Early growth response 1 regulates dual-specificity protein phosphatase 1 and inhibits cell migration and invasion of tongue squamous cell carcinoma

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Abstract. Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors in the head and neck, and among the OSCCs, tongue squamous cell carcinoma (TSCC) is one of the most common types. Although therapy strategies have recently advanced, the prognosis of TSCC has not substantially improved. Metastasis is one of the main causes of patient mortality in TSCC; therefore, it is necessary to elucidate the mechanism by which TSCC metastasis is regulated. In the present study, the early growth response 1 (Egr-1) expression in TSCC was analyzed based on GEO datasets and the effect of Egr-1 in TSCC tumor cell migration and invasion was measured using Transwell assay. By over-expressing dual-specificity protein phosphatase 1 (DUSP1) in cells with Egr-1 knockdown using lentivirus infection, the role of DUSP1 in Egr-1-regulated TSCC cell migration and invasion was determined. By using luciferase and ChIP assays, the mechanism behind how DUSP1 is regulated by Egr-1 was detected. In the present study, it was demonstrated that Egr-1 was downregulated in TSCC and the knockdown of Egr-1 increased TSCC cell migration and invasion. The expression of Egr-1 was also correlated with DUSP1. The overexpression of DUSP1 in Egr-1 knockdown cells, reduced the level of cell migration and invasion. Furthermore, it was demonstrated that knockdown of Egr-1 inhibited the promoter activity of DUSP1 and the site through which Egr-1 regulates DUSP1 transcription was identified. In conclusion, the present study demonstrated that Egr-1 regulates TSCC cell migration and invasion through modulating DUSP1, suggesting the potential of Egr-1 and DUSP1 as therapy targets for TSCC.

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Introduction

Oral squamous cell carcinoma (OSCC), one of the most common malignant tumors in the head and neck, accounts for ~4% of whole-body tumors and each year ~510,000 new OSCC cases are diagnosed worldwide (1,2). Tongue squamous cell carcinoma (TSCC) is one of the most common types of OSCC. Although tumor therapeutic strategies have advanced in recent years, the prognosis of TSCC has not substantially improvement. Metastasis is one of the main causes of mortality in patients with TSCC (3); therefore, elucidating the mechanism by which TSCC metastasis is regulated and developing novel therapeutics to inhibit this metastasis is a potential way of improving TSCC therapy (4).

The transcription factor, early growth response 1 (Egr-1), belongs to the early growth response protein family and is ubiquitously expressed in various tissues, such as cardiovascular (5), adipose (6), and muscle (7) tissues. Egr-1 is a zinc-finger protein and regulates gene expression through the binding of the C2H2-type zinc finger domain to GC-rich promoter regions of target genes. Egr-1 participates in several physiological and pathological processes, such as cell proliferation, cell apoptosis, cell invasion, angiogenesis and the immune response (8-10). Egr-1 is also associated with numerous diseases, including interstitial inflammation (11), diabetes (12), cardiovascular disease (5), and gastric cancer (13). Egr-1 primarily functions as a tumor suppressor in a number of tumor types, such as in colon cancer, mammary tumors and breast cancer (8,14,15), while in certain other tumors, such as skin cancer, Egr-1 is reported to be oncogenic (16). Therefore, the role that Egr-1 may have in TSCC is not clear.

Dual-specificity protein phosphatase 1 (DUSP1), also termed mitogen-activated protein kinase phosphatase 1 (17,18), is involved in numerous physiological and pathological

processes, including endoplasmic reticulum stress (19), microglial polarization (20), homeostasis and inflammation (21). Dysfunction of DUSP1 may induce several diseases, including neuro-inflammation (22), renal fibrosis (23) and rheumatoid arthritis (24). DUSP1 is also associated with tumor progression, and therefore, targeting DUSP1 has been suggested as a potential neoadjuvant strategy for tumor therapy (25). By inhibiting zinc finger protein SNAI1 (Snail), DUSP1 inhibits the migration and invasion of prostate cancer cells (26). Furthermore, micro RNA (miR)-133b inhibits bladder cancer cell proliferation through targeting DUSP1 (27). DUSP1 can also restrict myeloid cell-mediated OSCC progression (28). Long non-coding RNA (lncRNA) LINC01111 upregulates DUSP1 expression through miR-3924 and antagonizes pancreatic cancer aggressiveness (29). DUSP1 can also be regulated by several factors. For instance, lncRNAs such as LINC00702, regulate DUSP1 expression at the transcriptional level (30) and miR-152-3p downregulates DUSP1 and participates in the progression of acute lung injury (31). Other factors such as upframeshift 1 and platelet-derived growth factor receptor also regulate DUSP1 expression (32,33). However, the regulation mechanisms of DUSP1, especially at the transcriptional level, have not been fully elucidated.

The present studyThe present study aimed to demonstrate the role and mechanism of Egr-1 in regulating TSCC migration and invasion, in order to determine whether Egr-1 could be a potential therapeutic target for TSCC. To achieve this aim, Egr-1 expression in TSCC was analyzed based on GEO datasets and the effect of Egr-1 in TSCC tumor cell migration and invasion was measured using a Transwell assay. By overexpressing DUSP1 in cells with Egr-1 knockdown using Lentivirus infection, the role of DUSP1 in Egr-1-regulated TSCC cell migration and invasion was determined. Furthermore, by using luciferase assay and ChIP assays, the mechanism behind how DUSP1 was regulated by Egr-1 was detected.

Materials and methods

Cell lines. CAL-27 cells were purchased from the American Type Culture Collection and cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere with 5% CO_2.

Analyzing the expression of Egr-1 and DUSP1 in head and neck squamous cell carcinoma (HNSCC) based on the University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) database. The UALCAN database (http://ualcan.path.uab.edu/) is a comprehensive web resource for analyzing cancer omics data, which can be used for performing gene expression analysis in various tumor types (34). In the present study, the expression profiles of Egr-1, Egr-2, Egr-3 and Egr-4 in HNSCC samples were analyzed using the UALCAN database. The detail process was as follows: The TCGA→TCGA genes links were chosen, and 'Egr-1, Egr-2, Egr-3, or Egr-4' was entered in the 'Enter gene symbol(s)' dialog box. Next, in the 'TCGA dataset' drop-down list, 'Head and neck squamous cell carcinoma' was chosen. Differences between the normal and tumor groups were determined using unpaired Student's t-test, and P<0.05 was considered to indicate a statistically significant difference.

Analyzing Egr-1-associated genes based on ULCAN database. DUSP1-associated genes were screened in ULCAN database (http://ualcan.path.uab.edu/). The detailed process was as follows: The TCGA→TCGA genes links were chosen and 'Egr-1' was entered in the 'Enter gene symbol(s)' dialog box. Next, in the 'TCGA dataset' drop-down list, 'Head and neck squamous cell carcinoma' was chosen. After the correlation dialog box was chosen, the genes positively correlated with EGR1 in HNSC were provided (http://ualcan.path.uab.edu/cgi-bin/TCGAExCorrel.pl?genenam=EGR1&cancer=HNSC). The correlation was evaluated using Pearson's χ^2 test.

Relative Egr-1 expression analyzed using GEO dataset. The GSE31056 dataset was downloaded from GEO dataset (https://www.ncbi.nlm.nih.gov/) (35). The expression of Egr-1 in OSCC and normal tissue was analyzed using the R package DESeq2 based on the data of the GEO dataset. The data were analyzed using a paired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). CAL-27 cells were cultured in 6-well plates. To lyse the cells, 1 ml TRIzol™ reagent (Thermo Fisher Scientific, Inc.) was added to each well. The cell lysates were then transferred to new microtubes and RNA was extracted by adding 500 μl chloroform to each tube. RNA was then separated by adding 200 μl isopropanol. The RNA concentration was determined using a spectrophotometer (Implen GmbH). Total RNA was reverse transcribed to cDNA using a Reverse Transcriptase Kit (cat no. D7160S; Beyotime Institute of Biotechnology) following the manufacturer's instructions. Egr-1 and DUSP1 mRNA expression levels were determined by qPCR using a FastStart SYBR Green Master kit (cat. no. D7260; Beyotime Institute of Biotechnology) and the following thermocycling conditions: Initial denaturation at 95˚C for 10 min; 40 cycles of 58˚C for 15 sec and 72˚C for 30 sec. The primers for RT-qPCR are listed in Table SI. The experiment was repeated three times, and the expression of target genes was normalized to β-actin using the $2^{-\Delta\Delta Cq}$ method in accordance with a previous study (36).

Egr-1 knockdown. Egr-1 knockdown was conducted using the lentivirus transduction technique. The lentiviral plasmid, hU6-MCS-CBh-gcGFP-IRES-puromycin (Shanghai GeneChem Co., Ltd.), was used to construct the plasmid for Egr-1 knockdown. The sequence for knocking down Egr-1 was 5'-GCATCTGCATGCGCAACTTCA-3'. A non-targeting sequence served as the negative control (shCon; 5'-CCG GTTCTCCGAACGTGTCACGTCTCGAGTTCTGGAAGT TCTCACGGCTTTTT-3'). Both Lv-shEgr-1 and Lv-shCon were synthesized based on a 3rd lentivirus generation system (Shanghai GeneChem Co., Ltd.) using 293T cells (ATCC) as the interim cell line. For transfection, 1 μg lentivirus plasmid for Egr-1 knockdown or negative control and 1 μl lentivirus generation system were transfected into 293T cells at 37˚C for 4 h. After 48 h, the lentivirus particles were collected

using a Lentivirus concentration kit [Genomeditech; Jiman Biotechnology (Shanghai) Co., Ltd.] according to the manufacture's instruction. For lentivirus infection, CAL-27 cells were seeded in a 12-well plate at 30,000 cells/well. After 24 h of culture, lentivirus (MOI: 10) was added to each well. After 4 h of transduction, the medium containing lentivirus was replaced with lentivirus-free medium. After a 48-h incubation, the cells transduced with lentivirus were selected with 5 μg/ml puromycin (Sigma-Adrich; Merck KGaA). Each 3 weeks, the cells would be re-selected with 5 μg/ml puromycin. As the lentivirus contained a green fluorescent protein gene, green fluorescence was used to determine whether the cells had been transduced. RT-qPCR was then used to confirm that Egr-1 had been knocked down in transduced CAL-27 cells.

DUSP1 overexpression. DUSP1 overexpression was performed by lentivirus transduction. A lentivirus for the overexpression of DUSP1 (Lv-oeDUSP1) and a negative control lentivirus (Lv-oeCon) were synthesized based on a 3rd lentivirus generation system. The Lv-oeDUSP1 was synthesized by inserting DUSP1 cDNA into the plasmid backbone of pHBLV-CMV-MCS-IRES-Puro (Chemicalbook; Beijing Xilin Buke Network Technology Co., Ltd.). To construct Lv-oeCon the translation starting site codon (ATG) of Lv-oeDUSP1, was replaced by AAG to abolish translation. The lentivirus transduction was performed as aforementioned.

Construction of DUSP1 promoter luciferase reporter vectors. The human DUSP1 promoter sequence was obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The promoter (-1,500 to +20 bp, where the transcription start site was defined as +1) of human DUSP1 was amplified from the genomic DNA of CAL-27 cells using the following thermocycling conditions: Initial denaturation at 98˚C for 30 sec; 35 cycles of 98˚C for 10 sec, 50˚C for 10 sec, 72˚C for 40 sec; and a final extension step of 72˚C for 2 min. The PCR primers used for amplifying the DUSP1 promoter are shown in Table SII. After amplification, the promoter fragments were cut by *Sac*I and *Mlu*I restriction enzymes. Then, the fragment was re-cloned into the pGLuc-Dura plasmid (Beyotime Institute of Biotechnology) between the *Sac*I and *Mlu*I sites and the reconstructed plasmid was termed pGLuc-DUSP1. The pGLuc-DUSP1 was confirmed by DNA sequencing at Sangon Biotech Co., Ltd.

Identifying the putative binding sites of Egr-1 in the DUSP1 promoter. The putative binding sites of Egr-1 in the DUSP1 promoter were predicated in JASPAR[2022] software (https://jaspar.genereg.net/analysis). The explanation of the basis of the calculations and the mean of the indexes in the results outputted by the software are described in detail in a paper published by the manufacturer of the software (37). Briefly, Egr-1 was submitted to the search box and then human Egr-1 was added to the cart. The sequence of the DUSP1 promoter was inserted into the scanning box and the relative profile score threshold was set as 90%. The putative binding site of Egr-1 in the DUSP1 promoter was then presented.

Site-directed mutagenesis. Site-directed mutagenesis was performed according to a previous study (38). Briefly, Site A (-68/-81) and Site B (-1363/-1376) of pGLuc-DUSP1 were mutated with a Q5® High-fidelity DNA Polymerase (New England BioLabs, Inc.) and pGLuc-DUSP1 as the template, with the following thermocycling conditions: Initial denaturation at 98˚C for 30 sec; 35 cycles of 98˚C for 10 sec, 5˚C for 10 sec, 72˚C for 40 sec and a final extension at 72˚C for 2 min. The PCR primers used for mutating Site A (MTA) and Site B (MTB) are listed in Table SIII. After the template was digested with a *Dpn*I restriction enzyme, the products were transformed into TOP10 cells (Beyotime Institute of Biotechnology). The mutant pGLuc-DUSP1 plasmids were isolated using a Plasmid Mini Preparation Kit (Beyotime Institute of Biotechnology). The plasmid was confirmed by Sanger sequencing technique performed by Sangon Biotech, Co., Ltd.

Luciferase assay. The pGLuc-DUSP1 promoter reporter plasmid (1 μg) was co-transfected with pGMLR-TK luciferase reporter (1 μg) into CAL-27 cells using Lipofectamine™ 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). At 24-h post transduction, the cell culture supernatants were collected. The *Gaussia* luciferase activity was measured by a Promega GloMax luminometer (Promega Corporation) using a Gaussia-Dura luciferase kit following the manufacturer's instructions (Thermo Fisher Scientific, Inc.). *Renilla* luciferase activity was measured by the Promega GloMax luminometer (Promega Corporation) using a RenillaLumi™ Luciferase Assay Kit following the manufacturer's instructions (Beyotime Institute of Biotechnology). The experiment was repeated for three times. The promoter activity was calculated by normalized *Gaussia* luciferase activity with *Renilla* luciferase activity.

Transwell migration and invasion assays

Transwell migration assay. Cells were seeded into the upper chambers of Transwell plates at a density of $1x10^5$ cells/well in FBS-free DMEM, and DMEM with 10% FBS was added to the lower chambers. The cells were incubated for 10 h in an incubator containing 5% CO_2 at 37˚C. Subsequently, the cells on the top surface of the semipermeable membrane were removed, while the cells attached to the bottom surface of the membrane were stained with crystal violet for 5 min at 20˚C. Images of the stained cells were captured under a light microscope (Olympus Corporation). The experiment was repeated for three times and cells of three randomly selected visual fields were counted manually and averaged.

Transwell invasion assay. Transwell invasion assay shared same procedures as Transwell migration assay, except that each upper chamber of the Transwell plate was pre-coated with 20 μg Matrigel ECL (MilliporeSigma; Merck KGaA) for 4 h at 37˚C, and cells were seeded at a density of $2x10^5$ cells/well.

Western blotting. Cell lysates were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology). The concentration of the protein was determined using a BCA kit (Beyotime Institute of Biotechnology). Then, 30 μg of protein in each group was loaded per lane and separated on 10% gels using SDS-PAGE and transferred onto a nitrocellulose (NC) membrane. After blocking using 5% fat-free milk (Beyotime Institute of Biotechnology) for 30 min at 20˚C, the NC membrane was incubated with β-actin (1:1,000; cat. no. sc-8432; Santa Cruz Biotechnology, Inc.) or Egr-1 (1:1,000;

cat. no. ab194357; Abcam) antibodies. The membranes were then incubated with a Cy3-labelled goat anti-rabbit secondary antibody (1:10,000; cat. no. A0516; Beyotime Institute of Biotechnology) or a Cy3-labelled goat anti-mouse secondary antibody (1:10,000; cat. no. A0521; Beyotime Institute of Biotechnology) for 1 h at 20˚C. All antibodies were diluted in TBST (containing 0.1% Tween 20). Finally, the membrane was visualized in a Typhoon infrared scanning imager (Cytiva). The experiment was repeated three times. The densitometry was performed using ImageJ software (version 1.8.0; National Institutes of Health).

Chromatin immunoprecipitation (ChIP). The ChIP assay was conducted using a ChIP assay kit (Beyotime Institute of Biotechnology) following the manufacturer's instructions. Briefly, CAL-27 cells were cultured in a 10-cm dish. The chromatin in the cells was cross-linked by being incubated in 1% formaldehyde (Beyotime Institute of Biotechnology) at 20˚C for 10 min and then in 1% glycine (Beyotime Institute of Biotechnology) for 30 min. The cell were lysed in 0.1% SDS and then 500 μg cell lysates were incubated with a Micrococcal nuclease (cat. no. 9013-53-0; Merck KGaA) for 15 min at 37˚C to ensure the chromatin in the cell lysate was digested into fragments of 200-1,000 bp. The fragments were then incubated with 5 μg Egr-1 antibody (cat. no. 4154; Cell Signaling Technology, Inc.) at 4˚C for 12 h and pulled down using Protein A/G magnetic beads (cat. no. 26162; Thermo Fisher Scientific, Inc.). The chromatin fragments were also incubated with 5 μg anti-FLAG M2 antibody antibody (cat. no. 14793; Cell Signaling Technology, Inc.), which served as the negative control. After being washed in Immune Complex Wash Buffer (cat. no. P2080S; Beyotime Institute of Biotechnology), the cross-linked chromatin was released by incubation in 0.2 μM NaCl at 65˚C for 6 h. The presence of DUSP1 promoter fragments containing Site A were then determined by PCR amplification using a Taq DNA Polymerase (cat. no. D7209; Beyotime Institute of Biotechnology) under the following thermocycling conditions: Initial denaturation at 96˚C for 5 min; 35 cycles of 96˚C for 15 sec, 55˚C for 10 sec, 72˚C for 30 sec; and a final extension step of 72˚C for 5 min. The primers for amplifying the fragments containing Site A were as follows: Forward, 5'-ATTCAACGCAAAAAC-3' and reverse, 5'-AACATA AACATTGCGC-3'. The PCR products were analyzed using a 1.0% agarose gel electrophoresis with 0.01% Gel-Green reagent (cat. no. D0143; Beyotime Institute of Biotechnology) and then visualized using an ultraviolet imager (Tanon Science and Technology Co., Ltd.).

Statistical analysis. Data analysis was performed using GraphPad Prism 5.0 (Dotmatics) for Windows. For The Cancer Genome Atlas (TCGA) data comparing Egr-1 expression between normal tissues and HNSCC tissues, data were compared between two groups using an unpaired Student's t-test. For the Gene Expression Omnibus (GEO) dataset comparing Egr-1 expression between normal tissues and TSCC tissues, the data were analyzed using a paired Student's t-test. Data are presented as the mean ± standard deviation. Differences between groups with one variable were analyzed using the one-way ANOVA followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Egr-1 expression is decreased in TSCC tumor tissues. The expression levels of members of the EGR family in HNSCC were analyzed using the UALCAN database. The expression of Egr-1 in HNSCC tissues was significantly lower compared with normal tissues (Fig. 1A); however, there was no statistically significant difference in Egr-2, Egr-3 and Egr-4 mRNA expression between HNSCC tissues and normal tissues (Fig. 1B-D). Furthermore, Egr-1 expression in TSCC tissues and adjacent normal tissues from a GEO dataset (dataset no. GSE31056) (35) were compared, which demonstrated that Egr-1 expression in TSCC tissues was significantly decreased compared with normal tissues (Fig. 1E).

Knockdown of Egr-1 increases the migration and invasion of CAL-27 cells. To determine the role of Egr-1 in TSCC metastasis, the effects of Egr-1 knockdown on TSCC cell migration and invasion were evaluated. First, it was demonstrated that Egr-1 mRNA levels were significantly decreased in CAL-27 cells transduced with Lv-shEgr-1 compared with Lv-shCon (Fig. 2A). The knockdown of Egr-1 was also confirmed using western blotting, where the relative Erg-1 protein levels were significantly decreased in Lv-shEgr-1 cells compared with the Lv-shCon cells (Fig. 2B and C).

Transwell assays were used to evaluate the migration and invasion capacities of the transduced cells. The Lv-shEgr-1 group demonstrated a significantly higher migration and invasion capacity compared with the Lv-shCon group, indicating that the knockdown of Egr-1 increased the migration and invasion capacities of CAL-27 cells (Fig. 2D).

Egr-1 expression is correlated with DUSP1 expression in TSCC. To elucidate how Egr-1 regulates TSCC cell migration and invasion, Egr-1-associated genes were analyzed. According to the UALCAN database, 242 genes were correlated with Egr-1 in HNSCC. Among the correlated genes, FOS (Fig. 3A), DUSP1 (Fig. 3B), FOSB (Fig. 3C) and ZFP36 (Fig. 3D) had the highest Pearson correlation coefficients. Therefore, the expression of these genes in cells with Egr-1 knockdown were further analyzed.

The mRNA levels of FOS, DUSP1, FOSB and ZFP36 were all significantly reduced in the sh-Egr-1 group compared with the respective control, of which DUSP1 was the most reduced (Fig. 3E). Furthermore, the association between Egr-1 and DUSP1 in TSCC was analyzed using the GEO dataset, GSE31056, which demonstrated that Egr-1 was correlated with DUSP1 in TSCC tissues (Fig. 3F).

Egr-1-induced CAL-27 cell migration and invasion are reversed by DUSP1 overexpression. A lentivirus, Lv-oeDUSP1, was constructed for overexpressing DUSP1 in TSCC cells. Cells transduced with Lv-oeDUSP1 demonstrated higher mRNA expression of DUSP1 compared with cells infected with Lv-oeCon (Fig. 4A). In addition, Lv-oeDUSP1 CAL-27 cells underwent Egr-1 knockdown (Fig. 4B) and their migration and invasion capacities were

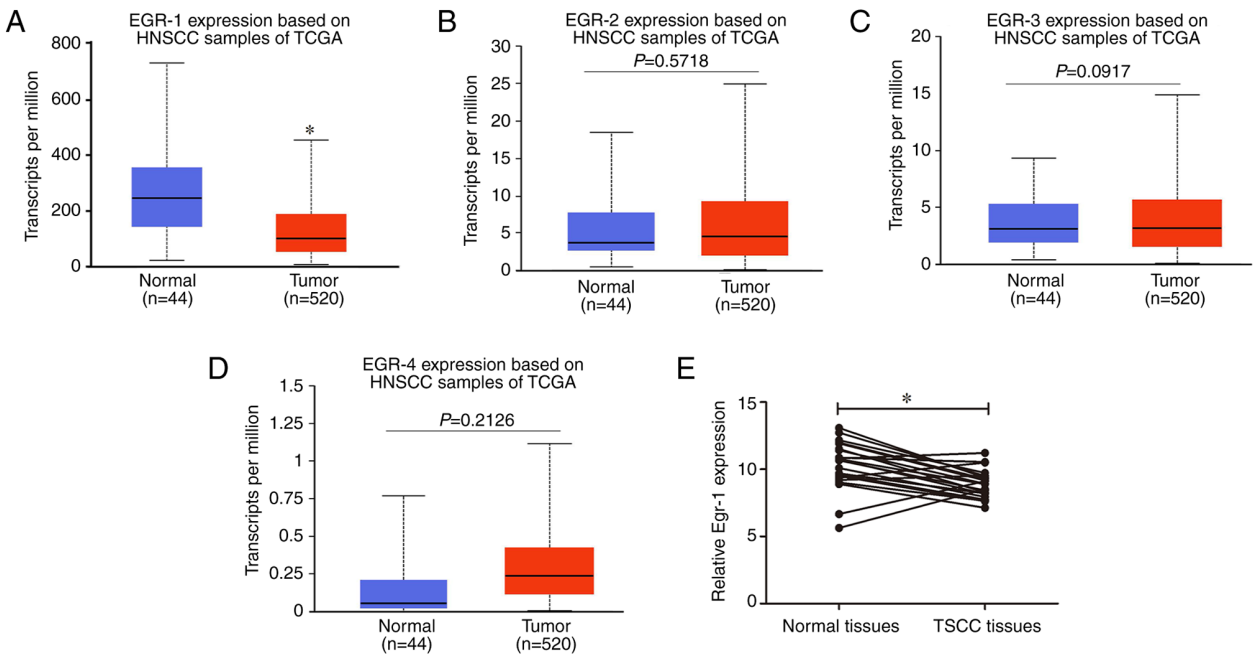

Figure 1. Egr-1 expression is decreased in TSCC tumor tissues. (A) Egr-1, (B) Egr-2, (C) Egr-3 and (D) Egr-4 expression in normal and HNSCC tissues based on the University of Alabama at Birmingham Cancer Data Analysis Portal database. (E) Expression of Egr-1 in TSCC and paired tumor-adjacent normal tissues based on the Gene Expression Omnibus dataset, GSE31056. *P<0.05 vs. normal tissues. TSCC, tongue squamous cell carcinoma; TCGA, The Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma; Egr, early growth response.

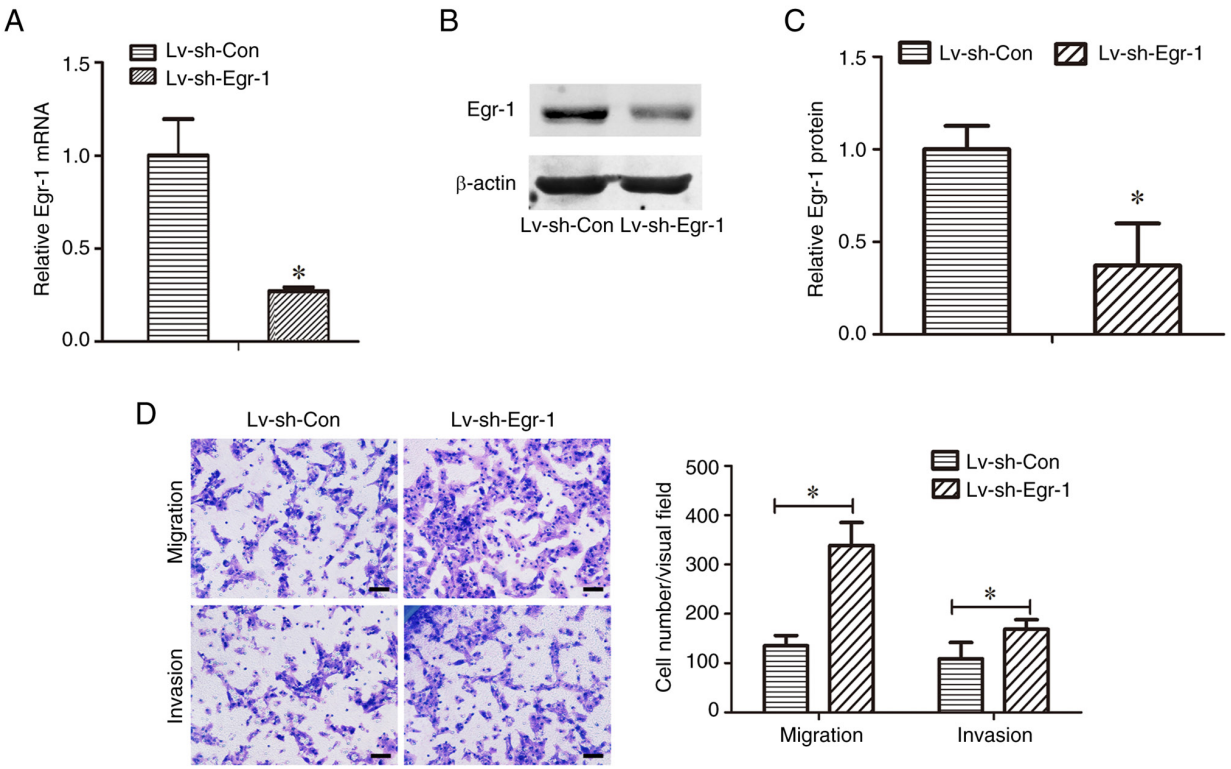

Figure 2. Knockdown of Egr-1 increases the migration and invasion of CAL-27 cells. (A) Egr-1 mRNA expression in CAL-27 cells with and without Egr-1 knockdown. (B) Egr-1 protein expression in CAL-27 cells with and without Egr-1 knockdown detected by western blotting. (C) The semi-quantification plot of Egr-1 protein expression levels. (D) CAL-27 cell migration and invasion capacity with and without Egr-1 knockdown, *P<0.05 vs. Lv-sh-Con. Lv, lentivirus; sh, short hairpin; Con, control; Egr-1, early growth response 1. Scale bar: 50 μm.

evaluated. Infection with Lv-shEgr-1 significantly increased the migration and invasion cell numbers of CAL-27 cells compared with the sh-Con; however, this increase was reversed by infection with Lv-oeDUSP1, suggesting that Egr-1 regulates CAL-27 cell migration and invasion through DUSP1 (Fig. 4C and D).

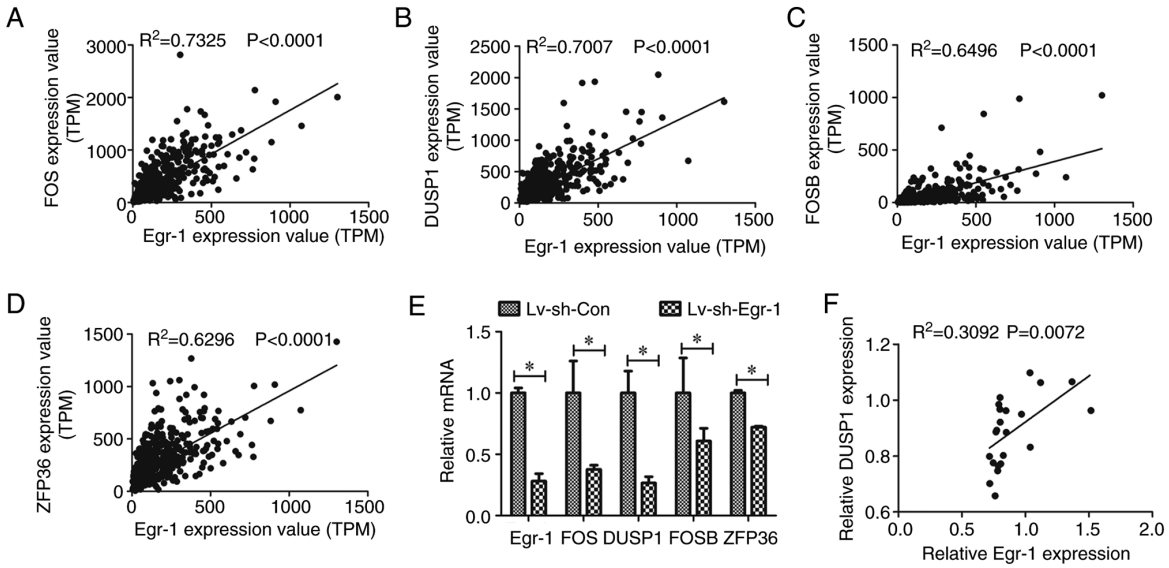

Figure 3. Egr-1 expression is correlated with DUSP1 expression in TSCC. Correlation analysis between Egr-1 expression and (A) FOS, (B) DUSP1, (C) FOSB and (D) ZFP36 based on the University of Alabama at Birmingham Cancer Data Analysis Portal database. (E) Egr-1, FOS, DUSP1, FOSB and ZFP36 mRNA expression in CAL-27 cells with and without Egr-1 knockdown, *P<0.05. (F) Correlation analysis between Egr-1 expression and DUSP1 based on the Gene Expression Omnibus dataset, GSE31056. TPM, transcripts per million; Lv, lentivirus; sh, short hairpin; DUSP1, dual-specificity protein phosphatase 1; Egr-1, early growth response 1.

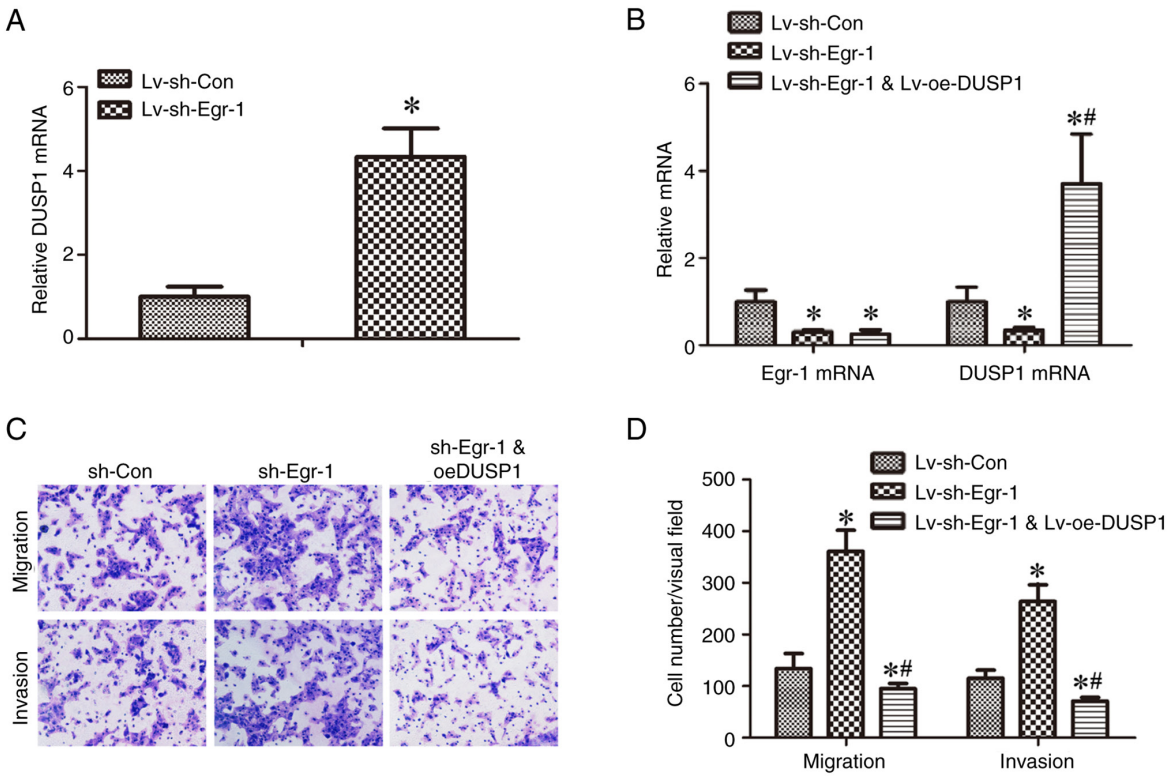

Figure 4. Egr-1-induced decreased CAL-27 cell migration and invasion is reversed by DUSP1 overexpression. (A) DUSP1 mRNA expression in CAL-27 transduced with Lv-oeCon and Lv-oeDUSP1 was detected by RT-qPCR, *P<0.05 vs. Lv-oeCon. (B) Egr-1 and DUSP1 mRNA expression in CAL-27 cells with Lv-shCon, Lv-shEgr-1 or Lv-shCon + Lv-oeDUSP1 was detected by RT-qPCR. *P<0.05 vs. sh-Con, #P<0.05 vs. sh-Egr-1. (C) CAL-27 cells transduced with Lv-shCon, Lv-shEgr-1 or Lv-shCon + Lv-oeDUSP1 were assessed by Transwell migration and invasion assays. (D) Cells in three randomly selected visual fields were counted. *P<0.05 vs. sh-Con, #P<0.05 vs. sh-Egr-1. RT-qPCR, reverse transcription quantitative-PCR; sh, short hairpin; Lv, lentivirus; oe, overexpression; DUSP1, dual-specificity protein phosphatase 1; Egr-1, early growth response 1.

Knockdown of Egr-1 decreases DUSP1 expression at the transcriptional level. Since it was demonstrated that Egr-1 expression was correlated with DUSP1 expression in TSCC, it was next determined whether the expression of DUSP1 is regulated by Egr-1. DUSP1 mRNA levels were measured in Egr-1 knockdown CAL-27 cells, and it was demonstrated that

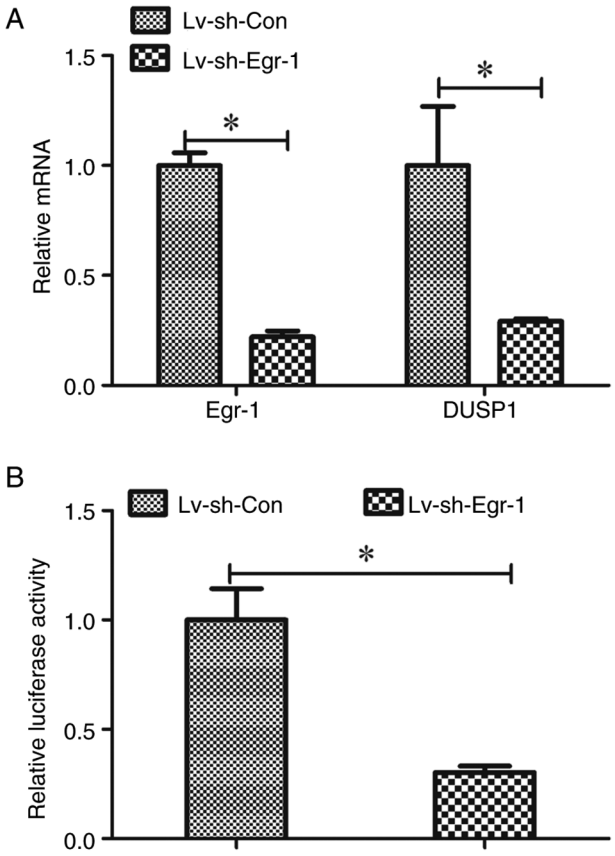

Figure 5. Knockdown of Egr-1 decreases DUSP1 expression at the transcriptional level. (A) Egr-1 and DUSP1 mRNA expression in CAL-27 cells with and without Egr-1 knockdown. (B) Relative luciferase activity of pGLuc-DUSP1 in CAL-27 cells with and without Egr-1 knockdown, *P<0.05 vs. Lv-sh-Con. DUSP1, dual-specificity protein phosphatase 1; Lv, lentivirus; sh, short hairpin; Con, control; Egr-1, early growth response 1.

these cells had significantly lower DUSP1 mRNA expression compared with the control cells (Fig. 5A). Considering that Egr-1 is a transcription factor, it was also assessed whether Egr-1 regulates DUSP1 at the transcriptional level. A DUSP1 promoter-reporter vector, pGLuc-DUSP1, was constructed and transduced into CAL-27 cells with or without Egr-1 knockdown and its activity was measured. The luciferase activity of pGLuc-DUSP1 in the cells with Egr-1 knockdown was significantly lower than the cells without Egr-1 knockdown (Fig. 5B).

Identifying the site in the DUSP1 promoter through which Egr-1 regulates DUSP1. The Egr-1 binding site in the DUSP1 promoter was predicted using JASPAR[2022] software. A total of two putative sites were predicted (Table I), which were termed Site A and Site B. To determine which site may be responsible for Egr-1 regulation of DUSP1 transcription, Site A and Site B in pGLuc-DUSP1 were mutated and termed pGLuc-DUSP1-MTA and pGLuc-DUSP1-MTB, respectively (Fig. 6A). pGLuc-DUSP1, pGLuc-DUSP1-MTA and PGLuc-DUSP1-MTB were transduced into CAL-27 cells with or without Egr-1 knockdown and the promoter activity was measured. pGLuc-DUSP1-MTA demonstrated significantly lower relative luciferase activity compared with pGLuc-DUSP1 and pGLuc-DUSP1-MTB in CAL-27 cells, while the activity of pGLuc-DUSP1-MTB was notably increased compared with pGLuc-DUSP1 (Fig. 6B). In addition, knockdown of Egr-1 significantly decreased the relative luciferase activity of pGLuc-DUSP1 (Fig. 5B) and pGLuc-DUSP1-MTB (Fig. 6D) compared with the relative controls; a slight decrease in the relative luciferase activity was also demonstrated in pGLuc-DUSP1-MTA (Fig. 6C), but this was not statistically significant. These results indicate that Egr-1 regulates DUSP1 transcription through the putative binding site A.

Furthermore, the results of the ChIP assay demonstrated that anti-Egr-1 antibodies immunoprecipitated chromatin fragments containing Site A (Fig. 6E), which was not demonstrated for the anti-Flag antibody control, suggesting that Egr-1 binds to Site A. Therefore, it can be suggested that Egr-1 may regulate DUSP1 via Site A.

Discussion

In the present study, the expression of Egr-1 in TSCC was analyzed. It was demonstrated that Egr-1 served a role in the downregulation of TSCC cell migration and invasion. Furthermore, it was also demonstrated that Egr-1 affected TSCC cell migration and invasion by regulating DUSP1 at the transcription level.

TSCC is one of the most common types of HNSCC. Metastasis is one of the main causes of the mortality of patients with TSCC, and it is therefore necessary to elucidate the mechanism of TSCC metastasis to identify possible therapeutic targets and biomarkers (4). Egr-1 is a ubiquitously expressed transcription factor and is dysregulated in various tumor types, including colon cancer (39), esophageal squamous cell carcinoma (ESCC) (40), breast cancer (15) and pituitary tumors (41); therefore, Egr-1 has been suggested as a potential biomarker for tumor prognosis and a candidate target for cancer therapy. In the present study it was demonstrated that Egr-1 expression was downregulated in HNSCC and TSCC and the knockdown of Egr-1 increased TSCC cell migration and invasion, indicating a potential role of Egr-1 in TSCC metastasis.

According to previous studies, Egr-1 has differing effects in different tumor types. It has been reported that knockdown of Egr-1 in ESCC significantly enhances cell migration and invasion (40), whereas Egr-1 inhibits cell proliferation, migration and invasion in colon cancer (8), suggesting that Egr-1 is a tumor suppressor in ESCC and colon cancer, which supports the results of the present study. However, in hepatocellular carcinoma cells, Egr-1 enhances radioresistance and chemoresistance (42,43), and Egr-1 is positively related to tumor size, lymph node metastasis, tumor stage and poor survival in gastric cancer (44), indicating that Egr-1 may exert an oncogenic role in these tumor types. The different roles of Egr-1 in tumor progression may be due to the histological diversity, but this opinion needs further research.

Egr-1 participates in tumor progression by transcriptionally regulating various target genes. Egr-1 regulates LC3 expression through binding to the promoter of LC3, promotes hypoxia-induced autophagy, and thus induces radioresistance in hepatocellular carcinoma cells (42). In addition, Egr1 inhibits colon cancer cell proliferation, migration and invasion by regulating CDKL1 at the transcriptional level (8). In the present study, to assess the mechanism by which Egr-1

Table I. Putative binding sites of Egr-1 in DUSP1 promoter predicted using the JASPAR[2022] software.

Matrix ID[a]	Name[b]	Score[c]	Relative score[d]	Start[e]	End[f]	Sequence[g]
MA0162.2	Egr-1	15.318353	0.9442267584698091	-81	-68	CCCCCTCCCCCTGC
MA0162.2	Egr-1	12.037096	0.9090872474628051	-1,376	-1,363	TCCCCTCCCCCAGG

Putative sites predicted with relative profile score threshold 90%. [a]Serial number of transcription factor in JASPAR[2022] software. [b]Transcription name. [c,d]Binding potential. [e]Start and [f]end location of the putative binding site, with the transcription starting site assigned as +1. [g]Sequence of the binding site. Egr-1, early growth response 1; DUSP1, DUSP1, dual-specificity protein phosphatase 1.

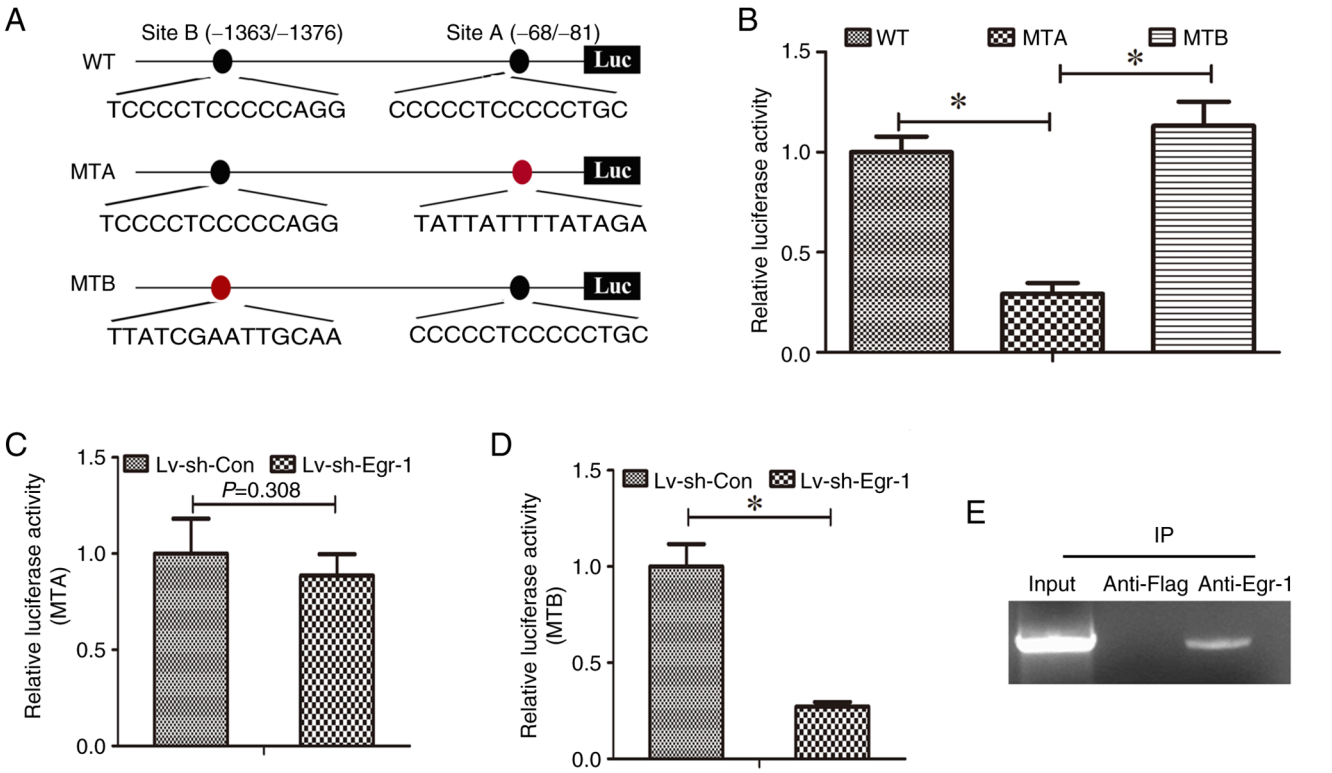

Figure 6. Identifying the Erg-1 binding site on the DUSP1 promoter. (A) The schematic of the DUSP1 promoter in pGLuc-DUSP1, pGLuc-DUSP1-MTA and pGLuc-DUSP1-MTB. The black oval represents the WT site and the red oval represents the mutant site. (B) Relative luciferase activity of pGLuc-DUSP1, pGLuc-DUSP1-MTA and pGLuc-DUSP1-MTB. MTA, pGLuc-DUSP1-MTA. MTB, pGLuc-DUSP1-MTB. Relative luciferase activity of pGLuc-DUSP1-MTA (C) and pGLuc-DUSP1-MTB (D) in CAL-27 cells with and without Egr-1 knockdown, *P<0.05. (E) Chromatin immunoprecipitation assay was used to verify whether Egr-1 bound to the Site A in DUSP1 promoter. WT, wild type; MTA, mutation Site A; MTB, mutation Site B; Lv, lentivirus; sh, short hairpin; Luc, luciferase; Con, control; DUSP1, dual-specificity protein phosphatase 1; Egr-1, early growth response 1.

regulates TSCC progression, Egr-1-associated genes were analyzed using bioinformatics methods. From this, four genes, DUSP1, FOS, FOSB and ZFP36, were selected for further screening. It was then demonstrated that the mRNA levels of these four genes were decreased following Egr-1 knockdown, of which DUSP1 was the most downregulated. It was further demonstrated that overexpression of DUSP1 reversed knockdown of Egr-1-induced TSCC migration and invasion, indicating that Egr-1 regulates TSCC migration and invasion through DUSP1. Furthermore, it was demonstrated that the DUSP1 binding site of Egr-1 was demonstrated to be within Site A of the promotor. Therefore, the present study demonstrated that DUSP1 may be a target of Egr-1 and suggested the mechanism through which Egr-1 is involved in TSCC metastasis. To regulate target gene expression in general, Egr-1 interacts with numerous transcription and regulatory factors, such as nuclear factor-κB, YAP-1 and Snail (45-48). However, the roles of other factors in the regulation of DUSP1 by Egr-1 requires further study.

In previous studies, DUSP1 has also been reported to be regulated by other transcription factors. For instance, LINC00702 was reported to promote DUSP1 expression by recruiting JunD to the DUSP1 promoter (30). In addition, AP-1 has been predicted to bind to the promoter region of DUSP1 and regulate its expression (48). Furthermore, FOXM1 was reported to directly activate the DUSP1 promoter in macrophages (49). Further studies are required to assess whether DUSP1 is regulated by the aforementioned transcription factors in TSCC, or whether these transcription factors have synergistic effects with Egr-1 in regulating DUSP1.

In the present study, other genes such as FOS, FOSB and ZFP36 were demonstrated to be associated with Egr-1. The FOS gene encodes a transcription factor that co-localise with members of the Jun family, including c-Jun, JunB and JunD, and regulate cell proliferation, tumor invasion, distant metastasis and angiogenesis (50). FOSB is upregulated in tumors and knockdown of FOSB decreases migration ability (51). ZFP36 inhibits prostate cancer progression by targeting CDK6 and oxidative stress (52). In addition, ZFP36 interacts with MCM3AP-AS1 to regulate the proliferation, apoptosis, migration and invasion of breast cancer cells (53). Therefore, FOS, FOSB and ZFP36 are also noteable regulators in tumor progression. However, whether these genes are associated with the role of Egr-1 in TSCC progression remains unknown, and this is a limitation of this study. There are other limitations to the present study; for example, the expression of Egr-1 in OSCC was analyzed based on data from a public database, whereas it would be more convencing if the expression of Egr-1 was detected in OSCC clinical tissues. Furthermore, the function of Egr-1 in OSCC progression was analyzed *in vitro*, whereas it will be better if there were also *in vivo* experiments.

In conclusion, the results of the present study demonstrated that Egr-1 was upregulated in TSCC and was associated with tumor cell migration and invasion. Moreover, it was demonstrated that DUSP1 may be a target gene of Egr-1 and DUSP1 participated in Egr-1-associated cell migration and invasion. The findings of the present study suggested that Egr-1 and DUSP1 are candidate biomarkers and therapeutic targets for the treatment of TSCC metastasis.

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Availability of data and materials

The data generated in the present study may be found in the Figshare repository at the following URL: https://figshare.com/search?q=10.6084%2Fm9.figshare.21801127. In addition, the data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZM and NG designed the study. LZ and JL performed the cell culture, Transwell assay and bioinformatics analysis. YS and ML performed the lentivirus infection, RT-qPCR and luciferase assay. ZM drafted the manuscript. ZM and NG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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