

PEG10/TRIB3 axis is associated with malignant behavior and Notch-related signaling in oral squamous cell carcinoma cells

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Abstract. Oral squamous cell carcinoma (OSCC) is an aggressive oral epithelial neoplasm for which the expression of paternally expressed gene 10 (*PEG10*) has notable prognostic implications. However, the functional role of this protein in OSCC remains to be fully elucidated. The present study aimed to explore the function of *PEG10* in regulating OSCC cell behavior and the potential mechanisms of this regulation. Cell Counting Kit-8, 5-ethynyl-2'-deoxyuridine, wound healing and Transwell assays were employed to estimate cell proliferation, migration and invasion, respectively. Furthermore, flow cytometry assays were used to detect the cell cycle status and apoptosis of OSCC cells. Western blot analysis was used to examine the expression of *PEG10* and Tribbles pseudokinase 3 (*TRIB3*), as well as cell cycle-, apoptosis- and Notch signaling-associated proteins. The HDOCK server (a hybrid docking server for protein and nucleic acid complex prediction) and co-immunoprecipitation assays were employed to investigate the relationship between *PEG10* and *TRIB3*. The present study found *PEG10* to be highly expressed in OSCC tissues and cells. *PEG10* knockdown reduced cell proliferation, migration and invasion, arrested the cell cycle in the G₀/G₁ phase and stimulated apoptosis. *TRIB3* expression was also shown to be elevated in OSCC cells and was decreased following *PEG10* knockdown. Concurrent *TRIB3* overexpression partially reversed the effects of *PEG10* knockdown on malignant phenotypes and Notch-related protein expression. These findings suggested that the *PEG10/TRIB3* axis may be associated with malignant behavior and Notch-related signaling in OSCC.

Introduction

Head and neck cancer encompasses a heterogeneous group of neoplasms of the throat, pharynx, nasal cavity, oral and maxillofacial regions, >90% of which are accounted for by oral squamous cell carcinoma (OSCC) (1-3). The total annual incidence of OSCC in 2018 was >377,713, globally, resulting in ~177,757 deaths (2); alcohol, tobacco, betel nuts and oncogenic viruses have been recognized as pathogenic factors (4). OSCC is usually treated by a combination of surgery, chemotherapy and radiotherapy (5), but outcomes for patients with advanced disease remain poor (6). Further scrutiny of pathological mechanisms may contribute to exposing therapeutic and prognostic targets, allowing for earlier diagnosis and more effective treatment.

Paternally expressed gene 10 (PEG10) is a maternally silenced imprinting gene (7) that is expressed in the human brain, kidney, lung, embryonic tissues and placenta (8). As an imprinted gene, *PEG10* is subject to epigenetic regulation, and its dysregulation may lead to the aberrant activation of oncogenic pathways, which highlights the importance of identifying its downstream effectors and its interacting partners in cases of cancer progression (9). Increased *PEG10* expression has also been linked to aggressive clinicopathological characteristics in various cancers (10,11) and has been implicated as a prospective prognostic biomarker for OSCC (12). However, the downstream molecular mechanisms through which *PEG10* contributes to OSCC progression remain poorly understood. Additionally, studies have yet to elucidate whether *PEG10* exerts its oncogenic effects through interactions with specific regulatory partners.

Tribbles pseudokinase 3 (*TRIB3*) is regarded as a stress sensor with notable roles in mitosis, cell activation and apoptosis (13). It has also been suggested that *TRIB3* represents an oncogene and prognostic gene for head and neck cancer (14-16) and that *TRIB3* stimulates OSCC cell proliferation (17). A potential interaction between *PEG10* and *TRIB3* was predicted using FpClass, a data mining-based method and database for the prediction of physical protein-protein interactions (<https://ophid.utoronto.ca/fpclass/>). However, although *PEG10* upregulation has previously been reported in OSCC (12) it remains ambiguous whether the oncogenic effects of *PEG10* expression may be mediated through

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interactions with stress-responsive regulators, such as *TRIB3*, and how such interactions may contribute to downstream signaling activation.

Notch is an evolutionarily conserved transmembrane receptor that is involved in the determination of cell fate and is known to affect proliferation and apoptosis. Activation of the Notch pathway involves the release of the Notch intracellular domain (NICD), for which hairy and enhancer of split-1 (HES1) is a downstream target (18,19). In head and neck cancers, Notch signaling has been shown to exhibit complex and context-dependent functions, acting either as an oncogenic driver or as a tumor suppressor depending on the cellular and molecular context (20,21). This highlights the complexity of Notch signaling in head and neck tumorigenesis. Accumulating evidence suggests that the dysregulation of Notch signaling is closely associated with the progression of OSCC (22,23). Previous studies have also suggested that *PEG10* and *TRIB3* may contribute to the activation of Notch1 signaling in ovarian cancer and triple-negative breast cancer (24,25). These findings raise the possibility that *PEG10* and *TRIB3* may influence OSCC progression through modulation of the Notch signaling pathway. Based on this premise, we hypothesized that *PEG10* may interact with *TRIB3* and regulate OSCC cell behavior via the Notch pathway. The present study investigated the effects of *PEG10* and *TRIB3* expression on OSCC cell behavior by knocking down *PEG10* and overexpressing *TRIB3* in OSCC cell lines, thereby aiming to elucidate the interaction between these two proteins and explore the potential tumor-promoting mechanism of this interaction.

Materials and methods

Bioinformatics tools. The GSE74530 microarray dataset of OSCC samples and adjacent non-tumoral oral mucosal tissues, originally reported by Oghumu *et al* (26), was downloaded from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) and used to analyze *PEG10* expression. The dataset was generated using the Affymetrix Human Genome U133 Plus 2.0 Array (GPL570; Affymetrix, Inc.). The potential interaction between *PEG10* and *TRIB3* was predicted using FpClass (<http://ophid.utoronto.ca/fpclass/>), a data mining-based method for proteome-wide prediction of physical protein-protein interactions (27). Molecular docking interactions between *PEG10* and *TRIB3* were subsequently predicted using the HDock server (Huang Laboratory, Huazhong University of Science and Technology; <http://hdock.phys.hust.edu.cn/>) (28), a web-based docking server that integrates template-based modeling and free docking for protein-protein and protein-DNA/RNA interactions. Docking was performed using the default settings of the HDock server without additional binding-site restraints or small-angle X-ray scattering (SAXS) information. Predicted complex models were evaluated and ranked using the knowledge-based iterative scoring functions implemented in HDock, with more negative docking scores indicating more favorable predicted binding models.

Cell culture. Human immortalized oral epithelial cells (HIOEC; The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were incubated in keratinocyte

serum-free medium (Thermo Fisher Scientific, Inc.). The human OSCC cell lines Cal-27 (human oral adenosquamous carcinoma) and SCC-9 (human lymph node metastasis from primary OSCC; BeNa Culture Collection; Beijing Beina Chunglian Institute of Biotechnology) were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom AG; MilliporeSigma) and the human OSCC cell line SCC-15 (American Type Culture Collection) was cultured in DMEM/F12 (Biochrom AG). All media contained 10% fetal bovine serum (FBS; Biochrom AG) and cells were cultured at 37°C with 5% CO₂. All cell lines were routinely tested and confirmed to be free of mycoplasma contamination.

Transient transfection. In total, two *PEG10*-targeting small interfering RNA (siRNA) sequences (siRNA-*PEG10*), a corresponding negative control siRNA sequence (siRNA-NC), a *TRIB3* overexpression lentiviral vector (Ov-*TRIB3*) and an empty overexpression vector control (Ov-NC) were custom synthesized by Charles River. The Ov-*TRIB3* construct was generated using the pLVX CMV MCS EF1α Puro lentiviral backbone and contained the human *TRIB3* coding sequence derived from the reference sequence NM_021158.4 (NP_066981.2; *Homo sapiens TRIB3* transcript variant 1). Transfection of SCC-15 cells was performed using Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For siRNA transfection, cells were transfected with 50 pmol siRNA-*PEG10* or siRNA-NC using 5 µl Lipofectamine® 3000 reagent. For plasmid transfection, cells were transfected with 2.5 µg Ov-*TRIB3* or Ov-NC plasmid using 5 µl Lipofectamine® 3000 reagent. Cells were incubated with the transfection mixture at 37°C for 4–6 h and subsequently cultured for 48 h before use in subsequent experiments. The sequences of siRNAs used in the present study were as follows: siRNA-*PEG10*-1, sense 5'-GCAGUCGGAGGAGACAAC-3', antisense 5'-GUUGUUCUCCUCCGACUGC-3'; siRNA-*PEG10*-2, sense 5'-GTCGCTGTCTGCTCTGATTTT-3', antisense 5'-AAAATCAGAGCAGACAGCGAC-3'; siRNA-NC-1, sense 5'-TTCTCCGACGTGTACAGT-3', antisense 5'-ACGTGACACGTTCCGAGAA-3'; and siRNA-NC-2, sense 5'-TGAAGGAGTTCTGATCTTTT-3', antisense 5'-AAAAGATCAGGAACCTCTCA-3'.

Cell viability assay. SCC-15 cells were divided into the following treatment groups: Untreated control, siRNA-NC, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* groups. The siRNA-*PEG10* group was transfected with siRNA-*PEG10*-1. Following transfection, cells were seeded into 96-well flat-bottomed plates at a density of 5×10³ cells/well and cultured for 24, 48 or 72 h. At each time point, 10 µl Cell Counting Kit-8 working solution (Signalway Antibody LLC) was added to each well, followed by incubation for 2 h at 37°C. Absorbance was subsequently measured at 450 nm using a CLARIOstar Plus microplate reader (BMG Labtech GmbH).

Cell proliferation assay. SCC-15 cells were implanted in 96-well plates at a density of 1×10⁴ cells/well, followed by the addition of 100 µl culture medium (DMEM/F12) containing 20 µM 5-ethynyl-2'-deoxyuridine (EdU) working solution

(Sangon Biotech Co., Ltd.). Cells were cultured for 2 h at 37°C, fixed with 4% paraformaldehyde for 30 min at room temperature and subsequently incubated with 0.5% Triton X-100 for 10 min at room temperature. Hoechst 33342 staining was subsequently performed and cells were evaluated under a fluorescence microscope (Leica Microsystems GmbH).

Wound healing assay. SCC-15 cells were seeded into 6-well plates at a density of 4×10^5 cells/well and cultured in DMEM supplemented with 10% FBS at 37°C until they reached ~80% confluency. The cell monolayer was subsequently scratched using a sterile 10- μ l micropipette tip, washed and cultured in serum-free DMEM to minimize the influence of serum-induced cell proliferation. Images of the wound area were captured immediately after scratching (0 h) and at 12 and 24 h using a light microscope (Leica Microsystems GmbH). The cell-free wound area was manually outlined and measured using ImageJ software [v1.53; National Institutes of Health]. The percentage of wound closure was calculated as follows: Wound closure (%) = [(wound area at 0 h - wound area at 12 or 24 h)/wound area at 0 h] $\times 100$.

Transwell assay. Transwell chambers were coated with Matrigel (Trevigen, Inc.; Bio-Techne), which was diluted in serum-free medium and added to the upper chamber, followed by incubation at 37°C for 2 h to allow polymerization. Subsequently, 6×10^4 SCC-15 cells/well were added to the upper chamber in serum-free DMEM (Biochrom AG), while 500 μ l DMEM supplemented with 10% FBS (Biochrom AG) was added to the lower chamber as a chemoattractant. Cells that had migrated to the lower chamber were fixed with 4% paraformaldehyde at room temperature for 30 min, stained with 0.1% crystal violet at room temperature for 30 min and counted in three randomly selected fields under a light microscope.

Flow cytometry analysis. Apoptosis was measured using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BioVision, Inc.; Abcam). SCC-15 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and transfected with siRNA-*PEG10*-1 or siRNA-NC, or co-transfected with siRNA-*PEG10*-1 and Ov-*TRIB3* or Ov-NC, as aforementioned. Following transfection for 48 h, cells were harvested, resuspended in 500 μ l 1X binding buffer and incubated with 5 μ l annexin V-FITC/PI for 5 min at room temperature in the dark. Apoptosis was analyzed using a CyFlow® Space flow cytometer (Sysmex Corporation) and the instrument's built-in software. Main cell populations were initially gated based on forward scatter area (FSC-A) and side scatter area (SSC-A), followed by doublet exclusion using FSC-A/forward scatter height (FSC-H) gating. Early apoptotic cells were defined as annexin V-positive/PI-negative, whereas late apoptotic or necrotic cells were defined as annexin V-positive/PI-positive. Fluorescence compensation between the FITC and PI channels was performed using single-stained controls.

For cell cycle analysis, SCC-15 cells were subjected to the same transfection treatments for 48 h as aforementioned. Cells were subsequently harvested and fixed with 70% ethanol at 4°C for 30-60 min. No additional permeabilization step

was performed. Following fixation, cells were incubated with 50 μ g/ml RNase A (Biotium, Inc.) and PI (Biotium, Inc.) for 30 min at room temperature. Flow cytometric analysis was performed using a CyFlow® Space flow cytometer (Sysmex Corporation) and the instrument's built-in software. After excluding debris and doublets through FSC-A/SSC-A and FSC-A/FSC-H gating, cell cycle distribution across the G0/G1, S and G2/M phases was determined based on PI fluorescence intensity histograms. As only PI staining was used, fluorescence compensation was not required.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from SCC-15 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the PrimeScript II 1st Strand cDNA Synthesis Kit (cat. no. 6210A/B; Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Reverse transcription was performed at 42°C, followed by enzyme inactivation at 65°C for 5 min and immediate cooling on ice. qPCR was subsequently performed using an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with TB Green® Premix Ex Taq Kit (Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: 95°C for 15 min, followed by 45 cycles at 95°C for 15 sec and 60°C for 1 min. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_q}$ method (29) and normalized to GAPDH expression. The primer sequences were used as follows: *PEG10*, forward 5'-TTGTAACAGCAATGCAATATTAGC-3', reverse 5'-CACATCTGATGAGACTGAAAGTTC-3'; *GAPDH*, forward 5'-AGGTCGGTGTGAACGGATTG-3', reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Co-immunoprecipitation (Co-IP). A Co-IP kit (Invent Biotechnologies, Inc.) was used according to the manufacturer's instructions. Briefly, SCC-15 cell lysates were incubated with 2 μ g anti-*PEG10* antibody (cat. no. ab215035; Abcam) or 2 μ g anti-*TRIB3* antibody (cat. no. ab75846; Abcam) per immunoprecipitation reaction overnight at 4°C with constant rotation. The immune complexes were subsequently incubated with protein A/G agarose beads for 2 h at 4°C. Following separation by SDS-PAGE, the immunoprecipitated proteins were analyzed by western blotting using goat anti-rabbit secondary antibody (1:2,000; cat. no. ab205718; Abcam) for 2 h at room temperature.

Western blotting. Proteins were extracted from the OSCC cell line SCC-15 using RIPA lysis buffer (Nacalai Tesque, Inc.) and quantified using the Protein Assay BCA Kit (Nacalai Tesque, Inc.). Protein samples (20-30 μ g) were separated by 10% SDS-PAGE, transferred onto PVDF membranes and blocked with 5% BSA (Nacalai Tesque, Inc.) in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h. The membranes were subsequently incubated at 4°C overnight with primary antibodies against *PEG10* (cat. no. ab215035; 1:1,000; Abcam), *TRIB3* (cat. no. ab75846; 1:5,000; Abcam), cyclin-dependent kinase (CDK)4 (cat. no. ab199728; 1:2,000; Abcam), cyclin D1 (cat. no. ab134175; 1:10,000; Abcam), B-cell lymphoma 2 (Bcl-2; cat. no. ab241548; 1:1,000; Abcam), Bcl-2-associated X (Bax; cat. no. ab182733; 1:2,000; Abcam), caspase 3 (cat. no. ab32351; 1:1,000; Abcam), cleaved

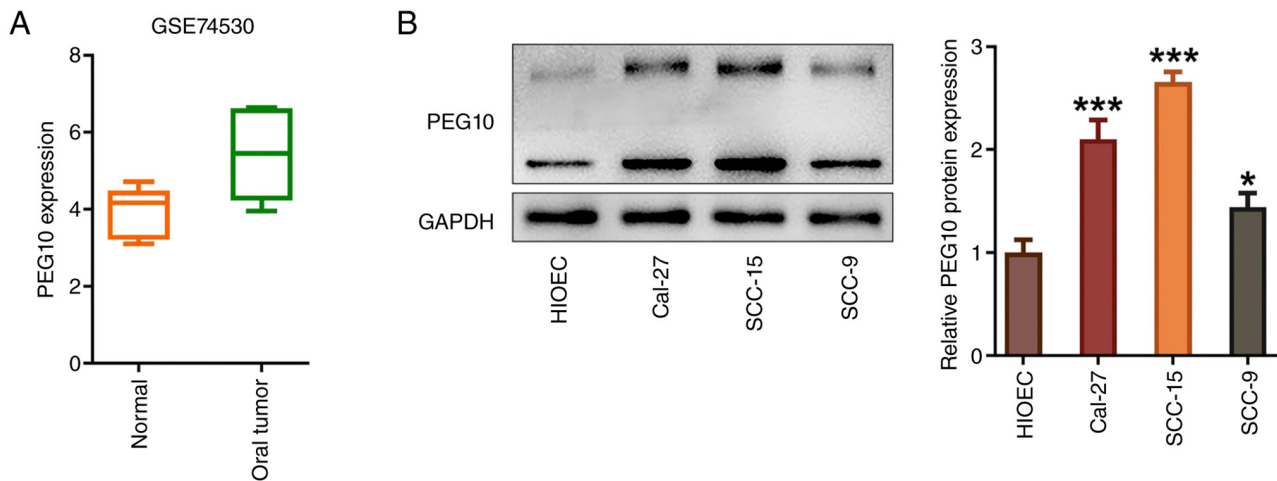


Figure 1. *PEG10* expression in OSCC and adjacent non-tumoral tissues and cell lines. (A) *PEG10* expression in OSCC and non-tumor adjacent patient tissue samples in the GSE74530 microarray dataset from the Gene Expression Omnibus database. (B) Western blotting (left) and semi-quantitative densitometric analysis (right) of the expression of *PEG10* in the OSCC cell lines Cal-27, SCC-15 and SCC-9 and the non-tumor HIOEC cell line. * $P < 0.05$ and *** $P < 0.001$ vs. controls. *PEG10*, paternally expressed gene 10; OSCC, oral squamous cell carcinoma; HIOEC, human immortalized oral epithelial cells. Data are presented as the mean $\pm$ SD from three independent biological replicates ($n=3$).

caspase 3 (cat. no. ab32042; 1:500; Abcam), Notch1 intracellular domain (NICD1; cat. no. ab52301; 1:500; Abcam), HES1 (cat. no. ab108937; 1:1,000; Abcam) and GAPDH (cat. no. ab128915; 1:10,000; Abcam). The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (cat. no. ab205718; 1:2,000; Abcam) or, for the Bcl-2 primary antibody, HRP-conjugated goat anti-rat IgG (H&L; cat. no. ab97057; 1:2,000; Abcam) at room temperature for 2 h. Protein bands were visualized using Radiance™ ECL Substrate (Azure Biosystems, Inc.) and analyzed using Image Studio 5.1 software (LI-COR Biosciences). Protein band intensities were quantified using ImageJ software (v1.53; National Institutes of Health) and normalized to GAPDH.

Statistical analysis. Each experiment was repeated to obtain at least three independent biological replicates. All data are presented as mean $\pm$ SD and analyses were performed using GraphPad Prism 8.0 software (Dotmatics). Data distribution was assessed for normality prior to statistical testing. For normally distributed data, inter-group comparisons for two groups were conducted using unpaired Student's *t*-test, whereas comparisons for multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

***PEG10* is expressed at higher levels in OSCC tissues and cells.** *PEG10* is expressed at higher levels in OSCC tissues and cells. Analysis of the GSE74530 dataset showed that *PEG10* expression was 1.5-fold higher in OSCC tissues compared with adjacent non-tumoral tissues (Fig. 1A). Western blotting showed significantly higher *PEG10* expression in the OSCC cell lines Cal-27, SCC-9 and SCC-15 compared with the non-tumoral HIOEC cell line (Fig. 1B). The cell line showing the highest *PEG10* expression was SCC-15, which was therefore selected for further experiments.

Knockdown of PEG10 reduces SCC-15 cell proliferation, migration and invasion. *PEG10* was significantly knocked down in SCC-15 cells by transfection with siRNA-*PEG10*-1, which reduced protein levels to ~25% those of the control, and with siRNA-*PEG10*-2, which reduced *PEG10* mRNA expression to ~40% of the control levels and reduced protein expression to ~50% (Fig. 2A and B). The more efficient siRNA sequence, siRNA-*PEG10*-1, was chosen for further experiments. Compared with controls, SCC-15 cell viability was reduced by >50% at 72 h following *PEG10* knockdown and cell proliferation was suppressed (Fig. 2C and D). In addition, SCC-15 cell migration was ~50% lower in wound healing assays following *PEG10* knockdown and invasion was reduced ~4.4-fold in Transwell assays (Fig. 2E and F). The data showed that *PEG10* knockdown was associated with reduced malignant phenotypes in OSCC cells in culture.

***PEG10* knockdown stimulates apoptosis of SCC-15 cells and causes cell cycle arrest.** *PEG10* knockdown resulted in a significantly increased number of SCC-15 cells in G₀/G₁ phase compared with controls, whereas the number of cells in S and G₂/M phase notably decreased (Fig. 3A). Furthermore, *PEG10* knockdown resulted in a marked increase in the apoptotic rate of SCC-15 cells compared with controls (Fig. 3B). Western blotting showed significantly reduced expression of the cell cycle proteins CDK4 and cyclin D1. Expression of the anti-apoptotic protein Bcl-2 was also significantly decreased, whereas those of the pro-apoptotic proteins Bax and cleaved caspase 3 were significantly increased (Fig. 3C). The aforementioned flow cytometry and gene expression data demonstrated that *PEG10* knockdown resulted in the stimulation of apoptosis and arrest of the cell cycle in G₀/G₁ phase in the OSCC cell line SCC-15.

***PEG10* interacts with *TRIB3* in OSCC cells.** The FPClass database was used to predict an interaction between the proteins *PEG10* and *TRIB3* based on the proteomic analysis of molecular binding site motifs. *TRIB3* showed a similar 1.7- to 2.5-fold pattern of upregulation in OSCC cell lines relative

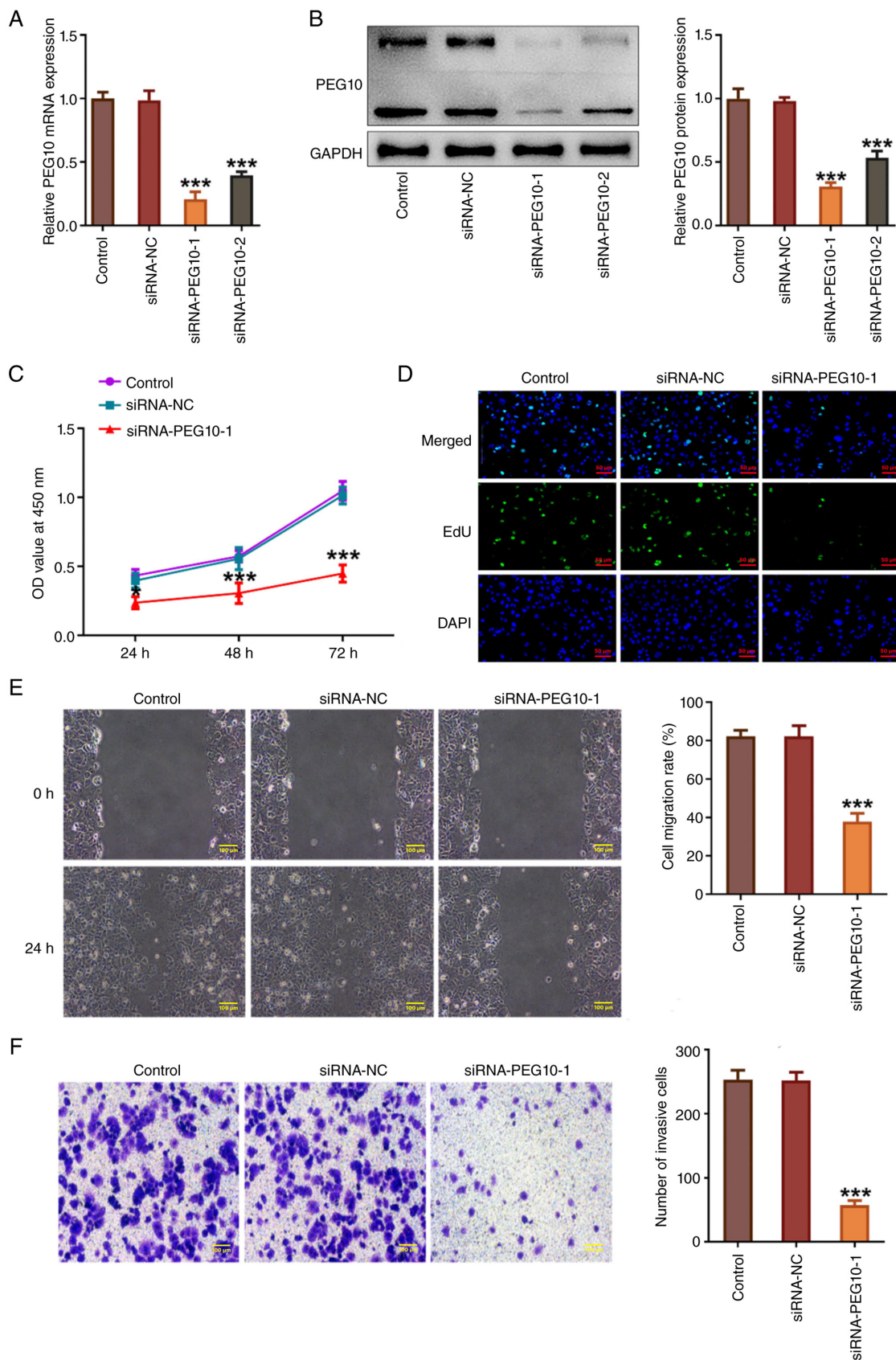


Figure 2. Effects of *PEG10* knockdown on SCC-15 cell proliferation, migration and invasion. (A) Reverse transcription-quantitative PCR for *PEG10* mRNA expression in untreated SCC-15 cells and SCC-15 cells after transfection with siRNA-*PEG10*-1, siRNA-*PEG10*-2 or siRNA-NC. (B) Western blotting for *PEG10* protein expression in untreated SCC-15 cells and SCC-15 cells after transfection with siRNA-*PEG10*-1, siRNA-*PEG10*-2 or siRNA-NC. (C) SCC-15 cell viability was measured via Cell Counting Kit-8 assay. (D) SCC-15 cell proliferation in different treatment groups was measured by EdU staining. (E) SCC-15 cell migration in different treatment groups was measured via wound healing assay and subsequently quantified. (F) SCC-15 cell invasion in different treatment groups was measured by Transwell assay and subsequently quantified. * $P < 0.05$ and *** $P < 0.001$ vs. siRNA-NC. *PEG10*, paternally expressed gene 10; EdU, 5-ethynyl-2'-deoxyuridine; siRNA-*PEG10*, *PEG10*-targeting small interfering RNA; siRNA-NC, negative control small interfering RNA sequence; OD, optical density. Data are presented as the mean $\pm$ SD from three independent biological replicates ($n=3$).

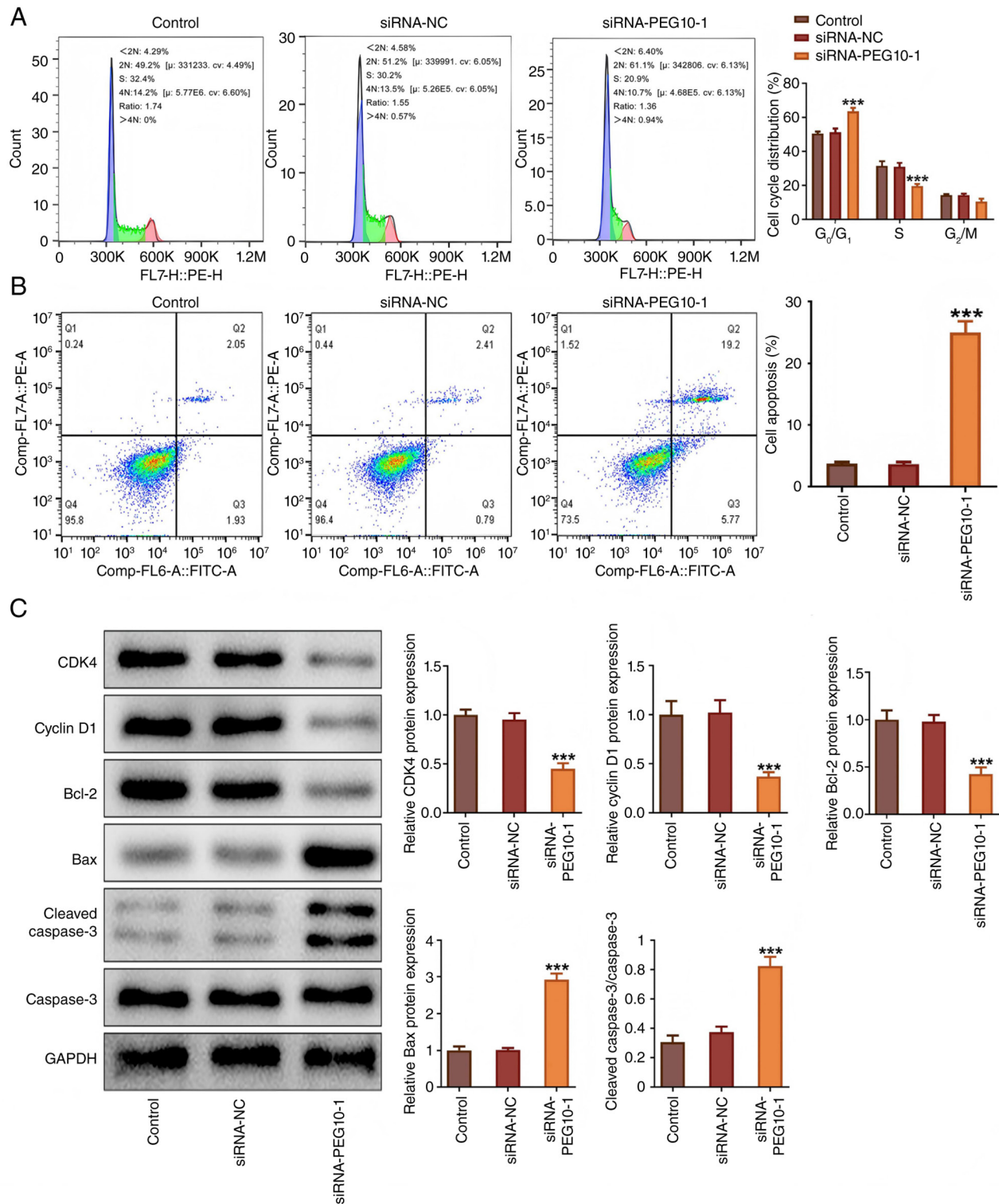


Figure 3. Effects of *PEG10* knockdown on apoptosis and cell cycle progression in SCC-15 cells. Flow cytometry analysis of (A) cell-cycle phase distribution and (B) the percentage of apoptotic cells across treatment groups. Flow cytometry plots are shown on the left and quantitative analysis is shown on the right. (C) Western blot analysis of CDK4, cyclin D1, Bcl-2, Bax, caspase 3 and cleaved caspase 3. Representative western blot images are shown on the left, and semi-quantitative densitometric analyses, including the cleaved caspase 3/total caspase 3 ratio, are shown on the right. *** $P < 0.001$ vs. siRNA-NC. *PEG10*, paternally expressed gene 10; siRNA-*PEG10*, *PEG10*-targeting small interfering RNA; siRNA-NC, negative control small interfering RNA sequence; CDK4, cyclin-dependent kinase 4; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X. Data are presented as the mean $\pm$ SD from three independent biological replicates ($n=3$).

to the non-tumoral HIOEC control when compared with the pattern shown by *PEG10* expression in these cell lines (Fig. 4A). Knockdown of *PEG10* with siRNA-*PEG10*-1 resulted in a 3-fold decrease in the expression level of *TRIB3* (Fig. 4B).

To further characterize the potential interaction between *PEG10* and *TRIB3*, protein-protein docking was performed using the HDOCK server. The top-ranked docking model exhibited a favorable docking score (-396.4 kcal/mol) with a high confidence

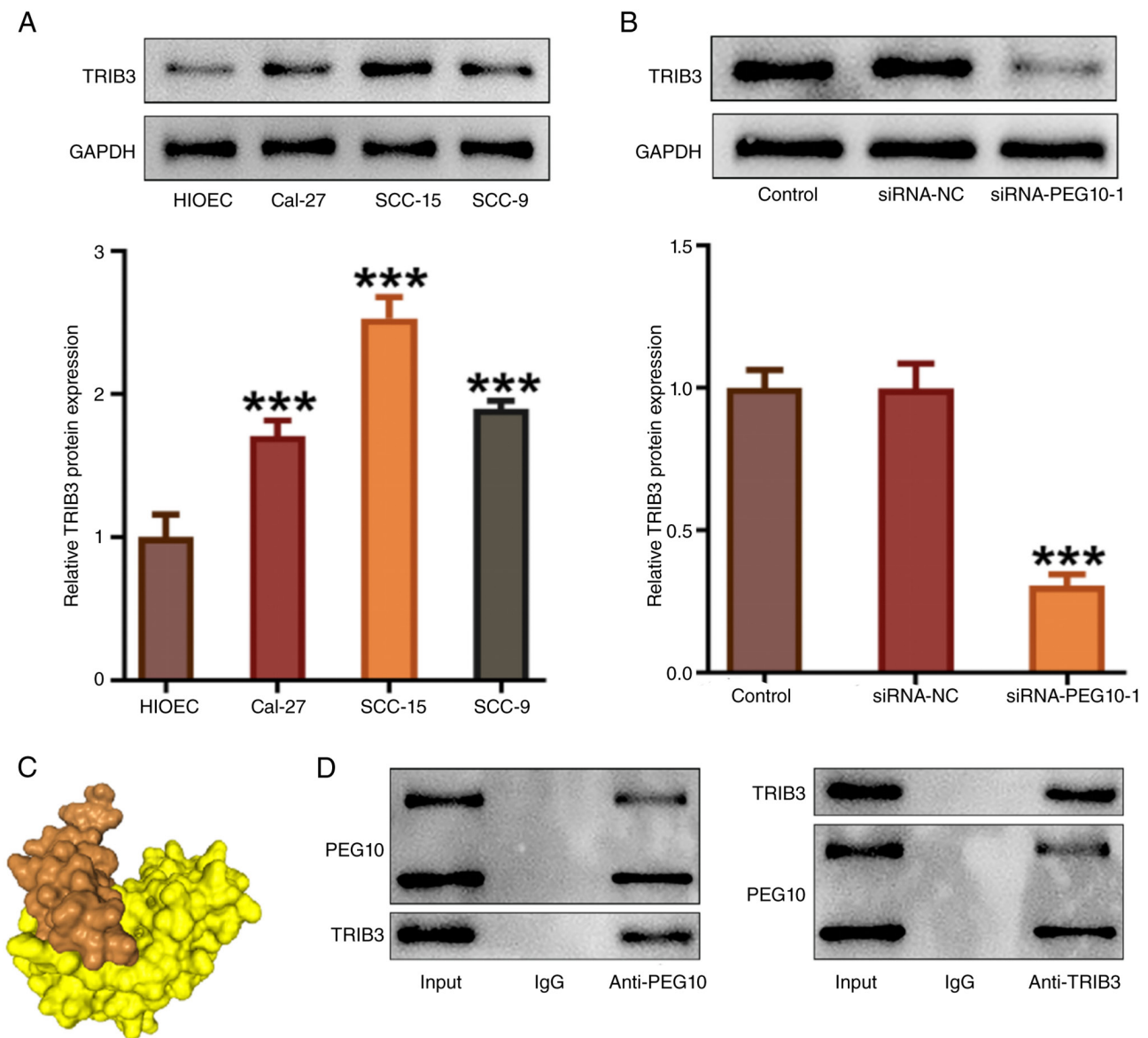


Figure 4. Effects of *PEG10* on *TRIB3* expression and interactions between *PEG10* and *TRIB3*. (A) Western blotting analysis of *TRIB3* expression in Cal-27, SCC-15 and SCC-9 OSCC cell lines in comparison with non-tumoral HIOEC control cells. Representative images are shown at the top and semi-quantitative densitometric analysis is shown below. (B) Western blotting analysis of *TRIB3* expression following *PEG10* knockdown in SCC-15 cells compared with untransfected control and siRNA-NC cells. Representative images are shown at the top and semi-quantitative densitometric analysis is shown below. (C) Prediction of the interaction between *PEG10* (brown) and *TRIB3* (yellow) using the HDOCK server. (D) Co-immunoprecipitation assay with anti-*PEG10* and anti-*TRIB3* antibodies showing the direct interaction between the two proteins. *** $P < 0.001$ vs. controls. *PEG10*, paternally expressed gene 10; *TRIB3*, Tribbles pseudokinase 3; OSCC, oral squamous cell carcinoma; HIOEC, human immortalized oral epithelial cells; siRNA-*PEG10*, *PEG10*-targeting small interfering RNA; siRNA-NC, negative control small interfering RNA sequence. Data are presented as the mean $\pm$ SD from three independent biological replicates ($n=3$).

score of 0.8, suggesting a potential interaction between *PEG10* and *TRIB3*. Structural analysis indicated that the interaction interface of these proteins was predominantly stabilized by hydrophobic interactions, together with four hydrogen bonds and a salt bridge. Specifically, residues Trp345, Phe367, Leu390 and Arg412 on *PEG10* formed the core binding region, interacting with residues Val223, Val224, Pro225 and Gln226 on *TRIB3*. Furthermore, a salt bridge was identified between the Arg412 residue on *PEG10* and Asp228 residue on *TRIB3* (Fig. 4C).

Although these residues have not been directly mapped to well-defined functional domains in *PEG10* or *TRIB3*, the identified interaction interface may contribute to the stability and specificity of the *PEG10*-*TRIB3* complex, thereby

potentially facilitating the functional association of these proteins. Notably, Co-IP assays further supported the interaction between *PEG10* and *TRIB3* in SCC-15 cells (Fig. 4D).

TRIB3 overexpression reverses the effects of *PEG10* knockdown, stimulating proliferation, migration and invasion by SCC-15 cells. Compared with controls, significant overexpression of *TRIB3* was achieved by transfection of SCC-15 cells with Ov-*TRIB3*, whereas transfection of cells with Ov-NC resulted in no significant changes in *TRIB3* expression compared with control cells (Fig. 5A). *TRIB3* overexpression partially reversed the suppression of cell viability, reduction in proliferation and inhibition of migration and invasion resulting

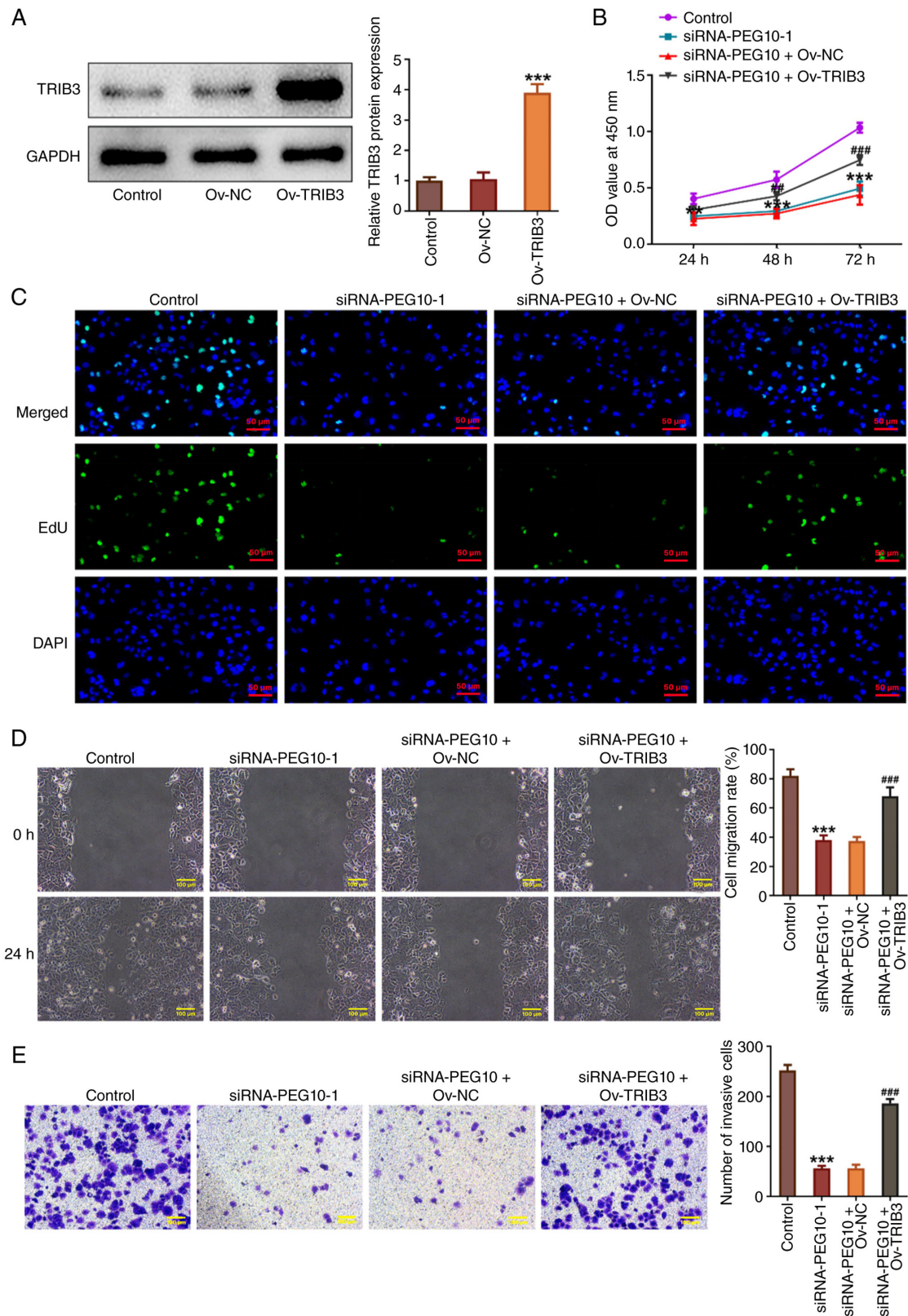


Figure 5. Effects of *TRIB3* overexpression in *PEG10*-knockdown SCC-15 cells on proliferation, migration and invasion rates. (A) Western blotting analysis of *TRIB3* expression in SCC-15 cells transfected with Ov-NC or Ov-*TRIB3*. Representative western blot images are shown on the left and semi-quantitative densitometric analysis is shown on the right. ***P < 0.001 vs. Ov-NC. (B) Cell viability was measured in SCC-15 cells in control, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* groups via Cell Counting Kit-8 assay. (C) Cell proliferation was measured in SCC-15 cells in control, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* groups via EdU staining. (D) Cell migration was measured in SCC-15 cells in control, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* treatment groups via wound healing assay. Representative images are shown on the left and quantitative analysis is shown on the right. (E) Cell invasion was measured in SCC-15 cells in control, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* treatment groups via Transwell assay. Representative images are shown on the left and quantitative analysis is shown on the right. **P < 0.01 and ***P < 0.001 vs. control; ##P < 0.01 and ###P < 0.001 vs. siRNA-*PEG10* group. *PEG10*, paternally expressed gene 10; *TRIB3*, Tribbles pseudokinase 3; OD, optical density; EdU, 5-ethynyl-2'-deoxyuridine; siRNA-*PEG10*, *PEG10*-targeting small interfering RNA; Ov-*TRIB3*, *TRIB3* overexpression lentiviral vector; Ov-NC, empty overexpression vector control. Data are presented as the mean ± SD from three independent biological replicates (n=3).

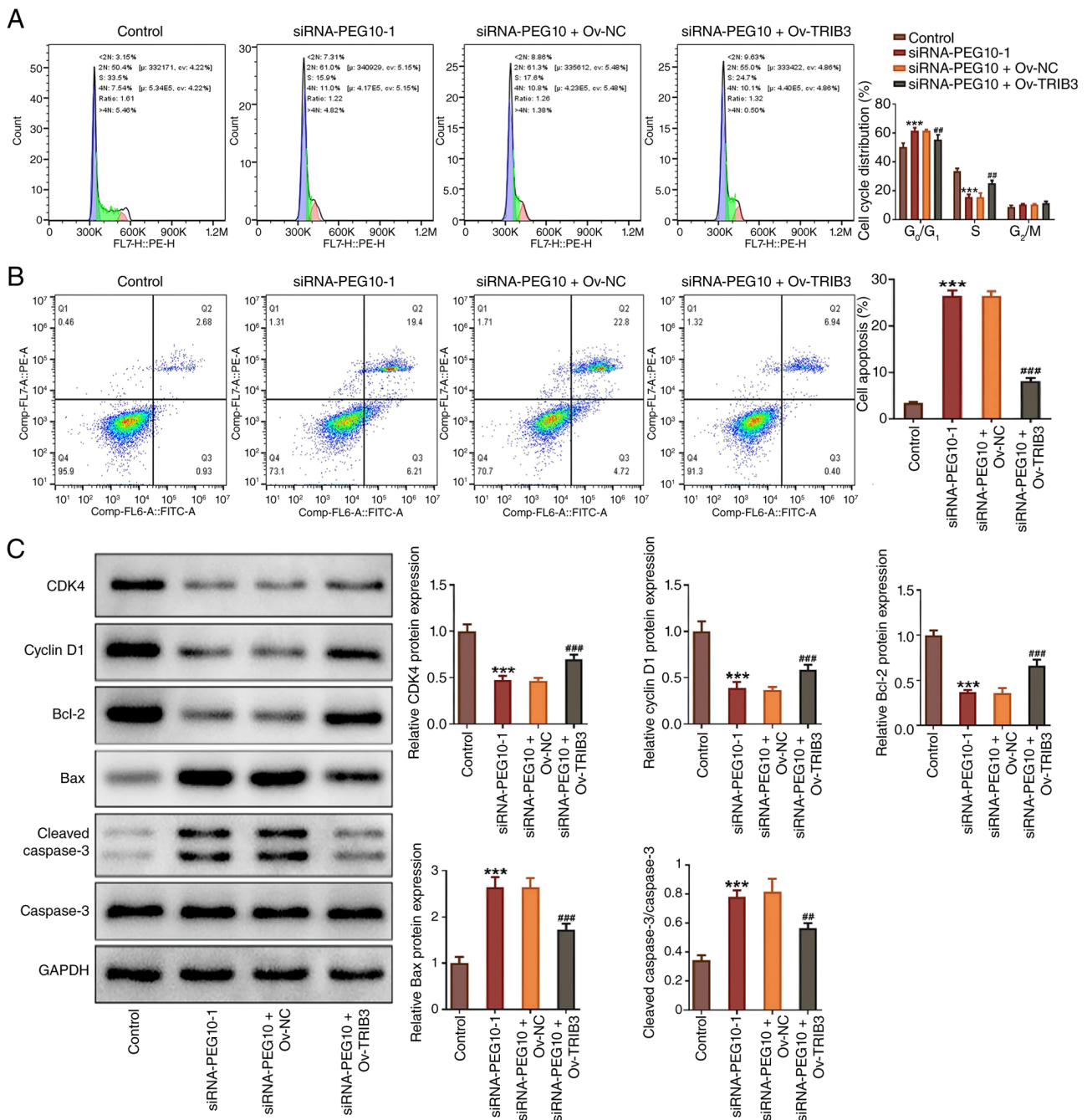


Figure 6. Effects of *TRIB3* overexpression in *PEG10*-knockdown SCC-15 cells on markers of cell cycle and apoptosis. Flow cytometry analysis showing (A) the proportions of SCC-15 cells in different cell cycle phases and (B) apoptotic SCC-15 cells in untreated controls, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* groups. Flow cytometry plots are shown on the left and quantitative analysis is shown on the right. (C) Western blot analysis of CDK4, cyclin D1, Bcl-2, Bax, caspase 3 and cleaved caspase 3 in SCC-15 cells in the control, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* groups. Representative western blot images are shown on the left, and semi-quantitative densitometric analyses, including the cleaved caspase 3/total caspase 3 ratio, are shown on the right. *** $P < 0.001$ vs. control cells; ## $P < 0.01$ and ### $P < 0.001$ vs. siRNA-*PEG10* group. *PEG10*, paternally expressed gene 10; *TRIB3*, Tribbles pseudokinase 3; siRNA-*PEG10*, *PEG10*-targeting small interfering RNA; Ov-*TRIB3*, *TRIB3* overexpression lentiviral vector; Ov-NC, empty overexpression vector control; CDK4, cyclin-dependent kinase 4; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X. Data are presented as the mean $\pm$ SD from three independent biological replicates (n=3).

from *PEG10* knockdown (Fig. 5B-E). These findings substantiated the existence of a functional association between *PEG10* and *TRIB3* that in turn produced downstream effects.

TRIB3 overexpression mitigates the effects of *PEG10* knockdown, suppressing apoptosis and stimulating cell cycle transition. *TRIB3* overexpression partially reversed the cell cycle arrest and stimulation of apoptosis induced by *PEG10*

knockdown. Concurrent *TRIB3* overexpression significantly decreased the proportion of cells in the G₀/G₁ phase and significantly increased the proportion of cells in the S phase compared with *PEG10* knockdown alone, whereas no notable change was observed in the G₂/M phase (Fig. 6A). The apoptotic rate of OSCC cells in the siRNA-*PEG10* + Ov-*TRIB3* group was also decreased 3-fold compared with the siRNA-*PEG10*-1 group (Fig. 6B). Furthermore, western

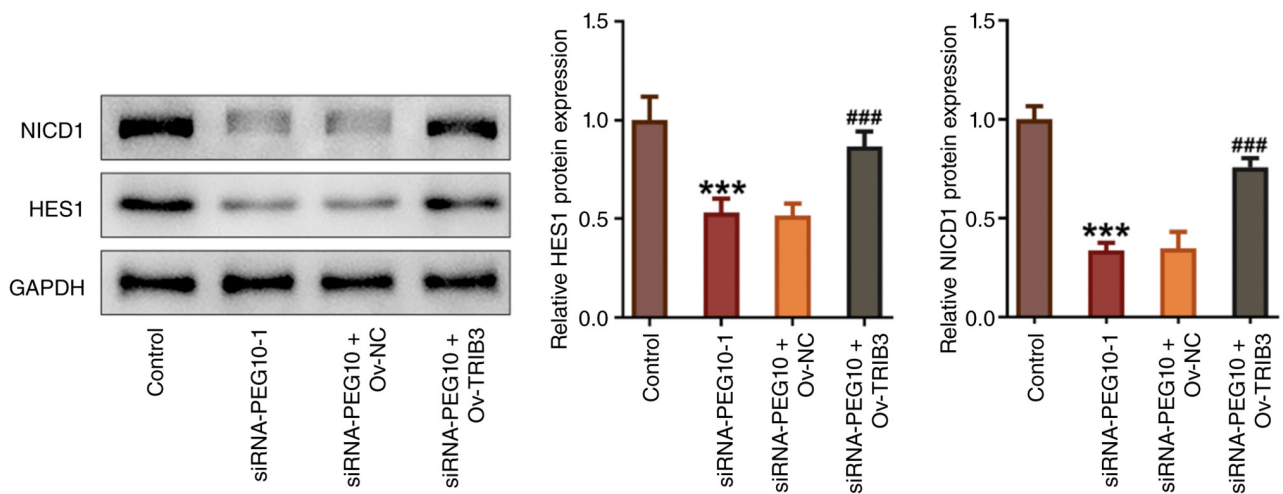


Figure 7. Effects of *PEG10* downregulation on the protein expression of the Notch signaling pathway components NICD1 and HES1. Western blotting analysis of NICD1 and HES1 in SCC-15 cells in control, siRNA-*PEG10*, siRNA-*PEG10* + Ov-NC and siRNA-*PEG10* + Ov-*TRIB3* groups. Representative western blot images are shown on the left and semi-quantitative densitometric analysis is shown on the right. *** $P < 0.001$ vs. control cells; ### $P < 0.001$ vs. siRNA-*PEG10* group. *PEG10*, paternally expressed gene 10; *TRIB3*, Tribbles pseudokinase 3; siRNA-*PEG10*, *PEG10*-targeting small interfering RNA; Ov-*TRIB3*, *TRIB3* overexpression lentiviral vector; Ov-NC, empty overexpression vector control; NICD1, Notch1 intracellular domain; HES1, hairy and enhancer of split-1. Data are presented as the mean $\pm$ SD from three independent biological replicates ($n=3$).

blotting analysis of the expression of proteins involved in the cell cycle and apoptosis produced results consistent with those of cell-cycle and apoptosis analyses. Compared with *PEG10* knockdown alone, the protein expression levels of CDK4, cyclin D1 and Bcl-2 were significantly increased following concurrent *TRIB3* overexpression, whereas Bax expression and the cleaved caspase 3/total caspase 3 ratio were significantly decreased following *TRIB3* overexpression (Fig. 6C). The role of *TRIB3* in stimulating cell cycle transition and reducing apoptosis reinforced the aforementioned findings supporting a functional association between *PEG10* and *TRIB3*.

PEG10 knockdown reduces Notch signaling by affecting *TRIB3*. Notch signaling is involved in the proliferation of multiple cell types, including cancer cells, and its dysregulation has been implicated in OSCC progression (22,23). The expression levels of the Notch effectors NICD1 and HES1 were significantly reduced when *PEG10* was knocked down in SCC-15 cells. Notably, this effect was significantly mitigated by concurrent *TRIB3* overexpression (Fig. 7). These findings indicated that the *PEG10*-*TRIB3* interaction played a role in regulating Notch signaling.

Discussion

Molecular alterations in OSCC cells drive the local invasion and lymph node metastasis that result in poor patient prognoses (30,31). In the present study, *PEG10* was shown to be upregulated in OSCC tissues and cells and was shown to interact with *TRIB3* on both a structural and functional level. Functional interactions between the two proteins were associated with the proliferation, migration and invasion of OSCC cells, potentially through modulation of Notch-related signaling.

Genomic imprinting achieves mono-allelic expression of a subset of genes in a parental-specific expression pattern via an

epigenetic mechanism (32), dysregulation of which has been implicated in cancer (33). A number of imprinting genes have been linked to cancers, such as head and neck squamous cell carcinoma (34), including *PEG10* (10). High *PEG10* expression has been shown to stimulate proliferation, migration and invasion in breast, lung and pancreatic cancer cells (35-37) and upregulated *PEG10* has been linked to poor prognosis in patients with OSCC (12). As such, *PEG10* has been suggested to represent an OSCC-associated oncogene. *PEG10* was upregulated in the OSCC tissues and cells of the present study, and *PEG10* knockdown reduced the proliferation, migration and invasion of OSCC cells.

Dysregulation of the cell cycle and evasion of apoptosis are hallmarks of cancer (38,39). The binding of cyclin D1 to CDK4/6 is required for the G_1 to S phase transition (40) and *PEG10* knockdown has been shown to delay this cell cycle transition in breast, pancreatic and bladder cancers (35,37,41). In the present study, *PEG10* knockdown caused SCC-15 cells to be arrested in G_0/G_1 phase and resulted in significant reductions in cyclin D1 and CDK4 expression. *PEG10* overexpression has been shown to reduce apoptosis in colorectal cancer and retinoblastoma cells and may act by changing the ratio of pro-apoptotic Bax/anti-apoptotic Bcl-2, therefore influencing downstream caspase 3 and caspase 9 (42-45). Notably, *PEG10* knockdown exerted a pro-apoptotic effect on the OSCC cells of the present study and resulted in reduced Bcl-2 expression and elevated Bax and cleaved caspase 3 expression.

A *PEG10*-*TRIB3* interaction was predicted by molecular docking. Furthermore, the expression level of *TRIB3* was higher in OSCC cells than non-tumoral cells, which was consistent with previous findings (17). The finding that *TRIB3* expression was downregulated following *PEG10* knockdown supported the view of a functional interaction between the two proteins. Notably, only *TRIB3* protein levels were assessed as a result of this interaction in the present study.

TRIB3 has been suggested to be a tumor promoter with effects on cell migration, invasion, epithelial-mesenchymal transition (EMT), stemness, cell cycle, angiogenesis and apoptosis (13,46); *TRIB3* has also been implicated in OSCC cell proliferation (17). The findings of the present study, which demonstrated the stimulation of proliferation, migration, invasion and cell cycle re-entry, as well as the suppression of apoptosis, upon *TRIB3* overexpression in *PEG10*-knockdown OSCC cells, were consistent with these previous reports. Therefore, the findings of the present study supported the role of *TRIB3* as a tumor promoter and suggested that *TRIB3* and *PEG10* interacted to exert downstream effects.

The abnormal expression of proteins in the Notch signaling pathway is closely related to the development of various cancers, such as colorectal and lung cancers (47,48). Among these proteins, NICD and HES1 are considered important biomarkers for OSCC (49,50). Research has demonstrated that targeting Notch signaling, particularly proteins such as Notch1, NICD and HES1, with cetuximab may inhibit the invasion and metastasis of OSCC (49). A study reported by Guo *et al* (51) enhanced the antiproliferative effect of Notch signaling inhibition in OSCC by inducing miR-211-5p upregulation and Alpinetin (ALP)-mediated Notch pathway inactivation, specifically NICD and HES1 inhibition. In the present study, *PEG10* knockdown in OSCC cells reduced NICD1 and HES1 levels, whereas concurrent *TRIB3* overexpression reversed these effects. These findings suggest that *PEG10* may regulate Notch signaling through *TRIB3*.

In addition, EMT, an important process driving tumor cell migration and invasion, may also be involved in the effects mediated by the *PEG10/TRIB3* axis (52). This was supported by the observed changes in migratory and invasive capacities of OSCC cells following *PEG10* knockdown and *TRIB3* overexpression, as well as a previous report implicating *TRIB3* in EMT regulation (53). However, EMT-related markers were not evaluated in the present study, and further investigations are required to validate this hypothesis.

Apart from its interaction with *PEG10*, *TRIB3* has been reported to regulate multiple oncogenic signaling pathways, including the AKT/mTOR pathway, which plays a central role in cell proliferation, survival and metabolism (54). A previous study demonstrated that *TRIB3* can modulate AKT signaling activity and thereby influence tumor progression (55). Notably, in OSCC, *TRIB3* has been shown to promote cell proliferation by activating AKT/mTOR signaling (17). Therefore, it is possible that the effects of signaling along the *PEG10/TRIB3* axis observed in the present study may also involve alternative downstream pathways beyond Notch signaling. Further investigation will be required to determine the relative contribution of these pathways in OSCC progression.

OSCC is a highly heterogeneous disease at the molecular level, with distinct genetic, epigenetic and signaling alterations across different subtypes (21,56). This heterogeneity may influence the functional role of specific molecular pathways in tumor progression. Therefore, the regulatory effects of the *PEG10/TRIB3* axis observed in the present study may vary across different OSCC contexts. Further studies using

additional cell models and clinical samples will be necessary to evaluate the broader applicability of the findings of the present study.

The present study also has several limitations. Primarily, all functional and mechanistic experiments were conducted in a single OSCC cell line, which may limit the generalizability of the findings. Furthermore, although an association between the *PEG10/TRIB3* axis and Notch signaling was observed in the present study, direct pathway validation experiments were not performed. Additionally, the mechanism by which *PEG10* regulated *TRIB3*, particularly regarding whether this regulation was transcriptional or post-translational, remains ambiguous. The lack of *in vivo* and clinical validation limits the translational applicability of the findings of the present study. Future studies incorporating additional cell models, *in vivo* experiments and clinical data will be required to further validate these observations. Furthermore, although public datasets, such as The Cancer Genome Atlas (TCGA)-Head-Neck Squamous Cell Carcinoma, could provide valuable clinical context for the *PEG10/TRIB3* axis in OSCC, the present study did not include bioinformatical analyses of *PEG10* and *TRIB3* expression using TCGA data. Future studies incorporating such analyses, alongside clinical sample validation, are warranted to further establish the clinical importance of the *PEG10/TRIB3* axis in OSCC.

In conclusion, *PEG10* was upregulated in OSCC, particularly in SCC-15 cells, and was associated with enhanced malignant behavior, such as proliferation, migration and invasion. *PEG10* was shown to interact directly with *TRIB3*, and this interaction may have influenced Notch-related signaling. As such, the *PEG10/TRIB3* axis may represent a potential regulatory mechanism in OSCC progression.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JZ designed the study, performed the experiments, analyzed the data, wrote the original draft, and contributed toward reviewing and editing the manuscript. YL performed the experiments and contributed toward data interpretation and manuscript revision. JZ and YL 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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