through in vitro experiments, this study found that *HMGB1* was linked to the proliferation and polarization of macrophages in ESCC, providing novel evidence for the role of *HMGB1* in ESCC development.

Keywords High mobility group box 1, Proliferation, Macrophage polarization, Insulin-like growth factor binding protein 3/DNA-dependent protein kinase catalytic subunit axis, Esophageal squamous cell carcinoma

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Introduction

Esophageal cancer (EC) is a frequently diagnosed tumor with high morbidity and mortality rates globally [1–3]. EC could be histologically classified into two primary subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), each with distinct management approaches [4]. At present, clinical treatment of ESCC remains challenging due to its unfavorable prognosis and limited therapeutic options [5, 6]. The development of monoclonal antibodies that inhibit programmed death ligand 1 (PD-L1) or programmed death 1 (PD-1) could improve the treatment responses in ESCC, yet clinical benefits are limited to few patients due to resistance [7]. Thus, identifying precise and accurate biomarkers to advance personalized immunotherapy in clinical practice is urgently needed.

Study has revealed a close link between the tumor microenvironment (TME) and the development and progression of ESCC cells, highlighting the TME as a crucial target for improving the prognosis of ESCC [8, 9]. The TME contains multiple types of immune cells and stromal components pivotal to the processes, such as EMT, immune escape, angiogenesis, formation of metastasis niche, and tumor invasion and metastasis [10]. Macrophages within the TME can polarize either into classically activated macrophages (M1) or alternatively activated macrophages (M2) [11]. It is currently widely acknowledged that tumor-associated macrophage (TAM) subtypes play crucial roles in cancer prognosis. Study showed that M2 TAMs are related to the malignant biological behavior of tumors [12], and that a high infiltration of M2 macrophages is indicative of a worse prognosis in ESCC [13]. In this study, *CD86* and *CD206* were considered as the markers for M1 and M2 macrophages, respectively, in our exploration of the infiltration status and significance of macrophages in ESCC.

Radiotherapy is extensively used in treating ESCC; however, radiation-induced DNA damage responses (DDRs) can trigger the release of cytokines and chemokines, intensifying inflammatory reactions and causing changes in the TME. The most lethal damage from ionizing radiation is DNA double-strand breaks, which could cause a range of cellular DDRs during cell recovery from radiation-induced injuries that confer radioresistance to tumor [14]. Hence, inhibiting DDRs has emerged as a promising anticancer strategy, potentially revolutionizing cancer therapy by selectively killing cancer cells while sparing normal ones [15]. Several factors such as *DNA–PKcs* and *IGFBP3* have been identified and investigated [16, 17]. In response to DNA damage, *IGFBP3* can interact with *DNA–PKcs* to form a complex in the regulation of DNA repair [18]. The role of *IGFBP3*/*DNA–PKcs* complex in modulating the sensitivity of tumor cells has been proved by previous studies [18–21], but the specific involvement of this complex in ESCC remain unexplored, which was the focus of the current research.

In our search for potential regulators that affect both DNA damage response and the progression of ESCC, *HMGB1* has come into focus. *HMGB1* is not only a broad cellular stress sensor that balances survival responses and cell death, but also a crucial mediator implicated in various pathological conditions, including cancers [22]. The expression level of *HMGB1* plays a critical part in the development of various cancers through enhancing metastasis [23]. For instance, *HMGB1* migrates into extracellular matrix and binds to immune cell receptor to affect their functions and differentiation in digestive tumors, thereby aggravating injury and promoting the tumor development [24]. Previous studies have found that overexpressed *HMGB1* triggers the radioresistance, whereas the knockdown of *HMGB1* promotes radiosensitization in ESCC [25, 26]. Moreover, *HMGB1* is regulated by genes, including *Epac1* and *PKA*, through the activation of *IGFBP3* [27, 28]. However, the casual relationship between *HMGB1* and *IGFBP3* in ESCC remained unclear, and the present research aimed to analyze the mechanisms through which *HMGB1* affected ESCC and the relationship between *HMGB1* and the *IGFBP3*/*DNA–PKcs* complex.

Materials and methods

Bioinformatics analysis

The impact of high and low expressions of *HMGB1* and *IGFBP3* on the survival data of ESCC patients was explored according to the Kaplan–Meier curve plotted based on the data from TCGA database.

Cell culture and transfection

Human esophageal epithelial cells (HEEC, IM-H467) and murine macrophage cell line RAW264.7 (IM-M028) were purchased from ImmoCell (Xiamen, China), and ESCC cell lines KYSE-140 (BNCC351870), KYSE-150 (BNCC359343), KYSE-410 (BNCC359845) and KYSE-510 (BNCC360126) were ordered from BeiNa Culture Collection (Xinyang, China). Dulbecco's modified Eagle's medium (IMC-201–2, ImmoCell, China) added with 10% FBS was used to culture HEEC cells and RAW264.7 cells, while ESCC cells were cultured in 90% Roswell Park Memorial Institute (RPMI)−1640 medium (IMC-202, ImmoCell, China) added with 10% fetal bovine serum (FBS, IMC-101–500, ImmoCell). All the cells were identified via short tandem repeat profiling and tested negative of mycoplasma contamination.

For the transfection via liposome, small interfering RNAs (siRNAs) against *HMGB1* (si-HMGB1-1 and

Table 1 Sequence for transfection

Gene	Sequence (5′–3′)
si-HMGB1-1	CCGTTATGAAAGAGAAATGAA
si-HMGB1-2	CGGCCTTCTTCTTGTTCTGTT
si-IGFBP3	GCCTCGATTTATATTTCTGTT
si-NC	TGTCTGTTCTTCGGCTCTTCT

si-HMGB1-2) and *IGFBP3* (si-IGBP3) and the negative control siRNAs (si-NC) were synthesized by RiboBio (Guangzhou, China). The overexpression plasmids of *HMGB1* (oe-HMGB1) and *IGFBP3* (oe-IGFBP3) were constructed using the pcDNA3.1 vector (V790-20, Invitrogen, Carlsbad, California, USA). Utilizing Lipofectamine 2000 reagent (11698–027, Invitrogen, USA), these constructed siRNAs and overexpression plasmids were transfected into ESCC cells following the protocols. All the cells were harvested after cell transfection for 48 h for subsequent assays. The target sequences applied are shown in Table 1.

Cell proliferation assay

To quantitatively assess the proliferative capacity of ESCC cells, CCK-8 assay (IMC-906, ImmoCell, China) and EdU incorporation assay were performed [29]. Briefly, ESCC cells were seeded into 96-well plates at 5×10^3 cells/well for 24-h and 48-h cell culture, followed by treatment with 10 μL CCK-8 working solution for another 4 h. An iMark microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, California, USA) was adopted to read the optical density (OD) value at 450 nm [30]. For EdU incorporation assay, the BeyoClick™ EdU-647 cell proliferation assay kit (C0081S, Beyotime, Shanghai, China) was employed to detect the synthesized DNA and evaluate the proliferation of ESCC cells. Images were taken under a confocal microscope (FV1000, Olympus, Tokyo, Japan) and the EdU-positive ESCC cells were quantified [31].

Immunofluorescence assay

Immunofluorescence assay was utilized to examine the effects of *HMGB1* and *IGFBP3* on the macrophage polarization [32]. In detail, the RAW264.7 cells inoculated in 24-well plates at 1×10^5 cells/well were fixed by 4% paraformaldehyde (P395744, Aladdin, Shanghai, China) for 10 min. After rinsing, RAW264.7 cells were incubated with the primary antibodies against CD11b (Alexa Fluor® 647, ab322209, 1:500, Abcam, Cambridge, UK), CD86 (Alexa Fluor® 488, ab290990, Abcam, UK) and CD206 (Alexa Fluor® 488, ab313398, 1:500, Abcam, UK) overnight at 4 ℃ and further incubated with the secondary antibody against rabbit IgG (1:2000, ab6708, Abcam, UK) for 1 h at room temperature. After staining the nuclei with DAPI solution (ab285390, Abcam, UK), the cells were visualized under a confocal laser microscope (LSM800, Zeiss, Oberkosen, Germany) and the MFI of *CD86* and *CD206* was calculated accordingly.

Quantitative real-time PCR (qRT-PCR)

The total RNA was extracted by RNA extractor kit (R0016, Beyotime, China) and the concentration was tested using a spectrophotometer (NanoDrop 2000, ThermoFisher, Waltham, Massachusetts, USA). The cDNA was synthesized using corresponding cDNA synthesis kit (1708896, Bio-Rad Laboratories, Inc., USA), and the q-PCR was performed using SYBR® Green qPCR Master Mix (1176202K, Invitrogen, USA) in CFX96 real-time PCR System (Bio-Rad Laboratories, Inc., USA). The relative mRNA expression was calculated using $2^{-\Delta\Delta ct}$, with GAPDH as the reference gene [33]. The primers used are presented in Table 2.

Co-immunoprecipitation (co-Ip) assay

The interaction relationships among *HMGB1*, *IGFBP3*, and DNA–PKcs proteins were analyzed by adopting co-Ip method [34]. Briefly, the cells were lysed by a commercial RIPA lysis buffer (P0013B, Beyotime, China) at 4 ℃ for 1 h. The homogeneous protein G-agarose beads (#37478, Cell Signaling Technology, Danvers, Massachusetts, USA) were used to reduce the non-specific binding. The antibodies against HMGB1 (ab18256, 1:2000, Abcam, UK), IGFBP3 (ab220429, 1:2000, Abcam, UK) and DNA–PKcs (ab32566, 1:2000, Abcam, UK) and the rabbit IgG antibody (ab313801, 1:2000, Abcam, UK) were incubated with the cell lysate at 4 ℃ overnight. Thereafter, the lysate with the antibodies were additionally incubated with 50 μL beads for another 10 h, followed by rinsing the beads in 1 mL lysis buffer. Denaturation

Table 2 Sequences of primers for qPCR

Gene	Primers (5′–3′)
HMGB1 forward	ATGTCTGCTAAAGAGAAAGG
HMGB1 reverse	ATACTCAGAGCAGAAGAGGA
CDH1 forward	GATGATGTGAACACCTACAA
CDH1 reverse	GTAGCTATGATTAGGGCTGT
CDH2 forward	TTTACAGTGCAGTCTTATCG
CDH2 reverse	CTTGGGAACACTATTTCTTC
VIM forward	GTCAGCAATATGAAAGTGTG
VIM reverse	GTCTTGGTAGTTAGCAGCTT
IGFBP3 forward	AGAGAAATGGAAGACACACT
IGFBP3 reverse	CATACTTATCCACACACCAG
GAPDH forward	CCTTCATTGACCTCAACTAC
GAPDH reverse	GAGGCTGTTGTCATACTTCT

was performed using 2 × SDS buffer (P0013G, Beyotime, China) at 100 ℃ for 20 min for subsequent immunoblotting assay [35].

Immunoblotting

To measure the expressions of *HMGB1* and *IGFBP3* proteins using Western Blot, the total protein was first separated by the RIPA lysis buffer and the concentration of the protein was quantified using a bicinchoninic acid assay kit (P0011, Beyotime, China). Subsequently, the proteins were separated via electrophoresis and electroblotted onto the PVDF membrane (FFP33, Beyotime, China). After incubating the membrane with 5% defatted milk at room temperature for 2 h, the primary antibodies against HMGB1, IGFBP3 and housekeeping control GAPDH (ab181602, 1:10000, Abcam, UK) were added to react with the membrane at 4 ℃ overnight. Subsequently, the corresponding secondary antibody against rabbit IgG (A0208, 1:1000, Beyotime, China) was applied to incubate the membranes at ambient temperature for 2 h. After rinsing, the membrane was exposed to the ECL visualization reagent (P0018S, Beyotime, China) to develop protein bands. Finally, the densitometry analysis was performed employing ImageJ 1.42 (Bio-Rad Laboratories, Inc., USA).

Statistical analyses

All the data from independent triplicates were analyzed using GrpahPad Prism 8 (GraphPad, LLC., La Jolla, California, USA) and displayed as mean ± standard deviation. Student's *t* test was applied to compare two-group differences, comparison among more than 2 groups was determined using one-way or two-way ANOVA test followed by Dunnett's multiple comparisons test. and the differences were considered statistically significant when p value < 0.05.

Results

The *HMGB1* expression in ESCC

First, the data on the effects of high and low expressions of *HMGB1* on the survival of ESCC patients were downloaded. It was found that high-expressed *HMGB1* was indicative of an unfavorable prognosis of ESCC (Fig. 1a, $p < 0.05$). Subsequently, the mRNA expression of *HMGB1* in HEEC and ESCC cell lines was quantified. The results showed remarkably higher mRNA expression of *HMGB1* in ESCC cells (KYSE-140, KYSE-150, and KYSE-410) in the control group (HEEC group) (Fig. 1b–d, $p < 0.05$). However, no significant difference in the mRNA expression of *HMGB1* between ESCC cells (KYSE-510) and the control group (HEEC group) was detected (Fig. 1e, $p > 0.05$). The immunoblotting results also demonstrated higher levels of HMGB1 protein in ESCC cells (KYSE-140, KYSE-150, KYSE-410, and KYSE-510) (Fig. 1f, g, $p < 0.05$).

Considering that *HMGB1* showed the highest expression in KYSE-410 cell line and the lowest expression in the KYSE-510 cell line, these two cell lines were used for subsequent knockout and overexpression experiments, respectively. It was observed that after knocking out *HMGB1*, the expression of *HMGB1* in KYSE-410 was significantly inhibited (Fig. 1 h, i, j, $p < 0.05$). Conversely, after overexpressing *HMGB1*, the expression of *HMGB1* in KYSE-510 was remarkably elevated (Fig. 1k, l, $p < 0.05$).

The effects of *HMGB1* on the proliferation and metastasis of ESCC cells

Subsequently, the potential effects of *HMGB1* on the proliferation and metastasis of ESCC cells were investigated using CCK-8 and EdU staining assays. According to the results, silencing *HMGB1* reduced the proliferation of ESCC cells KYSE-410 (Fig. 2a, b, $p < 0.05$), while overexpressed *HMGB1* enhanced the proliferation of ESCC cells KYSE-510 (Fig. 2c, d, $p < 0.05$). The quantification of the metastasis-related proteins cadherins (CDHs, including *CDH1* and *CDH2*) and Vimentin (VIM) demonstrated that the knockdown of *HMGB1* elevated the mRNA level of *CDH1* but inhibited that of *CDH2* and *VIM* in ESCC cells KYSE-410 (Fig. 2e, $p < 0.05$); however, the overexpression of *HMGB1* showed the opposite results (Fig. 2f, $p < 0.05$).

The effects of *HMGB1* on the macrophage polarization

After determining the MFI of *CD86* (M1 macrophage marker) and CD206 (M2 macrophage indicator) employing immunofluorescence assay, the effects of *HMGB1* on the macrophage polarization were examined. It was observed that silencing *HMGB1* enhanced the MFI of CD86 and suppressed the MFI of CD206 (Fig. 3a, b, $p < 0.01$) in KYSE-410 cells, while the overexpression of *HMGB1* suppressed the MFI of CD86 but promoted that of CD206 (Fig. 3c, d, $p < 0.05$) in KYSE-510 cells. The above results indicated that *HMGB1* maybe promote the polarization of M1 cells into M2 cells.

Interaction between *HMGB1* and *IGFBP3/DNA–PKcs* complex in ESCC cells

The interaction between *HMGB1* and *IGFBP3/DNA–PKcs* complex in ESCC cells was explored. The result from co-Ip assay showed that *IGFBP3/DNA–PKcs* had a strong affinity for *HMGB1* in ESCC cells (Fig. 4a, b), which proved the interaction between *HMGB1* and *IGFBP3/DNA–PKcs* complex in ESCC cells.

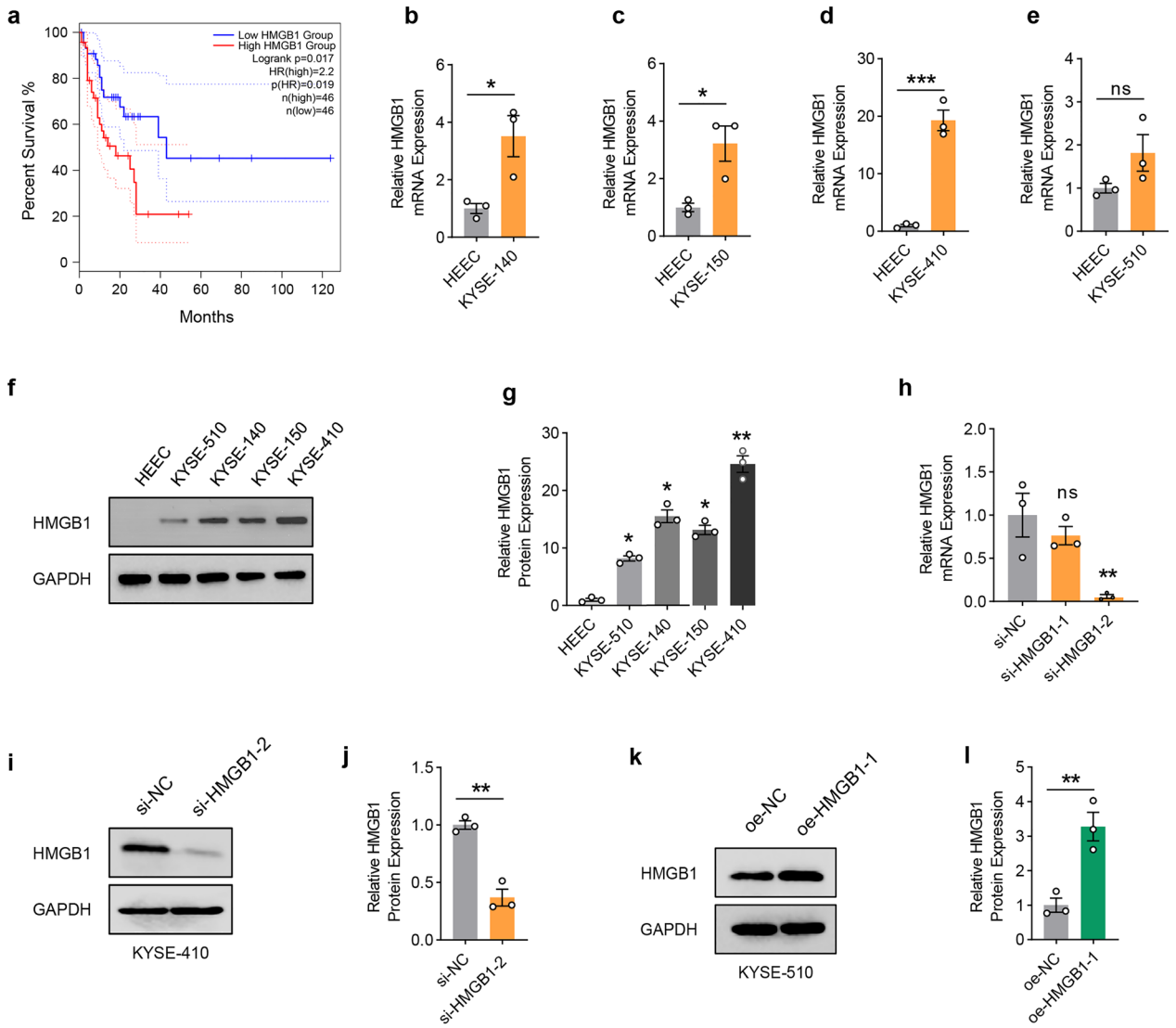

Fig. 1 Quantified *HMGB1* expression in ESCC. (**a**) Effects of high/low *HMGB1* expression on the survival of ESCC patients. **b**–**e** Quantified *HMGB1* mRNA level in human esophageal epithelial cells (HEEC) and ESCC cell lines (**b**) KYSE-140, (**c**) KYSE-150, (**d**) KYSE-410 and (**e**) KYSE-510. **f**, **g** Quantified HMGB1 protein level in HEEC cells as well as ESCC cell lines (KYSE-140, KYSE-150, KYSE-410 and KYSE-510) via immunoblotting. **h** Validation on the knockdown efficiency of *HMGB1*-specific siRNA. **i**, **j** Validation on the knockdown efficiency on *HMGB1* gene in ESCC cells KYSE-410. **k**, **l** Validation on the overexpression efficiency on *HMGB1* gene in ESCC cells KYSE-510. The si-HMGB1-1 means knockdown *HMGB1* in ESCC cells KYSE-510, si-HMGB1-2 means knockdown *HMGB1* in ESCC cells KYSE-410. In addition, oe-HMGB1-1 means overexpression of *HMGB1* in ESCC cells KYSE-510. All data of three independent tests were expressed as mean ± standard deviation and the data of linked group were compared with statistical significance shown as the asterisks (ns means $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

Effects of *IGFBP3* on the proliferation of ESCC cells

The effects of *IGFBP3* on the proliferation of ESCC cells were also examined, and the data related to the effects of high and low *IGFBP3* expression on ESCC survival were downloaded. It was fond that a high *IGFBP3* level was related to a more favorable ESCC prognosis (Fig. 5a, $p < 0.05$). Moreover, the expression of the IGFBP3 protein in ESCC cells KYSE-410 and KYSE-510 was relatively higher than in the control group (HEEC cells group) (Fig. 5b, c, $p < 0.01$).

The expression of IGFBP3 was relatively the most distinctly different in KYSE-510 cell line and the least different in KYSE-410 cell line between the control group and the experimental group. Thus, KYSE-510 cell line and KYSE-410 cell line were used for knockout and overexpression experiments, respectively. The

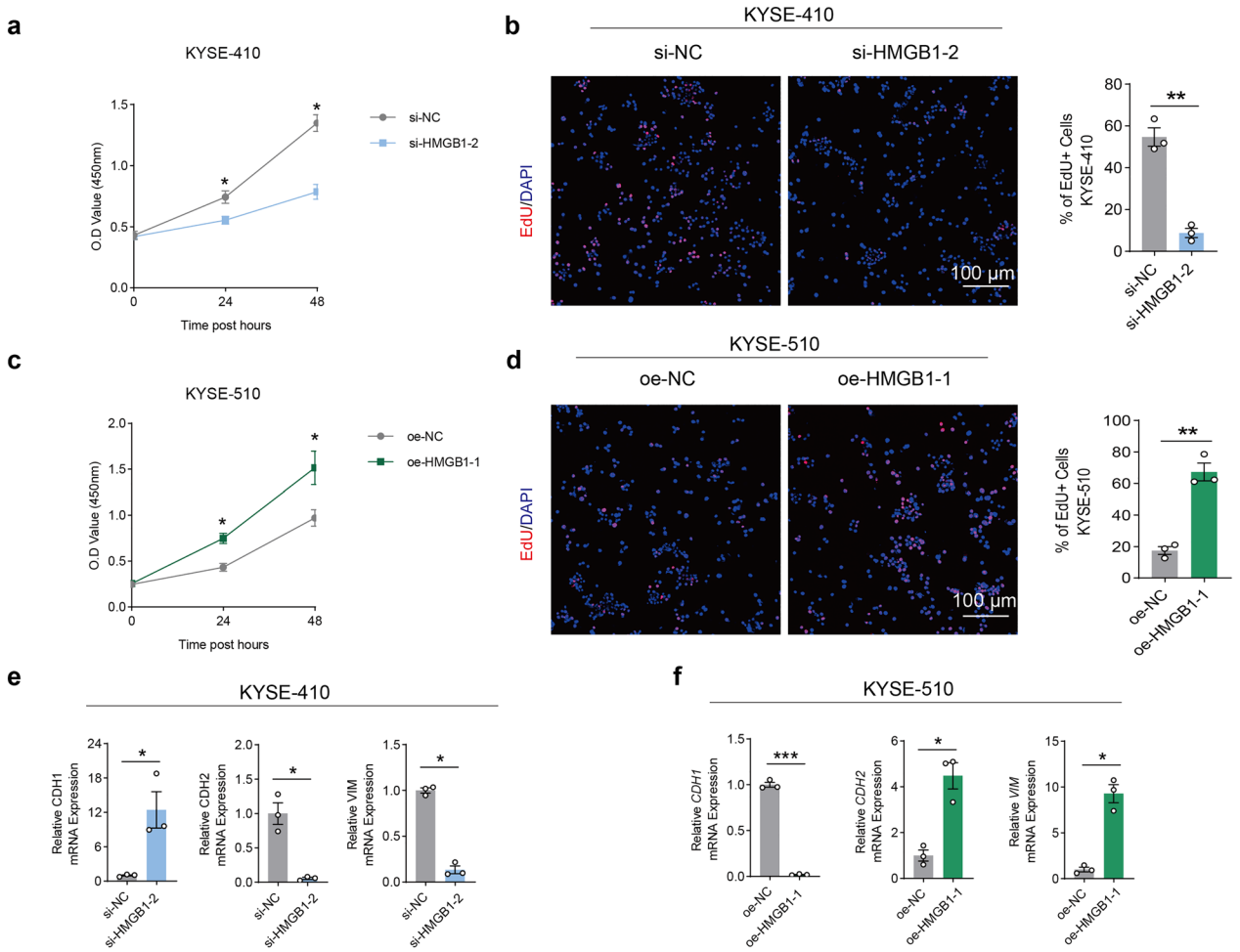

Fig. 2 Effects of *HMGB1* on the proliferation and metastasis of ESCC cells. **a**, **b** Effects of *HMGB1* silencing on the proliferation of ESCC cells KYSE-410 (scale bar: 100 μm). **c**, **d** Effects of *HMGB1* overexpression on the proliferation of ESCC cells KYSE-510 (scale bar: 100 μm). **e** Quantified mRNA levels of metastasis-related mediators in ESCC cells KYSE-410. **f** Quantified mRNA levels of metastasis-related mediators in ESCC cells KYSE-510. All data of three independent tests were expressed as mean ± standard deviation and the data of linked group were compared with statistical significance shown as the asterisks ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$)

results demonstrated that after knocking out *IGFBP3* (Fig. 5d, e, $p<0.01$), the cell viability and cell proliferation of KYSE-510 cells were notably enhanced (Fig. 5h–j, $p<0.05$). However, after overexpressing *IGFBP3* (Fig. 5f, g, $p<0.01$), the cell viability and cell proliferation ability of KYSE-410 were significantly reduced (Fig. 5k–m, $p<0.05$).

Interaction between *IGFBP3* and *HMGB1* on the proliferation of ESCC cells

First, we quantified the mRNA level of *HMGB1* following the silencing of *IGFBP3*. The results demonstrated that silencing *IGFBP3* elevated *HMGB1* level in ESCC cells KYSE-510 (Fig. 6a, $p<0.05$). The cell proliferation test showed that the co-silencing of *IGFBP3* and *HMGB1* further suppressed the proliferation of KYSE-510 cells (Fig. 6b, c, $p<0.05$). At the same time, co-silencing of *IGFBP3* and *HMGB1*upregulated the mRNA level of *CDH1* and downregulated that of *CDH2* in ESCC cells (Fig. 6d, $p<0.05$).

However, overexpressed *IGFBP3* downregulated the mRNA level of *HMGB1* (Fig. 6e, $p<0.05$), and co-overexpression of *IGFBP3* and *HMGB1* increased the proliferation, upregulated the mRNA level of *CDH2* and downregulated that of *CDH1* in KYSE-410 cells (Fig. 6f–h, $p<0.05$).

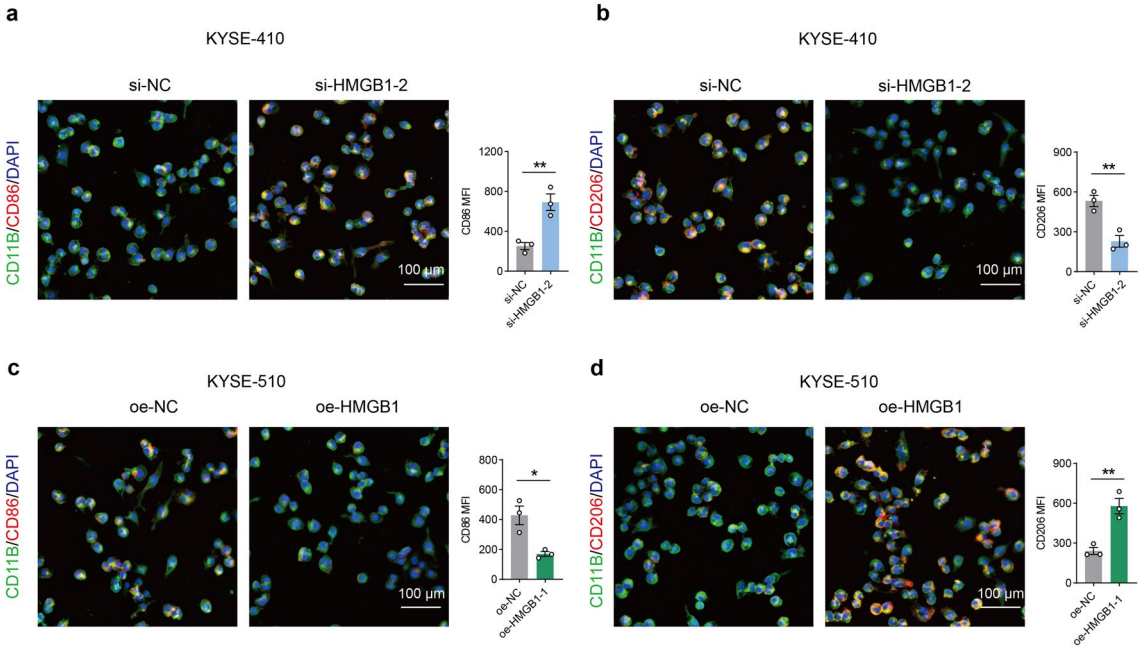

Fig. 3 Effects of *HMGB1* on the macrophage polarization. **a**, **b** Quantified mean fluorescence intensity of CD86 (**a**) and CD206 (**b**) following the silencing of *HMGB1*. **c**, **d** Quantified mean fluorescence intensity of CD86 (**a**) and CD206 (**b**) following the overexpression of *HMGB1*. All data of three independent tests were expressed as mean ± standard deviation and the data of linked group were compared with statistical significance shown as the asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$)

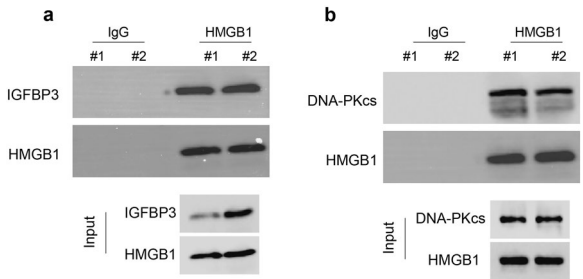

Fig. 4 Interplay between *HMGB1* and *IGFBP3/DNA–PKcs* complex in ESCC cells. **a**, **b** Co-immunoprecipitation assay was applied to explore the interplay between *HMGB1* and *IGFBP3/DNA–PKcs* complex in ESCC cells

Interplay between *IGFBP3* and *HMGB1* on the macrophage polarization

Finally, we determined the interaction between *IGFBP3* and *HMGB1* on macrophage polarization. Based on the results of immunofluorescence assay, the co-silencing of *IGFBP3* and *HMGB1* enhanced the MFI of *CD86* and reduced the MFI of *CD206* (Fig. 7a, b, $p < 0.01$). However, a higher MFI of *CD206* and a lower MFI of *CD86* were observed in the macrophages following the overexpression of *IGFBP3* and *HMGB1* (Fig. 7c, d, $p < 0.01$).

Discussion

HMGB1 functions crucially in the tumorigenesis of diverse tumors. Study observed that *HMGB1* released from gasdermin E (*GSDME*)-mediated pyroptotic epithelial cells is involved in the tumorigenesis of colorectal cancer related to colitis [36]. Moreover, *HMGB1* can form a feedback loop between tumor-associated macrophages (TAMs) and osteosarcoma cells, thereby promoting osteosarcoma cell migration and invasion [37]. In digestive cancers, an elevated expression of *HMGB1* can enhance tumor cell proliferation during EMT process and accelerate tumor vascular network formation [38–42]. Disruption of normal expression and cellular signaling pathways that involve genes with cancer-promoting or cancer-suppressive effects will lead to tumorigenesis [43]. The current study confirmed that *HMGB1* knockdown suppressed the proliferation and promoted the macrophage M1 polarization in ESCC via the *IGFBP3*/*DNA–PKcs* complex. The oncogenic effects of *HMGB1* on ESCC was additionally evidenced by decreased levels of *CDH2* and VIM and increased level of *CDH1*.

Silencing of *HMGB1* led to an upregulated expression of *CDH1* and a downregulated expression of *CDH2*. *CDH1* is a tumor-suppressive gene that encodes E-cadherin, a crucial protein in adherens junctions. Loss of the function of E-cadherin is contributes to in the progression of a variety of cancers, including gastric cancer [44].

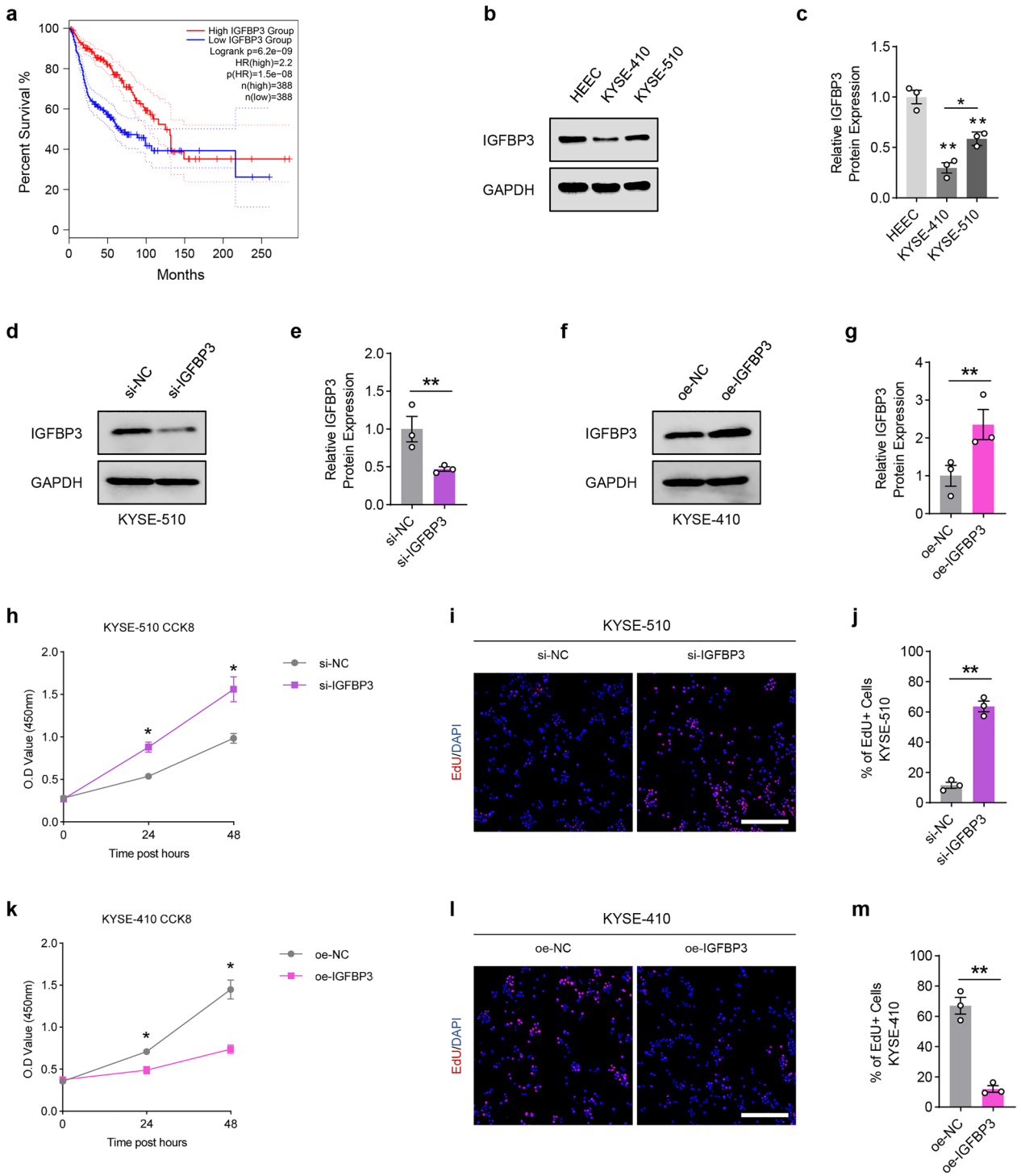

Fig. 5 Effects of *IGFBP3* on the proliferation of ESCC cells. **a** Effects of high/low *IGFBP3* expression on the survival of ESCC patients. **b**, **c** Quantified IGFBP3 protein level in HEEC cells as well as ESCC cell lines KYSE-410 and KYSE-510. **d**, **e** Validation on the knockdown efficiency on *IGFBP3* gene in ESCC cells KYSE-510. **f**, **g** Validation on the overexpression efficiency on *IGFBP3* gene in ESCC cells KYSE-410. **h**–**j** Effects of *IGFBP3* silencing on the proliferation of ESCC cells KYSE-510 (scale bar: 100 μm). **k**–**m** Effects of *IGFBP3* overexpression on the proliferation of ESCC cells KYSE-410 (scale bar: 100 μm). All data of three independent tests were expressed as mean ± standard deviation and the data of linked group were compared with statistical significance shown as the asterisks (ns means $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

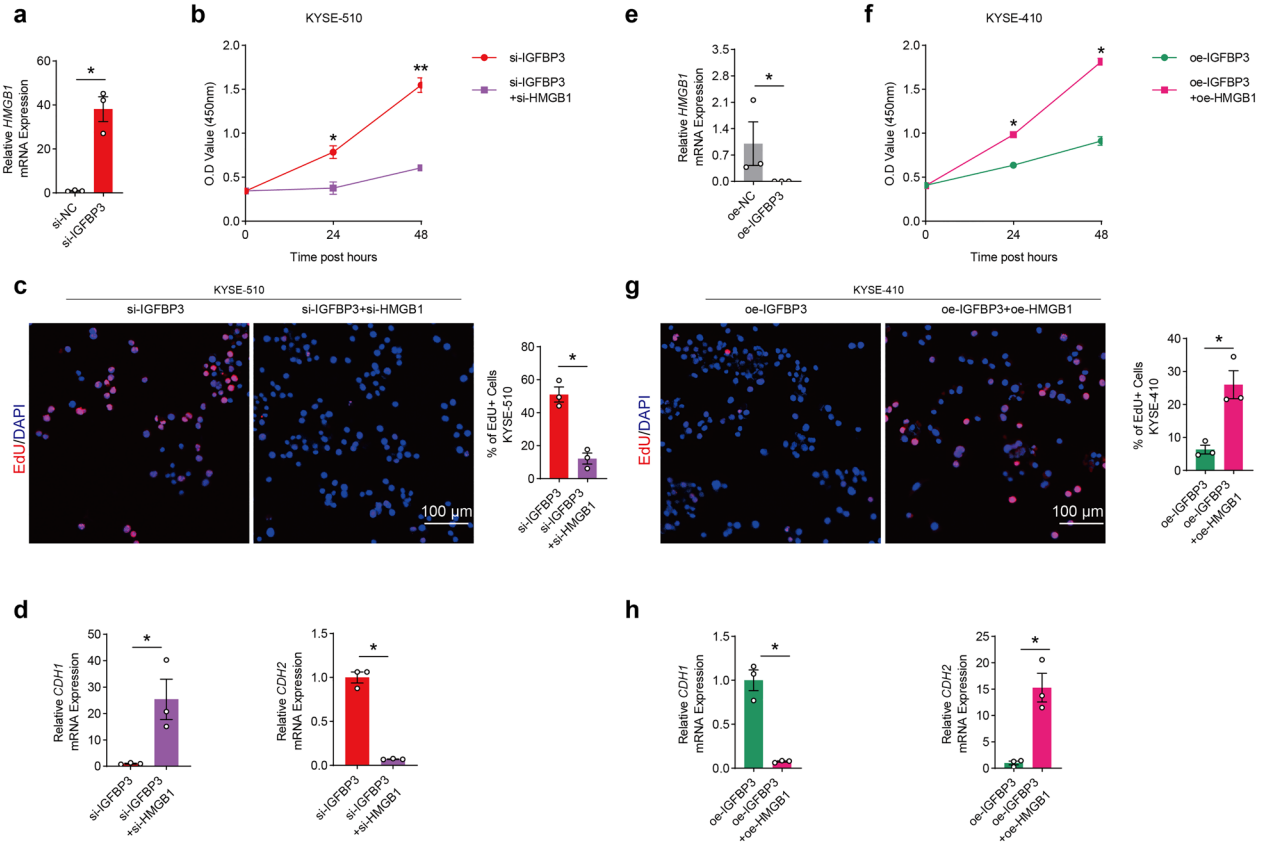

Fig. 6 Effects of *IGFBP3* and *HMGB1* on the proliferation of ESCC cells. **a** Effects of *IGFBP3* silencing on the mRNA level of *HMGB1* in ESCC cells KYSE-510. **b**, **c** Effects of *IGFBP3* and *HMGB1* silencing on the proliferation of ESCC cells KYSE-510 (scale bar: 100 μm). **d** Quantified mRNA levels of metastasis-related mediators in ESCC cells KYSE-510. **e** Effects of *IGFBP3* overexpression on the mRNA level of HMGB1 in ESCC cells KYSE-510. **f**, **g** Effects of *IGFBP3* and *HMGB1* overexpression on the proliferation of ESCC cells KYSE-510 (scale bar: 100 μm). **h** Quantified mRNA levels of metastasis-related mediators in ESCC cells KYSE-410. All data of three independent tests were expressed as mean ± standard deviation and the data of linked group were compared with statistical significance shown as the asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$)

In ESCC, a lower expression level of *CDH1* is linked to a worse prognosis [45]. *CDH2* is an oncogene that encodes N-cadherin, which typically promotes tumor progression [46]. In epithelial tumors, cells need to acquire motility for metastasis, a process known as EMT [47]. EMT transforms polarized epithelial cells into motile mesenchymal phenotypes and is frequently activated during tumor invasion and metastasis, contributing to the early dissemination of cancer cells [48]. The downregulation of *CDH1* and the upregulation of *CDH2* and *VIM* are the hallmarks of EMT [49]. Collectively, these findings suggested that overexpression of *HMGB1* may trigger EMT, further confirming the association between *HMGB1* and a poor prognosis in ESCC.

The heterogeneity in TME is linked to the therapeutic failure and varied prognostic outcomes of ESCC patients [50]. TAMs are the most predominant immune cells in the TME and can be polarized into M1 type (known for their v and phagocytosis effects) or M2 type (which support tumor progression by enhancing tumor cell survival and fostering an immunosuppressive environment) [51, 52]. In this study, following the co-culture of macrophages with *HMGB1*-silenced ESCC cells, we observed an increase in the MFI of *CD86* and a decrease in that of CD206. As surface markers, *CD86* and *CD206* can be used to label M1 and M2 macrophages, respectively [53]. Previous studies showed that in the cancer tissues of ESCC patients, a high infiltration of M2 macrophages (including CD206 + macrophages) is significantly associated with a poor prognosis in EC patients [54, 55]. M2 macrophages can promote the growth, invasion, and migration of EC cells and create a microenvironment conducive to tumor progression by secreting various cytokines and growth factors (such as *FGL2*, *IL-10*, *MMP9*, etc.) [56]. As a co-stimulatory molecule, the expression of *CD86* in ESCC tissues is significantly lower than that in the mucosa of healthy patients [57]. The results indicated that *HMGB1* may promote the

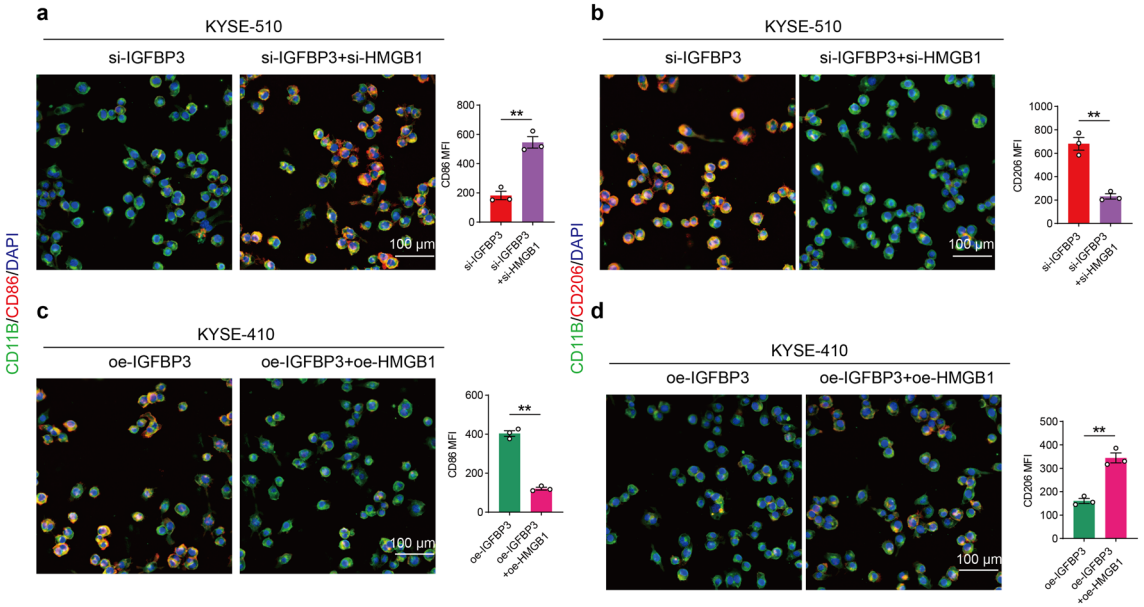

Fig. 7 Interplay between IGFBP3 and HMGB1 on the macrophage polarization. **a**, **b** Quantified mean fluorescence intensity of CD86 (**a**) and CD206 (**b**) following the knockdown of *IGFBP3* and *HMGB1*. **c**, **d** Quantified mean fluorescence intensity of CD86 (**c**) and CD206 (**d**) following the overexpression of *IGFBP3* and *HMGB1*. All data of three independent tests were expressed as mean ± standard deviation and the data of linked group were compared with statistical significance shown as the asterisks ($^{**}p < 0.01$)

polarization of M1 cells into M2 cells, thereby driving the development of EC. However, such a conclusion requires further verification in clinical trials.

Downstream pathways, including extracellular signal-regulated kinases 1/2 pathway, nuclear factor-kappa B pathway, and Wnt/beta-catenin pathway, have been found to be involved in the role of *HMGB1* in the malignant phenotypes of tumor cells [58–60]. The current study observed that the silencing of *IGFBP3* promoted the level of *HMGB1* in ESCC cells. *IGFBP3* is an uncommon secreted proteins than can enter the nucleus, acting as a signal peptide and a nuclear localization signal, with varied expression levels between different cell lines and tumor tissues [61]. The research results showed that *IGFBP3* acted in a way opposite to *HMGB1*. *IGFBP3* can reduce the proliferation and viability of ESCC cells. Downregulated *IGFBP3* expression might be linked to advanced clinical stages of ESCC and poor prognosis [62].

Therefore, we further investigated the joint impact of *HMGB1* and *IGFBP3* in ESCC. It was observed that compared to the knockout of *IGFBP3*, a simultaneous knockout of *IGFBP3* and *HMGB1* l resulted in a more pronounced decrease in cell proliferation and viability. In the co-knockout group, upregulated *CDH1* and downregulated *CDH2* and *VIM* indicated that the cell motility and invasive characteristics of ESCC cells were inhibited. As for cell polarization, the increase in CD86 and decrease in CD206 in the co-knockout group suggested that their combined action enhanced the polarization effect of M2 cells, further confirming the impact of *HMGB1* on the polarization of ESCC cells. Hence, we have demonstrated an affinity between *HMGB1* and the *IGFBP3/DNA–PKcs* complex in ESCC cells. In addition, a previous study revealed that *IGFBP3* is a substrate of *DNA–PKcs* and can transactivate epidermal growth factor receptor (*EGFR*), forming a complex termed *IGFBP3/EGFR/DNA–PKc* [63]. *DNA–PKcs* plays an important role in DNA damage and cell-cycle progression and can regulate the activity of AKT by phosphorylating the Ser473 site of AKT [64]. Both the PI3K/Akt signaling pathway and *DNA–PKcs* are key components of the DDR in influencing the development of various cancers [65]. Collectively, these findings suggested that this complex was highly likely to be involved in the regulation of key processes in ESCC cells via *IGFBP3* and through activating relevant downstream pathways. However, the specific molecular mechanisms require further investigation. Importantly, the role of this complex was closely related to *HMGB1*, and an antagonistic effect may exist between them.

This study revealed an affinity between *HMGB1* and the *IGFBP3/EGFR/DNA–PKcs* complex formed with *IGFBP3*. *HMGB1* and *IGFBP3* may jointly participate in regulating some key processes of ESCC cells, such as cell proliferation, motility, and invasion. This provided a new perspective for further exploring the pathogenesis

of ESCC, helping to improve the understanding of tumor cell behaviors. In addition, we elucidated the mechanism through which *HMGB1* promoted the polarization of M1 macrophages into M2 macrophages, a process driving the development of ESCC. By modulating HMGB1, we might control macrophage polarization, opening avenues for novel treatment strategies in ESCC. Considering the correlation between high infiltration of M2 macrophages and a worse prognosis of ESCC patients, measuring the level of *HMGB1* in ESCC patients could potentially facilitate the prognostic assessment of the patients and the development of the treatment plans.

It should be equally noted that the present experiment had certain limitations. Our research primarily relied on cell lines, which provide a simplified environment that cannot fully replicate the complexity of physiological conditions. Although we confirmed that *HMGB1* and *IGFBP3* had antagonistic effects on ESCC cell proliferation and macrophage polarization, in vivo animal experimental models should be used to observe the effects of *HMGB1* and *IGFBP3* on ESCC mice to further confirm the mechanisms of *HMGB1* and *IGFBP3* in the cancer. In addition, a lack of clinical trial currently prevents a direct translation of these findings to patient care. Our future study will include clinical trials to bridge this gap, facilitating the transition from research to clinical practice. Moreover, the detailed interaction mechanism between the *IGFBP3/EGFR/DNA–PKcs* complex and *HMGB1* and how *HMGB1* induced macrophage polarization in ESCC cells to promote ESCC progression require further verification through in vivo experiments and clinical trials.

Conclusion

To conclude, through in vitro experiments, our current research analyzed the roles of *HMGB1* and *IGFBP3*/*DNA–PKcs* complex in esophageal carcinogenesis. These findings improved the understanding on the mechanism of *HMGB1* and *IGFBP3*/*DNA–PKcs* in ESCC, potentially informing clinical management of ESCC. Clinically, the current discoveries can guide the treatment plans based on the expression levels of these molecules in individual patients, ultimately contributing to the overall treatment of ESCC and patients' prognosis.

Abbreviations

EC	Esophageal cancer
EAC	Esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
DDRs	DNA damage responses
TME	Tumor microenvironment
DNA–PKcs	DNA-dependent protein kinase catalytic subunit
IGFBP3	Insulin-like growth factor binding protein 3
HMGB1	High mobility group box 1
TCGA	The Cancer Genome Altas
HEEC	Human esophageal epithelial cells
RPMI	Roswell Park Memorial Institute
FBS	Fetal bovine serum
siRNAs	Small interfering RNAs
si-NC	Negative control siRNAs
CCK-8	Cell counting kit-8
OD	Optical density
EdU	5-Ethynyl-2′-deoxyuridine
DAPI	4′,6-Diamidino-2-phenylindole
MFI	Mean fluorescence intensity
q-PCR	Quantitative real-time PCR
cDNA	Complementary DNA
co-Ip	Co-immunoprecipitation
CDHs	Cadherins
VIM	Vimentin
GSDME	Gasdermin E
EMT	Epithelial–mesenchymal transition
TAMs	Tumor-associated macrophages
EGFR	Epidermal growth factor receptor

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Author contributions
All authors contributed to this present work: [LLL] & [SXX] designed the study, [CJ] & [LLL] acquired the data. [LLL] & [SXX] drafted the manuscript, [LLL], [SXX] and [CJ] revised the manuscript. I certify that all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The raw data is available upon reasonable request from the corresponding author Songxi Xie.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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