

Histone deacetylase 7 inhibits plakoglobin expression to promote lung cancer cell growth and metastasis

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Abstract. Plakoglobin is a tumor suppressor gene in lung cancer; however, the mechanism by which it is downregulated in lung cancer is largely unknown. The aim of the present study was to investigate whether histone deacetylases (HDACs) regulate plakoglobin expression in lung cancer. The effects of overexpression or knockdown of HDAC7 on plakoglobin were determined using stably transfected lung cancer cell lines. Chromatin immunoprecipitation assays were performed to elucidate the mechanisms underlying the HDAC7-induced suppression of plakoglobin. A Cell Counting Kit-8 and Transwell assays were performed, and a nude mouse *in vivo* model was established to investigate the role of the HDAC7/plakoglobin pathway in cell migration, invasion and metastasis. Ectopic expression of HDAC7 was identified to suppress mRNA and protein levels of plakoglobin in lung cancer cells, whereas silencing HDAC7 with short hairpin RNA increased the expression of plakoglobin. HDAC7 was proposed to suppress plakoglobin by directly binding to its promoter. Overexpression or knockdown of HDAC7 promoted or inhibited cell proliferation, migration and invasion, respectively. Furthermore, knockdown of HDAC7 significantly

suppressed tumor growth and metastasis *in vivo*. In addition, overexpression of plakoglobin significantly reduced the enhanced cell proliferation, migration and invasion induced by ectopic HDAC7. In conclusion, suppression of plakoglobin by HDAC7 promoted the proliferation, migration, invasion and metastasis in lung cancer. This novel axis of HDAC7/plakoglobin may be valuable in the development of novel therapeutic strategies for treating patients with lung cancer.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1,2), with an associated 5-year survival rate of <16% (3). Advancements in treatment have been made recently (4); however, the molecular mechanisms underlying the pathogenesis of lung cancer remain unknown. Therefore, there is an urgent requirement to identify the molecular mechanisms underlying lung cancer in order to develop novel treatment strategies and improve patient survival.

Plakoglobin is a member of the armadillo family of proteins, and is a structural and functional homolog of β -catenin (5-7). Notably, previous studies have reported that a decreased level of plakoglobin is associated with shorter disease-free survival (DFS) and worse overall survival (OS) in non-small cell lung cancer (NSCLC) (8-10). On the contrary, He *et al* (11) demonstrated that high cytoplasmic plakoglobin expression in tumor tissues is associated with poor DFS and OS in patients with resected lung adenocarcinoma. Unlike β -catenin, which has an oncogenic function, plakoglobin generally acts as a tumor/metastasis suppressor (12,13). Plakoglobin is weakly expressed in numerous NSCLC cell lines and overexpression of plakoglobin in these cell lines has been demonstrated to inhibit the proliferation of lung cancer cells (14). Overexpressing plakoglobin in SCC-9 squamous carcinoma cells was observed to induce a mesenchymal to epidermoid phenotypic transition (15). Decreased expression of plakoglobin is regulated by promoter hypermethylation in renal cell carcinoma (16), prostate cancer (17), embryonal

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rhabdomyosarcoma (18) and mammary carcinoma (19); however, the factors affecting low plakoglobin expression are largely unknown, and whether plakoglobin is involved in other signaling pathways in lung cancer remains unclear.

Aberrant expression of histone deacetylases (HDACs) is a hallmark of numerous types of cancer, and is involved in mediating the proliferation, migration, invasion and genotoxic chemotherapy resistance of cancer cells (20). HDACs are potential anticancer drug targets that may be used to treat patients with lung cancer (21,22). HDACs act as transcriptional repressors by interacting with corepressor complexes and regulating the acetylation state of histones, resulting in condensed chromatin (23). Plakoglobin regulates HDAC4 expression by binding to its promoter via interactions with the T-cell factor/lymphoid enhancer-binding factor family of transcription factors (24). Plakoglobin expression is reduced in tumor tissues compared with in normal tissues, and HDACs have been demonstrated to inhibit plakoglobin expression in cancers (25,26); however, which HDACs directly inhibit plakoglobin expression in lung cancer require further investigation.

In the present study, the role of HDACs in regulating plakoglobin expression in lung cancer was investigated. HDAC7 was demonstrated to directly bind to the promoter of plakoglobin to deacetylate H3K9 and H4K5 and inhibit plakoglobin expression in lung cancer. This novel HDAC7/plakoglobin axis may serve as a promising target for treating patients with lung cancer.

Materials and methods

Cells and reagents. A total of three human lung cancer cell lines (H460, H1299 and A549) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; cat. no. 11965-092) supplemented with 10% fetal bovine serum (FBS) (cat. no. 10270-106) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at 37°C in an incubator containing 5% CO₂. Dimethyl sulfoxide, Trichostatin A (TSA) and nicotinamide (NAM) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and 7.5 mM NAM or 0.5 nM TSA was applied to A549, H1299 or H460 cells for 24 h at 37°C in an incubator containing 5% CO₂.

Plasmids. The full-length cDNA of human HDAC7 and plakoglobin were cloned into the pSin-puro vector (Focus Bioscience Co., Ltd., Nanchang, China), and the restriction sites were *EcoRI* and *NheI*. The full-length cDNA of human HDAC (1-11) was constructed by inserting a Flag tag into the HDAC N-terminus in a pcDNA3.1(+) vector (cat. no. V790-20, Invitrogen; Thermo Fisher Scientific, Inc.) and the plasmids were transfected into H460 cells using Lipofectamine 2000 (no. 11668019; Thermo Fisher Scientific, Inc.). All recombinant plasmids were verified by DNA sequencing (Ruiboxingke Biotech Co., Ltd., Beijing, China).

Antibodies. Human anti-plakoglobin (cat. no. 75550), anti-GAPDH (cat. no. 5174), anti-HDAC7 (cat. no. 33418), anti-Flag (cat. no. 14793) and the Acetyl-Histone Antibody Sampler kits containing anti-H3K9, -H3K18, -H3K27 and -histone 3 (cat.

nos. 9927), and anti-H4K5, H4K12, histone 4 (cat. no. 8346) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-β-actin (cat. no. 66009-1-Ig) was procured from ProteinTech Group, Inc. (Chicago, IL, USA).

Stable lines. H1299 cells stably expressing scrambled (Scr) or HDAC7 short hairpin RNA (shRNA) were established using the Sigma-Aldrich shRNA system (Merck KGaA), according to the manufacturer's protocols. Scr shRNA (with no known targets in the human genome) with the following sequence: 5'-GGGCGAGGAGCTGTTCACCG-3', and the oligonucleotides for human HDAC7 shRNA#1 and #2 were 5'-GATCCG GGTGCAAGTAAATA-3' and 5'-CCACTATTCCTGGCT CTGCAA-3', respectively. A total of 3 μg pSin-puro delivering HDAC7 or plakoglobin, 3 μg pSin-puro-empty vector, 3 μg shRNA-NC, 3 μg shRNA-HDAC7#1 or #2 was co-transfected with 3 μg pMD2.G and 3 μg psPAX2 into 293 cells (ATCC) for 48 h. using Lipofectamine 2000. The recombinant viruses were subsequently collected and applied to H1299, A549 or H460 cells cultured with 8 μg/ml Polybrene for 24 h. The stable lines were selected with 1 μg/ml puromycin for 2 weeks.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as described (27). Briefly, total RNA obtained from cell lines was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. First-strand cDNA was synthesized at 42°C for 60 min using the Revert Aid™ First Strand cDNA Synthesis kit (cat. no. 6110A; Takara Bio, Inc., Otsu, Japan). Subsequently, the qPCR reaction was performed in a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR®-Green mix (Tiangen Biotech Co., Ltd., Beijing, China). Thermal cycling of the qPCR reaction was initiated with a denaturation step at 95°C for 15 min, and consisted of 40 cycles (denaturation at 95°C 15 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec). The amplified products were examined using the 2^{-ΔΔC_q} method (28), and each sample was calibrated to the expression levels of the housekeeping gene GAPDH. The primers used for amplifying HDAC7, plakoglobin and GAPDH were as follows: HDAC7, forward, 5'-GGCGGCCCTAGAAAGAACAG-3' and reverse, 5'-CTTGGGCTTATAGCGCAGCTT-3'; plakoglobin, forward, 5'-TCTCCAACCTGACATGCAACA-3' and reverse, 5'-CATAGTTGAGACGCACAGAGTTC-3'; and GAPDH, forward, 5'-ACAGTCAGCCGCATCTTCTT-3' and reverse, 5'-GACAAGCTTCCCCTTCTCAG-3'.

Cell proliferation assay. *In vitro* cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay. For cell proliferation, cells were seeded in 96-well plates at a density of 1,000 cells/well and incubated for 1, 2, 3, 4 or 5 days; 10 μl of CCK-8 reagent (Beyotime Institute of Biotechnology, Haimen, China) was then added to each well, followed by incubation for 1.5 h at 37°C. The absorbance value (optical density) of each well was measured at 450 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc.).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using the ChIP kit (cat. no. 53008, Active Motif,

Carlsbad, CA, USA) as described previously (29). Briefly, to fix the cells, Complete Cell Fixative Solution (included in kit) was added to the existing culture medium for the cells at 80% confluence at room temperature, and the fixation reaction was stopped by adding Stop Solution (included in kit) to the existing culture medium. The cells were collected by centrifugation at 1,000 x g for 5 min at 4°C. Subsequently, the nuclear pellet was resuspended in ChIP Buffer (included in kit). The cell lysate was subjected to shearing using a sonication instrument (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) to a fragment length of 200-500 bp. Total genomic DNA (input) was quantified and 20 µg of chromatin from each sample was immunoprecipitated overnight at 4°C with 5 µg anti-HDAC7 (cat. no. 33418; Cell Signaling Technology, Inc.), anti-acetyl-H3K9 (cat. no. 39917) or anti-acetyl-H4K5 (cat. no. 39699) (both from Active Motif), or normal IgG as a negative control. Then, nucleosome complexes were isolated with the protein G agarose beads for 3 h at 4°C. Bound DNA-protein complexes were eluted and cross-links were reversed after a series of washes using the washing reagent contain in the ChIP kit. Purified DNA was resuspended in TE buffer. Subsequently, the PCR reaction was performed using PrimeSTAR[®] Max DNA Polymerase (cat. no. R045A; Takara Bio, Inc.). Thermal cycling of the qPCR reaction was initiated with a denaturation step at 94°C for 2 min, and consisted of 35 cycles (denaturation at 98°C 10 sec, annealing at 60°C for 15 sec and elongation at 72°C for 30 sec). The primers for the plakoglobin were as follows: Plakoglobin-ChIP, forward, 5'-GGACAGTCAGGCGAGATAGC-3' and reverse, 5'-CGAACAAAAGGCCGAAGAGAC-3'

Transwell assays. For the Transwell migration assay, 4.0x10⁴ (A549) or 1.5x10⁵ (H1299) cells in 200 µl serum-free DMEM were added to cell culture inserts with an 8-µm microporous filter, without an extracellular matrix coating (BD Biosciences, Franklin Lakes, NJ, USA). DMEM medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10270-106) was then added to the bottom chamber. After 24 h of incubation at 37°C in an incubator containing 5% CO₂, the cells on the lower surface of the filter were fixed, stained and examined under a light microscope. The number of migrated cells in three random optical fields (magnification, x10) from triplicate filters was averaged. For the Transwell invasion assay, 8.0x10⁴ (A549) or 3.0x10⁵ (H1299) cells resuspended in 200 µl serum-free DMEM were added to cell culture inserts, which contained 8-µm microporous filters and were coated with Matrigel (BD Biosciences; cat. no. 354480). DMEM containing 10% FBS was then added to the bottom chamber. After 24 h of incubation at 37°C in an incubator containing 5% CO₂, the cells on the lower surface of the filter were fixed with 10% formalin for 15 min at room temperature, stained with 0.1% crystal violet for 60 min at room temperature and examined under a light microscope. The number of migrated cells in three random optical fields (magnification, x100) from triplicate filters was averaged.

Western blotting. Western blotting procedures were performed as described previously (3), including the experimental conditions of the gels. Briefly, cultured cells from all cell lines were lysed in ice-cold radioimmunoprecipitation assay lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology)

at 4°C for 30 min. Following centrifugation (12,000 x g) at 4°C for 20 min, the lysates were obtained and protein concentration was determined with the BCA method. Equal amounts of protein (30 µg/lane) were separated by 10% SDS-PAGE. The conditions were as follows: Voltage of 100 V for 2.5 h at room temperature. Proteins were then transferred to polyvinylidene fluoride membranes with an electrical current of 250 mA at 4°C for 2 h. To block the non-specific binding sites, the membranes were incubated with 5% non-fat milk (in Tris-buffered saline with 0.1% Tween-20) at room temperature for 60 min, and then membranes were then incubated with the following primary antibodies: Human anti-plakoglobin (cat. no. 75550, anti-GAPDH (cat. no. 5174), anti-HDAC7 (cat. no. 33418), anti-Flag (cat. no. 14793) and the Acetyl-Histone Antibody Sampler kit (cat. nos. 9927 and 8346) (all from Cell signaling Technology, Inc.), anti-β-actin (cat. no. 66009-1-Ig; ProteinTech Group, Inc.) at a dilution of 1:1,000 overnight at 4°C. Subsequently, the membranes were incubated with anti-rabbit IgG Secondary antibody peroxidase-conjugated (cat. no. W401B; Promega Corporation, Madison, WI, USA) and anti-Mouse IgG Secondary Antibody Peroxidase Conjugated (cat. no. W402B; Promega Corporation) with the dilution 1:10,000 at room temperature for 2 h. Specific protein bands were visualized using an enhanced chemiluminescence detection system (cat. no. P0018F; Beyotime Institute of Biotechnology) and exposed to radiographic film (Carestream Health, Rochester, NY, USA; cat. no. 6535876).

Animal experiments. All animal studies were performed in accordance with protocols approved by the Research Animal Resource Center of Sun Yat-Sen University (Guangzhou, China). The mice were maintained in specific pathogen free conditions at a temperature of 20-25°C, a 50-70% humidity, under a light/dark cycle of 12 h, with free access to water and food. A total of 18 male athymic nude mice at 4 weeks of age were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). For subcutaneous injection, 2x10⁶ H1299 cells with stably expressing Scr or shHDAC7-1 were mixed with 0.2 ml PBS (pH 7.4) and 30% (v/v) Matrigel matrix (BD Biosciences). Suspensions were injected subcutaneously into the flanks of 4-week-old male athymic nude mice, which were monitored over 5 weeks. For tail vein injection, 3x10⁶ H1299 cells with stably expressing Scr or shHDAC7-1 were resuspended in 300 µl PBS (Biological Industries, Beit Haemek, Israel) and injected into the lateral tail vein of mice that did not receive a subcutaneous injection of cells (3x10⁶ cells/animal). Mice were sacrificed 7 weeks following injection, and nodules were counted and compared between the H1299-scr and H1299-shHDAC7-1 groups.

Immunohistochemistry (IHC) and histological evaluation. Samples were fixed in 10% formalin for 10 h at room temperature and embedded in paraffin. Sections (3-µm thick) were prepared and mounted onto positively-charged glass slides. Sections for HDAC7 staining were incubated in 10 mM citrate buffer (pH 6.0) and boiled in a microwave oven for 15 min. After incubation with Protein Block Serum-Free (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), sections were incubated overnight with anti-HDAC7 (1:250)

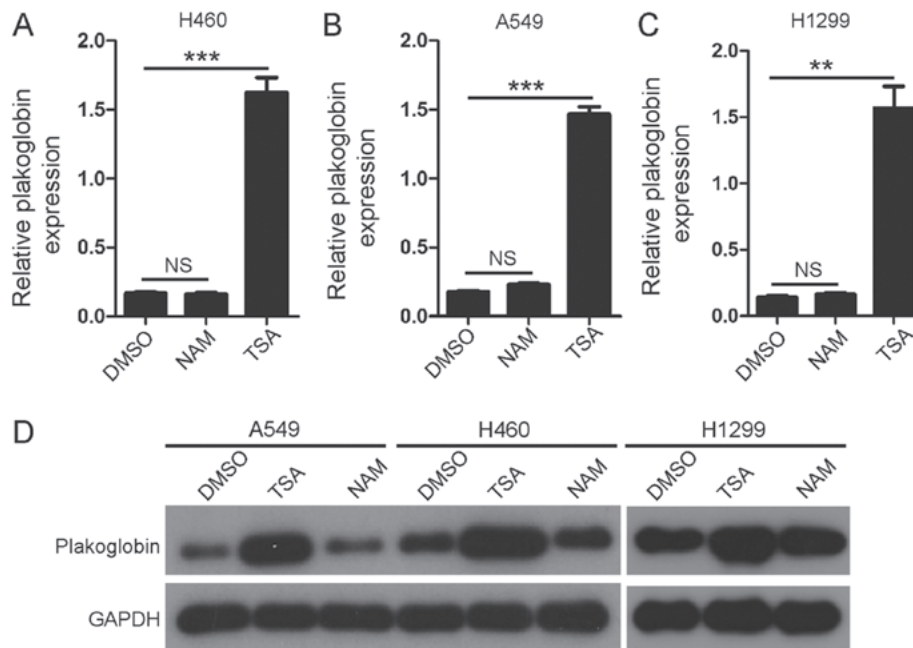


Figure 1. Plakoglobin expression is upregulated following treatment with histone deacetylase inhibitor TSA. (A-C) mRNA expression levels of plakoglobin were determined via reverse transcription-quantitative polymerase chain reaction in H460, A549 and H1299 cells treated with TSA or NAM for 24 h. GAPDH was used as an internal control. Data are presented as the mean \pm standard error (n=3). **P<0.01, ***P<0.001. (D) Protein expression levels of plakoglobin were determined via western blot analysis in H460, A549 and H1299 cells treated with TSA or NAM for 24 h. GAPDH was used as an internal control. DMSO, dimethyl sulfoxide; NAM, nicotinamide; TSA, trichostatin A.

at 4°C in a humidified container. Following washing with PBS three times, the tissue slides were treated with a non-biotin horseradish peroxidase detection system according to the manufacturer's protocols (Dako; Agilent Technologies, Inc.). IHC staining was evaluated by two independent pathologists who were experts in diagnosing lung cancer. HDAC7 detection was quantified as described (3). Briefly, the HDAC7 signal was detected in the cytoplasm and the nucleus, and the intensity in tissues was categorized into four categories: 0, absent; 1, weak; 2, moderate; or 3, strong. The percentage of stained cells was categorized as: 0, no staining; 1, 1-10%; 2, 11-50%; 3, 51-80%; and 4, 81-100%. The staining score for each tissue was calculated by multiplying the intensity value by the percentage value, and the average of the scores from the two pathologists was used as the final score.

Clinical dataset analysis. The association between HDAC7, and the clinical characteristics or survival of lung cancer patients were analyzed using the online KMplot database (30). The median value of expression was selected as the cutoff in the online database; Cox's proportional hazards model was used to estimate the hazard ratio.

Study approval. A total of 35 pairs of specimens were collected from patients with lung cancer (median age, 45 years; age range, 33-78 years; male/female ratio, 3:4) with resection between March 2014 and September 2016, and inclusion criteria were as follow: Initial diagnosis of lung cancer. Exclusion criteria included: Previous radiotherapy, chemotherapy or surgery, and previous malignancy or other concomitant malignant disease. Normal lung tissues were collected at >5 cm from the edge of the tumor. The use of human lung cancer tissues was reviewed and approved by the Ethics Committee of the Sun Yat-Sen

University Cancer Center. Informed consent was obtained from patients.

The correlation between plakoglobin and HDAC7 expression. The raw data for the correlation between plakoglobin and HDAC7 expression were obtained from the MethHC 1.0.3. software (<http://methhc.mbc.nctu.edu.tw/php/index.php>).

Statistical analysis. All statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS, Inc., Chicago, IL, USA). All values from the *in vitro* assays were expressed as the mean \pm standard deviation or standard error of the mean of at least three independent experiments or replicates. A paired Student's t-test was used for the comparison of two groups, multiple group comparisons were performed by using one-way analysis of variance followed by a Tukey-Kramer post-hoc test. The correlation between plakoglobin and HDAC7 expression was analyzed by a Pearson's correlation analysis. The Mann-Whitney test was used to analyze the difference expression of HDAC7 in lung cancer tissues and paired normal tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

Treating lung cancer cells with TSA increases the mRNA and protein expression of plakoglobin. H460, A549 and H1299 cells were treated with HDAC inhibitors TSA and NAM for 24 h; these reagents are inhibitors of class I and II HDACs and class III HDACs (31,32), respectively. It was identified that the addition of TSA, but not NAM, significantly increased the mRNA expression levels of plakoglobin compared with the control (Fig. 1A-C); notable increases in the protein expression

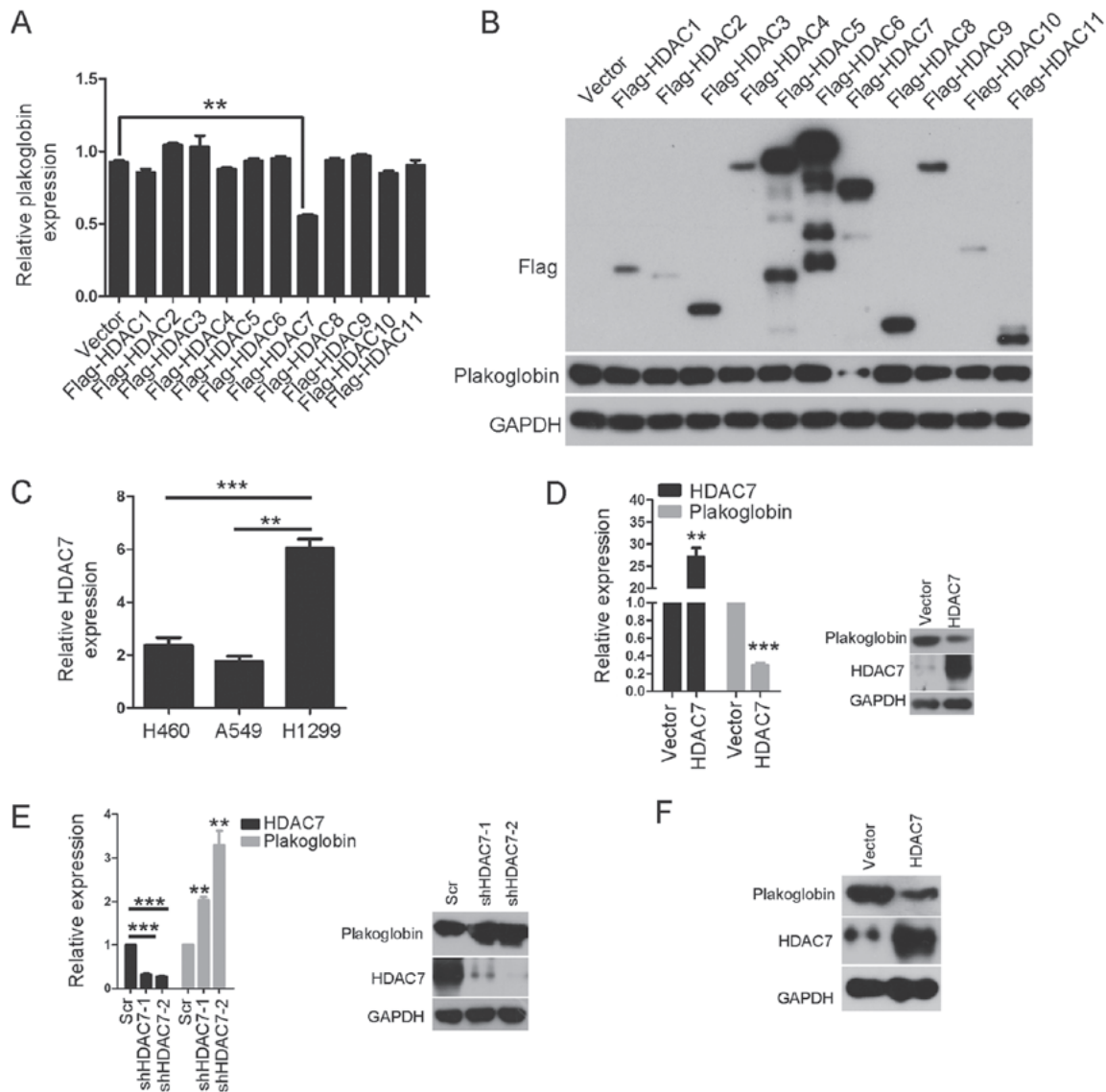


Figure 2. HDAC7 inhibits plakoglobin expression. (A and B) H460 cells were transfected with empty vector or Flag-tagged HDACs, as indicated, for 48 h. (A) mRNA expression levels of plakoglobin were evaluated via RT-qPCR. Data are presented as the mean \pm standard deviation. ** $P < 0.01$. (B) Protein expression levels of plakoglobin and the HDACs were determined via western blotting. (C) mRNA levels of HDAC7 were detected by RT-qPCR. Data are presented as the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$. (D) Overexpression of HDAC7 in A549 suppressed the mRNA and protein levels of plakoglobin. ** $P < 0.01$, *** $P < 0.001$ vs. vector. (E) Silencing HDAC7 in H1299 increased the mRNA and protein levels of plakoglobin. Data are presented as the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$ vs. Scr. (F) Overexpression of HDAC7 in H1299 suppressed the protein levels of plakoglobin. HDAC, histone deacetylases; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Scr, scrambled.

levels of plakoglobin were observed following TSA treatment. The results indicated that plakoglobin may be regulated by class I and II HDACs in lung cancer cells.

HDAC7 is a novel negative regulator of plakoglobin in lung cancer. In order to determine which class I and II HDAC was responsible for the suppression of plakoglobin expression, class I and II HDACs were ectopically expressed in lung cancer cells. As presented in Fig. 2A and B, only ectopic HDAC7 significantly suppressed the mRNA levels of plakoglobin in H460 cells; the protein expression levels of HDAC7 were notably downregulated. Furthermore, the expression levels of HDAC in three cancer cell lines was determined (Fig. 2C). It was evaluated whether overexpression of HDAC7 could decrease plakoglobin expression in other lung cancer cells according to the endogenous levels of HDAC7 in the H460, H1299 and

A549 cell lines. RT-qPCR indicated that stable expression of HDAC7 significantly suppressed the mRNA expression levels of plakoglobin in A549 cells compared with the control; the protein expression levels of plakoglobin following HDAC7 overexpression were notably reduced (Fig. 2D). Thus, plakoglobin expression was downregulated by overexpression of HDAC7. The opposite effect was observed with HDAC7 knockdown in H1299 cells. As indicated in Fig. 2E, the two shRNA sequences effectively decreased the expression of endogenous HDAC7 levels compared with the control, whereas the mRNA and protein expression levels of plakoglobin increased. Furthermore, the protein expression levels of plakoglobin decreased following stable overexpression of HDAC7 in H1299 cells (Fig. 2F).

HDAC7 directly binds to the promoter region of plakoglobin and deacetylates histones H3 and H4 in lung cancer. To investigate

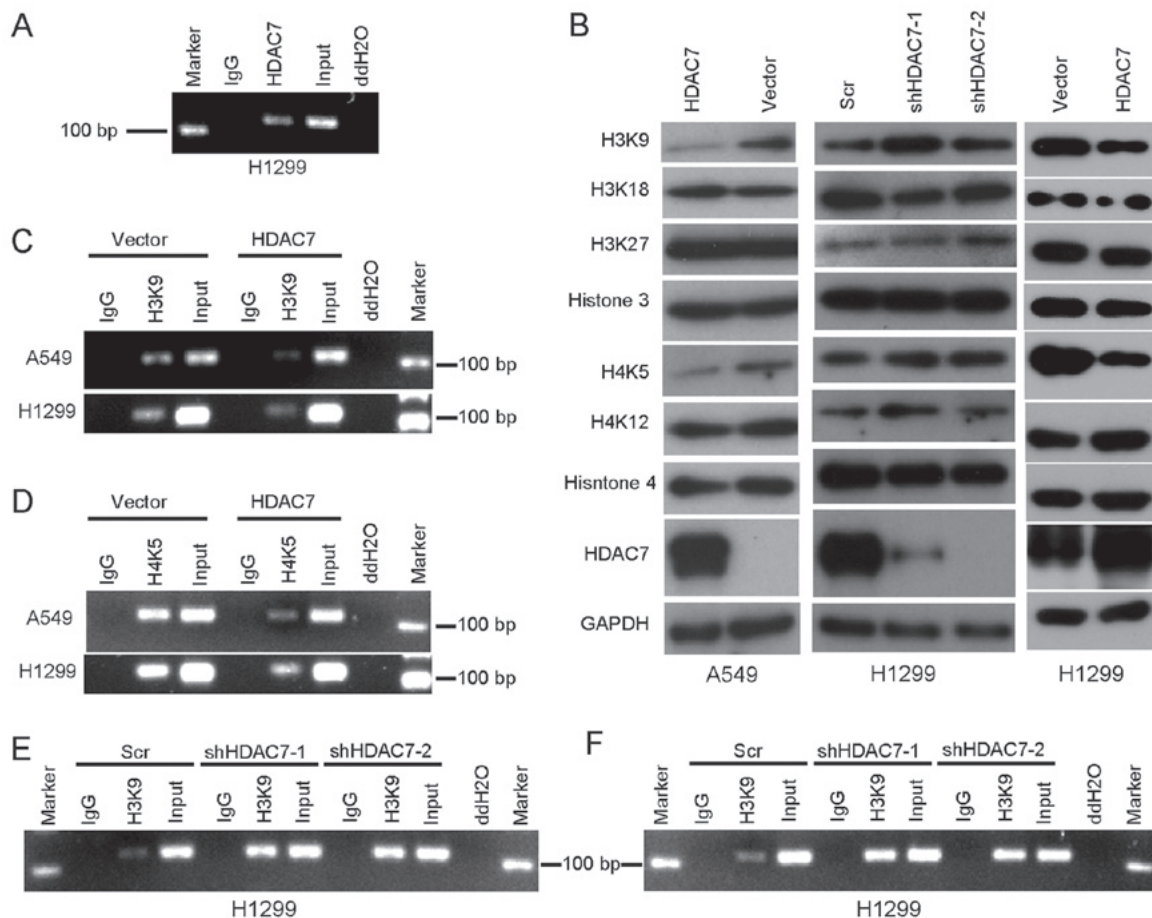


Figure 3. HDAC7 directly binds to the promoter region of plakoglobin and deacetylates histone H3 and histone H4 in lung cancer. (A) ChIP assays in H1299 cells were performed using anti-HDAC7 or negative control IgG. (B) The levels of acetylation at the corresponding residues of histone H3 and H4 were decreased or increased following overexpressing or knockdown, respectively. (C and D) ChIP analysis of the occupancies of H3K9 and H4K5 on the plakoglobin promoter in stably transfected vector or HDAC7 cells. The levels of H3K9 and H4K5 on the plakoglobin promoter decreased following overexpressing HDAC7 in A549 and H1299 cells. (E and F) ChIP analysis of H3K9 and H4K5 on the plakoglobin promoter in H1299-Scr, H1299-shHDAC 7-1 and H1299-shHDAC7-2 groups. The levels of H3K9 and H4K5 on the plakoglobin promoter increased after knocking down HDAC7 in H1299 cells. HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; Scr, scrambled; sh, short hairpin RNA.

the mechanisms by which HDAC7 participates in silencing plakoglobin expression, a ChIP assay was performed to analyze whether HDAC7 directly binds to the plakoglobin promoter. The results indicated that HDAC7 binds to the plakoglobin promoter in H1299 cells (Fig. 3A). Acetylation of histones 3 and 4 was assessed by western blot analysis using antibodies targeting lysine residues that are known to be acetylated. It was identified that the levels of acetylation at H3K9 and H4K5 markedly increased following knockdown of HDAC7 with shDAC7 #2 in H1299 cells compared with the control (Fig. 3B); however, overexpression of HDAC7 decreased the levels of the H3K9 and H4K5 acetylation in A549 and H1299 cells (Fig. 3B). To investigate whether H3K9 and H4K5 acetylation was decreased in the promoter of plakoglobin, a ChIP assay was performed to analyze H3K9 and H4K5 acetylation within the plakoglobin promoter regions. The results indicated that the degree of histone acetylation at this promoter was decreased in HDAC7-overexpressing A549 and H1299 cells (Fig. 3C and D). By contrast, the level of histone acetylation at this promoter increased in HDAC7-knocked down H1299 cells (Fig. 3E and F). Acetylation of H3K9 and H4K5 contributes to chromatin decondensation (33), and the enrichment of acetylated H3K9 and H4K5 near a transcription initiation site is associated with the transcriptional activity of the

gene (33,34). Thus, HDAC7 may directly inhibit the transcription of plakoglobin via binding to the promoter region of plakoglobin and deacetylating histones H3K9 and H4K5 in lung cancer.

HDAC7 expression is increased in lung cancer samples and higher HDAC7 levels predict a poor outcome. As plakoglobin is downregulated in lung cancer tissues (9), the expression of HDAC7 in lung cancer tissues was evaluated using IHC. The results indicated that HDAC7 expression significantly increased in lung cancer tissues compared with paired normal tissues (Fig. 4A); representative images were presented (Fig. 4B). Using an online database (35), an inverse correlation was identified between HDAC7 and plakoglobin in tissues (Fig. 4C). In addition, whether the mRNA expression levels of HDAC7 is clinically relevant in lung cancer was determined in the present study. Based on the HDAC7 mRNA levels, lung cancer samples were subdivided into two groups, and the associated OS was analyzed. Individuals with high HDAC7 levels were observed to exhibit shorter OS compared with those with low levels (Fig. 4D) using a large public clinical microarray database of lung tumors from 2,170 patients (30). Therefore, HDAC7 expression levels may be a clinical predictor in lung cancer.

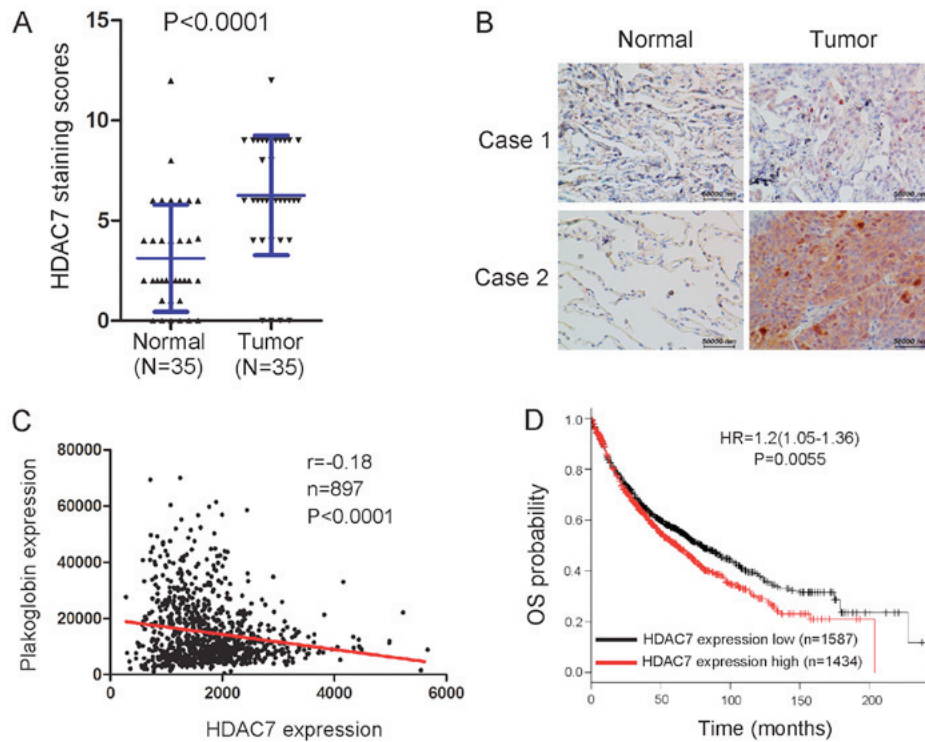


Figure 4. HDAC7 expression is increased in lung cancer samples and higher HDAC7 levels predict a poor outcome. (A and B) Immunohistochemical analysis lung cancer tissues (n=35) and paired normal tissues (n=35). Panel B presents representative images, and panel A comprises the statistical results based on a Mann-Whitney test. The dots represent the scores, and the bars indicate the standard deviation. Scale bar, 50 μ m. (C) A negative correlation between HDAC7 expression and plakoglobin expression was observed in the lung cancer tissues. r is a measure of the linear correlation between two variables HDAC7 expression and plakoglobin expression; $r=-0.18$ stands for a negative correlation between HDAC7 expression and plakoglobin expression in lung cancer tissues. The correlation between plakoglobin and HDAC7 expression was analyzed by a Pearson's correlation analysis. The raw data were obtained from the MethHC database. (D) Lung cancer patients with higher HDAC7 expression exhibited significantly shorter OS compared with those with low expression ($P=0.0055$). Cox's proportional hazards model was used to estimate HR. $HR>1$ indicates that the patients with high HDAC7 expression exhibited a poor prognosis. HDAC, histone deacetylase; HR, hazard ratio; OS, overall survival.

HDAC7 promotes lung cancer growth and metastasis. Subsequently, it was investigated whether HDAC7 regulates the biological behaviors of lung cancer cells. Stable cell lines with either ectopic expression of HDAC7 in A549 and H1299 cells, or silenced HDAC7 in H1299 cells, were constructed (Fig. 2C-E). Cell proliferation assays indicated that the ectopic expression of HDAC7 significantly promoted cell proliferation of A549 cells compared with the control (Fig. 5A). The migration and invasion of lung cancer cells was analyzed. The ectopic expression of HDAC7 significantly enhanced cell migration and invasion abilities of A549 cells compared with the control (Fig. 5B and C). The proliferative ability was notably increased following HDAC7 overexpression in H1299 cells (Fig. 5D); however, significant increases in migration and invasion were observed compared with the control (Fig. 5E and F). On the contrary, knockdown of HDAC7 markedly reduced cell proliferation (Fig. 5G), and significantly inhibited H1299 cell migration and invasion compared with the control (Fig. 5H and I). In summary, these findings indicate that HDAC7 promotes lung cancer cell proliferation, migration and invasion.

As HDAC7 was observed to promote lung cancer cell proliferation, migration and invasion *in vitro*, whether HDAC7 promotes lung cancer cell tumorigenesis and metastasis was investigated *in vivo*. To confirm the promoting effects of HDAC7, an *in vivo* tumorigenesis study was performed by inoculating shHDAC7 H1299 cells into nude mice. Mice

in the shHDAC7#1-H1299 and Scr groups were sacrificed 5 weeks after inoculation, with average tumor weights of 0.04 and 1.313 g, respectively ($P<0.01$; Fig. 5J-L). These results suggested a significant inhibitory effect of decreased HDAC7 on *in vivo* tumorigenesis.

To further evaluate the role of HDAC7 in lung cancer cell migration and invasion *in vivo*, lung cancer cells with or without HDAC7 knockdown were injected into nude mice via the tail vein. Cells with endogenous HDAC7 formed colonies in the lungs within 7 weeks of injection, whereas the HDAC7 knockdown cells resulted in significantly reduced pulmonary metastatic colonization ability (Fig. 5M and N). These results suggest that HDAC7 promotes lung tumor growth and metastasis *in vivo*.

Suppression of plakoglobin by HDAC7 promotes cell proliferation, migration and invasion in lung cancer. As the aforementioned experiments demonstrated that plakoglobin was a direct target of HDAC7, whether loss of plakoglobin contributes to the oncogenic role of HDAC7 in lung cancer was determined. Plakoglobin was stably induced into HDAC7-overexpressing lung cancer cells to observe whether restoration of plakoglobin expression could rescue the promoted cell proliferation, migration and invasion of HDAC7-overexpressing cells *in vitro* (Fig. 6A). Notably, overexpression of plakoglobin significantly reduced the effects of HDAC7 overexpression on cell proliferation, migration and

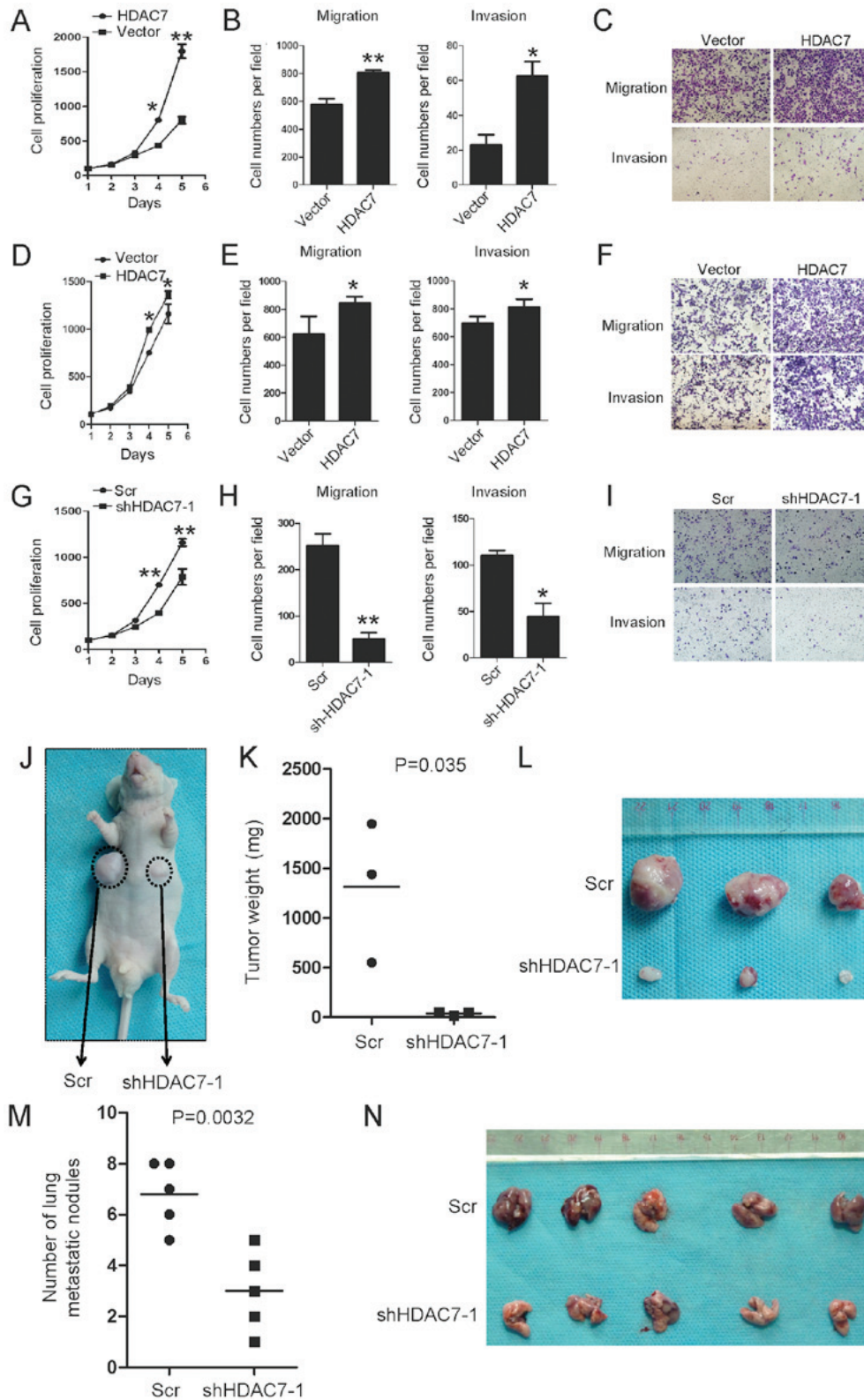


Figure 5. HDAC7 promotes tumor growth and metastasis. (A and D) Cell proliferation of A549 and H1299 cells transfected with HDAC7-overexpressing or empty vector *in vitro* was measured at different time-points, as indicated by a CCK-8 assay. Data are presented as the mean \pm standard error. * $P < 0.05$, ** $P < 0.01$ vs. Vector. The migration and invasion abilities of (B and C) A549 and (E and F) H1299 cells transfected with HDAC7-overexpressing or empty vector were measured at 24 h by Transwell assays. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. Vector. The number of cells passing via the membrane in each cell was statistically analyzed in triplicate and repeated three times with similar results. Magnification, $\times 100$. (C) and (F) are representative images. (G) Cell proliferation of H1299 cells expressing sh-HDAC7-1 or Scr *in vitro* was measured at different time-points, as indicated by a CCK-8 assay. Data are presented as the mean \pm standard error. ** $P < 0.01$ vs. Scr. (H and I) Cell migration and invasive abilities of H1299 cells expressing sh-HDAC7-1 and Scr were measured at 24 h by Transwell assays. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. Scr. The number of cells passing via the membrane in each cell was statistically analyzed in triplicate and repeated three times with similar results. (I) Is a representative image. (J-L) Stable H1299 with HDAC7 knockdown cells were injected into nude mice, which were randomly divided into two groups. Mice were sacrificed at 5 weeks following inoculation. Tumors were excised from the mice and weighed. (M and N) Stable H1299 cells with HDAC7 knockdown were injected via the tail vein, and the number of visible lung metastases was counted 7 weeks later ($P = 0.0032$). HDAC, histone deacetylase; CCK-8, Cell Counting Kit-8; Scr, scramble; sh, short hairpin RNA.

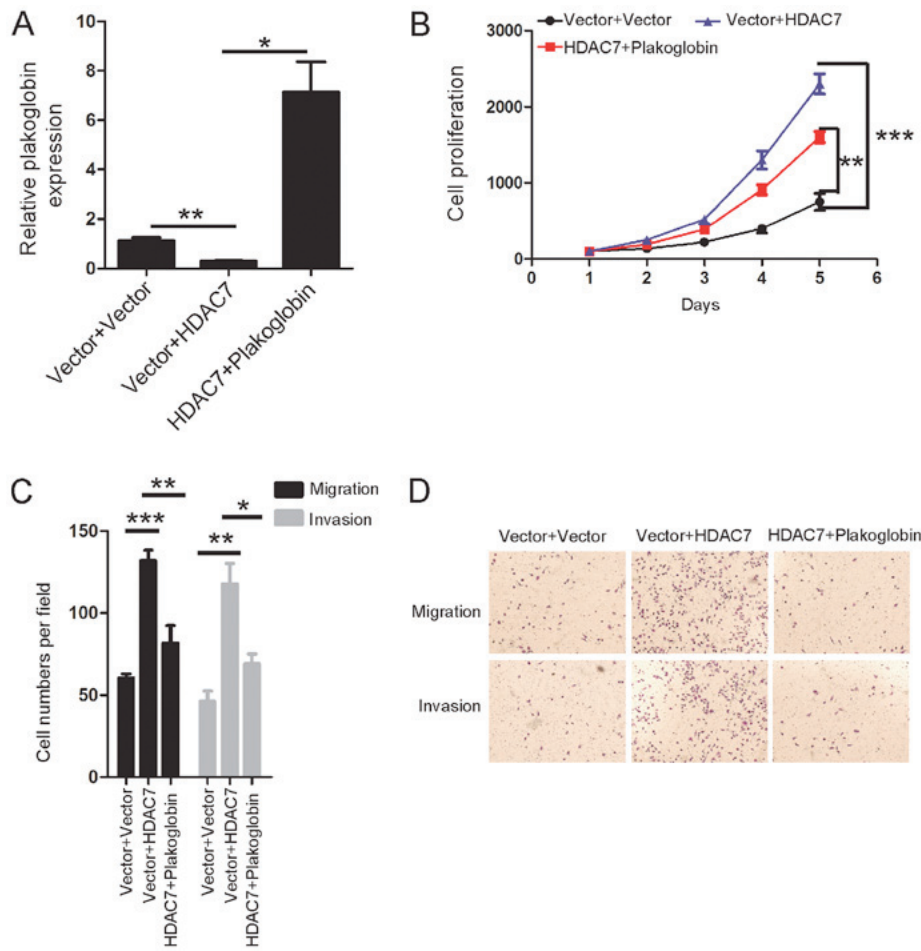


Figure 6. Suppression of plakoglobin by HDAC7 promotes cell proliferation, migration and invasion in A549 cells. (A) mRNA levels of plakoglobin were detected in the indicated cells by reverse transcription-quantitative polymerase chain reaction, with GAPDH as the control. *P<0.05, **P<0.01. (B) Cell proliferation of the indicated cells *in vitro* was measured at different time-points, as indicated by a Cell Counting Kit-8 assay. **P<0.01, ***P<0.001. (C and D) Migration and invasive abilities of the indicated cells were measured at 24 h by Transwell assays. Panel (D) indicates a representative image. Data are presented as the mean \pm standard deviation. The number of cells passing via the membrane in each cell was statistically analyzed in triplicate and repeated three times with similar results. *P<0.05, **P<0.01 and ***P<0.001. HDAC, histone deacetylase.

invasion compared with the control (Fig. 6B-D). These results indicated that suppression of plakoglobin by HDAC7 promoted cell proliferation, migration and invasion in lung cancer.

Discussion

Plakoglobin is a structural and functional homolog of β -catenin (5); these proteins serve two major roles in the cell, including the mediation of cell-cell adhesion and cell signaling (5). As adhesive proteins, plakoglobin and β -catenin can tether cadherin proteins to the cytoskeleton by interacting with the cytoplasmic domain of cadherins (5). Unlike β -catenin, plakoglobin has been demonstrated to be absent or weakly expressed in NSCLC cells and tumor tissues (5). Hypermethylation of the plakoglobin promoter mediates repression of its transcription in lung cancer (18); however, the mechanisms that contribute to the inactivation of plakoglobin expression in lung cancer cells have not been fully investigated. The present study aimed to determine which HDACs directly regulate plakoglobin expression in lung cancer cells. The results indicated that HDAC7 could serve as a novel negative regulator of plakoglobin expression in lung cancer.

HDACs (1, 2, 5, 7 and 10) are overexpressed in lung cancer and promote lung cancer progression (36-40). HDAC7 promotes the proliferation of cancer cells, including HeLa, HCT116 and MCF-7 cells, which is likely to occur via stimulating c-Myc and inhibiting p21 and p27 expression (41-44); however, HDAC7 depletion had no effect on the proliferation of glioblastoma U87 cells *in vitro* (45). Therefore, its role in cell proliferation may vary in different cancer cell types and act via different mechanisms. Lei *et al* (41) reported that HDAC7 has positive effects on lung tumorigenesis in mice and cell proliferation/soft agar colony formation of human lung cancer cell lines by inhibiting signal transducer and activator 3 activation via its deacetylation. The present study also indicated that HDAC7 promoted lung cancer cell proliferation, migration and invasion *in vitro*, and promoted H1299 cell tumor growth and metastasis *in vivo*. Mechanistically, it was demonstrated that HDAC7 can directly suppress plakoglobin expression in lung cancer by promoting the deacetylation of H3 and H4 histones at the promoter region; a negative correlation between HDAC7 and plakoglobin in lung cancer tissues was reported in the present study. These findings indicate a novel mechanism by which plakoglobin expression is lost in lung cancer cells.

The results also suggested that HDAC7 promotes lung cancer cell proliferation, migration and invasion, at least partly via inhibiting plakoglobin expression, as demonstrated by rescue experiments. In addition, plakoglobin was identified as a target gene of HDAC in human fibrosarcoma HT1080 cells (26), but the subtype of HDAC was unclear. Therefore, further investigation was conducted in the present study. The novel axis of HDAC7/plakoglobin may provide a basis for the development of novel therapeutic strategies for human lung cancer in the future.

To the best of our knowledge, this study is the first to demonstrate plakoglobin is a target of HDAC7. It was also demonstrated that plakoglobin inhibits HDAC7-mediated lung tumor growth and metastasis, and this axis may be applied in the development of novel therapeutic strategies for treating patients with lung cancer.

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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

YS, YL and SWL made substantial contributions to the conception and design of the present the study. YS LS, YW and WY developed the methodology, and acquired, analyzed and interpreted the data. YL and SWL wrote and reviewed the manuscript. SWL supervised the study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Animal Resource Center and the Ethical Committee of Sun Yat-Sen University. Informed consent was obtained from patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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