Lysine-Specific Histone Demethylase 1 Promotes Oncogenesis of the Esophageal Squamous Cell Carcinoma by Upregulating DUSP4

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Abstract—Esophageal squamous cell carcinoma (ESCC) is a predominant subtype of esophageal cancer (EC) and has a poor prognosis due to its aggressive nature. Accordingly, it is necessary to find novel prognostic biomarkers and therapeutic targets for ESCC. Lysine-specific histone demethylase 1 (LSD1) plays a core role in the regulation of ESCC oncogenesis. However, the detailed mechanism of LSD1-regulated ESCC growth has not been elucidated. This study aims to explore molecular mechanism underlying the LSD1-regulated ESCC's oncogenesis. After LSD1 silencing, we detected differentially expressed genes (DEGs) in human ESCC cell line, TE-1, by transcriptome sequencing. Subsequently, we investigated expression pattern of the selected molecules in the ESCC tissues and cell lines by qRT-PCR and Western blotting. Furthermore, we explored the roles of selected molecules in ESCC using gene silencing and overexpression assays. Transcriptome sequencing showed that the expression of dual specificity phosphatase 4 (DUSP4) in TE-1 was significantly attenuated after the *LSD1* silencing. In addition, the *DUSP4* mRNA expression level was significantly higher in the ESCC tissues, especially in those derived from patients with invasion or metastasis. Moreover, the DUSP4 expression was positively associated with the LSD1 expression in the ESCC tissues. DUSP4 overexpression promoted proliferation, invasion, and migration of the ESCC cells, while *DUSP4* silencing had an opposite effect. DUSP4 overexpression also enhanced tumorigenicity of the ESCC cells *in vivo*, while DUSP4 silencing inhibited tumor growth. Importantly, inhibition of cell proliferation, invasion, and migration by the LSD1 inhibitor (ZY0511) was reversed by DUSP4 overexpression. Conclusively, we found that LSD1 promotes ESCC's oncogenesis by upregulating DUSP4, the potential therapeutic and diagnostic target in ESCC.

INTRODUCTION

As the 8th most common malignancy and one of the main digestive tract tumors, esophageal cancer (EC) is characterized mainly by high invasiveness and poor prognosis, which poses a great threat to public health [1, 2]. Esophageal squamous cell carcinoma (ESCC) is the main subtype of EC. Clinical outcome for ESCC is often poor

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because of its malignant behavior such as the invasive attribute and lymph node metastasis [3]. Accordingly, improving therapeutic efficacy and prognosis of patients with ESCC is a major focus of oncological research.

The lysine-specific histone demethylase 1 (LSD1) is a nuclear histone demethylase that is a member of the amine oxidase (AO) family. LSD1 is a flavin-containing AO that catalyzes demethylation of histone H3 lysine 4 through FAD-dependent oxidation. LSD1 is involved in embryonic differentiation, proliferation of pluripotent stem cells, and HIV infection [4-6]. It is known that the expression of LSD1 is increased in a variety of cancer tissues and contributes greatly to oncogenesis of a variety of tumors [7-10]. Moreover, the expression of LSD1 in ESCC and its effect on proliferation, invasion, and migration of ESCC have been reported in many studies. Yu et al. revealed that the expression of LSD1 increased in the ESCC cancer tissues, which was associated with the lymph node metastases and poor survival in ESCC patients [11]. An *in vitro* study also found that the LSD1 expression is dysregulated in the invasive ESCC cell lines, and gene silencing and pharmacological inhibition of LSD1 inhibit migration and invasion of the ESCC cells [11]. Previous studies also confirmed growth inhibition and induction of apoptosis by the LSD1 inhibitor in ESCC cells [12, 13]. Similar findings were also reported in other investigations [14, 15]. Taken together, it makes clear that LSD1 contributes greatly to the diagnosis and treatment of ESCC. Hou et al. showed that LSD1 plays a positive role in regulating Notch signaling and PI3K/Akt/mTOR pathway by binding to the promoter regions of the related genes in the Notch pathway in the ESCC cells [16]. However, the role of LSD1-regulated pathways in the ESCC growth was not discussed in detail in the above study. Therefore, the detailed mechanism of LSD1-regulated ESCC growth remains unclear.

In this study, we silenced *LSD1* gene in the ESCC cell line TE-1 and found by transcriptome sequencing that the expression level of dual specificity phosphatase 4 (DUSP4) was significantly reduced. Moreover, overexpression of DUSP4 in the ESCC tumor tissues and its effect on the proliferation, function, and tumorigenicity of ESCC cells were also verified. The ability of DUSP4 to compensate for the LSD1 inhibition was also confirmed. Therefore, our study provides the first evidence demonstrating that LSD1 promotes ESCC growth through potential downstream signals, which improved our understanding of the molecular mechanism of LSD1-regulated ESCC growth.

MATERIALS AND METHODS

Cell lines and culture. A human normal esophageal epithelial cell line Het-1A and ESCC cell lines TE-1, TE-13, and KYSE-450 were obtained from the American Type Culture Collection (ATCC). Het-1A was incubated in a Keratinocyte Serum Free Medium (KSFM) (Invitrogen, USA). TE-1, TE-13, and KYSE-450 were incubated in a Roswell Park Memorial Institute (RPMI)-1640 Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). All cells were cultivated in a humidified atmosphere at 37°C with 5% CO_2. A novel LSD1 inhibitor ZY0511 (provided by State Key Laboratory of Biotherapy, Sichuan University, Sichuan, China) was used to treat TE-1 cells at a level of 2 µM [17, 18].

Clinical tissue specimens. A total of 43 pairs of ESCC tissues and adjacent noncancerous tissues were obtained via surgical resection at Fujian Provincial Hospital (Fuzhou, Fujian, China) with written informed consent from the patients. There were 28 male and 15 female patients. The patients had not received chemotherapy and/or radiation therapy prior to the surgery. All tissue specimens were histopathologically examined by three independent pathologists. Fresh tissue specimens were frozen in liquid nitrogen and stored at −80°C until use. This study received all patient consent and was in accordance with the hospital ethical guidelines. The Institutional Review Board of Fujian Provincial Hospital reviewed and approved this study [No. (DL) 2019-01]. All clinical studies were conducted according to the principles expressed in the Helsinki Declaration.

siRNA transfection. Control siRNA and siRNA against human *LSD1* or *DUSP4* were obtained from Invitrogen. The target sequences were as follows:

Control, 5′-UUCUCCGAACGUGUCACGUTT-3′,
LSD1, 5′-CCGGAUGACUUCUCAAGAATT-3′;

Control, 5′-CCCATCCTTCAACGAGCAT-3′,
DUSP4, 5′-CCCAGTACCTTACCAGCAT-3′.

The cells were cultured in 6-well plates and then transfected with siRNA (100 pmol/well) using a RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. After 48 h, endogenous LSD1 or DUSP4 levels were examined by qRT-PCR assays.

Vectors construction and transfection. A DUSP4 expression vector (pCMV-myc-DUSP4) was obtained from BioVector Science laboratory (Beijing, China). The expression vector was stably expressed in TE-1 cells. A pCMV-myc-control was used as a negative control in the experiments. Transfection of the vectors was performed using a Lipofectamine 3000 reagent (Invitrogen) in accordance with the manufacturer's protocol. DUSP4 expression was evaluated by qRT-PCR assays.

RNA sequencing. Total RNA was prepared from approximately 5 million LSD1-silenced TE-1 cells and control cells by using a TRIzol Reagent (Ambion, USA) according to the manufacturer's instructions. Total RNA (2 µg) was subsequently used to prepare RNA-seq library using a NEBNext UltraTM RNA Library Prep Kit from

Illumina (NEB, USA) following the manufacturer's protocol. The library preparations were sequenced with an Illumina Hiseq 4000 platform and paired-end 150 bp reads were generated. Raw data (raw reads) in FASTQ format were processed through in-house Perl scripts. Reads were aligned to release GRCh38 of the human genome using TopHat 2 (v. 2.1.0). Gene expression levels (FPKM, fragments per kilobase of exon model per million mapped reads) were calculated with HTSeq (v. 0.5.4 p3). All downstream statistical analyses, including PCA, were performed with edgeR program package.

Western blotting analysis. Whole-cell lysates of cells with indicated interventions were prepared from 6-well plates. Lysates were loaded onto and separated in a 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes, which were incubated with antibodies against LSD1 (#2139), DUSP4 (#5149), histone H3 di-methylation at lysine 4 (H3K4me2, #9725), and GAPDH (#5174) (Cell Signaling Technology, USA). Horseradish peroxidase-linked secondary antibodies (#3999) were used to visualize immunoreactivity using a chemiluminescence system (Amersham Image 600, General Electric, USA).

Quantitative real-time PCR (qRT-PCR) assay. Total RNA was extracted and purified by the TRIzol method. Synthesis of cDNA and qRT-PCR measurements were carried out as described previously [19]. The designed primer sequences for qRT-PCR were as follows:

LSD1, 5′-CCAGAGATATTACTGCCGAGTT-3′ (sense) and 5′-GCTTTCCTTGTGTTTCAGCTAA-3′ (anti-sense);

DUSP4, 5′-CTACATCCTAGGTTCGGTCAAC-3′ (sense) and 5′-TAGACGATGACCGCCGAGTA-3′ (anti-sense);

β-actin, 5′-GGGAAATCGTGCGTGACATTAAG-3′ (sense) and 5′-TGTGTTGGCGTACAGGTCTTTG-3′ (anti-sense).

Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

qRT-PCR was performed using a SYBR Premix Ex TaqTM kit (TakaRa, Japan) and an ABI7500 PCR system (Applied Biosystems, Thermo Fisher Scientific, USA).

Measurement of cell viability. To evaluate cell proliferation, cell counting Kit-8 (CCK-8) was used. For CCK-8 assay, cells with the indicated interventions were plated into 96-well plates (2000 cells/well). After the indicated time, the treated cells were incubated with 10 μl of CCK-8 reagent (Dojindo, Japan). Following 2 h incubation, optical density at 450 nm (OD450) was measured with a Varioskan Flash reader (Thermo Fisher Scientific).

Cell invasion assay. The invasion level of TE-1 cells was evaluated by the Transwell migration assay. For the Transwell migration assay, the indicated cells suspended in a serum-free DMEM supplemented with 1 mg/ml mit-omycin C (inhibitor of cell proliferation) and ZY0511 (added according to the experimental design) were seeded into an upper chamber of a Transwell. DMEM containing 20% FBS was added to the lower chamber. After 36 h incubation, the cells migrating into the lower section of the inserts were fixed, stained with 1% crystal violet, and photographed. The invasion levels were measured by counting the number of stained cells.

Cell migration assay. The migration level of TE-1 cells was measured using the scratch test. For the scratch test, cells were seeded in 12-well plates and cultured until 75% confluence. Then, a wound was created by manually scratching the cell monolayer with a 200-microliter pipette tip. The cultures were washed to remove floating cells, and then the adherent cells with indicated interventions were incubated in DMEM containing 1% FBS and ZY0511 (added according to the experimental design). Cell migration into the wound was observed at 0, 36 h for each group. The scratch area was calculated using ImageJ software.

Animal experiments. Four-five-week-old male athymic BALB/c nude mice (20-22.5 g) were obtained from the Animal center of Gem Pharmatech Co., Ltd. (Nanjing, China). DUSP4-siRNA-transfected, DUSP4-vector-transfected, and corresponding control TE-1 cells were inoculated subcutaneously on the ventral side of the right rib at the density of 5×10^6 cells per mouse (6 mice per group). Tumor volumes in all mice were measured every three days to observe the tumor growth. The shortest diameter (A) and the longest diameter (B) were measured with a caliper to determine the tumor volume. The volume (V) was calculated using the formula V = $(A^2 \times B)/2$. After 30 days, all mice were anesthetized with isoflurane (2%, inhalation anesthesia), and then sacrificed via cervical dislocation. All mice were considered dead, when their hearts and breathings stopped. Their tumors were removed and weighted. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Fujian Academy of Medical Sciences. The mice were housed in a specific pathogen-free (SPF) facility with a barrier at temperature 20~30°C and humidity 60~80% and fed a SPF mouse chow and sterile water *ad libitum*.

Statistical analysis. All statistical analyses were performed using the GraphPad Prism Software 6. For comparisons, Wilcoxon rank sum test, one-way ANOVA test, or Student's *t*-test were performed as indicated. Bonferroni test was used for Post Hoc Multiple Comparisons. Pearson correlation analysis and Pearson chi-square test were performed for correlations. $p < 0.05$ was considered as statistically significant.

RESULTS

LSD1 promoted expression of DUSP4 in the ESCC cells. In order to investigate potential mechanisms associ-

ated with the LSD1-regulated ESCC carcinogenesis, we tested the global transcriptional response of the LSD1-silenced TE-1 cells. Therefore, we performed RNA sequencing of the siRNA-control-transfected and siRNA-LSD1-transfected TE-1 cells (Fig. 1a). Transcriptome analysis revealed a large number of differentially expressed genes (DEGs) (Fig. 1, a and b). Compared with the control cells, there were 439 upregulated genes and 69 downregulated genes in the siRNA-LSD1-transfected TE-1 cells (Fig. 1b).

Of note, we found a significantly decreased expression of DUSP4 in the LSD1-silenced TE-1 cells (Fig. 1c and Table 1). The decreased expression of DUSP4 in the LSD1-silenced TE-1 cells was confirmed by Western blotting and qRT-PCR assays (Fig. 1d). Furthermore, in the presence of LSD1 inhibitor, ZY0511, the LSD1 expression was not changed, but the DUSP4 expression was sig-

nificantly decreased (Fig. 1e). Previous studies showed that demethylation of the H3K4me2 could be used to indicate LSD1 activity [20, 21]. It was observed that ZY0511 enhanced the production of H3K4me2 (Fig. 1e), which indicated that the experimental system was reliable. Overall, these results demonstrated the inhibitory effect of LSD1 on DUSP4 expression in ESCC cells.

DUSP4 was overexpressed in the ESCC specimens. DUSP4 levels in 43 pairs of ESCC tissues and adjacent noncancerous tissues were determined using qRT-PCR. As shown in Fig. 2a, DUSP4 was significantly overexpressed in the ESCC tissue specimens compared with the adjacent noncancerous tissues. The analyses of correlation between the DUSP4 levels and clinicopathological characteristics of the ESCC patients revealed that the high expression level of DUSP4 was positively correlated with the larger ESCC tumors and advanced pathological
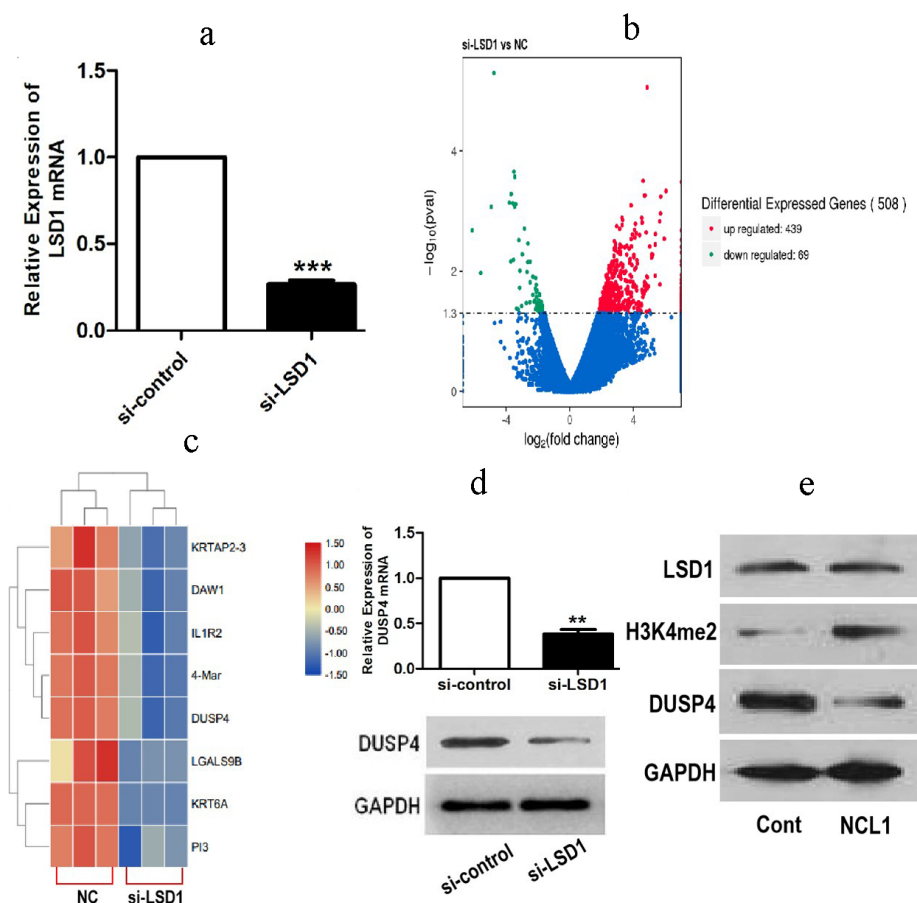

Fig. 1. LSD1 promoted DUSP4 expression in ESCC cells. a) mRNA levels of LSD1 in the TE-1 cells transfected with the indicated siRNAs for 48 h. b) Volcano plot representing differentially expression analysis of the genes based on the results of RNA sequencing of the siRNA-control-transfected and siRNA-LSD1-transfected TE-1 cells ($n = 3$, each group). X-axis shows $\log_2$ fold change in expression, negative $\log_{10}$ of the p-value was plotted on the Y-axis. Each gene was represented by one point on the graph. c) Heat map representing differential expression analysis of several representative genes between the siRNA-control-transfected and siRNA-LSD1-transfected TE-1 cells ($n = 3$, each group). d) mRNA or protein levels of DUSP4 in the siRNA-control-transfected and siRNA-LSD1-transfected TE-1 cells. e) After treatment with or without 2 μM ZY0511 for 48 h, the protein levels of LSD1, H3K4me2, and DUSP4 were evaluated by Western blotting. Results are presented as mean ± SEM from three independent experiments. ** $p < 0.01$, *** $p < 0.001$ by Student's t-test. NC, si-control-transfected cells; Cont, control group.

Table 1. Representative DEGs on transcriptome analysis (ReadCount)

Group	si-Cont-1	si-Cont-2	si-Cont-3	si-LSD1-1	si-LSD1-2	si-LSD1-3	$\log_2$ (FC)	p-value
Downregulated genes								
DUSP4	3360	2597	2840	232	307	585	−3.407	0.000771
KRTAP2-3	118	69	57	4	8	12	−3.8013	0.000728
DAW1	252	151	250	11	21	38	−3.6926	0.000521
IL1R2	302	196	232	12	24	50	−3.595	0.000744
4-Mar	648	522	524	36	54	125	−3.4751	0.000845
KRT6A	1911	1707	1824	84	80	72	−4.7817	5.10E-06
PI3	988	710	658	109	82	31	−3.5168	0.000222
Upregulated genes								
AGXT	1	3	7	120	51	50	4.2234	0.020883
VTCN1	4	6	10	116	394	274	4.8795	8.79E-06
TRANK1	2	10	17	42	119	214	3.1454	0.001990
FOXN1	1	2	1	32	37	19	4.2008	0.002346
ZCCHC12	6	22	19	191	271	105	3.3335	0.001416

Note. FC, Fold Change. si-Cont, siRNA-control-transfected cells; si-LSD1, siRNA-LSD1-transfected cells.

T/N stage (Table 2). Moreover, 18 ESCC specimens with metastases had higher DUSP4 expression than 25 non-metastatic ESCC specimens (Fig. 2b). Furthermore, compared with 21 ESCC specimens with a diameter less than 3 cm, 22 ESCC specimens with diameter more than 3 cm showed higher DUSP4 expression (Fig. 2c). Importantly, correlation analyses between the DUSP4 and LSD1 levels in ESCC tissues revealed that DUSP4 expression levels were positively associated with that of LSD1 in the ESCC tissues (r = 0.8996, p < 0.0001) (Fig. 2d), suggesting the positive effect of LSD1 on DUSP4 expression *in vivo*. Moreover, the DUSP4 mRNA levels and the protein levels of LSD1, DUSP4, and H3K4me2 in the normal esophageal epithelial cell line Het-1A and ESCC cell lines TE-1, TE-13, and KYSE-450 were determined. As shown in Fig. 2, e-i, LSD1 and DUSP4 were significantly overexpressed in the ESCC cell lines. Nevertheless, the change of the level of H3K4me2 expression was opposite to that of LSD1 (Fig. 2, f-i), indicating demethylation of H3K4me2 in the ESCC cell lines, which confirmed the function of LSD1. It is remarkable that the DUSP4 levels were the highest in the aggressive cancer cell line KYSE450 (Fig. 2, f-i), proving contribution of DUSP4 to the malignancy of ESCC.

Table 2. Correlation between DUSP4 and clinicopathological characteristics in ESCC patients

Characteristics	Cases	Cases with corresponding level of DUSP4		p-value
		Low	High	
Total	43	21	22	
Sex				$p = 0.2838$
Male	28	12	16	
Female	15	9	6	
Age				$p = 0.2797$
≥65	20	8	12	
<65	23	13	10	
ESCC diameter				$p = 0.0223$
≥3.0 cm	22	7	15	
<3.0 cm	21	14	7	
Pathological stage (T)				$p = 0.0191$
T2	25	16	9	
T3-T4	18	5	13	
Pathological stage (N)				$p = 0.0075$
N0	30	18	12	
N1-N3	13	3	10	

Note. DUSP4 median expression level was used as the cut-off. p-value was determined by Pearson chi-square test.

Overexpression of DUSP4 promoted proliferation, invasion, and migration of ESCC cells, whereas knockdown of *DUSP4* exhibited opposite effect. To explore the roles of DUSP4 in ESCC oncogenesis, we overexpressed or silenced DUSP4 by transfecting the DUSP4-encoding vector (DUSP4-vector) or siRNA-DUSP4 in the TE-1 cells, respectively (Fig. 3a). CCK-8 assay showed that DUSP4 overexpression enhanced proliferation of the TE-1 cells (Fig. 3b). Also, the results of Transwell migration assay showed that DUSP4 overexpression increased the number of invasive cells. The scratch test showed that DUSP4 overexpression increased the number of migratory cells (Fig. 3, c-f). In contrast, the DUSP4 knockdown exhibited the opposite results (Fig. 3, b-f). Overall,

DUSP4 played a significant role in enhancing proliferation, invasion, and migration of the ESCC cells. We also silenced DUSP4 in the ESCC cell line, KYSE-450, through siRNA transfection (Fig. S1a in the Supplement). As shown in Fig. S1, b-f in the Supplement, DUSP4-silencing KYSE-450 showed a lower level of proliferation, invasion, and migration, further verifying the results presented above.

DUSP4 overexpression promoted tumorigenicity of ESCC cells while DUSP4 silencing showed the reverse effect. The effect of DUSP4 on tumorigenicity of the ESCC cells was also examined *in vivo*. As shown in Fig. 4, a and b, overexpression or silencing efficiency of DUSP4 was measured by detecting mRNA or protein levels. Remarkably, the sizes and weights of tumors

formed by the DUSP4-overexpressing TE-1 in nude mice were significantly larger than those observed in the mice inoculated with the corresponding control TE-1 cells (Fig. 4, c and e). In addition, the increase of tumor volume in the nude mice inoculated with the DUSP4-overexpressing TE-1 was more significant than that of the corresponding control group inoculated with the TE-1 (Fig. 4d). However, compared with the corresponding control TE-1, DUSP4-silenced TE-1 had a weaker tumorigenicity, based on the abovementioned parameters (Fig. 4, c-e). These results suggested that DUSP4 contributed to tumorigenesis of the ESCC cells *in vivo*.

DUSP4 overexpression partially restored proliferation, invasion, and migration reduced by LSD1 inhibitor in the ESCC cells. Can DUSP4 mediate LSD1-regulated
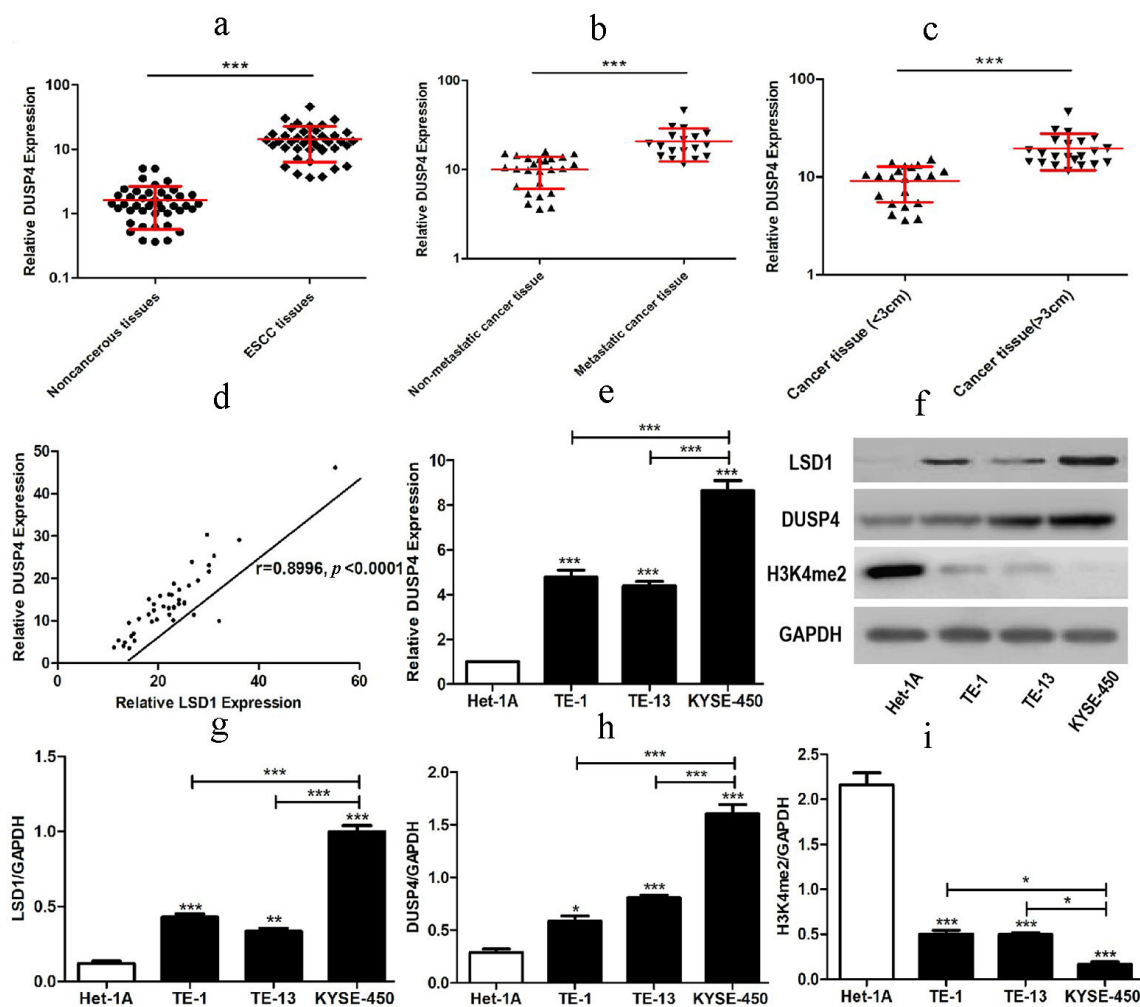

Fig. 2. DUSP4 expression in the ESCC specimens. a) mRNA levels of DUSP4 in 43 pairs of ESCC tissues and adjacent noncancerous tissues. b) mRNA levels of DUSP4 in 18 ESCC specimens with metastasis and 25 non-metastatic ESCC specimens. c) mRNA levels of DUSP4 in 22 ESCC specimens with a diameter more than 3 cm and 21 ESCC specimens with a diameter less than 3 cm. a-c) Results are presented as median with interquartile range. *** $p < 0.001$ by Wilcoxon rank sum test. d) Association between the mRNA levels of LSD1, and DUSP4 in ESCC tissues (analyzed by Pearson correlation analysis, $n = 43$, r = 0.8996, $p < 0.0001$). e) mRNA levels of DUSP4 in normal esophageal epithelial cell line, Het-1A, and ESCC cell lines TE-1, TE-13, and KYSE-450. f-i) Protein levels of LSD1, DUSP4 and H3K4me2 in Het-1A, TE-1, TE-13, and KYSE-450 evaluated by Western blotting. e-i) Results are presented as mean ± SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA test.
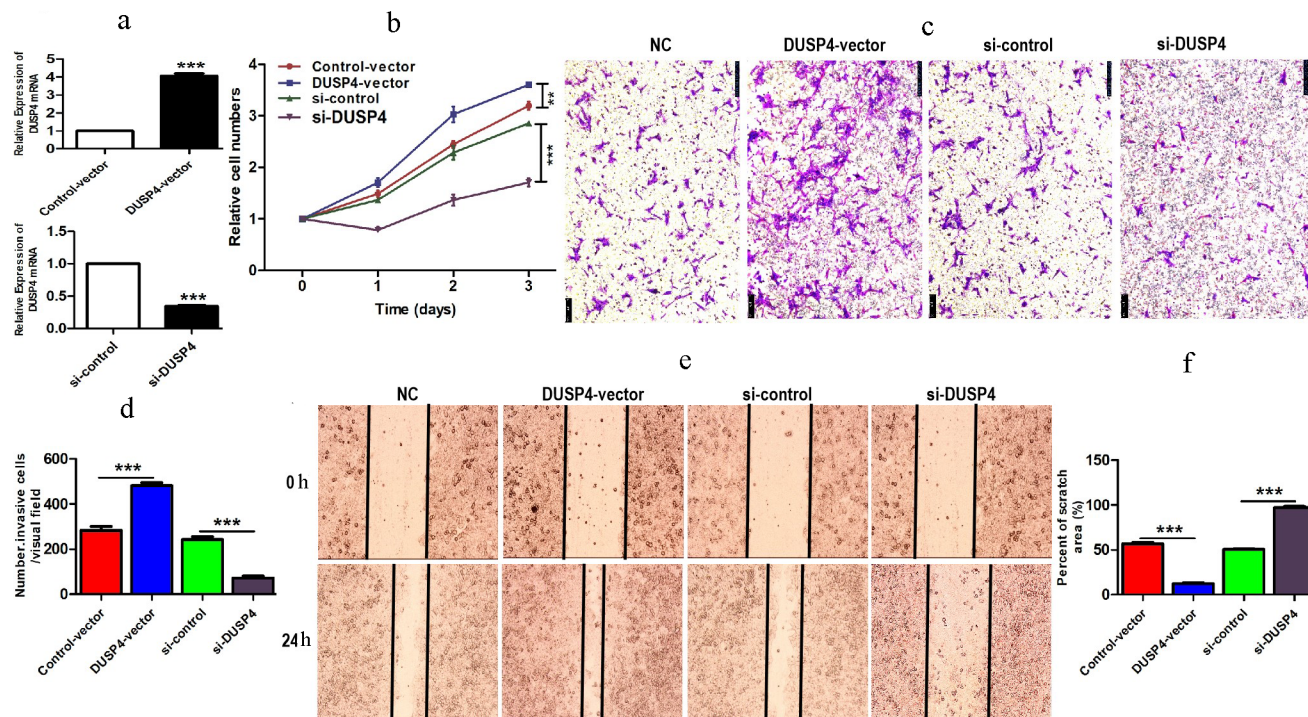
Fig. 3. DUSP4 overexpression promoted proliferation, invasion and migration of ESCC cells, while the DUSP4 knockdown exhibited opposite effect. a) mRNA or protein levels of DUSP4 in the TE-1 cells transfected with the indicated siRNAs or plasmids for 48 h. b) Proliferation of TE-1 cells transfected with the indicated siRNAs or plasmids measured by CCK-8 assay. c and d) Invasion of TE-1 cells transfected with the indicated siRNAs or plasmids evaluated by the Transwell migration assay (scale bars, 200 μm). e) Migration of TE-1 cells transfected with the indicated siRNAs or plasmids assessed by the scratch tests. The images were acquired under the microscope at 0 and 24 h. f) Cell migration ratios (24 h/0 h) were shown in the histogram. Results are presented as mean ± SEM from three independent experiments. ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA test. NC, group with control-vector; DUSP4, group with DUSP4-vector.

ESCC's oncogenesis? After using ZY0511 to inhibit LSD1 activity, it was observed that proliferation, invasion, and migration of TE-1 cells were significantly decreased, which was similar to the DUSP4 knockdown (Fig. 5, a-e). DUSP4 overexpression significantly reversed the inhibitory effect of ZY0511 on proliferation, invasion, and migration of the TE-1 cells (Fig. 5, a-e). These results suggested that DUSP4 served as a downstream molecule for the LSD1-regulated ESCC's oncogenesis *in vitro*.

DISCUSSION

The important role of LSD1/KDM1A in oncogenesis has attracted much attention [7-10]. Remarkably, an increasing number of studies regarding ESCC also focus on LSD1 [11-16]. These previous studies demonstrated critical roles of LSD1 in the ESCC pathogenesis [11-16]. However, how LSD1 regulates the emergence and development of ESCC has not been clarified, leaving an interesting scientific problem for current research.

After silencing *LSD1*, we found using transcriptome analysis that the DUSP4 gene in the ESCC cells was sig-

nificantly downregulated, which confirmed that LSD1 upregulated expression of DUSP4 in the ESCC cells. The same results were confirmed through detection of mRNA or protein. DUSP4 has been widely recognized as a biological marker of multiple malignant tumors. DUSP4 is a member of the family of dual specificity phosphatases and can negatively regulate the activity of MAP kinases [22]. Alterations in the DUSP4 expression are involved in oncogenesis of a variety of tumors. DUSP4 expression is upregulated in colorectal, breast, rectal, liver carcinomas, and pancreatic cancer, indicating that it may be a marker of adverse prognosis in various malignant tumors [23-26]. Zhang et al. revealed that sanguinarine represses growth and invasion of the gastric cancer cells through inhibiting DUSP4 signaling [27]. Xu et al. demonstrated that miR-122-5p inhibits cell migration and invasion of the gastric cancer cells by targeting DUSP4 [28]. De et al. verified that high DUSP4 expression is associated with worse overall survival and with clinical characteristics typical of highly invasive colorectal cancer (CRC) [29]. Moreover, DUSP4 overexpression leads to increased proliferation in the CRC cell lines [23]. It is thus clear that DUSP4 is not only highly expressed in various tumour tissues but also serves as a promoter of the growth and spread of some

digestive tract tumors. However, there are few reports regarding DUSP4 in EC (especially ESCC) studies. As expected, DUSP4 was upregulated in the ESCC tissues and cell lines compared with noncancerous tissues and normal esophageal epithelial cell line, respectively. Even more interestingly, DUSP4 expression in the cancerous tissues was further enhanced with the growth and metastasis of ESCC. Correlation analyses between the DUSP4 expression and clinicopathological features showed that the enhanced DUSP4 expression indicated poorer overall survival. Accordingly, the above data suggest that DUSP4 may be a novel prognostic biomarker for ESCC.

Functional *in vitro* and *in vivo* assays showed that the DUSP4 overexpression promoted proliferation, invasion, migration, and tumorigenicity of ESCC cells in animal models. Conversely, the *DUSP4* knockdown repressed proliferation, invasion, migration, and tumorigenicity of the ESCC cells in animal models. These results suggest that DUSP4 may serve as a potential therapeutic target for ESCC. DUSP4 can play a dual role in preventing and promoting carcinogenesis; however, DUSP4 exerts a positive effect on some digestive tract tumors. ESCC tumorigenesis promoted by DUSP4 may be related to the ESCC tumor property, which requires further investigation. It is worth noting that higher DUSP4 expression is a feature of a larger tumor size based on the examination of the clinical tissue specimens and *in vivo* assays. It is well established that the high level of hypoxia can significantly promote tumor growth. Previous study showed that hypoxia can cause insufficient demethylase activity, thus leading to gene methylation, ultimately hindering expression of the tumor suppressor genes and promoting tumor growth [30]. Remarkably, DUSP4 overexpression can prevent hypoxia/reoxygenation-induced cell death by upregulating endothelial nitric oxide synthase (eNOS) [31]. Accordingly, we speculate that DUSP4 contributes to the ESCC tumor growth upon hypoxia, which may be a potential mechanism of the DUSP4-regulated ESCC tumorigenesis. The relationship between the DUSP4, hypoxia level, and ESCC tumorigenesis
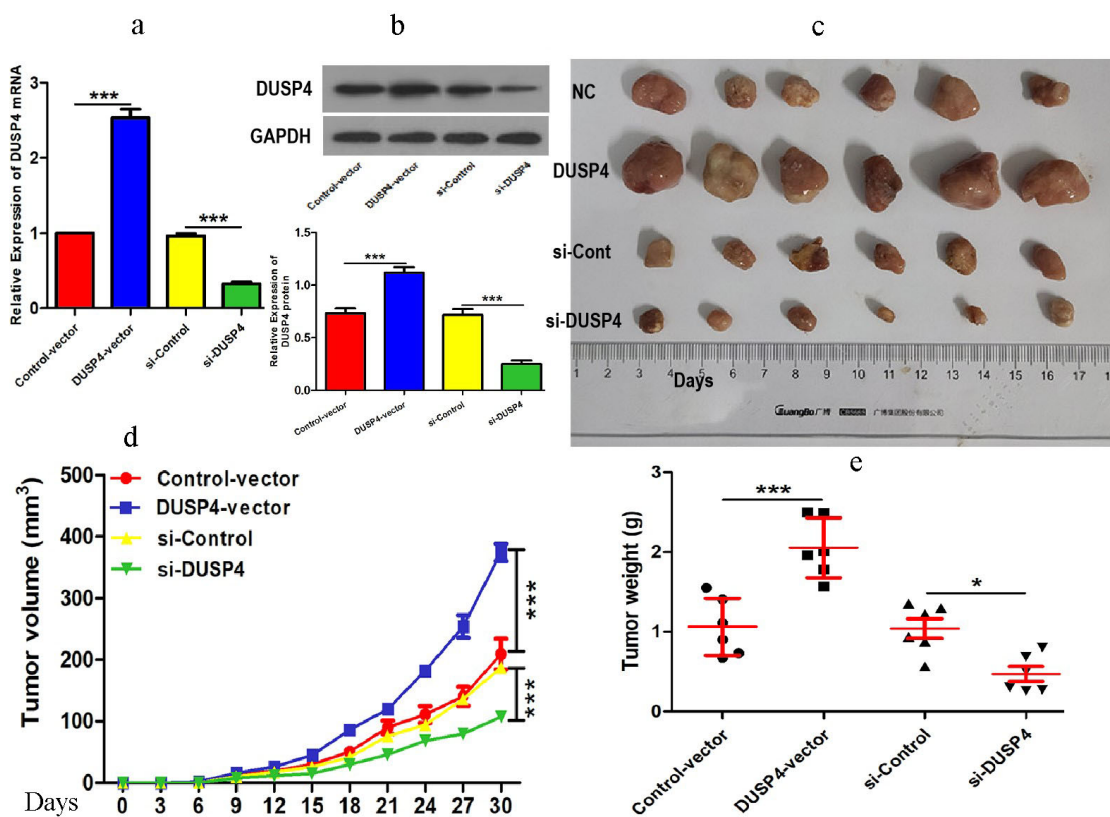

Fig. 4. DUSP4 overexpression promoted tumorigenicity of ESCC cells, while the DUSP4 knockdown exhibited opposite effect. DUSP4-vector-transfected, siRNA-DUSP4-transfected, or corresponding control TE-1 cells were inoculated into nude mice. 30 days later, tumour-bearing mice were killed, and all tumors were removed and weighted. a) DUSP4 mRNA expression in tumor tissues from four group of mice assessed by qPCR assays ($n = 6$). b) DUSP4 protein expression of tumour tissues from four group of mice evaluated by Western blotting ($n = 6$). c) Representative images of the removed tumors from the tumor-bearing mice. d) Tumor volume was measured every three days for each mouse and the tumor growth curve was plotted ($n = 6$). e) Comparison of the tumor weight ($n = 6$). Results are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ by one-way ANOVA test. NC, the removed tumors with control-vector-transfected cells; DUSP4, the removed tumors with DUSP4-vector-transfected cells.
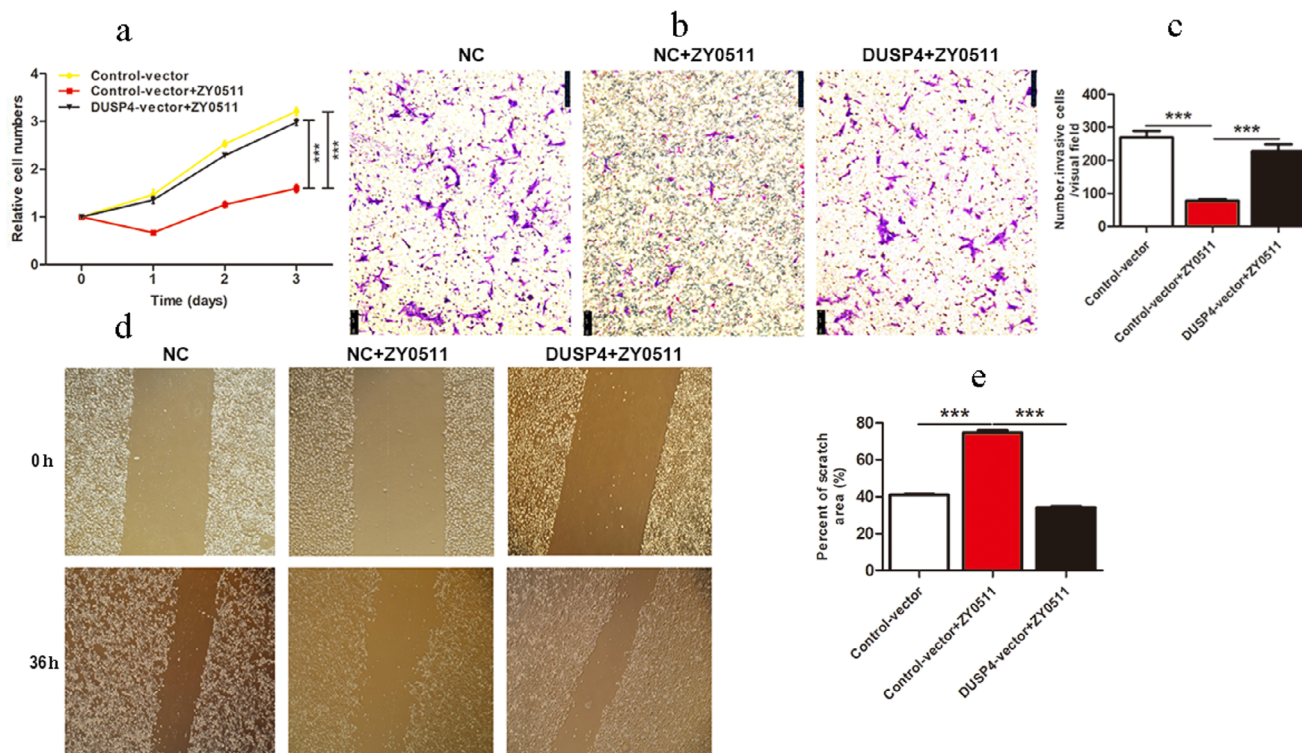
Fig. 5. DUSP4 overexpression reversed ZY0511-inhibited proliferation, invasion, and migration of ESCC cells. a) Proliferation of TE-1 cells transfected with the indicated plasmids measured using CCK-8 assay in the presence or absence of 2 μM ZY0511. b and c) Invasion of TE-1 cells transfected with the indicated plasmids evaluated using the Transwell migration assay in the presence or absence of 2 μM ZY0511 (scale bars, 200 μm). d) Migration of TE-1 cells transfected with the indicated plasmids assessed by the scratch tests in the presence or absence of 2 μM ZY0511. The images were acquired under the microscope at 0 and 36 h. e) Cell migration ratios (36/0 h) are shown in the histogram. Results are presented as mean ± SEM from three independent experiments. *** $p < 0.001$ by one-way ANOVA test. NC, control-vector-transfected cells group; NC+ZY0511, control-vector-transfected cells treated with ZY0511; DUSP4+ZY0511, DUSP4-vector-transfected cells treated with ZY0511.

needs to be comprehensively and deeply investigated in future.

We demonstrated the promoting effect of LSD1 on DUSP4 expression in ESCC and contribution of DUSP4 to the growth and development of ESCC. LSD1 may also play a key role in the ESCC pathogenesis through DUSP4 signaling. As expected, ZY0511 attenuated proliferation, invasion, and migration of ESCC cells to the level similar to the observed for the DUSP4 knockdown. Importantly, DUSP4 overexpression reversed the inhibitory effect of ZY0511 on the above parameters in the ESCC cells. The above data indicate that the positive regulation of DUSP4 at least partially mediates the roles of LSD1 in the pathogenesis of ESCC. However, the limitation of this study is that it does not explore in depth how the overexpressed LSD1 promotes DUSP4 expression in the ESCC. Accordingly, the detailed mechanisms underlying the LSD1-regulated DUSP4 expression in ESCC require further study. Demethylating function of LSD1 makes HIF-1α resistant to the methylation-mediated degradation [32]. In addition, LSD1 indirectly represses HIF-1α hydroxylation-mediated degradation [32]. Furthermore, demethyla-

tion of RACK1 by LSD1 inhibits the RACK1-mediated HIF-1α degradation [32]. Accordingly, LSD1 is also a key regulatory signal for the cancer cells to adapt to hypoxic microenvironment [32]. Therefore, enhancing adaptation of the ESCC cells to hypoxia is a possible mechanism of ESCC tumorigenesis regulated by the LSD1/DUSP4 axis, which also needs to be explored in future.

In conclusion, the current study identified DUSP4 as an oncogenic molecule downstream of LSD1 in the ESCC, which, to some extent, explains the ESCC pathogenesis regulation by LSD1. More importantly, by revealing the downstream molecules regulated by LSD1, our experimental data not only allows us suggesting a promising prognostic biomarker for ESCC but also shed light on the improvement of therapeutic strategies in the treatment of ESCC, i.e., DUSP4 may be a potential effective target in repressing ESCC.

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