Zinc-finger protein 750 mitigates malignant biological behavior of oral CSC-like cells enriched from parental CAL-27 cells

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Abstract. Oral squamous cell carcinoma (OSCC) is the most commonly occurring oral malignancy. Cancer stem cells (CSCs) are known to be responsible for cancer recurrence and metastasis. Zinc-finger protein 750 (ZNF750) has been reported to inhibit OSCC cell proliferation and invasion. The present study aimed to elucidate the role of ZNF750 in the inhibition of the renewal ability of CSCs derived from the OSCC cell line, CAL-27. The effects of ZNF750 on CSC-like properties were examined using aldehyde dehydrogenase (ALDH), tumor sphere formation and colony formation assays. Reverse transcription-quantitative PCR and western blotting were performed to detect the expression levels of octamer-binding transcription factor 4, sex-determining region Y-box 2, the enhancer of zeste homolog 2 (Ezh2), embryonic ectoderm development (EED) and SUZ12 polycomb repressive complex 2 subunit (SUZ12), and for the identification of genes associated with metastasis. ZNF750 effectively attenuated CSC-like cell self-renewal abilities; ZNF750 decreased the ALDH-positive cell population, tumor sphere and colony formation abilities, cell viability and stemness factors. Furthermore, the expression levels of Ezh2, EED and SUZ12 were decreased by ZNF750. ZNF750 inhibited MMP1, 3, 9 and 13 expression levels, and decreased the cell invasion and migratory abilities. Moreover, the expression of tissue inhibitors of matrix metalloproteinases-1 was increased by ZNF750. However, opposite effects were observed following the knockdown of the ZNF750 gene. Overall, the present study demonstrated that ZNF750 has the potential to inhibit the renewal of CSC-like cells enriched from parental CAL-27 cells.

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Introduction

Oral squamous cell carcinoma (OSCC) accounts for >90% of the total oral cancer cases (1). The prognosis of patients with OSCC is affected by tumor recurrence and metastasis; metastasis is the main causative factor for mortality in 90% of all cancer-related deaths (1). Cancer stem cells (CSCs) are implicated in tumor progression, metastasis, recurrence and the high mortality rate (2).

CSCs are a minority subpopulation of cells within a tumor that can sustain long-term tumor maintenance and growth through their ability of self-renewal and their high differentiation potential (3). CSCs have been investigated as prognostic markers in OSCC (4), and are considered the main factor responsible for the failure of traditional anticancer therapies (such as surgery, radiotherapy, chemotherapy or combined therapies). Tumor recurrence is a major issue in OSCC therapy and the elimination of CSCs is a target of therapies (5). Thus, various antitumor strategies have been developed to eliminate CSCs, including focusing on the identification of specific CSC markers, such as aldehyde dehydrogenase (ALDH), CD44, CD133, octamer-binding transcription factor 4 (Oct4), sex-determining region Y-box 2 (Sox2), homeobox protein NANOG, keratins, E-cadherin and integrins, phosphorylated STAT3 and spalt-like transcription factor 4 (6). Other studies have focused on molecular signaling pathways involved in the CSC niche, such as autophagy, apoptosis or the differentiation of CSCs (7-9). The search for novel cancer therapeutic strategies is still ongoing.

Zinc-finger protein 750 (ZNF750) is a potential antitumor gene that may be used to eliminate CSCs. ZNF750 has been validated as a lineage-specific tumor suppressor gene in squamous cell carcinoma (10). Previous studies have revealed that the decreased or deleted expression of ZNF750 is responsible for cell proliferation, invasion and migration in esophageal squamous cell carcinoma and OSCC (11,12). Furthermore, the overexpression of ZNF750 has been demonstrated to decrease the cancer stem cell marker $CD44^+$ cell population in OSCC (11). In addition, RNA sequence analysis (GSE134835) has revealed that ZNF750 functions as a tumor suppressor gene, suppressing the expression of histone lysine methyltransferase enhancer of zeste homolog 2 (Ezh2) in the CAL-27 OSCC cell line (13). Ezh2 has been implicated in promoting cancer cell proliferation, migration, invasion and metastasis, as well as CSC self-renewal (14).

However, to the best of our knowledge, the effects of ZNF750 on Ezh2-mediated CSC renewal have not yet been investigated. The identification of novel antitumor strategies with which to reduce or eliminate CSCs would be invaluable for cancer treatment. CSC-like cells comprise only a fraction of the total tumor cell population, which display stem cell-like characteristics with an efficient self-renewal ability and can be defined by functional analysis (15). Therefore, the present study aimed to examine the effects of ZNF750 on the renewal and invasive abilities of CSC-like cells derived from OSCC cells.

Materials and methods

Cell lines and plasmids. The present study used 293T cells and the OSCC cell line CAL-27 purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. and Procell Life Science & Technology Co., Ltd., respectively. OSCC cell line TCA-83 was provided from Dr Zhen Meng (Peking University School and Hospital of Stomatology, Beijing, China). All experiments were performed with mycoplasma-free cells. The cells were cultured in DMEM complemented medium with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin, followed by incubation at 37˚C in a humidified incubator containing 5% CO_2. The lentiviral packaging plasmids, psPax2 and pRSV-Rev, and the VSV-G envelope-encoding plasmid, pMD2.G, were provided from Dr Padraig Strappe (Central Queensland University, Queensland, Australia). The pLVX-PGK-Puro lentiviral vector backbone (oe-control) and lentiviral vector pLVX-ZNF750-PGK-Puro (oe-ZNF750) were purchased from Biowit Technologies, Ltd. The Hu6-CMV-puro lentiviral vector backbone (sh-control) and Hu6-shZNF750-CMV-puro (sh-ZNF750), which were used to knockdown the ZNF750 gene, were purchased from Shanghai GeneChem Co., Ltd. The lentiviral vector backbones (oe-control and sh-control) were used as a control for investigating the changes in the oe-ZNF750 and sh-ZNF750 groups, respectively.

CSC-like cell enrichment and identification. CSCs in OSCC are possibly isolated via cell-surface markers or specific practical features. Yet, no specific marker or CSC feature can specifically isolate oral CSC populations from OSCC (5). Spheroid assays are used to determine the self-renewal ability of CSCs (16). It has been reported that tumor spheres extracted from OSCC cells exhibit higher stem-like features (5), for example they show greater volume of expression of pluripotent transcription agents, such as Oct2, Sox2, Nanog and Kruppel-like factor 4 (17,18). Therefore, in the present study, CSC-like cells were enriched from tumor spheres following several cycles of passage.

For spheroid formation assay, CAL-27 cells (5,000 cells/well) and TCA-83 cells (5,000 cells/well) was cultured in an ultra-low adherent six-well plate (Corning, Inc.) with serum-free DMEM (Boster Biological Technology) supplemented with B27 serum-free supplements (1:50; Invitrogen; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor and 20 ng/ml human recombinant epidermal growth factor (all from PeproTech, Inc.). The first-generation spheroids were dissociated to single cells, and reseeded for a second and third round of spheroid formation to obtain CSC-like cells (the five cycles of passage cells were used in the following study). The spheroid formation was imaged and counted under a light microscope (CKX71; Olympus Corporation) following 7 days of culture. The rate of tumor sphere formation=number of tumor spheres/input cells x100%. Following tertiary generation sphere formation, tumor spheres were dissociated using trypsin digestion and the cells were re-plated to verify whether the enriched CSC-like cells from the tumor spheres exhibited stem cell properties. In addition, the effects of ZNF750 on ALDH activity, cell viability and CSC markers were further investigated. For investigating the effects of ZNF750 on tumor malignant biological behavior, CSC-like cells enriched from CAL-27 cells were used in the corresponding experiments.

Lentiviral gene transfer. The lentiviral particles were produced by using the 3rd generation system as previously described (11). Briefly, the three plasmids 7 μg psPax2, 3 μg pRSV-Rev and 3 μg VSV-G were co-transfected with a lentiviral vector (7 μg) into 293T cells using Lipofectamine 2000® (Thermo Fisher Scientific, Inc.). Lipofectamine 2000®/DNA complexes (2.5:1) were added to the 293T cells with the addition of caffeine (final concentration, 4 mM) to achieve a higher titer lentivirus (19). The lentivirus-containing supernatant was harvested at 48 and 72 h post-transfection, centrifuged at 4˚C, 1,751 x g for 10 min, filtered through the Steriflip-HV0.45 μm PVDF Filter Unit (MilliporeSigma) and concentrated using the one-step virus concentration solution, PEG-it™ (System Biosciences, LLC). The pellet was resuspended in cold PBS. The CSC-like cells were randomly divided into four groups (oe-control, oe-ZNF750, sh-control and sh-ZNF750 groups), transduced with viral stock (at MOI of 10) that expressed ZNF750 or silenced the ZNF750 gene, and supplemented with polybrene (3 μg/ml, Sigma-Aldrich; Merck KGaA) for 6 h. Cells were collected for subsequent experimentation after transduction 5 days later.

Flow cytometry analysis. For the investigation of ALDH activity in the CSC-like cells and the effects of ZNF750, the cells were stained with Aldefluor™ reagent (Stemcell Technologies, Inc.) according to the manufacturer's recommendations. The Aldefluor™ reagent system was used to identify stem/progenitor cells on the basis of their ALDH activity; cells expressing high levels of ALDH become brightly fluorescent (ALDHbr) and can be identified and enumerated using a flow cytometer. Briefly, $1x10^6$ cells were suspended in 1 ml Aldefluor assay buffer containing the ALDH substrate, with or without the ALDH specific inhibitor, diethylaminobenzaldehyde (DEAB). DEAB is used to control for background fluorescence. The cells were then incubated at 37˚C for 40 min. $ALDH^+$ cells were analyzed using a flow cytometer (FACSCalibur; BD Biosciences) to compare the fluorescence intensity with the ALDH inhibitor DEAB-treated negative control. BD FACSDiva software version 8.0.1 (BD Biosciences) was used for analysis. A high fluorescence was associated with a high ALDH activity ($ALDH^+$ cells).

Colony formation assay. To evaluate the effects of ZNF750 on the self-renewal and tumor propagation potency of CSC-like

cells (enriched from CAL-27 cells), colony formation assays were performed. The cells were seeded in six-well plates at a low density (500 cells/well) in triplicate, and cultured at 37˚C for 10 days. The plates were then washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min, followed by staining with 0.5% crystal violet at room temperature for 1 min. The plates were then washed with PBS, and images of each well were captured (Nikon Corporation) and the colonies consisting of >50 cells were counted using AlphaView version 3.4.0 (ProteinSimple). The experiment was repeated three times.

Cell viability assay. The effects of ZNF750 on the viability of the oral CSC-like cells were detected using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) assay according to the manufacturer's instructions. Briefly, CCK-8 solution (50 μl) was added to each well (1x10^5/ml) during the 2 h of culture at 37˚C, and the absorbance in each well at 48 and 72 h was measured at 450 nm using a 96-well Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.). The experiment was repeated three times.

cDNA preparation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the parental cells (CAL-27 and TCA-83 cells) and CSC-like cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.). cDNA was synthesized from 1 μg total RNA using the PrimeScript™ RT reagent kit with gDNA Eraser, according to the manufacturer's instructions, and amplified using the SYBR® Premix Ex Taq™ II kit (all from Takara Biotechnology Co., Ltd.) on an ABI 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed to examine the expression of stemness factors (Oct4 and Sox2), Ezh2, embryonic ectoderm development (EED), SUZ12 polycomb repressive complex 2 subunit (SUZ12), metastasis-related genes (MMP1, 3 and 13) and tissue inhibitors of matrix metalloproteinases (TIMP1 and 2). All PCR primers were designed for SYBR Green-based qPCR detection and purchased from Shanghai Shenggong Biology Engineering Technology Service, Ltd. The sequences of the PCR primers are presented in Table I. The thermocycling conditions were as follows: Initial incubation at 95˚C for 30 sec, then 40 cycles alternating in turn at 95˚C for 5 sec and 60˚C for 34 sec. Comparative gene expression analysis was performed using the $2^{-\Delta\Delta Cq}$ method (20) with normalization to the level of the internal control gene, β-actin (21).

Western blotting. Total protein was extracted from each group via homogenization in ice-cold RIPA lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) (all from Beyotime Institute of Biotechnology). Protein quantitation was performed by using a bicinchoninic acid assay. Equal amounts of protein (15 μg) were subjected to 10% SDS-PAGE and were transferred to polyvinylidene difluoride (PVDF) membranes (MilliporeSigma) after electrophoresis. Non-specific bindings to the membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween-20 at room temperature for 1 h, followed by incubation with the following antibodies: Anti-ZNF750 (1:1,000; cat. no. ab121685; Abcam), anti-Ezh2 (1:1,000; cat. no. ab191250; Abcam), anti-MMP9 (1:500; cat. no. 27306-1-AP; ProteinTech Group, Inc.) and monoclonal antibody anti-β-actin (1:10,000; cat. no. 66009-1; ProteinTech Group, Inc.) overnight at 4˚C. The appropriate secondary antibodies used were goat anti-mouse HRP-conjugated (1:10,000; cat. no. SA00001-1; ProteinTech Group, Inc.) or goat anti-rabbit HRP-conjugated (1:10,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at 4˚C for 1 h. All primary and secondary antibodies were diluted in TBS containing 0.1% Tween-20 at 4˚C. The blots were developed using the ECL western blotting kit (MilliporeSigma). The semi-quantification of the protein bands was performed using the AlphaView analysis system, version 3.4.0 (ProteinSimple). The values of protein expression were normalized against β-actin.

Cell invasion, migration and wound healing assays. To examine the effects of ZNF750 on the invasion of oral CSC-like cells, the Transwell system with a filter (polycarbonate membrane, 8-μm pore size) was used with Matrigel (BD Biosciences) as the substrate for invasion. The cells (1x10^5/ml) were dispersed onto a Matrigel-coated polycarbonate membrane at a dilution of 1:6 in serum-free culture DMEM (Boster Biological Technology) in the upper chamber. The lower chamber comprised of DMEM containing 10% FBS. Cell invasion was allowed to proceed for 24 h at 37˚C in 5% CO_2. After 24 h, the cells remaining on the upper surface of the filter (non-invading cells) were removed using cotton swabs. Cells that had invaded to the lower surfaces of the membrane were fixed with 4% formaldehyde at room temperature for 15 min and stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 1 min at room temperature. Filters were visualized under a light microscope (CKX71; Olympus Corporation). The assays were performed in triplicate and the average numbers of invaded cells per field (five randomly selected fields were counted) were assessed using Image-Pro Plus 6 software (National Institutes of Health). Compared with the cell invasion assay, the vertical cell migration assay used a similar approach without Matrigel coating.

To evaluate the effects of ZNF750 on the lateral migratory capacity of CSCs, a wound healing assay was performed. The CSC-like cells (enriched from CAL-27 cells, 2x10^5/well) were cultured with DMEM medium containing 2% FBS and seeded in six-well plates and cultured to almost 80% confluency. The similar distance linear scratch wounds in each group were created on the cell monolayer using a sterile 200 μl pipette tip at 0 h, and the cells were then rinsed with PBS and incubated at 37˚C for 24 h. To visualize migrating cells and wound healing, images were acquired using a light microscope at 0 and 24 h after scratching. Cell migration characteristics were measured and analyzed using the manual tracking tool of Image-Pro Plus 6 software (National Institutes of Health) and calculated as a percentage of wound healing according to the equation: Wound healing (%)=[1-(wound area at T24 h/wound area at T0 h)] x100%, where T0 is the time immediately after wounding and T24 is the detection time point. The cells in three wells of each group were quantified. In total, three independent experiments were performed.

Statistical analysis. Values are presented as the means ± SD of at least three independent experiments. Data were analyzed using SPSS 23.0 software (SPSS, Inc.) with an unpaired

Table I. Sequences of primers.

Gene	Ref seq accession number	Direction	Sequence (5'-3')
ZNF750	NM_024702.3	Forward	GCCACCATCTACTCGCCTTA
		Reverse	GCAGGAAGTGTCTCGGGTCT
Oct4	NM_002701.6	Forward	GTGAGAGGCAACCTGGAGAA
		Reverse	AACCACACTCGGACCACATC
Sox2	NM_003106.4	Forward	CACAACTCGGAGATCAGCAA
		Reverse	TATAATCCGGGTGCTCCTTC
MMP1	NM_002421.4	Forward	TGGGCTGAAAGTGACTGGGA
		Reverse	ATGGCATGGTCCACATCTGC
MMP3	NM_002422.5	Forward	GGCCAGGGATTAATGGAGAT
		Reverse	TGAAAGAGACCCAGGGAGTG
MMP13	NM_002427.3	Forward	CACTTTATGCTTCCTGATGACG
		Reverse	TCTGGCGTTTTTGGATGTTTAG
TIMP1	NM_003254.3	Forward	CATCACTACCTGCAGTTTTGTG
		Reverse	TGGATAAACAGGGAAACACTGT
TIMP2	NM_003255.5	Forward	GTCACAGAGAAGAACATCAACG
		Reverse	GATGTCGAGAAACTCCTGCTT
Ezh2	NM_004456.4	Forward	AAATCAGAGTACATGCGACTGA
		Reverse	GTATCCTTCGCTGTTTCCATTC
EED	NM_003797	Forward	GTCCTGTGGTATGGATCATTCT
		Reverse	GTATCAAATCGCCTAACCATCG
SUZ12	NM_015355	Forward	CAAACTGAAGCAAGAGATGACC
		Reverse	GCTATGGCAGAGTTTAAGATGC
GAPDH	NM_002046.5	Forward	TGCACCACCAACTGCTTAGC
		Reverse	GGCATGGACTGTGGTCATGAG

ZNF750, zinc-finger protein 750; Oct4, octamer-binding transcription factor 4; Sox2, sex-determining region Y (SRY)-box 2; TIMP, tissue inhibitors of matrix metalloproteinases; Ezh2, enhancer of zeste homolog 2; EED, embryonic ectoderm development; SUZ12, SUZ12 polycomb repressive complex 2 subunit.

Student's t-test for differences between two groups. When comparing >2 groups, data were analyzed using one-way ANOVA followed by a Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CSC-like cell properties. The stem cell properties of CSC-like cells enriched from CAL-27 and TCA-83 cells were investigated. Tumor spheres exhibit higher levels of CSC-specific markers, including ALDH, and higher expression levels of pluripotent transcription factors, such as Oct4 and Sox2 (5). In the present study, the spheroid assay indicated that the tumor sphere formation abilities of the cells were enhanced after several cycles of passage, demonstrated by the more rapid and larger sphere formation at the same culture time points (Fig. 1A). With DEAB (ALDH inhibitor), the ALDH activity was 0±0% and 0.1±0.1% in the parental cells (CAL-27 cells) and CSC-like cells, respectively. Without DEAB, the ALDH activity was 0.1±0.1% in the CAL-27 cells, whereas it was increased (17.8±1.0%) in the CSC-like cells (enriched from CAL-27 cells; Fig. 1B; upper panel). Similar results were also revealed in CSC-like cells enriched from TCA-83 cells without DEAB, as indicated by increased ALDH activity (17.3±0.9%) compared with the TCA-83 cells (0.1±0.1%) (Fig. 1B; lower panel). Furthermore, compared with their matched parental cells, the oral CSC-like cells derived from tumor spheres exhibited a significantly increased cell viability (Fig. 1C), as well as a significantly increased Oct4 and Sox2 expression levels (Fig. 1D).

ZNF750 decreases the percentage of ALDH$^+$ cells. A high ALDH activity can be used to identify CSC-like cells characterized by a significantly higher proliferation rate (22). In the present study, to demonstrate the inhibitory effects of ZNF750 on CSC maintenance, the CSC-like cells (enriched from CAL-27 cells) were transduced with a lentiviral vector encoding the ZNF750 gene; a significant decrease in the number of ALDH$^+$ cells from 15.9±0.6% (oe-control group) to 11.7±0.5% (oe-ZNF750 group) in the CSC-like cell population without DEAB was observed. Nevertheless, the knockdown of the ZNF750 gene increased the number of ALDH$^+$ cells from 13.9±0.9% (sh-control group) to 23.0±2.5% (sh-ZNF750 group) in the CSC-like cell population without DEAB (Fig. 2A and B). The similar results were also revealed in CSC-like cells which enriched from TCA-83 cells (Fig. 2A and C).

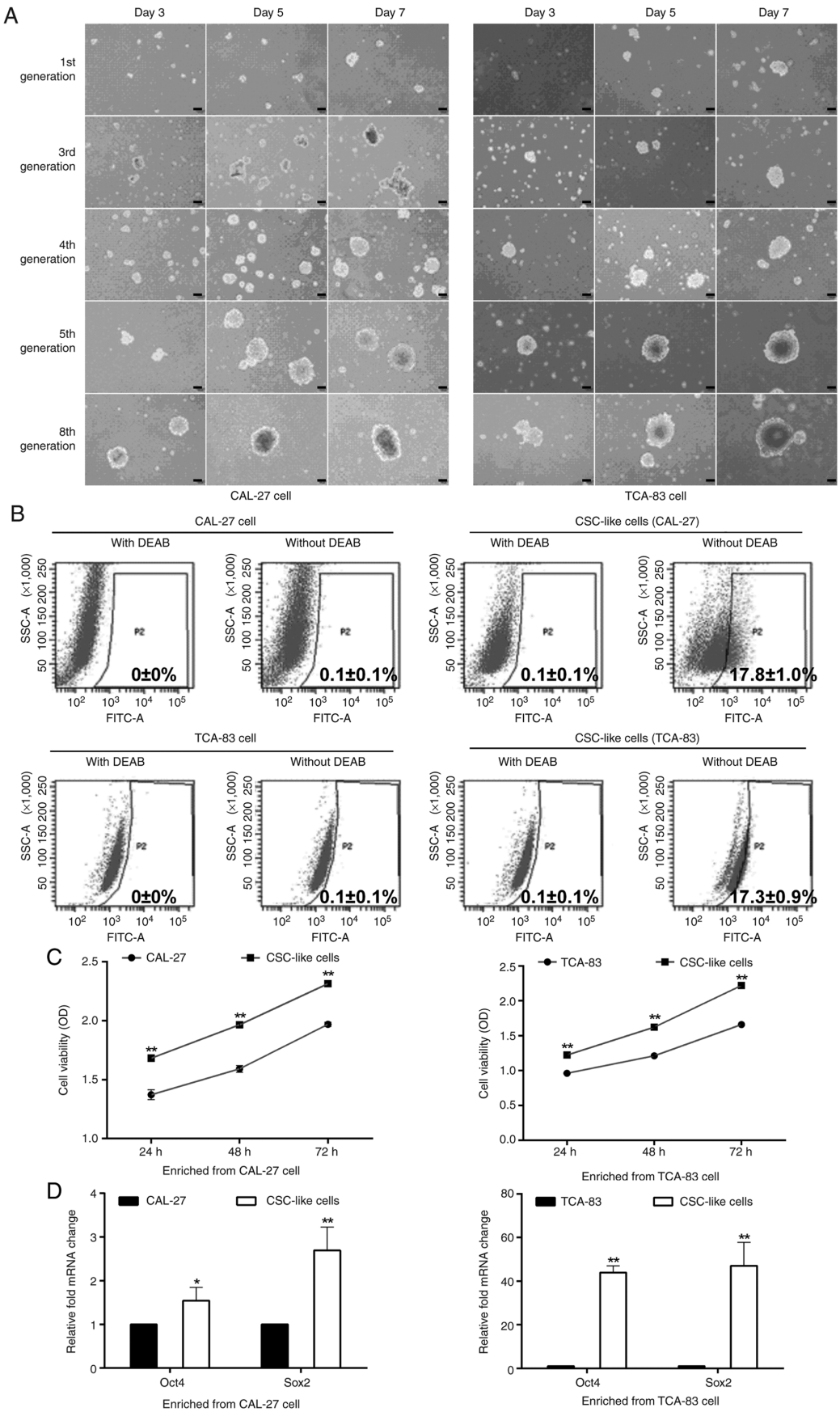

Figure 1. Identification of CSC-like cells. (A) Tumor sphere formation. At the same culture time points, tumor sphere formation was enhanced after several cycles of passage. Scale bar, 100 μm. (B) ALDH activity. ALDH$^+$ cells (P2) were analyzed using flow cytometry. CSC-like cells exhibited a high ALDH activity. A specific inhibitor of ALDH, DEAB, was used to control for background fluorescence. CAL-27 cells and TCA-83 cells were the parental cells. (C) Viability of the parental cells and CSC-like cells. (D) mRNA expression levels of Oct4 and Sox2. *P<0.05 and **P<0.01 vs. parental cells. CSC, cancer stem cell; ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde; OD, optical density; Oct4, octamer-binding transcription factor 4; Sox2, sex-determining region Y (SRY)-box 2.

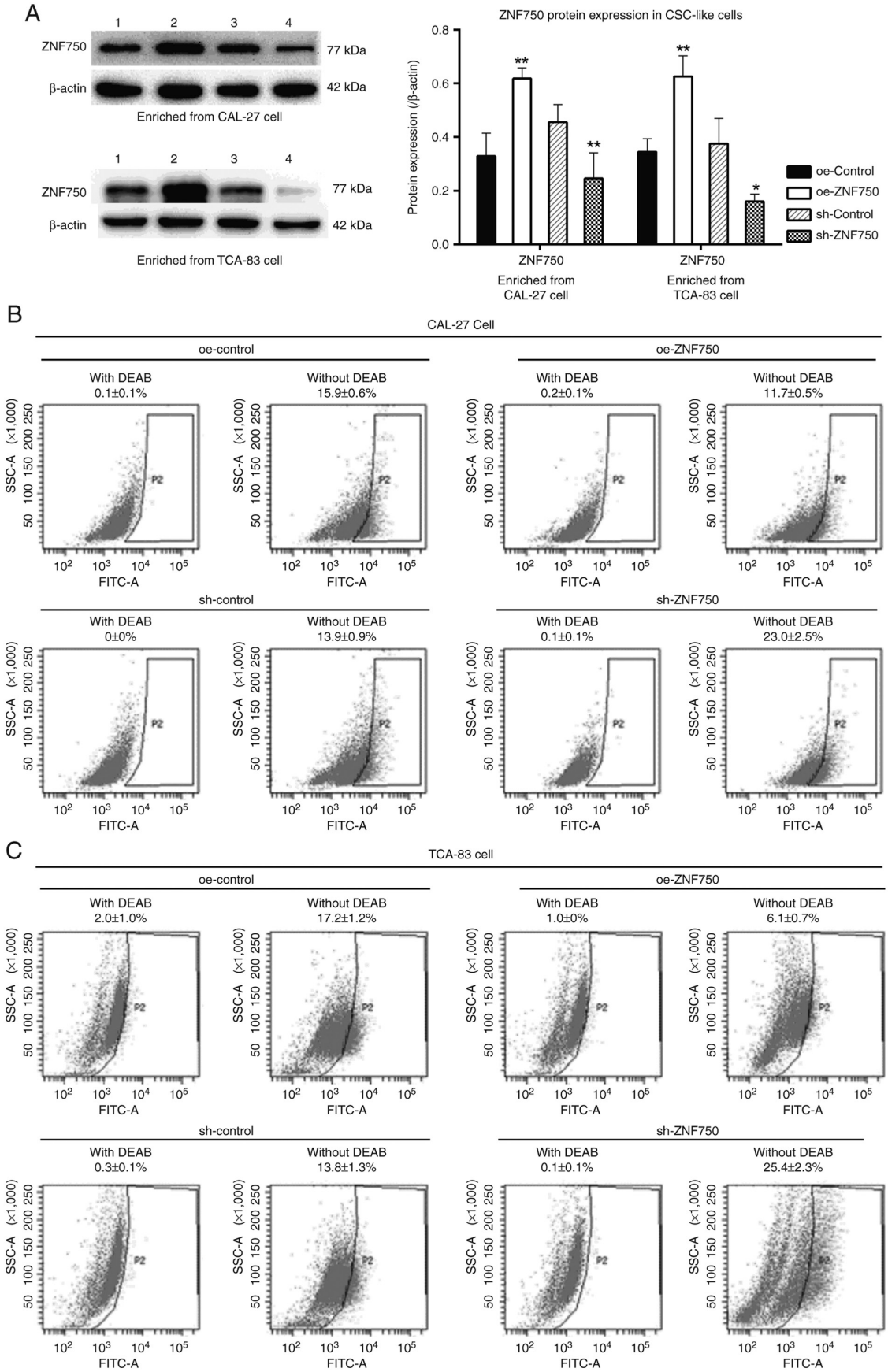

Figure 2. Inhibitory effects of ZNF750 on the ALDH activity of oral CSC-like cells. (A) ZNF750 protein expression level. 1, oe-control group; 2, oe-ZNF750 group; 3, sh-control group; 4, sh-ZNF750 group. (B) ALDH activity in CSC-like cells (enriched from CAL-27 cells). (C) ALDH activity in CSC-like cells (enriched from TCA-83 cells). ZNF750 effectively eliminated the CSC specific marker, ALDH. $ALDH^+$ cells (P2) were analyzed using flow cytometry. ALDH specific inhibitor, DEAB, was used to control for background fluorescence. Cells were treated with or without DEAB. $^*P<0.05$, $^{**}P<0.01$ vs. oe-control or sh-control group. ZNF750, zinc-finger protein 750; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; oe-, overexpression; sh-, short hairpin; DEAB, diethylaminobenzaldehyde.

ZNF750 inhibits the self-renewal ability of oral CSC-like cells. Tumor sphere- and colony-formation assays were performed to evaluate the self-renewal ability and tumor propagation potency of oral CSC-like cells (enriched from CAL-27 cells). Compared with the oe-control group, the tumor sphere and colony formation in the oe-ZNF750 group was significantly decreased, characterized by the decreased number and size of cell spheres and colonies. However, the knockdown of the ZNF750 gene significantly enhanced the number and size of the cell spheres and colonies (Fig. 3A and B). Furthermore, cell viability was decreased by ~20% in the oe-ZNF750 group at 24, 48 and 72 h compared with the oe-control group; however, it was increased by 1.24-, 1.31- and 1.30-fold at 24, 48 and 72 h, respectively, in the sh-ZNF750 group compared with the sh-control group (Fig. 3C). The increased cell viability observed in the sh-ZNF750 group suggested that ZNF750 exerts inhibitory effects on the self-renewal ability of oral CSC-like cells.

Effect of ZNF750 on stemness-related transcription factors. To determine the effects of ZNF750 on cancer stemness, the transcriptional activities of the genes encoding Oct4 and Sox2 were investigated using RT-qPCR. The expression levels of Oct4 and Sox2 were significantly increased in the sh-ZNF750 group by ~3.8- and 2.3-fold, respectively, compared with the sh-control group, although it was significantly reduced in the oe-ZNF750 group compared with the oe-control group (Fig. 3D).

ZNF750 decreases the expression of genes associated with epigenetic regulation and metastasis. The present study revealed that ZNF750 mRNA and protein was effectively overexpressed or silenced in the oe-ZNF750 and sh-ZNF750 groups, respectively (Figs. 2A and 3E). The mRNA expression levels of epigenetic regulatory genes (Ezh2, EED and SUZ12) were significantly decreased in the oe-ZNF750 group compared with the oe-Control. However, this was significantly reversed by the knockdown of the ZNF750 gene compared with the sh-Control group. CSCs are considered to be involved in tumor metastasis (23). The expression levels of the metastasis-related genes, MMP1, MMP3 and MMP13, were significantly downregulated compared with the oe-Control groups; whereas that of TIMP1 was significantly increased by ZNF750 overexpression. The opposite effect was observed in the sh-ZNF750 group (Fig. 3F-H). With regards to TIMP2 expression, no significant differences were observed between the groups. As the aberrant expression of Ezh2 is a frequent occurrence in tumors (14), and TIMP1 exerts potent inhibitory effects against MMP9 (24), Ezh2 and MMP9 protein expression levels were investigated. The present study found that Ezh2 and MMP9 protein expression levels were elevated in the sh-ZNF750 group compared with the sh-control group, however, they were decreased in the oe-ZNF750 group compared with the oe-control group (Fig. 3I and J).

ZNF750 inhibits the invasive and migratory abilities of CSC-like cells. In order to examine the effects of ZNF750 on the metastatic ability of CSCs, the cell invasive and migratory abilities were analyzed. The results revealed that the numbers of cells that had invaded or migrated to the lower chamber of the Transwell in the oe-ZNF750 group were significantly lower compared with those in the oe-control group. However, the invasive and migratory abilities were increased by 1.72- and 1.86-fold, respectively, in the sh-ZNF750 group compared with the sh-control group. Moreover, the wound closure ability of the CSC-like cells was significantly reduced in the oe-ZNF750 group (19.50±0.04%) compared with the oe-control group (41.25±0.11%). However, knockdown of the ZNF750 gene led to a more rapid wound closure; the wound had almost healed in the sh-ZNF750 group, in which the cells exhibited high migration, covering 68.46±0.04% of the scratch, which was significantly higher compared with the coverage observed in the sh-control group (41.16±0.08%) at 24 h (Fig. 4A-D). The aforementioned findings indicated that the ZNF750 gene significantly regulated the invasive and migratory capacity of CSC-like cells.

Discussion

CSCs are hypothesized to be involved in tumor metastasis and recurrence, thus contributing to the high mortality rate of patients with cancer. Therefore, they have become a potential target for anticancer therapy (5). The functional relevance of ZNF750 in oral CSC-like cells has not yet been studied, at least to the best of our knowledge. In the present study, it was revealed that ZNF750 inhibited the cancer stem cell-like behaviors of oral CSC-like cells enriched from tumor spheres. The overexpression of ZNF750 was efficient in repressing the ALDH-positive cell population and impairing the self-renewal capability of CSC-like cells. It also inhibited the expression levels of stemness factors (Oct4, Sox2), epigenetic regulatory genes (Ezh2, SUZ12 and EED) and cell invasion and metastasis related-genes, whereas it increased the expression of TIMP1.

The present study revealed that ZNF750 efficiently suppressed the stemness characteristics of CSC-like cells, as evidenced by the reduced numbers of ALDH-positive cells. ALDH is an intracellular enzyme involved in detoxification and drug resistance via the oxidation of aldehydes; its activity has been widely used as a functional stem cell marker in various cell lines, including oral cancer cells (25). In human head and neck squamous cell carcinoma, ALDH-positive cells have been reported to exhibit typical CSC behavior and an increased tumorigenic ability (26). The findings of the present study demonstrated that ALDH activity in CSC-like cells was reduced by the overexpression of ZNF750, whereas it was increased following the knockdown of the ZNF750 gene. This indicated that ZNF750 had the ability to suppress the stemness characteristics of oral CSC-like cells.

Furthermore, both sphere and colony formation assays have been used to evaluate the proliferative ability of CSCs by measuring the ability of single CSCs to form spheres over multiple passages (27). Consistent with these findings, the present study demonstrated the potent anti-proliferative effects of ZNF750 on oral CSC-like cells, manifested by the inhibition of cell viability, and the tumor sphere and colony forming abilities of the cells in the oe-ZNF750 group compared with the control group. By contrast, the self-renewal ability of the CSC-like cells was enhanced by the knockdown of the ZNF750 gene. This was accompanied by the loss of their differentiated phenotype, as assessed by the increased

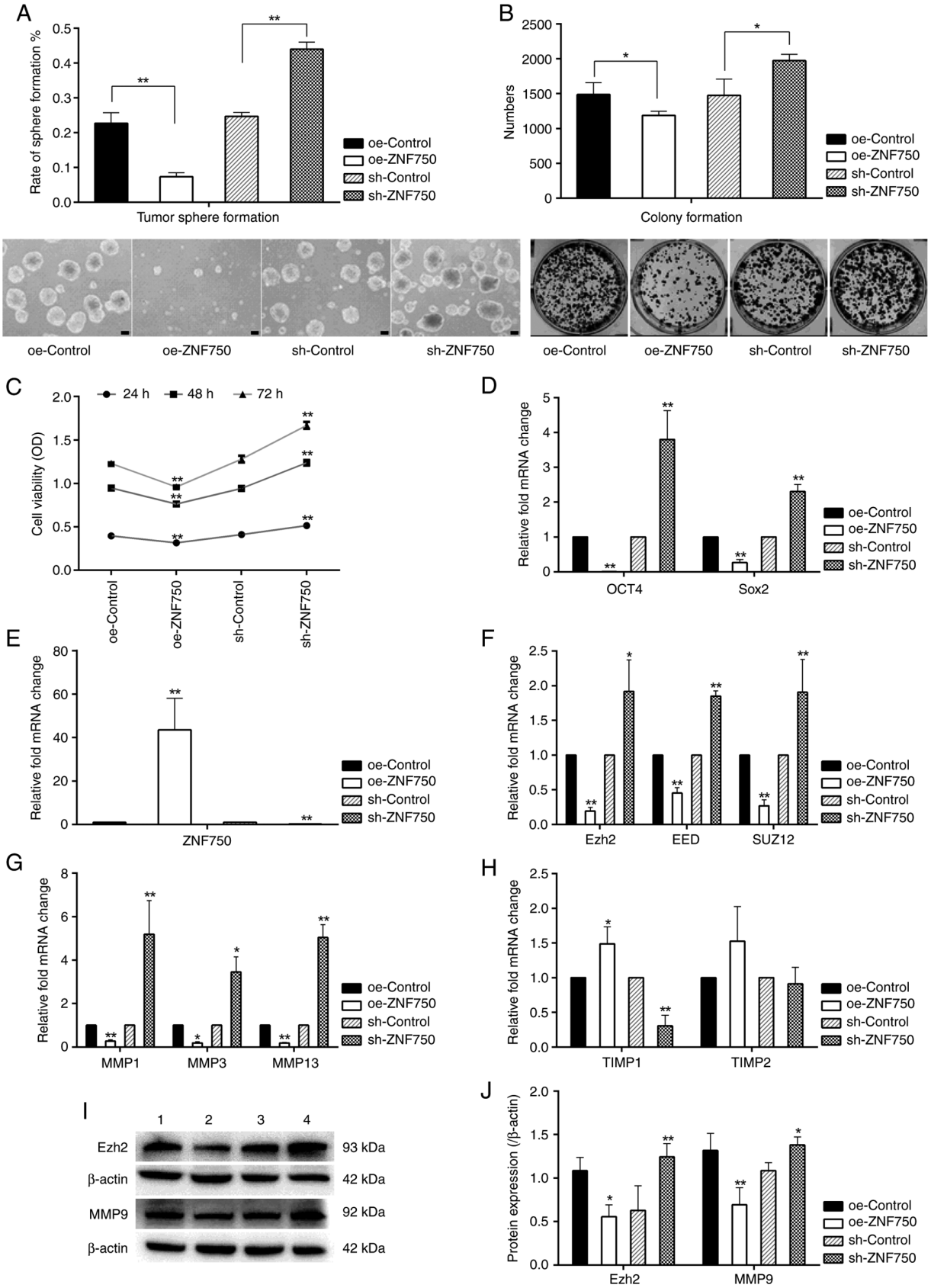

Figure 3. Regulatory effects of ZNF750 on stem cell properties, Ezh2, SUZ12 and EED and metastasis-related genes. (A) Tumor sphere and (B) colony formation ability. ZNF750 significantly suppressed the ability of tumor sphere (Scale bar, 100 μm) and colony formation (magnification, x1). (C) Effect of ZNF750 on cell viability. (D) mRNA expression profiles of stemness factors (Oct4 and Sox2). (E) Verification of ZNF750 mRNA expression in the oe-ZNF750 and sh-ZNF750 groups. mRNA expression profiles of (F) epigenetic regulatory genes (Ezh2, SUZ12 and EED), and metastasis-related genes (G) MMP1, MMP3, MMP13 and (H) TIMP1 and TIMP2. (I and J) Ezh2 and MMP9 protein expression. 1, oe-control groups; 2, oe-ZNF750 groups; 3, sh-control groups; 4, sh-ZNF750 groups. *P<0.05 and **P<0.01 vs. oe-control or sh-control group. ZNF750, zinc-finger protein 750; Ezh2, enhancer of zeste homolog 2; SUZ12, SUZ12 polycomb repressive complex 2 subunit; EED, embryonic ectoderm development; Oct4, octamer-binding transcription factor 4; Sox2, sex-determining region Y (SRY)-box 2; oe-, overexpression; sh-, short hairpin; TIMP, tissue inhibitors of matrix metalloproteinases.

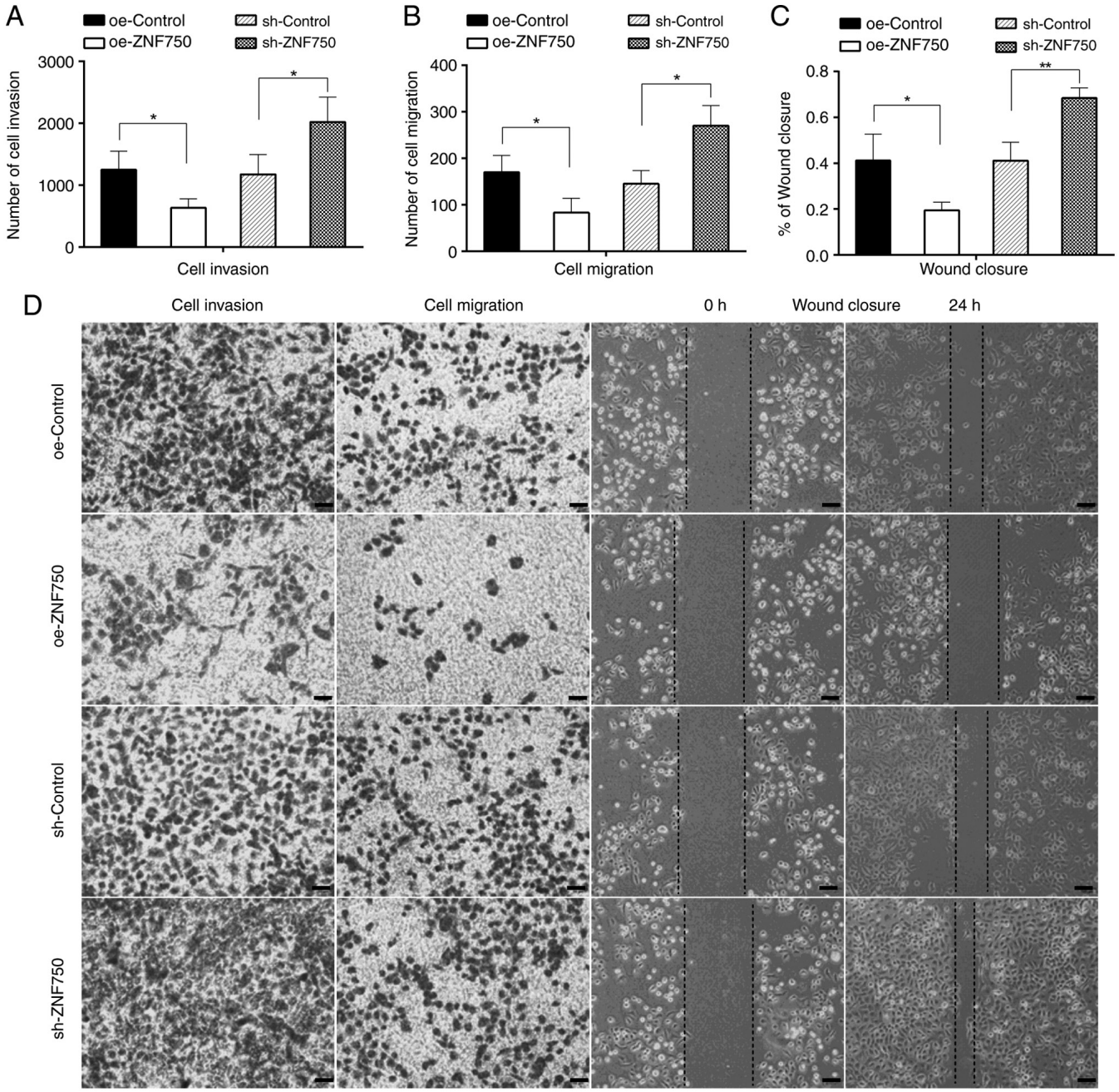

Figure 4. ZNF750 inhibits CSC-like cell invasion and migration. Changes in (A) cell invasion, (B) migration and (C) wound closure in each group. (D) Representative images of cell invasion (left panel; scale bar, 50 μm), migration (middle left panel; scale bar, 50 μm) and wound closure (two right panels; scale bar, 100 μm). The 0 h time point was immediately after wounding and 24 h was the detection time point. *P<0.05 and **P<0.01. ZNF750, zinc-finger protein 750; CSC, cancer stem cell; oe-, overexpression; sh-, short hairpin.

expression of Oct4 and Sox2, two human embryonic stem cell pluripotency markers (28). However, the expression levels of the stemness factors, Oct4 and Sox2, were downregulated by ZNF750 overexpression. The expression levels of the Oct4 and Sox2 transcription factors have been demonstrated to be associated with metastasis and a poor prognosis in OSCC (29). As recently demonstrated, Sox2 expression in tumors is associated with increased tumor invasive, metastatic and proliferative activities in cancer, and contributes to drug resistance, tumor aggressiveness and a poor prognosis (30). Sox2 can enhance the expression of key pluripotent factors, such as Oct4, driving cancer cells toward a stem-like state (31). Therefore, these results indicated that ZNF750 exerted an inhibitory effect on the stemness characteristics of oral CSC-like cells.

Multiple molecular abnormalities may contribute to aggressive behavior, possibly via the epigenetic regulation of CSCs (32). The present study demonstrated that the expression levels of Ezh2, SUZ12 and EED, which are components of the polycomb-repressive complex 2 (PRC2), were decreased by ZNF750 overexpression. Ezh2 is a catalytic subunit of PRC2, and functions as an epigenetic silencer of several tumor suppressor genes through histone 3 lysine 27 trimethylation (H3K27me3) (33). Histone lysine methyltransferases have been linked to the pathogenesis of cancer (34). The aberrant

expression of Ezh2 is a frequent occurrence in multiple tumors, and there is evidence to suggest that the upregulation of Ezh2 promotes cancer cell proliferation, migration, invasion and metastasis, as well as CSC self-renewal (14). The present study revealed that oral CSC-like cells expressed higher levels of Ezh2 in the sh-ZNF750 compared with the sh-control group. However, the overexpression of ZNF750 decreased the Ezh2 protein expression in oral CSC-like cells. Moreover, SUZ12 and EED mRNA expression levels were also decreased by ZNF750 overexpression, whereas they were increased in the sh-ZNF750 group. Therefore, the increased PRC2 components (Ezh2, SUZ12 and EED) may trimethylate H3K27me3, hence increasing the repression of transcription and leading to tumor-suppressor gene inactivation in the sh-ZNF750 group. This consequently resulted in an increased self-renewal, migratory and invasive abilities of the CSC-like cells. The aforementioned results suggested that ZNF750 may down-regulate the expression of the epigenetic silencer, Ezh2, to suppress the self-renewal ability of oral CSC-like cells.

The self-renewal and pluripotency properties of CSCs are considered to enable primary tumors to metastasize (35). Activated MMPs can degrade the highly rigid extracellular matrix and basement membrane components, thus mediating matrix degradation and leading to tumor cell invasion (36). All MMPs are specifically inhibited by TIMPs, a family of proteins that includes TIMP1, TIMP2, TIMP3 and TIMP4. The balance between MMPs and their inhibitors is an important factor that affects tumor invasion and metastasis (37). It has been reported that TIMPs have the ability to both stimulate and suppress tumor growth (24). TIMP1 exerts potent inhibitory effects against MMP9. With regards to TIMP2, its low expression can lead to the activation of MMP2, whereas its high expression inhibits MMP2 activity (24). Consistent with these aforementioned studies, the present study demonstrated that the expression levels of the matrix remodeling genes, MMP1, MMP3, MMP9 and MMP13, were decreased, whereas that of TIMP1 was elevated by the overexpression of ZNF750. The present study revealed that TIMP2 expression was not altered by ZNF750. Additionally, the CSC-like behaviors were also inhibited by the overexpression of ZNF750, and cell invasion and migration were attenuated in the oe-ZNF750 group. By contrast, these effects were reversed by the knockdown of ZNF750. These observations provided further evidence of the suppressive effects of ZNF750 on oral CSC-like cell invasion.

In conclusion, the present study demonstrated that ZNF750 had the ability to eradicate the cancer stemness and stem cell-like behaviors of oral CSC-like cells enriched from parental CAL-27 cells. However, the limitation of the present study is absence of *in vitro* and *in vivo* data considering the mechanism of ZNF750 on epigenetic genes and metastatic genes regulation. Future studies are required to further investigate the suppressive effects of ZNF750 *in vivo* in order to fully elucidate the role of ZNF750 in OSCC tumor development.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HYC and LP contributed to the study conception and design. CX, HLY, YKY, HYC and LP conducted experiments and data analysis. CX and HYC wrote the first draft of the manuscript. HYC, CX, HLY, YKY and LP confirm the authenticity of all the raw data. All authors have read and approved the final 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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