# COL4A1 promotes the proliferation and migration of oral squamous cell carcinoma cells by binding to NID1

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**Abstract.** Collagen type IV α1 chain (COL4A1) is a collagen protein that acts as a tumor-promoting factor in several types of cancer. However, the role and the potential mechanisms involving COL4A1 in oral squamous cell carcinoma (OSCC) remain unclear. Using reverse transcription-quantitative PCR and western blotting, the expression levels of COL4A1 and (nidogen-1) NID1 in OSCC cells were assessed. Cell Counting Kit-8, EdU staining and colony formation assays were used to evaluate cell proliferation. Cell migration and invasion were assessed using wound healing and Transwell invasion assays, respectively. The expression levels of proteins involved in epithelial-mesenchymal transition (EMT) were assessed using western blotting. In addition, the association between COL4A1 and NID1 was analyzed using TNMplot and the STRING database and verified by co-immunoprecipitation analysis. COL4A1 expression was found to be significantly increased in OSCC cells. Knockdown of COL4A1 expression decreased SCC-4 cell proliferation, migration and invasion, as well as the progression of EMT. In addition, COL4A1 was shown to be significantly positively associated with NID1 in OSCC and to bind to NID1. NID1 overexpression reversed the inhibitory effects of COL4A1 knockdown on cell proliferation, migration and invasion as well as on the progression of EMT in OSCC cells. In summary, the present findings demonstrated that COL4A1 promoted cell proliferation and migration as well as the progression of EMT in OSCC cells by binding to NID1, highlighting a potential avenue for therapeutic management of OSCC.

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*Key words:* collagen type IV α1 chain, nidogen-1, oral squamous cell carcinoma, epithelial-mesenchymal transition, metastasis

## Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy of the head and neck (1). It is estimated that ~350,000 individuals are diagnosed with OSCC every year worldwide, accounting for 2-4% of all malignant tumors (2,3). Among patients with OSCC, ~50% are diagnosed in the first instance with advanced-stage cancer. This delay in diagnosis and lack of specific markers for the prediction of OSCC development and progression may also explain the high global mortality rate in 2018 (4,5). Despite notable progress made in the diagnosis and treatment of tumors, surgery, postoperative radiotherapy and chemotherapy remain the conventional therapeutic approaches and prognosis of OSCC remains poor (6). Although OSCC, oropharyngeal and esophageal carcinoma are classified as head and neck squamous cell carcinomas, the differences in the microenvironment and pathogenesis between these cancers lead to a large difference in the gene expression profile between OSCC and other types of squamous cell carcinoma (7,8). OSCC carcinogenesis is a global burden that needs to be addressed; therefore identifying novel effective biomarkers and therapeutic targets for patients with OSCC should be a priority.

Collagen type IV α1 chain (COL4A1), a type of collagen that belongs to the type IV collagen family, is a key component of the basement membrane and several studies have found that it acts as a cancer-promoting factor in several types of cancer (9-12). Recent studies have found that COL4A1 is abnormally expressed in invasive ductal carcinoma of breast and bladder tumors and is associated with tumor invasion and metastasis (13,14). Furthermore, COL4A1 is reported to be upregulated in patients with OSCC (15). Nevertheless, the specific role that COL4A1 plays in OSCC progression remains unclear. Thus, the present study aimed to identify the biological role and the potential mechanism of COL4A1 in OSCC cells.

## Materials and methods

*Bioinformatics analysis*. The TNMplot database (tnmplot.com) was used to perform the association analysis between COL4A1 and Nidogen-1 (NID1) in OSCC. In addition, STRING database (cn.string-db.org/) was used to predict the binding between COL4A1 and NID1.

*Cell culture and treatment*. Human OSCC cell lines HN4, HN6, SCC-4 and Cal-27, as well as human oral keratinocyte (HOK) cells were provided by the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37˚C in a humified incubator with 5% $CO_2$.

*Cell transfection*. For knockdown of COL4A1, the specific small interfering (si)RNAs targeting COL4A1 (si-COL4A1-1, 5'-AGG ACAAGCUCAAGUUCAAGA-3'; and si-COL4A1-2, 5'-GGAG CGAGAUGUUCAAGAAGC-3') and the corresponding negative control siRNA (si-NC, 5'-UUCUCCGAACGUGUCACG U-3') were synthesized by Shanghai Gene Pharma Co., Ltd. To overexpress NID1, pc-DNA3.1 vector containing the full-length NID1 gene (Oe-NID1) and the empty vector (Oe-NC) were synthesized by Shanghai Gene Pharma Co., Ltd. A total of 100 nM plasmids/siRNAs were transfected into SCC-4 cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. A total of 48 h post-transfection, cells were collected for use in subsequent experiments.

*Cell Counting Kit-8 (CCK-8) assay*. Following transfection, SCC-4 cells were plated into 96-well plates at a density of $5x10^3$ cells/well and cultured at 37˚C for 24, 48 or 72 h. Subsequently, 10 $\mu$l CCK-8 (Beyotime Institute of Biotechnology) was added and cells were further cultured at 37˚C with 5% $CO_2$ for 4 h. Absorbance was measured using a microplate reader at a wavelength of 450 nm as a measure of proliferation (Bio-Rad Laboratories, Inc).

*EdU incorporation cell proliferation assay*. SCC-4 cells were plated in a 6-well plate at a density of $4x10^5$ cells/well and cultured overnight at 37˚C. The following day, cells were fixed in 4% polyformaldehyde at room temperature for 15 min and permeabilized with 0.5% Triton X-100 at room temperature for 15 min. Then, cells were stained using a Cell-Light™ EdU Cell Proliferation Detection Assay (Thermo Fisher Scientific, Inc.) at room temperature for 30 min according to the manufacturer's protocol and counterstained with 5 mg/ml DAPI at room temperature for 10 min. The stained cells were counted using a fluorescence microscope (Nikon Corporation; magnification, x100).

*Colony formation assay*. Transfected cells were plated at a density of 500 cells/well in 6-well plates and incubated in DMEM with 10% bovine calf serum (Thermo Fisher Scientific, Inc.) at 37˚C. Following 10 days of culture, cells were fixed using 4% paraformaldehyde for 15 min at room temperature and stained with 1.5% crystal violet (FUJIFILM Wako Pure Chemical Corporation) at room temperature for 10 min. Colonies were counted with ImageJ software (Version 146; National Institutes of Health) using a light microscope (Olympus Corporation; magnification, x1). Colonies consisted of ≥50 cells.

*Wound healing assay*. The migration of SCC-4 cells was assessed using a wound healing assay. Transfected cells were plated into a 6-well plate at a density of $5x10^5$ cells/well and cultured at 37˚C until they reached 80-90% confluence. A 20-$\mu$l tip was used to create a scratch in the monolayer, after which cells were cultured in serum-free DMEM (Gibco; Thermo Fisher Scientific, Inc.). After 24 h incubation at room temperature, the scratched area was observed using a light microscope (Olympus Corporation; magnification, x100). The migratory rate (%) was calculated as follows: (Wound width at 0 h-wound width at 24 h)/wound width at 0 h x 100. Analysis was based on five randomly selected fields of view.

*Transwell assay*. Transwell chambers (Corning, Inc.) were precoated with 0.1 ml Matrigel (Becton, Dickinson and Company) at 37˚C for 1 h. SCC-4 cells were collected and suspended to a final concentration of $2x10^5$ cells/ml in DMEM containing 1% FBS (HyClone; Cytiva). The cell suspensions were placed in the upper chamber and DMEM supplemented with 10% FBS was added to the lower chamber. After incubation at 37˚C for 24 h, a cotton swab was used to remove cells from the upper chamber that had not invaded. Cells that had invaded were fixed using 70% ethanol at 4˚C for 30 min and stained using 0.5% crystal violet for 10 min at room temperature. The number of cells that had invaded was counted using a light microscope (Olympus Corporation; magnification, x200) in five randomly selected fields of view.

*Reverse transcription-quantitative (RT-q)PCR*. Total RNA was extracted from $1x10^4$ SCC-4 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and this was used to synthesize cDNA using cDNA Synthesis kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using SYBR Premix ExTaq kit (Takara Bio, Inc.) with amplification performed on an ABI PRISM 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The qPCR thermocycling conditions were as follows: Initial denaturation at 95˚C for 3 min; followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. The following primer pairs were used for qPCR: COL4A1 forward, 5'-AGGAGTGCCATTGCTTTTTCAA-3' and reverse, 5'-TGG AAACCAGTCCATGCTCG-3'; NID1 forward, 5'-CTTTGA GGTACCCACCGTCC-3' and reverse, 5'-GGGAGGGGCTGC TCATTATC-3' and GAPDH forward, 5'-GGGAAACTGTGG CGTGAT-3' and reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'. The relative mRNA level was calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene GAPDH (16).

*Co-immunoprecipitation (Co-IP)*. Total protein was extracted from SCC-4 cells using IP lysis buffer (20 mM Tris-HCl, 150 mM NaCl and 1% Triton X-100, pH 7.5). The supernatant was collected after centrifugation at 13,000 x g for 10 min at 4˚C. Protein A agarose beads (0.2 mg; cat. no. 20366; Thermo Fisher Scientific, Inc.) washed with 100 $\mu$l PBS were added to 500 $\mu$g cell lysates and incubated with 2 $\mu$g IgG antibody (cat. no. ab6715; Abcam) or COL4A1 antibody (cat. no. ab6586; Abcam) overnight at 4˚C. Following rinsing with PBS, precipitated protein was resuspended and boiled for 5 min at 100˚C. Finally, the eluates were collected by magnetic separation and subsequently subjected to western blotting.

*Western blotting*. Total protein was extracted from SCC-4 cells using RIPA buffer (Auragene) and quantified using a BCA Protein Assay kit. Equal amounts of protein (20 $\mu$g/lane)

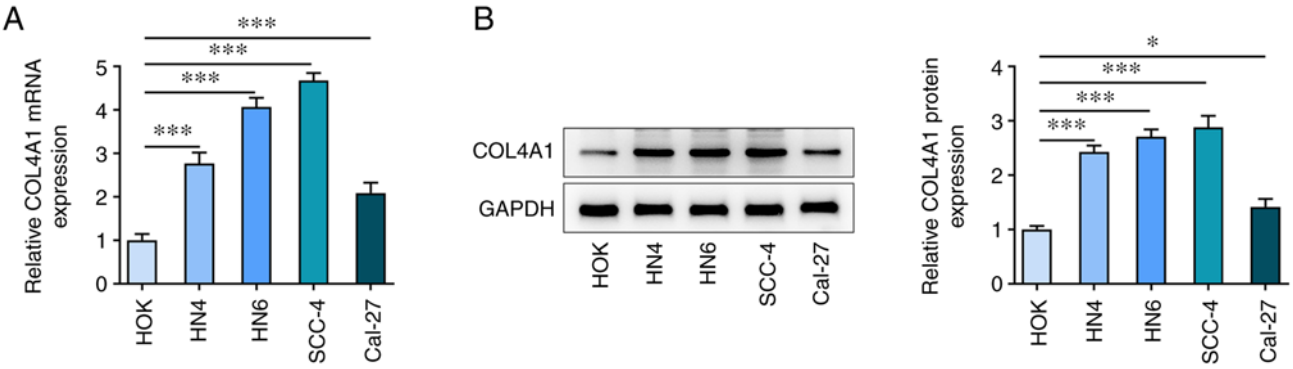


Figure 1. COL4A1 is upregulated in OSCC cells. (A) mRNA expression of COL4A1 in OSCC cell lines measured via reverse transcription-quantitative PCR. (B) COL4A1 protein level in OSCC cell lines semi-quantified using western blot assay. Results are presented as the mean ± standard deviation. *P<0.05 and ***P<0.001. COL4A1, collagen type IV α1 chain; OSCC, oral squamous cell carcinoma.

were loaded on a 10% SDS gel (Bio-Rad Laboratories, Inc.), resolved using SDS-PAGE and transferred to PVDF membranes (MilliporeSigma) at 25 V for 30 min. Membranes were blocked for 2 h at room temperature using 5% non-fat milk in 0.1% tris-buffered saline with Tween-20 and incubated with primary antibodies against COL4A1 (cat. no. ab226485; 1:500; Abcam), N-cadherin (cat. no. ab76011; 1:5,000; Abcam), Vimentin (cat. no. ab92547; 1:1,000; Abcam), E-cadherin (cat. no. ab40772; 1:10,000; Abcam), NID1 (cat. no. ab254325; 1:1,000; Abcam) and GAPDH (cat. no. ab9485; 1:2,500; Abcam) at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (cat. no. ab6721; 1:2,000; Abcam) for 2 h at room temperature. Signals were visualized using an ECL detection system (Beyotime Institute of Biotechnology) and densitometry analysis was performed using QuantityOne version 4.5.0 (Bio-Rad Laboratories, Inc.).

*Statistical analysis*. All experiments were performed at least three times. All data were analyzed using SPSS version 22.0 software (IBM Corp) and are presented as the mean ± standard deviation. Differences between two groups were compared using unpaired Student's t test whereas differences between multiple groups were compared using one-way ANOVA with post hoc Bonferroni's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*COL4A1 is upregulated in OSCC cells*. To determine the role that COL4A1 serves in OSCC progression, COL4A1 expression in OSCC cells was initially assessed. mRNA expression levels of COL4A1 in OSCC cell lines including HN4, HN6, SCC-4 and Cal-27 were increased compared with those in HOK cells (Fig. 1A). In addition, results from western blot analysis demonstrated that COL4A1 levels were increased in OSCC cell lines compared with those in HOK cells (Fig. 1B). Among these OSCC cell lines, SCC-4 showed the highest expression of COL4A1, thus this cell line was used for all subsequent experiments.

*Knockdown of COL4A1 suppresses proliferation of SCC-4 cells*. To determine the biological role that COL4A1 serves in OSCC cells, si-COL4A1-1/2 was used to knock down COL4A1 expression in SCC-4 cells. RT-qPCR and western blot analysis showed that si-COL4A1-2 exhibited a greater knockdown efficiency. Thus, si-COL4A1-2 (henceforth referred to as si-COL4A1) was selected for all subsequent experiments (Fig. 2A and B). CCK-8 assay showed that cell proliferation was significantly inhibited following transfection with si-COL4A1 compared with NC (Fig. 2C). In addition, data from Edu staining also revealed the number of positive cells in the si-COL4A1 group was decreased compared with that in the control (Fig. 2D). As shown in Fig. 2E, the colony-forming ability of SCC-4 cells was reduced by COL4A1 knockdown compared with that in the si-NC-transfected cells.

*COL4A1 knockdown inhibits migration, invasion and expression of epithelial-mesenchymal transition (EMT)-associated protein in SCC-4 cells*. Next, the effects of COL4A1 knockdown on migration, invasion and EMT-associated protein expression in SCC-4 cells were assessed. Knockdown of COL4A1 decreased cell migration compared with NC (Fig. 3A and B). Transwell invasion assay results showed that the invasive ability was decreased in the COL4A1 knockdown cells compared with NC (Fig. 3C and D). Western blot analysis showed that COL4A1 knockdown resulted in a decrease in the levels of N-cadherin and vimentin while increasing expression levels of E-cadherin (Fig. 3E).

*COL4A1 interacts with NID1 in OSCC cells*. mRNA and protein expression levels of NID1 were significantly higher in SCC-4 cells compared with those in the HOK cells (Fig. 4A and B). In addition, TNMplot database showed that COL4A1 was highly positively associated with NID1 in OSCC (Fig. 4C). STRING database analysis also showed that COL4A1 and NID1 may form a complex (Fig. 4D). Co-IP analysis showed that COL4A1 and NID1 were present in the IP assay with anti-COL4A1 and anti-NID1 antibody but not with control IgG (Fig. 4E).

*Upregulation of NID1 reverses the inhibitory effect of COL4A1 silencing on SCC-4 cells*. To identify the role of NID1 in COL4A1 regulation in SCC-4 cells, NID1 was overexpressed in SCC-4 cells. RT-qPCR and western blot analysis confirmed that NID1 expression was successfully increased in the NID1 cells (Fig. 5A and B). CCK-8 assay showed that

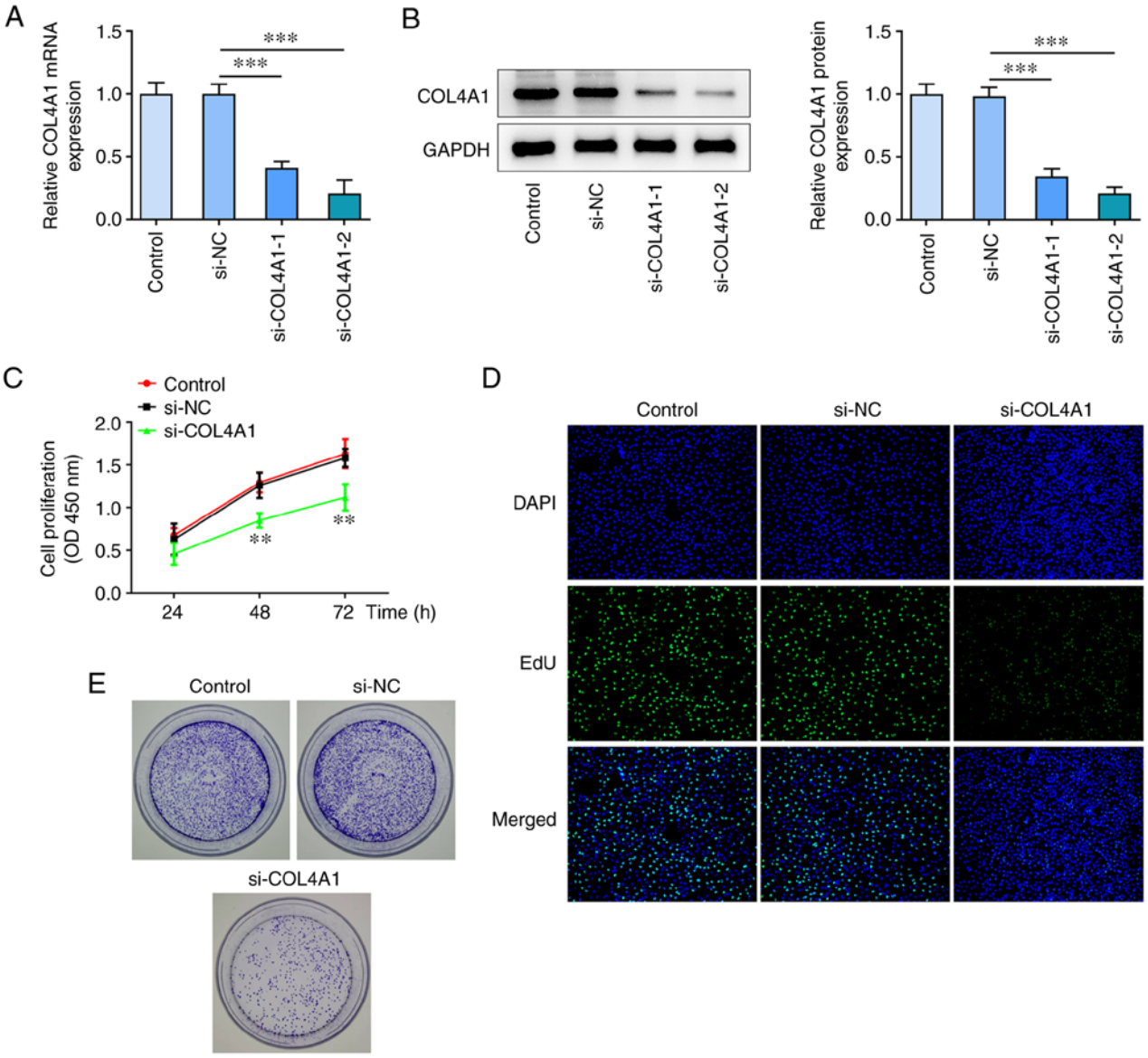


Figure 2. Knockdown of COL4A1 suppresses proliferation of SCC-4 cells. (A) mRNA expression of COL4A1 in SCC-4 cells measured via reverse transcription-quantitative PCR. ***P<0.001. (B) COL4A1 protein level in SCC-4 cells semi-quantified using western blot assay. ***P<0.001. Cell proliferation in SCC-4 cells was evaluated using (C) CCK-8 (**P<0.01) and (D) EdU incorporation (magnification, x100). (E) Colony formation assay (magnification, x1) was used to measure the colony-forming ability of SCC-4 cells. Results are presented as the mean ± standard deviation. COL4A1, collagen type IV α1 chain; NC, negative control; si, small interfering; OD, optical density.

NID1 overexpression reversed the decrease in cell proliferation compared with NC (Fig. 5C). Similarly, the Edu assay showed that transfection with Oe-NID1 notably increased the number of positive cells (Fig. 5D). Results obtained from the colony formation assays showed that the number of colonies was higher in NID1-overexpressing cells (Fig. 5E). Furthermore, cell migration and invasion were increased following transfection with Oe-NID1 (Fig. 5F-I). Finally, an increase in levels of N-cadherin and vimentin and decrease in E-cadherin levels were observed in the cells transfected with Oe-NID1 (Fig. 5J).

## Discussion

Oral cancer is a common malignancy that poses a notable threat to human health. In the past decade, the occurrence of oral cancer has increased markedly worldwide (17). OSCC, a common type of oral cancer, has been characterized by a high degree of malignancy and poor prognosis (18). It has been shown that OSCC has high a potential for metastasis (19). The invasion and metastasis of a tumor is a complex process that requires the involvement of numerous genes (20). Tumor cells detach from the primary lesion and invade the basement membrane to infiltrate the surrounding interstitium, enter the lumen of blood vessels through the local capillary blood or lymphatic vessel wall, forming small tumor emboli and are transported in blood or lymphatic fluid (21-23). When they are transferred to a target organ, these cells adhere to endothelial cells of the blood or lymphatic vessels and proliferate continuously at the secondary site to form a metastasis (24). In the present study, the molecular mechanism underlying OSCC migration and invasion was explored. It was demonstrated that COL4A1 may interact with NID1 to promote proliferation and migration of OSCC cells.

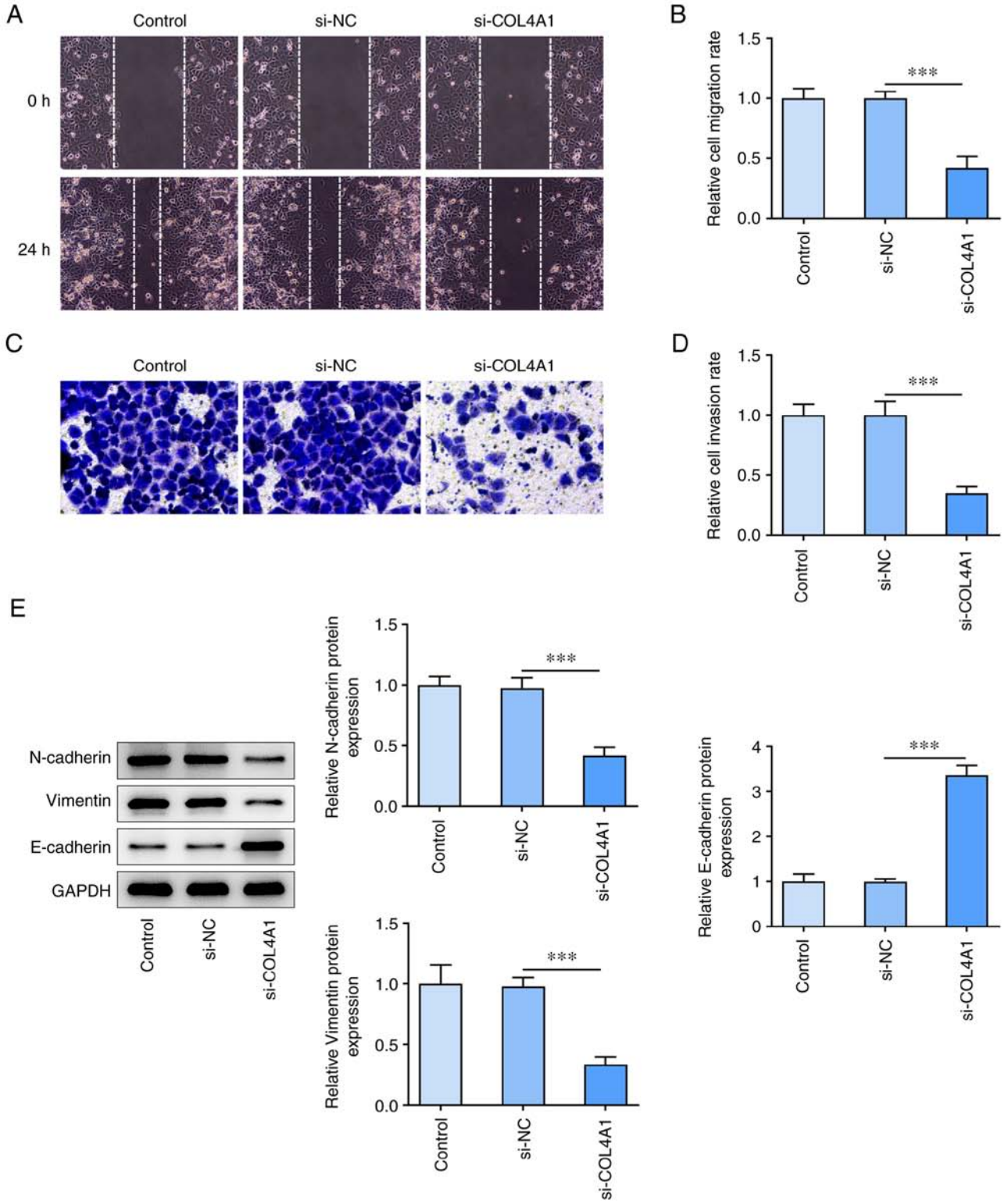


Figure 3. COL4A1 silencing inhibits migration, invasion and expression of epithelial-mesenchymal transition-associated protein of SCC-4 cells. (A) Representative wound healing assay of SCC-4 cells transfected with si-COL4A1 (magnification, x100). (B) Cell migration rate measured using wound healing assay. (C) Representative Transwell invasion assay of SCC-4 cells transfected with si-COL4A1 (magnification, x100). (D) Cell invasion rate measured via Transwell invasion assay. (E) Protein levels of N-cadherin, vimentin and E-cadherin in SCC-4 cells transfected with si-COL4A1 semi-quantified using western blot assay. Results are presented as the mean ± standard deviation. ***P<0.001. NC, negative control; COL4A1, collagen type IV α1 chain; si, small interfering.

Collagens are the most abundant protein in the extracellular matrix and are primarily involved in formation of basement membranes, fibrillar and microfibrillar networks, as well as other structures in the extracellular matrix (25). A previous study has shown that aberrantly expressed collagens affect the biological behavior of cancer cells (26). Biosynthesis of collagens can be modulated by cancer cells by targeting transcription factors, mutated genes and receptors, as well as signaling pathways.

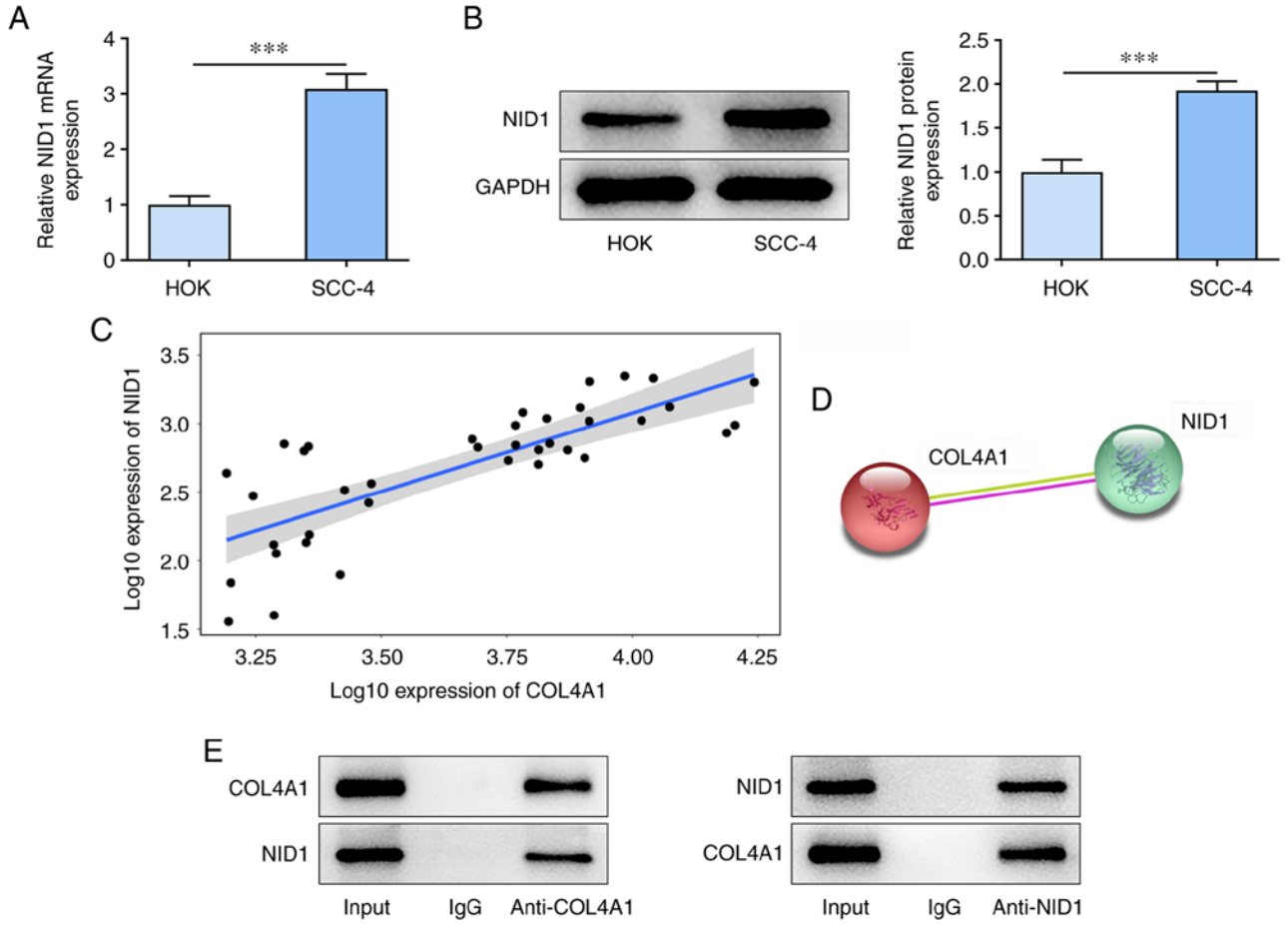


Figure 4. COL4A1 interacts with NID1 in oral squamous cell carcinoma cells. (A) mRNA expression of NID1 in SCC-4 cells measured via reverse transcription-quantitative PCR. (B) NID1 protein level in SCC-4 cells semi-quantified using western blot assay. (C) Association between COL4A1 and NID1, analyzed using the TNMplot database. (D) Binding of COL4A1 and NID1 analyzed using the STRING database. (E) Experimental verification of the binding between COL4A1 and NID1 using co-immunoprecipitation assay. Results are presented as the mean ± standard deviation. ***P<0.001. NID, nidogen-1; COL4A1, collagen type IV α1 chain.

Conversely, collagens bind to integrins, discoidin domain receptors and tyrosine kinase receptors to influence the behavior of tumor cells (27). COL4A1, which belongs to the collagen family, serves a tumor-promoting role in several types of cancer (28,29). Wang *et al* (30) showed that COL4A1 exerts a promoting effect on proliferation and metastasis in hepatocellular carcinoma via the activation of a FAK-Src signaling pathway. Jin *et al* (31) used functional enrichment analysis to screen genes associated with improving invasive ductal carcinoma treatment and COL4A1 was found to be a key gene that influenced proliferation and invasion of the invasive ductal carcinoma cells. Other studies have shown that COL4A1 is upregulated in head and neck squamous cell carcinoma and may serve as a novel prognostic biomarker for the recurrence of OSCC (32,33), indicating that COL4A1 expression is associated with OSCC. In the present study, COL4A1 was highly expressed in several OSCC cell lines and COL4A expression was highest in SCC-4 cells, which may be associated with the high invasiveness, as well as migration, proliferation and differentiation, of SCC-4 (34,35). In addition, COL4A1 knockdown suppressed the ability of SCC-4 cells to proliferate, migrate and invade. COL4A1 knockdown also suppressed the expression levels of the EMT-related proteins N-cadherin and Vimentin, and elevated E-cadherin expression in SCC-4 cells.

Nidogens are the primary components of the basement membrane and function as a linker connecting collagen IV and laminin networks to stabilize the three-dimensional structure of the basement membrane (36). NID1 and NID2 belong to the nidogen family (37). Studies have shown that NID1 is involved in promoting tumor development, such as gastric, breast cancer and colorectal cancer (38-40). Xu *et al* (41) found that microRNA-1298-3p suppresses the ability of glioma cells to proliferate and invade via downregulation of NID1. Yuan *et al* (42) reported that NID1 is upregulated in ovarian cancer cells and NID1 overexpression reverses the inhibitory effect of long non-coding RNA-ATB silencing on progression of ovarian cancer. Hsu *et al* (43) showed that NID1 expression is enhanced in OSCC tissues and that it could be used as a biomarker for OSCC. Based on TNMplot and STRING databases, the present analysis found that NID1 was highly positively associated with COL4A1 and bound to COL4A1. Thus, co-IP was used to verify the binding between NID1 and COL4A1. Moreover, the present study indicated that NID1 overexpression reversed the suppressive effects of COL4A1 knockdown on OSCC cell proliferation, migration and invasion, as well as on EMT-associated protein expression. There are several limitations in the present study. Only one OSCC cell line was used to explore the biological role of COL4A1;

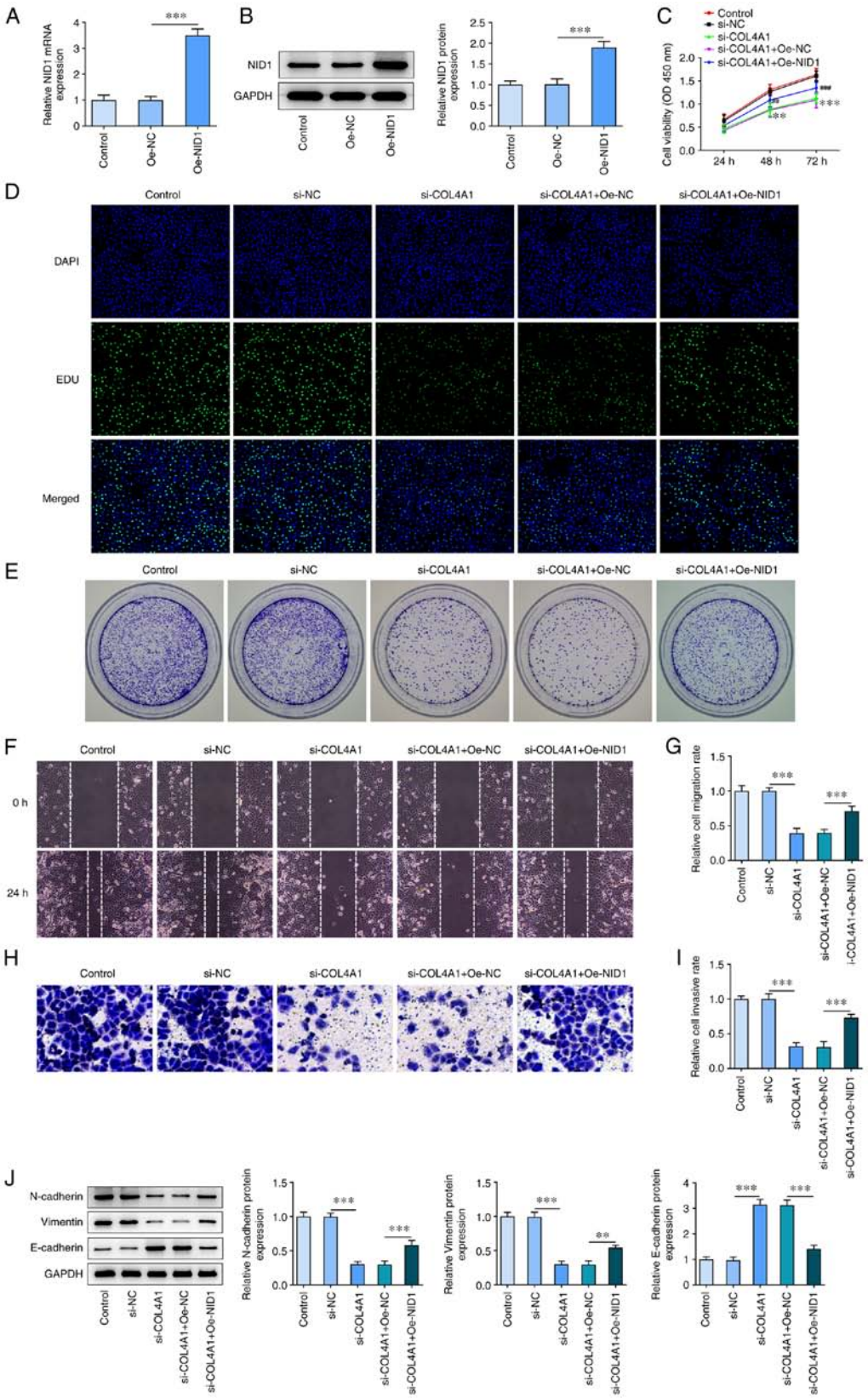


Figure 5. Upregulation of NID1 reverses the inhibitory effect of COL4A1 silencing on SCC-4 cells. (A) mRNA expression of NID1 in SCC-4 cells transfected with Oe-NID1 was measured via reverse transcription-quantitative PCR. ***P<0.001. (B) NID1 protein levels in SCC-4 cells transfected with Oe-NID1 measured using western blot assay. ***P<0.001. Cell proliferation in SCC-4 cells was evaluated using (C) Cell Counting Kit-8 (**P<0.01, ***P<0.001 vs. si-NC; ##P<0.01, ###P<0.001 vs. si-COL4A1+ Oe-NC) and (D) EdU incorporation (magnification, x100). (E) Colony formation assay (magnification, x1) was used to measure the colony-forming ability of SCC-4 cells. (F) Representative image of the wound healing assay of SCC-4 cells transfected with Oe-NID1 (magnification, x100). (G) Migration rate of SCC-4 cells transfected with Oe-NID1 measured using a wound healing assay. ***P<0.001. (H) Representative image of Transwell invasion assay of SCC-4 cells transfected with Oe-NID1 (magnification, x100). (I) Invasion rate of SCC-4 cells transfected with Oe-NID1 measured via Transwell invasion assay. ***P<0.001. (J) Protein levels of N-cadherin, vimentin and E-cadherin in SCC-4 cells transfected with Oe-NID1 semi-quantified using western blot assay. Results are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001. NID1, nidogen-1; Oe, overexpression; NC, negative control; COL4A1, collagen type IV α1 chain; si, small interfering; OD, optical density.

the effects of COL4A1 in other OSCC cell lines and other potential mechanisms are currently being investigated. In addition, the results of the present would be further supported by NID1 silencing experiments as no significant difference was demonstrated for cell proliferation in the Oe-NID1 group.

In conclusion, the molecular mechanisms by which COL4A1 exerts its effects in OSCC were determined in the present study. The results revealed that COL4A1 increased the proliferation and migration of OSCC cells by interacting with NID1, highlighting a potential novel avenue for therapeutic targeting in the management of OSCC.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

XT and KZ designed the research and drafted and revised the manuscript. XT, JS, CL and KZ performed the experiments. JS and CL searched the literature and analyzed the data. KZ guided the experiments. XT and KZ 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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