

Differentiation-related zinc finger protein 750 suppresses cell growth in esophageal squamous cell carcinoma

SHEYNE STA ANA CHOI, JOSEPHINE MUN-YEE KO, VALEN ZHUOYOU YU,
LVWEN NING and MARIA LI LUNG

Department of Clinical Oncology, The University of Hong Kong, Hong Kong, SAR, P.R. China

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Abstract. Esophageal squamous cell carcinoma (ESCC) is a deadly squamous cell carcinoma (SCC) of the esophagus. Development of SCCs is associated with the deregulation of the squamous cell lineage program and/or keratinocyte terminal differentiation by genomic and genetic aberrations; thus, these processes must be tightly controlled to maintain normal squamous cell development. Zinc finger protein 750 (*ZNF750*) is a gene involved in keratinocyte terminal differentiation and is frequently mutated and putatively silenced in ESCC, which implicates its function as a potential differentiation-related suppressor of ESCC. The present study aimed to elucidate the relationship between *ZNF750* function to induce keratinocyte differentiation and tumor suppression in ESCC. The results demonstrated that chemical manipulation of esophageal keratinocyte differentiation in mouse normal esophageal epithelial organoids (mNEEO) implicated the involvement of the mouse homologue of *ZNF750*, *Zfp750*, in keratinocyte differentiation in premalignant cells. Bioinformatics analyses of data from high *ZNF750*-expressing ESCC tumors obtained from public databases and *ZNF750*-overexpressing ESCC cells compared with low *ZNF750*-expressing ESCC tumors and *GFP*-expressing ESCC cells, respectively, revealed enrichment of keratinocyte differentiation-related gene sets in these samples. Finally, the induction through to terminal differentiation of the keratinocyte by all-trans retinoic acid on parental ESCC cell lines led to the upregulation of the terminal differentiation marker *Involucrin* and a decrease in cell viability similar to that observed in *ZNF750*-overexpressing ESCC cells. The results of the present study demonstrated a functional link between the ability of *ZNF750* to induce cell differentiation through to terminal differentiation and its

function as a growth suppressor in ESCC. This study provides improved understanding of the role of *ZNF750*, a frequently mutated differentiation-related gene in ESCC, and its effects in ESCC pathogenesis.

Introduction

Esophageal cancer is a deadly type of cancer that ranks 8th in terms of worldwide cancer mortality (1). The predominant histological subtype in most parts of the world, especially in East and Central Asia and parts of East Africa and South Africa, is esophageal squamous cell carcinoma (ESCC) (2). The development of squamous cell carcinomas (SCCs) is associated with various genomic and genetic aberrations that lead to the deregulation of the squamous cell lineage and terminal differentiation program; thus, these processes must be tightly controlled to maintain normal squamous epithelium development (3).

Zinc finger protein 750 (*ZNF750*) is a member of the zinc finger family proteins, which are involved in numerous biological processes, including keratinocyte and epithelial cell differentiation, as well as adipogenesis (4). These proteins have also been implicated in different diseases including diabetes, psoriasis and cancer (4,5). *ZNF750* has been reported to be involved in the maintenance of epithelial homeostasis and the epidermal terminal differentiation process in primary and organotypic human keratinocyte culture through its interaction with transcription factors and epigenetic modifiers (6,7). In addition, knockout of *ZNF750* in squamous cells silenced the expression levels of differentiation-related genes Krüppel-like factor 4, S100 calcium-binding protein A8 and loricrin (8). Frequent somatic mutations within the exonic regions have been reported in cervical, head and neck, lung and esophageal SCCs (7-9), implicating the silencing of *ZNF750* in these malignancies.

The whole-exome sequencing analysis in our previous study demonstrated the clinical relevance of *ZNF750* in ESCC (10). *ZNF750* levels are downregulated in ESCC tumors compared with those in distant non-tumor tissues, and mutations in this gene are significantly more frequent in primary ESCC tumors with lymph node (LN) metastasis compared with those without LN metastasis (10). The levels of differentiation-related genes, including small proline-rich protein 3 (*SPRR3*) and transglutaminase 3 (*TGM3*), have been reported to be downregulated

Correspondence to: Professor Maria Li Lung, Department of Clinical Oncology, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, SAR, P.R. China
E-mail: mlilung@hku.hk

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in primary tumor samples compared with those in adjacent non-tumor tissues (11,12). In addition, SPRR3 exhibits tumor-suppressive functions in ESCC cells (13), indicating a link between silenced differentiation-related genes and tumor-suppressive functions in ESCC. Therefore, we hypothesized that *ZNF750* as a frequently mutated and downregulated differentiation-related gene may function similarly to other downregulated differentiation-related genes, such as SPRR3, and act as a tumor suppressor in ESCC.

The function of *ZNF750* in the suppression of SCCs, including ESCC, has been demonstrated in an *in vitro* cell viability and an *in vivo* tumorigenesis models (9,14), whereas its functions in keratinocyte differentiation induction have been reported in keratinocyte cell line and organoid models (5-7); however, the functional link between keratinocyte differentiation induction and tumor suppression in ESCC has not been fully explored. The aim of the present study was to elucidate this relationship.

Materials and methods

Cell culture. The KYSE-series cell lines (ACC 351, ACC 375, ACC 379, ACC 363, ACC 374 ACC 380, ACC 387; DSMZ) were cultured in RPMI-1640 medium (cat. no. 23400) supplemented with 10% fetal bovine serum (FBS; cat. no. 10270) and 1% penicillin-streptomycin (cat. no. 15070). NE-1 cells were cultured in Defined Keratinocyte SFM medium with Defined Keratinocyte SFM Growth Supplement (cat. no. 10744019). 293T, HKESC-2 and SLMT cells were cultured in DMEM (cat. no. 12100) supplemented with 5% FBS and 5% Newborn Calf Serum (NCS; cat. no. 16010), and 1% penicillin-streptomycin. 293T cells served as host cells for lentiviral production. Cells were seeded and incubated until full confluence and passaged every 2-3 days using Trypsin/EDTA (cat. no. 25200) for continuous culture at 37°C with 5% CO₂. All reagents used for cell culture were purchased from Invitrogen; Thermo Fisher Scientific, Inc. The cell lines were genotyped, authenticated and tested for mycoplasma prior to the experiments.

Mouse normal esophageal epithelial organoid (mNEEO) generation and culture. Mouse esophageal tissue isolation, organoid generation and culture were performed as previously described with modifications (15). In brief, 8-10-week old BALB/c/nu/nu athymic mice (n=4) were sacrificed through anesthetic overdose by intraperitoneal injection of ketamine/xylazine (300 mg/kg ketamine and 30 mg/kg xylazine) followed by carbon dioxide asphyxiation by placing mice in a carbon dioxide-filled container. The esophagus was surgically removed, and the mucosa was pulled away from the submucosa layer using forceps. The mucosa layer was cut into small pieces with a scalpel, dissociated in Trypsin-EDTA at 37°C for 30 min and subsequently neutralized with RPMI-1640 supplemented with 10% FBS. To generate organoids, the dissociated cells were suspended in Matrigel® (cat. no. 356234; Corning, Inc.) and placed in 24-well low-bind culture plates (cat. no. 174930; Thermo Fisher Scientific, Inc.) at 37°C to solidify. The mNEEO was cultured in growth medium comprising advanced DMEM/F12 (cat. no. 12634), 1X N2 (cat. no. 17502) and 1X B27 Supplements (cat. no. 17504), 1X Glutamax (cat. no. 35050), 1X HEPES (cat. no. 15630),

1X penicillin/streptomycin, 1 mM N-acetyl-L-cysteine (cat. no. A9165, Sigma-Aldrich; Merck KGaA), 100 μM gastrin (cat. no. G9020, Sigma-Aldrich; Merck KGaA), 10 mM nicotinamide (cat. no. N0636, Sigma-Aldrich; Merck KGaA), 10 μM SB202190 (cat. no. S7067, Sigma-Aldrich; Merck KGaA), 50 ng/ml epidermal growth factor (cat. no. PHG0314), 100 ng/ml Noggin (cat. no. 120-10C; PeproTech, Inc.), 100 ng/ml Wnt3A (cat. no. 315-20; PeproTech, Inc.), 100 ng/ml R-Spondin 2 (cat. no. 120-43; PeproTech, Inc.) and 500 nM A8301 (cat. no. 2939; Tocris Bioscience) as previously described (15) and passaged every 7 days for continuous culture. All reagents were supplied by Thermo Fisher Scientific, Inc. unless otherwise specified. Ethics approval for animal studies was obtained from the Animal Ethics Committee of The University of Hong Kong (approval no. CULATR 3377-14).

Chemically induced manipulation of mNEEO differentiation. For all-trans retinoic acid (ATRA; cat. no. R2625; Sigma-Aldrich; Merck KGaA) and Compound E (cat. no. sc-221433; Santa Cruz Biotechnology, Inc.) treatment of mNEEO, 2,000 dissociated esophageal epithelial cells were embedded in 4 wells/group in a low-bind 96-well culture plate (cat. no. 15237905; Thermo Fisher Scientific, Inc.). The esophageal epithelial cells were cultured in growth medium for 24 h prior to ATRA and Compound E treatment. Growth medium was replaced with growth medium containing 100 nM ATRA, 10 μM Compound E or DMSO (cat. no. D418; Sigma-Aldrich; Merck KGaA) vehicle after 24 h, and the cells were cultured for 9 days to generate organoids.

Molecular cloning of *ZNF750*. *ZNF750* (NM_024702.3) was amplified using Q5 High-Fidelity DNA Polymerase (cat. no. M0491; New England Biolabs, Inc.) from KYSE30 cell line cDNA by PCR. The PCR procedure was performed using a Veriti™ 96-Well Thermal Cycler (cat. no. 4375786; Thermo Fisher Scientific, Inc.) under the following cycling conditions: Initial denaturation at 98°C for 30 sec, followed by 30 cycles of annealing at 56°C for 30 sec and extension at 72°C for 2 min. The oligonucleotide pairs used for amplification were *ZNF750* forward, 5'-GGAATTCGCCACCATGAGTCTCCTCAAAGA GCGG-3' and reverse, 5'-GCTCTAGATTACTTGTCTGTCATCGTCTTTGTAGTCGGACACCCGGGCGCCTC-3'. The amplified DNA was subsequently cloned into a pLVX-EF1α-puro vector. The *ZNF750* sequence (pLVX-*ZNF750*) was validated by Sanger sequencing (16) at the Centre for PanorOmic Sciences (CPOS) at the University of Hong Kong (Hong Kong, SAR, China). pLVX-puro (cat. no. 632164; Clontech Laboratories, Inc.) was modified by removing the CMV promoter and replacing it with an EF1α promoter to construct a pLVX-EF1α-puro vector (17). The pLVX-EF1α-puro vector was used to insert a GFP gene to create the pLVX-GFP control plasmid (17).

Lentivirus production and infection. 293T cells were seeded in preparation for viral production in T25 culture flasks. When 293T cells reached 60% confluence, the transfection mixture was prepared using 8 μl X-tremeGENE™ HP DNA Transfection Reagent (cat. no. 6366546001; Roche Molecular Systems, Inc.), 2 μg plasmid DNA (pLVX-GFP or pLVX-*ZNF750*), 1.5 μg viral packaging vector psPAX2 (cat. no. 12260; Addgene, Inc.) and 0.5 μg viral envelope vector

pMD2.G (cat. no. 12259; Addgene, Inc.) in 400 μ l serum-free DMEM and incubated for 20 min at room temperature. The transfection mixture was added dropwise to the culture flask of HEK293T cells and incubated for 72 h at 37°C. Subsequently, the viral supernatant was passed through a 0.45- μ m filter and aliquoted. The virus was stored at -80°C until further experiments. For lentiviral infection, KYSE30 and KYSE180 cells were seeded to achieve a confluency of ~30% at 24 h post-seeding. A 1 ml aliquot of pre-prepared virus expressing GFP or ZNF750 with 0.006 μ l 5 μ g/ml polybrene (cat. no. H9268; Sigma-Aldrich; Merck KGaA) was added to cells in tissue culture flasks to facilitate lentiviral infection. The medium was changed at 24 h post-infection to allow the cells to recover. The cells were passaged at day 2 post-infection for expansion and prepared for assays after the second passage.

RNA isolation, reverse transcription (RT) and quantitative (q)PCR. RNA was extracted from cells and organoids using the TRI Reagent™ solution (cat. no. AM9738; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions and reverse-transcribed to complementary DNA using PrimeScript RT Master Mix (Perfect Real Time) (cat. no. RR036; Takara Bio, Inc.) according to the manufacturer's instructions. FastStart Universal SYBR®-Green Master (Rox) (cat. no. 4913914001; Roche Life Science) was used to determine the mRNA expression levels of target genes. Each reaction comprised 1X FastStart Universal SYBR® Green Master mix, 30 ng/ μ l cDNA, and 0.4 mM each of forward and reverse primer. The reactions were performed in the LightCycler® 480 Instrument II (cat. no. 05015243001; Roche Life Science) using the default SYBR®-Green program. mRNA expression levels were quantified using the $2^{-\Delta\Delta C_q}$ method (18). Human GAPDH forward, 5'-AAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3', was used as the internal control. The primer sequences were as follows: Human: *ZNF750* forward, 5'-AAAGCACAGAATGCCTACCTG-3' and reverse, 5'-GGGTCTCCGTTTACAACATT-3'; keratin 4 (*KRT4*) forward, 5'-GATGCTCTTCTGGGGGAT T-3' and reverse, 5'-GCTCTGGTTGATGGTGACCT-3'; *SPRR1A* forward, 5'-CCGTATACCAGCTTTCTGTCTC-3' and reverse, 5'-GCTGGCAAGGTTGTTTCAC-3'; *SPRR2E* forward, 5'-TGGTACTTGAGCACTGATCTGC-3' and reverse, 5'-TGCAGTCTGCTGTTGATAA-3'; *TGM3* forward, 5'-TGG AAGGACTCTGCCACAAT-3' and reverse, 5'-TGAGCG TACGAGATCTTTATGG-3'; and involucrin (*IVL*) forward, 5'-GCAGGAGGGACAGCTGAA-3' and reverse, 5'-ATCTGC TCCTCTGGGACCTC-3'; mouse: *Zfp750* forward, 5'-GAG CCAGCGTGAGAACAGA-3' and reverse, 5'-CAGGAGAGT TCCTTCCGTCA-3'; *Ivl* forward, 5'-CCCTAGTTGCTGCTC AGCTC-3' and reverse, 5'-TGGAAGCTTCTGTAGACCAAA A-3'; *Krt4* forward, 5'-AACAAATTGCGTCCTTCAT-3' and reverse, 5'-CTGCTGGAGGAGGTTCCAT-3'; and *Krt14* forward, 5'-ATCGAGGACCTGAAGAGCAA-3' and reverse, 5'-TCGATCTGCAGGAGGACATT-3'.

Protein extraction and western blotting. Protein lysates were obtained by lysing KYSE30 and KYSE180 cells for 10 min at 4°C in RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) supplemented with 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (cat. no. 78442; Thermo Fisher Scientific,

Inc.), and 1 unit of Pierce Universal Nuclease (cat. no. 88700; Thermo Fisher Scientific, Inc.). Protein lysates were collected by centrifugation at 12,000 x g at 4°C for 10 min. The proteins were denatured at 95°C for 10 min prior to electrophoresis. A total of 50 μ g protein per sample were electrophoresed using 12% SDS-PAGE at 80V for 30 min and 120V for 1.5 h using the Mini Gel Tank (cat. no. A25977; Thermo Fisher Scientific, Inc.). Following electrophoresis, the proteins within the gel were transferred to methanol-activated 0.45- μ m PVDF membranes (cat. no. IPVH00010; MilliporeSigma) using a Mini Blot system (cat. no. B1000; Thermo Fisher Scientific, Inc.) at 30V for 1 h. Subsequently, the membranes were blocked with 3% bovine serum albumin (v/v) in Tris-buffered saline with 0.1% Tween (TBST) for 45 min at room temperature. The membranes were hybridized with the primary antibodies at 4°C overnight with constant shaking. The membranes were briefly washed with TBST and probed with a horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:5,000 for 1 h at room temperature with gentle agitation, followed by extensive washing with TBST prior to signal detection. Signals were detected by WesternBright ECL HRP substrate (cat. no. K-12045; Advanta, Inc.) and Super RX X-ray films (Fujifilm Corporation). Band intensities were measured using ImageJ software version 1.53a (National Institutes of Health) (19) and quantified by normalization to the GFP control. The primary antibodies used were polyclonal rabbit anti-human ZNF750 (1:1,000; cat. no. 21752-1-AP; Proteintech Group, Inc.), polyclonal rabbit anti-human SPRR3 (1:1,000; cat. no. A12041; ABclonal, Inc.), monoclonal mouse anti-human IVL (1:1,000; cat. no. MA5-11803; Thermo Fisher Scientific, Inc.), monoclonal mouse anti-human p84 (1:1,000; cat. no. GTX70220; GeneTex, Inc.), monoclonal rabbit anti-human GAPDH (1:5,000; cat. no. 5174; Cell Signaling Technology, Inc.) and monoclonal rabbit anti-GFP (1:1,000; cat. no. 2956; Cell Signaling Technology, Inc.). The secondary antibodies used were polyclonal anti-mouse IgG (1:5,000; cat. no. GTX213111-01) and polyclonal anti-rabbit IgG (1:5,000; cat. no. GTX213110-01) (both from GeneTex, Inc.).

RNA sequencing (RNA-seq) and transcriptomic analysis of ZNF750-upregulation in ESCC cells. Total RNAs of pLVX-GFP- or pLVX-ZNF750-transduced KYSE180 cells were extracted with the AllPrep DNA/RNA Micro kit (cat. no. 80284; Qiagen, Inc.) according to the manufacturer's instructions for RNA sequencing. Library preparation and Illumina sequencing (paired-end sequencing of 151 bp) were performed at CPOS. Bioanalyzer RNA Nano 6000 (Agilent Technologies) was used to determine the quality of the RNA. Ribosomal RNA (rRNA) depletion was performed using NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) (cat. no. E6310; New England BioLabs, Inc.). cDNA libraries were prepared using the NEBNext® Ultra II Directional RNA Library Prep Kit (cat. no. E7760; New England BioLabs, Inc.). A total of 500 ng RNA was used as starting material for ribosomal RNA depletion. Cytoplasmic and mitochondrial rRNA were firstly hybridized to ssDNA capture probes and then digested by Ribonuclease H. Subsequently, the residual ssDNA probes were digested by DNase I. The rRNA-depleted RNA was purified by Agencourt® RNAClean™ XP beads (cat. no. A63987; Beckman Coulter, Inc.). The purified rRNA-depleted RNA was

fragmented to 200–500 bp by incubating at 94°C for 10 min in the presence of magnesium ions (NEBNext® Magnesium RNA Fragmentation Module; cat. no. E6150; New England BioLabs, Inc.). The fragmented rRNA-depleted RNA was then applied as a template to synthesize the first-strand cDNA using random hexamer-primer and a reverse transcriptase. In the second strand cDNA synthesis, the mRNA template was removed, and a replacement strand was generated to form the blunt-end double-stranded (ds) cDNA. The ds cDNA underwent 3'adenylation and indexed adaptor ligation, followed by treatment with User Enzyme. The adaptor-ligated libraries were enriched and indexed using NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) (cat. no. E6440; New England BioLabs, Inc.) by 8 cycles of PCR according to the manufacturer's instructions. The concentration was determined using qPCR following library construction, and the libraries were denatured and diluted to 1.2 nM. Illumina NovaSeq 6000 Reagent Kit (cat. no. 20028312; Illumina, Inc.) was used for paired-end 151bp sequencing. Raw RNA-Seq data were cleaned and aligned to the hg19 reference genome using Tophat (version 2.0.14; bowtie version 2.2.4) (20) with library-type fr-firststrand parameter. Gene expression profile and differentially expressed genes were calculated by Cufflinks (version 2.2.1) with the Ensemble gene annotation file and Cuffdiff (21), respectively. Differential gene expression was calculated by log₂ fold-change (FC) expression. Genes with log₂ (FC) ≥ 2 were used for further analysis.

Determination of enriched pathways in ZNF750-upregulated ESCC cells. Gene Set Enrichment Analysis (GSEA) (22) and ToppFun (<https://toppgene.cchmc.org/enrichment.jsp>) (23) were used for the analysis of enriched pathways in ZNF750-overexpressing cells. Using GSEA version 4.1.0, transcriptomic data were analyzed to identify the enriched gene sets in the ZNF750-overexpressing group compared with the GFP-expressing group to identify the genes driving the enrichment. In ToppFun, differentially upregulated genes in the ZNF750-overexpressing group compared with the control group were used for analysis. The gene sets from the Molecular Signatures Database (MsigDB; <http://www.broad.mit.edu/gsea/msigdb/index.jsp>) were used for both analyses. The consensus gene sets between the two analyses with a false discovery rate (FDR) ≤ 0.25 were considered to be enriched.

Extraction and analysis of the cancer genome atlas (TCGA) esophageal carcinoma (ESCA) RNA-seq data. TCGA data were downloaded from the National Cancer Institute Genomic Data Commons (<https://gdc.cancer.gov/>) (24). Normalized gene expression profiles of SCC samples with RNA-seq data from the TCGA-ESCA project (dbGaP study accession no. phs000178) (25) were extracted using R version 4.0.2 (26) and the TCGAAbiolinks R/Bioconductor package (27) (<http://bioconductor.org/packages/release/bioc/html/TCGAAbiolinks.html>). ESCC data were divided into high-ZNF750 expressing tumors (ZNF750-high; n=40) and low-ZNF750 expressing tumors (ZNF750-low; n=41) using median normalized expression levels as the cut-off value. GSEA was used to determine the enriched gene sets in the ZNF750-high compared with the ZNF750-low group using the same cut-off values as aforementioned.

MTT cell viability assay. To assess cell viability, 2.5x10³ KYSE30 and 5x10³ KYSE180 cells per well were seeded in a 96-well plate with 100 µl medium. For visualization, 30 µl/well 5 mg/ml MTT dye (cat. no. M6494; Thermo Fisher Scientific, Inc.) was added to the cells and incubated at 37°C for 2 h. Subsequently, the medium was aspirated, and 100 µl DMSO was added to dissolve the formazan crystals. Absorbance was measured at 540 nm using an Ao Absorbance Microplate Reader (Azure Biosystems) daily for 3 days post-seeding.

ATRA treatment of parental ESCC cell lines. For the ATRA treatment, parental KYSE30 and KYSE180 cells were seeded as aforementioned and cultured for 24 h. Subsequently, the culture medium in each well was replaced with medium containing 50 or 100 nM ATRA or DMSO and cultured for 3 days. Concurrently, cells from the same batch were seeded in T25 culture flasks and treated with ATRA. RNA was extracted as aforementioned from the ATRA-treated cells at 48 h post-treatment and subjected to RT-qPCR analysis. Experiments were performed on KYSE30 and KYSE180 cell lines using previously reported ATRA concentrations as a reference to determine the optimal ATRA concentration for each cell line (28).

Colony formation assay. For the colony formation assay, 1x10³ KYSE30 or KYSE180 cells were seeded into a single well of a 6-well culture plate to form colonies. The colonies were fixed with 37% formaldehyde for 30 min at room temperature and stained with 1:20 Giemsa solution in water overnight at room temperature. The solution was washed away with 1X PBS until the buffer was clear. Colonies were counted using the Gel Doc XR+ system (cat. no. 170-8195; Bio-Rad Laboratories, Inc.).

Subcutaneous tumor mouse model. ESCC tumorigenicity was evaluated by subcutaneous inoculation of ESCC cells into the flanks of 8–10-week-old BALB/c/nu/nu athymic nude mice. A total of 1x10⁶ KYSE30 cells were resuspended in 150 µl of serum-free medium and injected in both flanks of each mouse (n=4 mice per group). Tumor growth was monitored weekly for ≥ 4 weeks and measured by calipers to calculate the tumor volume as follows: Volume=(length x width x height). At the experimental endpoint, mice were sacrificed by anesthetic overdose through intraperitoneal injection of ketamine/xylazine (300 mg/kg ketamine and 30 mg/kg xylazine) followed by carbon dioxide asphyxiation by placing mice in a carbon dioxide-filled container. Cessation of cardiac function was used as the method for verification of death. All experimental procedures were approved by the Committee on the Use of Live Animals in Teaching and Research at The University of Hong Kong (approval no. CULATR 3377-14).

Statistical analysis. Data are presented as the mean ± SEM. Each *in vitro* experiment was repeated at least three times in duplicate, unless otherwise stated. Unpaired Student's t-test was performed for all statistical analyses using GraphPad Prism version 8.4.3 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

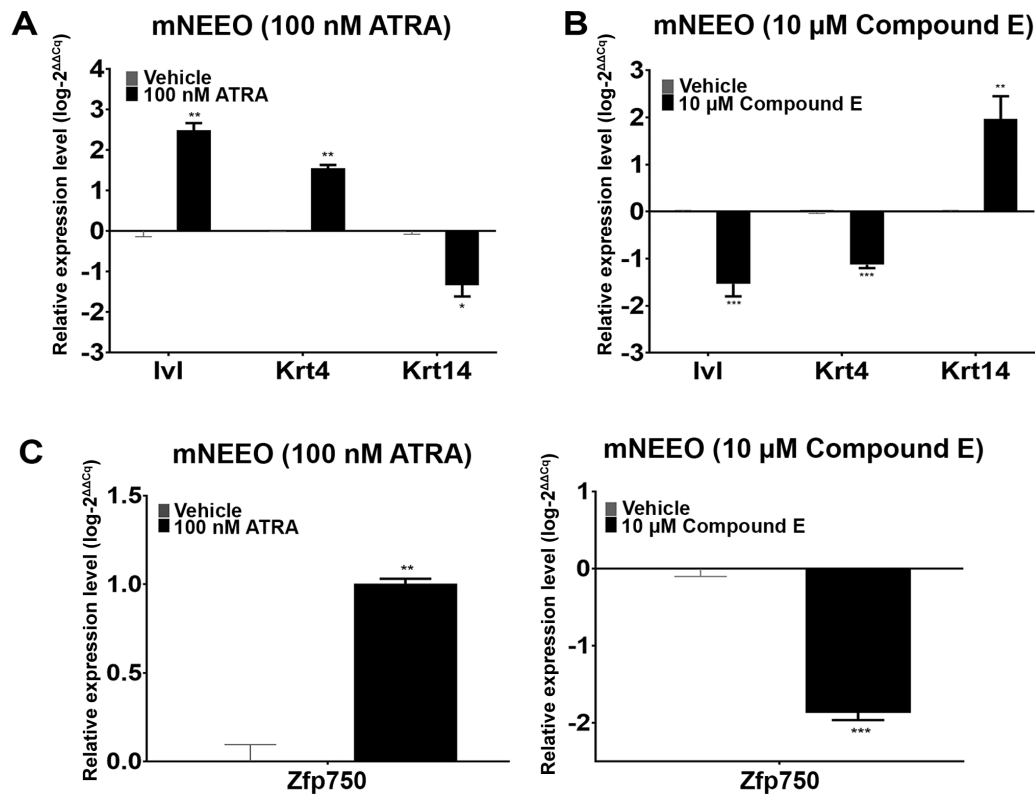


Figure 1. *Zfp750* is involved in the esophageal keratinocyte differentiation program in mNEEO cells. (A) ATRA treatment induced the upregulation of *Ivl* and *Krt4* and the downregulation of *Krt14* expression levels compared with those in the vehicle-treated cells. (B) Compound E treatment resulted in the downregulation of *Ivl* and *Krt4* and upregulation of *Krt14* expression levels compared with those in the vehicle-treated cells. (C) *Zfp750* expression was upregulated upon induction of differentiation through ATRA treatment and downregulated upon inhibition of differentiation through Compound E treatment. Data are presented as the mean \pm SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle. mNEEO, mouse normal esophageal organoid; *Zfp750*, zinc finger protein 750; ATRA, all-trans retinoic acid; *Ivl*, involucrin; *Krt*, keratin.

Results

Zfp750 is involved in the esophageal keratinocyte differentiation program in mouse epithelial cells. mNEEOs were used to determine the role of *Zfp750* in the esophageal keratinocyte differentiation program. Following ATRA treatment, the organoids demonstrated increased expression levels of *Ivl* and *Krt4* and decreased levels of *Krt14* compared with those in the vehicle-treated mNEEOs (Fig. 1A), suggesting an induction of the keratinocyte differentiation program and subsequent decrease in the basal cell layers within the organoid. By contrast, following Compound E treatment, the expression levels of *Ivl* and *Krt4* were markedly decreased, whereas those of *Krt14* were increased compared with those in the vehicle-treated group (Fig. 1B), suggesting the inhibition of the differentiation program and an increase in the basal cell layers in these organoids. Changes in *Zfp750* expression corresponded with the expression levels of the markers of differentiation, *Ivl* and *Krt4*, after both treatments (Fig. 1C). These results suggested that *Zfp750* expression may be proportionally affected by the stimulation and inhibition of the esophageal keratinocyte differentiation program, and that *Zfp750* may be involved in this cellular process in esophageal keratinocytes.

High ZNF750 expression in ESCC tumors is associated with the enrichment of keratinocyte and epidermal cell

differentiation gene sets. To determine whether *ZNF750* expression may affect the keratinocyte differentiation program in ESCC tumors, RNA-seq data of ESCC tumor samples (n=81) from the TCGA-ESCA dataset were analyzed using GSEA. The samples were stratified into *ZNF750*-high [median, 20.07; interquartile range (IQR), 19.03-21.11] and *ZNF750*-low (median, 17.86; IQR, 16.45-19.27) groups (Fig. 2A). The results of GSEA confirmed that epithelial and epidermal differentiation-related cellular processes including 'cornification', 'keratinization', 'keratinocyte differentiation' and 'epidermal cell differentiation' (Table SI) were enriched in the *ZNF750*-high group compared with the *ZNF750*-low group. 'Keratinocyte differentiation' and 'epidermal cell differentiation' gene sets were among the most enriched sets (Fig. 2B), whereas *SPRR*, *TGM* and *IVL* were among the most enriched genes (Table SII). These results supported the involvement of *ZNF750* in keratinocyte differentiation in ESCC.

ZNF750 overexpression suppresses ESCC cell viability and growth in vitro and in vivo. *ZNF750* expression levels were downregulated in the ESCC cell line models compared with those in the immortalized human esophageal epithelial cell line NE1 (Fig. 3A); therefore, to study the suppressive function of *ZNF750* in ESCC, *ZNF750* was overexpressed in KYSE30 and KYSE180 cells by lentiviral transduction of pLVX-GFP and pLVX-ZNF750. These cell lines were selected as the models for further experiments as they exhibited decreased expression

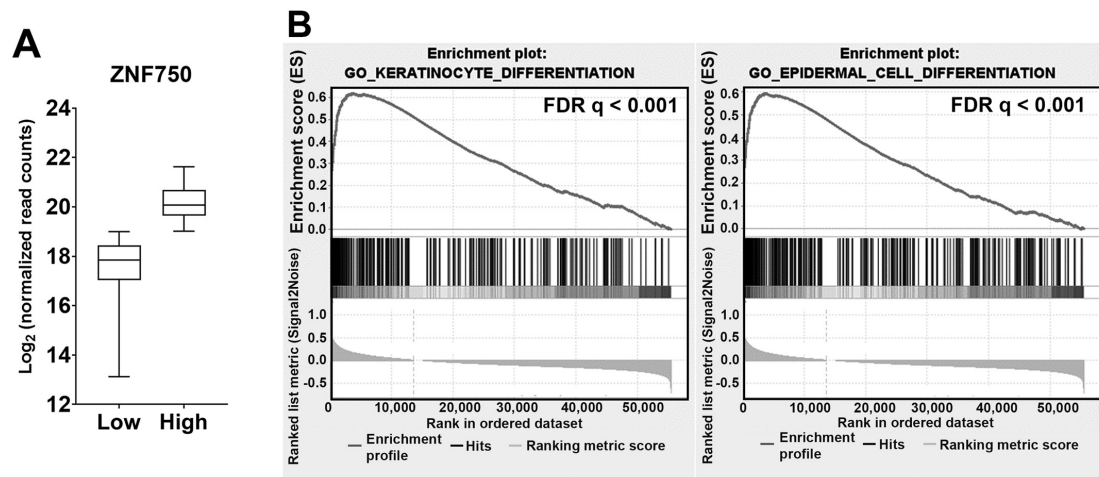


Figure 2. ESCC tumors with high *ZNF750* expression levels in The Cancer Genome Atlas exhibit upregulated genes involved in keratinocyte and epidermal differentiation. (A) *ZNF750* expression in ESCC tumors with normalized to the median expression as the cut-off. The box and whisker plot represents the normalized median expression and the interquartile range. (B) Enrichment plots of 'keratinocyte differentiation' and 'epidermal cell differentiation' gene sets in the *ZNF750*-high group compared with the *ZNF750*-low group. *ZNF750*, zinc finger protein 750; ESCC, esophageal squamous cell carcinoma.

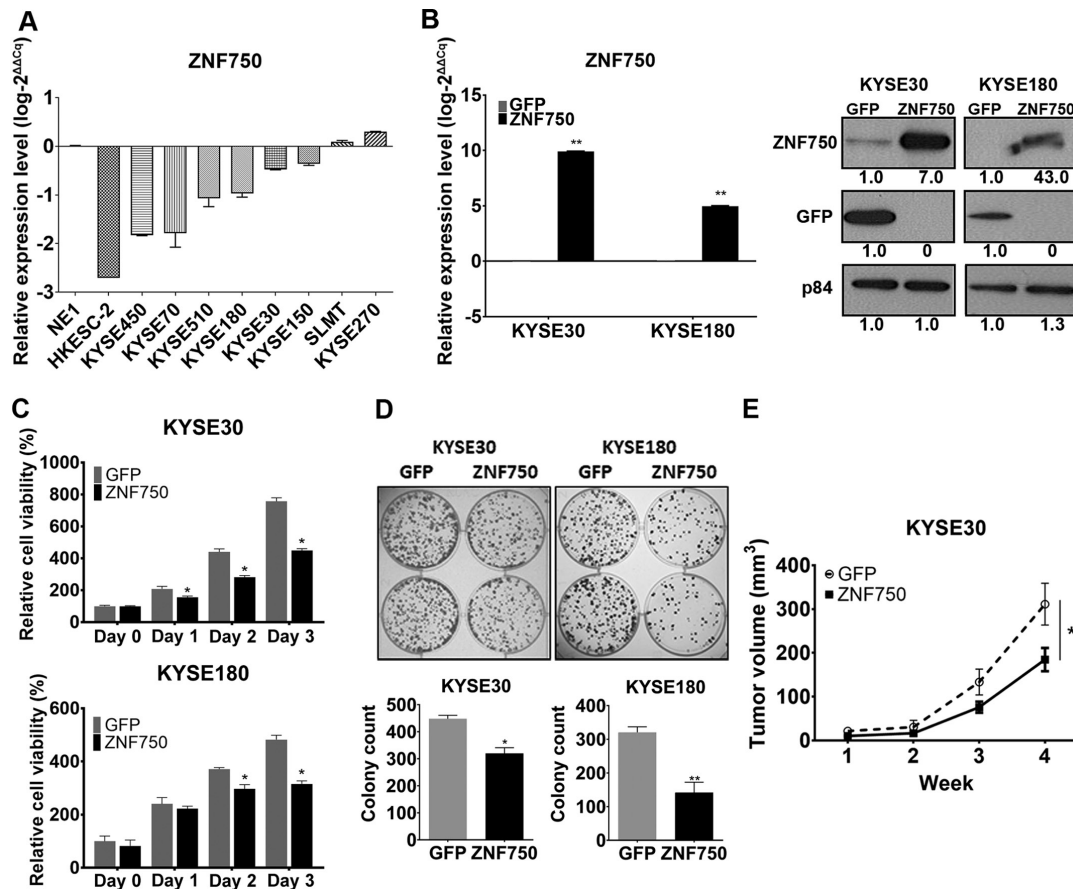


Figure 3. Overexpression of *ZNF750* suppresses ESCC cell viability and proliferation *in vitro* and *in vivo*. (A) *ZNF750* expression levels were downregulated in the majority of the tested ESCC cell lines compared with those in the immortalized normal esophageal cell line NE1. (B) *ZNF750* overexpression in KYSE30 and KYSE180 cells was confirmed by reverse transcription-quantitative PCR and western blotting. Numbers under the blots represent quantified band intensities normalized to the GFP control for each presented blot. (C) Overexpression of *ZNF750* decreased ESCC cell viability, as determined by the MTT assay. (D) Colony formation assay demonstrated that overexpression of *ZNF750* decreased the colony formation ability of ESCC cells compared with that of the control cells. (E) *In vivo* tumor formation assay in nude mice demonstrated that *ZNF750* overexpression inhibited ESCC tumorigenicity. Data are presented as the mean \pm SEM. **P*<0.05, ***P*<0.01 vs. GFP. *ZNF750*, zinc finger protein 750; GFP, control green fluorescent protein lentivirus; ESCC, esophageal squamous cell carcinoma.

of *ZNF750* at the mRNA and protein level (Fig. 3A and B). *ZNF750* expression levels were upregulated in both cell

lines in the pLVX-*ZNF750* groups compared with those in the corresponding pLVX-GFP groups, as demonstrated

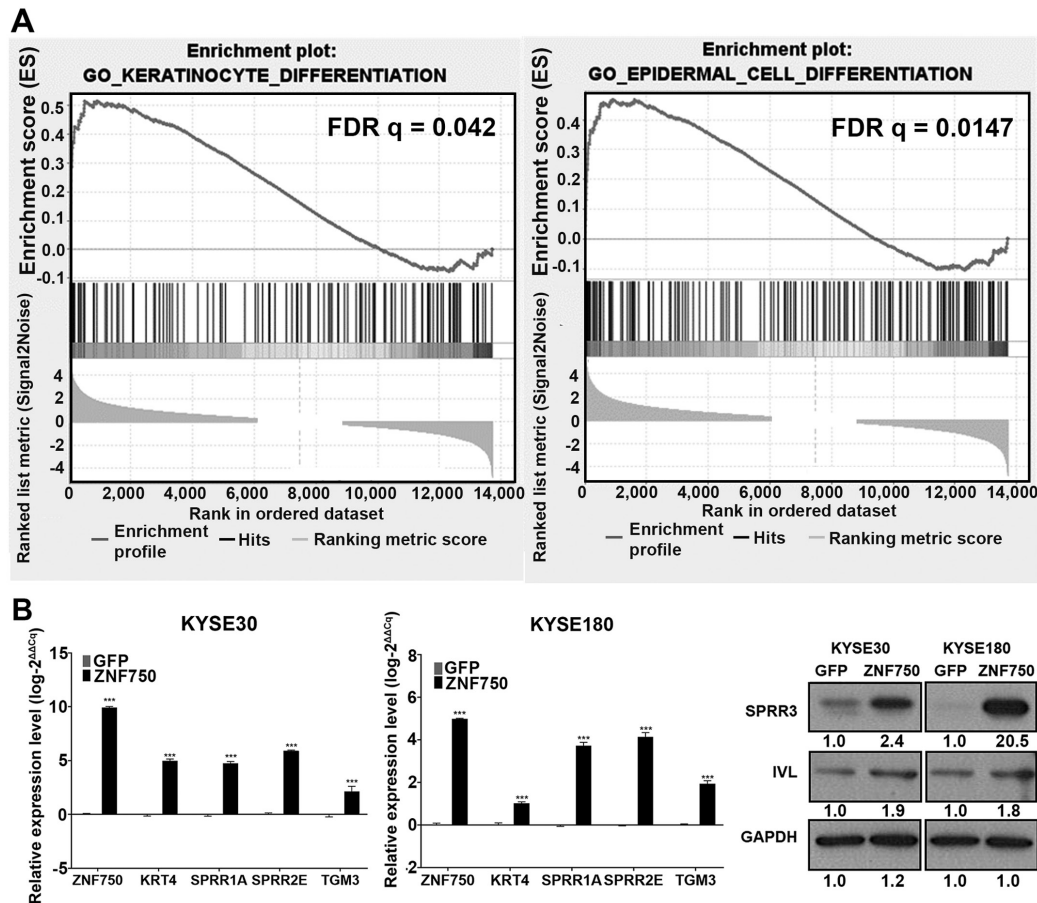


Figure 4. Overexpression of *ZNF750* leads to enrichment of differentiation-related gene sets in ESCC cells. (A) Enrichment plots of 'keratinocyte differentiation' and 'epidermal cell differentiation' gene sets analyzed by Gene Set Enrichment Analysis and Toppfun with a false discovery rate <0.25. (B) Reverse transcription-quantitative PCR and western blot analyses confirmed the upregulation of differentiation-related gene *KRT4*, *SPRR1A*, *SPRR2E*, *SPRR3*, *TGM3* and *IVL* expression levels in *ZNF750*-overexpressing ESCC cells compared with those in the control group. Numbers represent band intensities normalized to the GFP control for the presented blot. Data are presented as the mean \pm SEM. ***P<0.001 vs. GFP. *ZNF750*, zinc finger protein 750; GFP, control green fluorescent protein lentivirus; ESCC, esophageal squamous cell carcinoma; *SPRR*, small proline-rich protein; *TGM3*, transglutaminase 3; *IVL*, involucrin.

by RT-qPCR and western blot analyses (Fig. 3B). *In vitro* MTT cell viability and colony formation assays and *in vivo* subcutaneous tumorigenicity model were used to assess the tumor-suppressive ability of *ZNF750*. *In vitro*, *ZNF750* overexpression suppressed ESCC cell viability compared with that in the control group (Fig. 3C). Colony formation ability was also reduced in the *ZNF750*-overexpressing groups compared with that in the controls (Fig. 3D). In the *in vivo* subcutaneous tumor model, mice in the *ZNF750* overexpression group developed smaller tumors (n=8; mean \pm SEM, 184 \pm 26.5 mm³) in mice compared to the GFP control (n=8; mean \pm SEM, 311.17 \pm 47.76 mm³) (P<0.05; Fig. 3E). These results demonstrate the ability of *ZNF750* to suppress cell viability and growth *in vitro* and *in vivo*.

***ZNF750* overexpression in ESCC cells induces the expression of esophageal keratinocyte differentiation genes.** To determine the pathways affected by *ZNF750* overexpression in the *in vitro* model, *ZNF750* and GFP lentivirus-transduced KYSE180 cells were prepared for RNA-seq and subjected to subsequent bioinformatics analyses. The results of the analyses using the MSigDB revealed enrichment of gene sets involved in epithelial differentiation including 'epithelial cell differentiation', 'epidermal cell differentiation' and 'keratinocyte

differentiation' (Table SIII). Consistent with the GSEA analysis of *ZNF750*-high ESCC tumors, these results confirmed the enrichment of keratinocyte differentiation and epidermal cell differentiation gene sets in *ZNF750*-overexpressing ESCC cells compared with the controls (Fig. 4A). The upregulation of differentiation-related genes *KRT4*, *SPRR1A*, *SPRR2E*, *TGM3*, *IVL* and *SPRR3* were validated by RT-qPCR or western blotting (Fig. 4B). These results suggested that *ZNF750* overexpression induced the expression of differentiation-related genes in the ESCC cell line model.

***ATRA*-induced terminal differentiation decreases the viability of ESCC cells.** To determine the effects of the induction of the keratinocyte differentiation program on ESCC cell viability, parental KYSE30 and KYSE180 cells were treated with *ATRA* and subsequently tested for changes in cell viability using the MTT assay. The results demonstrated a significant suppression in the viability of the *ATRA*-treated parental ESCC cells compared with that in the vehicle-treated group at 48 h post-treatment (Fig. 5A). RT-qPCR analysis revealed that *ATRA* treatment upregulated the expression levels of *IVL* compared with those in the vehicle group (Fig. 5B), indicating the induction of terminal differentiation. These results suggested that induction of the keratinocyte differentiation

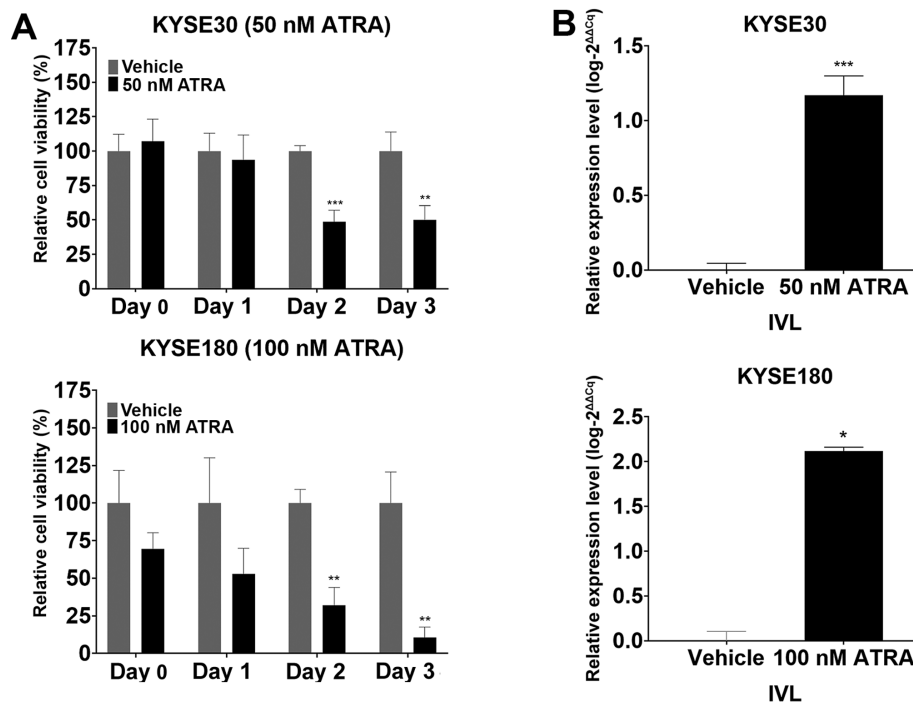


Figure 5. Induction of keratinocyte differentiation through to terminal differentiation contributes to suppression of ESCC cell viability. (A) MTT assay of KYSE30 and KYSE180 cells demonstrated the suppression of ESCC cell viability following ATRA treatment. (B) Reverse transcription-quantitative PCR analysis of ESCC cells treated with ATRA for 48 h revealed significant upregulation of the mRNA levels of the terminal differentiation marker *IVL*. Data are presented as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle. ATRA, all-trans retinoic acid; *IVL*, involucrin.

program through to terminal differentiation may contribute to the suppression of ESCC cell viability following ATRA-induced stimulation of differentiation.

Discussion

ESCC is a highly malignant SCC of the esophagus (29). Although SCCs arise from various organ systems, they have been reported to share common genetic, genomic and epigenetic modifications relevant to malignancy, and the development of SCCs is associated with the deregulation of squamous cell lineage commitment that may ultimately lead to carcinogenesis (3). Numerous landscape studies have aimed to identify the genetic and genomic changes that contribute to the development of ESCC (9,14,30,31). These studies identified frequently mutated genes in ESCC including common cancer-related (*TP53*, *RBI*, *CDKN2A*, *PIK3CA*, *NOTCH1* and *NFE2L2*) and previously unreported (*ADAM29*, *FAM135B*, *ZNF750* and *FAT1*) Genes. *ZNF750* is a gene that encodes a transcription factor involved in the terminal differentiation of SCCs (8,14) and has been reported to be one of the most frequently mutated genes in ESCC, suggesting that it may function as a differentiation-related tumor suppressor gene. Although *in vitro* and *in vivo* studies have provided evidence for *ZNF750* functioning as a tumor suppressor and as an inducer of differentiation in ESCC (9,14), the link between the functions of *ZNF750* in inducing differentiation and tumor suppression in ESCC is yet to be fully explored.

The results of the present study demonstrated the involvement of *Zfp750* in the esophageal keratinocyte differentiation program through the upregulation and downregulation of *Zfp750* expression with chemical stimulation and inhibition,

by ATRA and Compound E, respectively, of the differentiation program in mNEEOs. ATRA is a chemical promoter of keratinocyte differentiation in esophageal cells (28,29) and was used in the present study to induce the epithelial differentiation program. Alternatively, γ -secretase inhibitor Compound E was used to mimic the inhibition of the keratinocyte differentiation program through the inhibition of the NOTCH signaling pathway (30,31). Through the analysis of public data, the present study also confirmed the ability of *ZNF750* to induce differentiation-related gene expression through the enrichment of differentiation-related gene sets in *ZNF750*-high ESCC tumors. In addition, the results of the present study demonstrated that *ZNF750* suppressed ESCC cell viability and proliferation *in vitro*, and its overexpression resulted in smaller tumors in the mouse model compared with those in the corresponding controls. RNA-seq study of the ESCC cell line model further confirmed the involvement of *ZNF750* in keratinocyte differentiation in ESCC cells through the upregulation of differentiation-related genes *SPRR1A*, *SPRR2E*, *SPRR3*, *TGM3*, *KRT4* and *IVL*. Finally, chemically induced stimulation of keratinocyte differentiation through to terminal differentiation by ATRA functionally suppressed ESCC through the observed decrease in cell viability in ATRA-treated cells compared with the controls. This decrease in cell viability was similar to that of *ZNF750*-overexpressing ESCC cells. Collectively, these results demonstrated the ability of *ZNF750* to induce ESCC cell differentiation through to terminal differentiation, as well as the link between this process and the suppression of ESCC cell viability.

The surface of the esophagus is lined with stratified squamous epithelium, which comprises the basal proliferative (keratinocyte) layer that gives rise to the layers of non-dividing

terminally differentiated cells that form the stratified layer (3). The regulation of this proliferation-differentiation gradient ensures homeostasis within the epithelium (32). The squamous cell commitment to differentiate involves the regulation of the expression levels of genes relevant to this process, and deregulation of this system has been reported to initiate malignancy (33). NOTCH is a major player in epithelial differentiation and one of the most frequently mutated genes in ESCC (3,9,30), suggesting that it may be a candidate tumor suppressor. Differentiation-related genes including *SPRR1A*, *SPRR2E*, *SPRR3*, *TGM3* and *IVL* have been reported to be downregulated in ESCC (11,34). *SPRR3*, also termed esophagin, is one of the genes upregulated by ZNF750 in ESCC cells in the current study, and has been implicated as a tumor suppressor in ESCC (13). Exogenous expression of *SPRR3* attenuates ESCC tumorigenicity by promoting apoptosis (13) and enhances sensitization to DNA damage-related apoptosis (35). In addition, *TGM3* has been reported as a potential prognostic marker in ESCC (12). Proteomics analysis in tissues from patients with ESCC has revealed that the presence of *TGM3* in ESCC tissues is correlated with a longer survival time (12). These results suggest that epithelial differentiation-related genes are downregulated or silenced in ESCC and may function as putative tumor suppressors or prognostic markers, providing evidence of the relevance of this process in ESCC development.

The ability of ZNF750 to induce keratinocyte terminal differentiation indicated by the expression of *IVL* in the present study resembled the chemically induced differentiation stimulation that caused increased *IVL* expression in ESCC cell lines. ESCC viability suppression was consistently observed in ZNF750-overexpressing and differentiation-induced cells, suggesting a functional association between the two groups. This provides a novel functional indication of the link between the differentiation-related functions of ZNF750 and its tumor-suppressive ability in ESCC.

In conclusion, the results of the present study demonstrated that ZNF750 may be a differentiation-related silenced tumor suppressor gene in ESCC. Overexpression of ZNF750 in ESCC cells led to the upregulation of differentiation-related genes that may restore epithelial homeostasis by driving differentiation through to terminal differentiation and, thus, restoring the proliferation-differentiation balance. In addition, the ability of ZNF750 to induce the expression of genes such as *SPRR3*, which exhibit tumor-suppressive functions in ESCC, may contribute to its ability to suppress ESCC. Further studies are required to fully elucidate the link between the differentiation-related functions of ZNF750 and its growth-suppressive ability in ESCC.

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Availability of data and materials

The RNA sequencing data generated and/or analyzed during the current study are available in the National Center for Biotechnology Information Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under the accession no. PRJNA707329.

Authors' contributions

SSAC contributed to the design and execution of the study and data interpretation, and wrote the manuscript. JMYK and VZY contributed to the design of the study, data interpretation and the preparation of the manuscript. SSAC and JMYK confirm the authenticity of the raw data. LN contributed to the processing of the raw RNA-sequencing data and its subsequent analysis. MLL contributed to the study design, preparation of the manuscript and overall supervision of the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval for animal studies was obtained from the Animal Ethics Committee of The University of Hong Kong (approval no. CULATR 3377-14). The tumor burden did not exceed the recommended dimensions set by The University of Hong Kong, and the animals were anesthetized and sacrificed using acceptable methods/techniques.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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