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Inhibitors of differentiation-1 promotes nitrosopyrrolidine-induced transformation of HPV 16-immortalized cervical epithelial cell

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Key words

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Our previous study implied a correlation between inhibitors of differentiation-1 (Id-1) and cervical cancer development. However, how Id-1 contributes to cervical carcinogenesis is unknown. In the present study, we used an *in vitro* transformation model to investigate the role of Id-1 in the transformation of cervical cells. Human papillomavirus (HPV)-immortalized cervical epithelial cells (H8) were successfully transformed by exposure to the carcinogen N-nitrosopyrrolidine (NPYR). The expression of both Id-1 RNA and protein was significantly increased in transformed H8 cells, suggesting a possible role of Id-1 in cervical cell transformation. Ectopic expression of Id-1 in H8 cells potentiated NPYR-induced cell transformation. In contrast, silencing of Id-1 suppressed NPYR-induced H8 cell transformation. In addition, the expression of HPV E6 and E7 oncoproteins was upregulated while that of the tumor suppressors p53 and pRb was suppressed after H8 cell transformation. Our results suggest that Id-1 plays an oncogenic role in HPV-related cervical carcinogenesis, which sheds light on cervical cancer development mechanisms and implies that Id-1 is a potential target for cervical cancer prevention and therapy.

Cervical cancer is the third most common neoplastic disease afflicting women worldwide, with an estimation of 530 000 new cases and 275 000 deaths every year.[1] Although the incidence and mortality in developed countries have declined due to cervical cytologic screening campaigns, the fact that more than 85% of the global cervical cancer patients reside in undeveloped countries should not be ignored.[2] It is now clear that human papillomavirus (HPV) is the most important risk factor for cervical cancer development.[3] More than 100 different HPV genotypes are detected in the female reproductive tract, among which at least 15 genotypes have high oncogenic potential.[4] The most oncogenic HPV, type 16, is responsible for over 70% of cervical carcinomas and up to 90% of HPV-correlated extra-cervical carcinomas.[5–7] By integration into host genomic DNA, the HPV encodes E6 and E7 proteins which promote cancer development by interacting with certain oncoproteins or tumor suppressors such as *p53* and *RB*.[8] However, the detailed mechanism for HPV-induced cervical carcinogenesis is not yet fully understood because the majority of HPV infections vanish before cancer development, partly due to immunological responses, while only 1% of HPV infections progress into carcinoma.[9,10]

Inhibitors of DNA binding proteins, also known as inhibitors of differentiation (Id) proteins, are helix-loop-helix (HLH) proteins and dominant inhibitors of the basic HLH transcription factors.[11] The Id protein family consists of four components: Id-1, Id-2, Id-3 and Id-4, of which Id-1 is most intensively studied. Id-1 has multiple functions, such as regulating cell differentiation, inducing cell immortalization, inhibiting cell apoptosis and promoting tumor angiogenesis.[11] Recent studies have demonstrated increased expression of Id-1 in several types of human tumors including gastric carcinoma, breast cancer, prostate carcinoma and ovarian cancer.[12–15] Our previous studies have shown that Id-1 expression was increased in cervical carcinoma tissues, which was correlated to HPV infection, indicating a possible role of Id-1 in cervical cancer development.[16,17] However, the detailed mechanism for HPV-induced cervical carcinogenesis and how Id-1 is involved in the whole carcinogenic process still remain unclear. It is critical to understand the molecular mechanisms involving Id-1 in the initiation and progression of cervical cancer, which will help to develop effective approaches for prevention and therapy of this malignancy.

The N-nitrosopyrrolidine (NPYR), a product of vaginal anaerobic metabolism and a possible co-factor for cervical carcinogenesis, is known to be a strong carcinogen.[18–20] In this study, we established an *in vitro* cell transformation model with NPYR as a carcinogen to induce malignant transforma-

tion of HPV-16 immortalized human cervical epithelial H8 cell. With this model, we demonstrated a promoting role of Id-1 in the transformation of cervical epithelial cells and investigated possible mechanisms by which Id-1 contributes to HPV-related cervical cancer development.

Materials and Methods

Cell cultures. The HPV16 immortalized but not transformed human cervical epithelial cell line H8 originated from the Institute of Virology of Chinese Academy of Medical Sciences and was a generous gift from Professor Yong Zhao from the Department of Pathology of Chongqing Medical University. H8 cells cannot form tumors in nude mice or colonies in soft agar.[21] The human cervical cancer-derived cell line used in the present study, SiHa, was from the American Type Culture Collection. Both H8 and SiHa cells were cultured in (DMEM) (Gibco/BRL, Grand Island, NY, USA) supplemented with 100 μg/mL of streptomycin, 100 U/mL of penicillin and 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA). The cultures were maintained in a humidified 5% CO_2 incubator at 37°C. The medium was changed three times a week.

Cell transformation. N-nitrosopyrrolidine (Sigma Chemical, St. Louis, MO, USA) was used to induce malignant transformation. To determine the dosage of NPYR to be used in the transformation assay, a preliminary cytotoxic test was performed as reported previously.[22] Generally, cells were seeded at a density of 1×10^5 cells in 5 mL of culture medium per 60-mm dish (three dishes per group). After 3 or 4 days, when cells were in the log growth phase, the cells were fed 5 mL of fresh media and exposed to different concentrations of NPYR for 24 h. Cells were counted and cytotoxicity was determined by comparing viable cell numbers in exposed cultures to that of untreated controls. A dose of 80 μM NPYR that killed approximately 50% of cells was used for transformation assays following the procedure described in a previous report.[23] Exponentially growing H8 cells were plated at a density of 1×10^5 cells per 60-mm dish and incubated for 24 h. The cells were exposed to NPYR for 48 h, followed by culture in fresh medium for another 48 h. This treatment cycle was repeated seven times followed by a subsequent incubation in fresh medium for 3–4 weeks before further experiments.

Soft agar assay. Anchorage-independent growth was determined by a soft agar assay as described previously.[24] Briefly, a bottom layer of 1.4 mL (0.6%) was prepared with a 1:1 mixture of 1.2% low melting-point agarose (AMRESCO, OH, USA) (approximately 50°C) and warm 2 × DMEM, and was poured into each well of six-well plates. A top layer, which was a mixture of 500 μL of warm 2 × DMEM, 500 μL of the 0.6% base agar and cells at the density of 1×10^3 cells per well, was poured on top of the solidified bottom layer. The cultures were fed 1 mL of DMEM supplemented with 10% FBS, which was gently refreshed twice a week. The plates were kept in a cell culture incubator maintained at 37°C under 5% CO_2 to allow colony growth. After 2 weeks of culture, the colony assay was terminated and cell colonies of more than 50 cells in a cluster were counted using a graduated eyepiece fitted in a transmission light microscope at a magnification of 40.[25]

Tumorigenic assays in nude mice. The tumorigenic ability of the transformed cells was examined in 6-week-old female nude mice obtained from the Animal Experimental Center of Sichuan University. The cells (1×10^6) were suspended in PBS (pH 7.4, 0.1 mL) and injected subcutaneously in the sub-axilla of the mice. Ten mice were used in each group. The mice were palpated every 3 days to detect tumor development. Tumor sizes were measured in two dimensions with a Vernier caliper and recorded in mm^3 (length × width^2).[26] The mice were killed at week 8 and tumors were excised and weighed. All the experimental procedures were carried out in accordance with the Institutional Ethical Guidelines for Animal Experiments approved by the Animal Ethic Commission of the University.

Western blot. Cells were collected and whole cell lysates were prepared by lysing cells in RIPA lysis buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Equal amounts of protein extracts (50 μg of protein) were separated in 15% polyacrylamide gels, electrotransferred to polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA, USA). The membranes were incubated overnight at 4°C in PBS containing 5% defatted milk powder, 0.1% Tween-20 and primary antibodies: Id-1 polyclonal rabbit antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HPV16 E6 polyclonal goat antibody (1:500, Santa Cruz Biotechnology), HPV16 E7 monoclonal mouse antibody (1:500, Santa Cruz Biotechnology), p53 monoclonal mouse antibody (1:500, Santa Cruz Biotechnology), Retinoblastoma (Rb) monoclonal mouse antibody (Bio-Rad Laboratories, Hercules, CA, USA) and β-actin monoclonal mouse antibody (1:500, Santa Cruz Biotechnology). The membranes were further incubated with secondary antibodies conjugated with HRP (Santa Cruz Biotechnology). A luminol reagent detection kit (Santa Cruz Biotechnology) was used for visual detection of proteins of interest.

Quantitative real time-PCR. Total RNA was isolated from 1×10^7 cells with the RNeasy Mini kit (QIAGEN, Alameda, CA, USA) according to the manufacturer's instructions, and the quality of the RNA samples was confirmed by electrophoresis in 1% agarose gels. The RNA were reversely transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instruction. Reactions were performed in a 10-μL SYBR GREEN PCR reaction volume in a 96-well optical reaction plate. The PCR mixture contained: 5 μL Eva Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1 μL forward and reverse primers, 2 μL ddH_2O and 2 μL cDNA. B2M was detected as an internal control. The sequences of primers were as follows: for Id-1, forward: 5′-TCTACGACATGAACGGCTG-3′; reverse: 5′-GGTCCCTGATGTAGTCGAT-3′; for B2M, forward: 5′-TG CCGTGTGAACCATGTGA-3′; reverse: 5′-CCAAATGCGGC ATCTTCAA-3′. PCR was performed on the CFX96 fluorescent quantitative PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with the following parameters: initial denaturation at 98°C for 30 s, followed by 40 cycles of 95°C for 5 s and 65°C for 5 s, and then a final extension at 95°C for 10 s. All samples were performed in triplicate and the results were fitted to standard curves. The presence of a single specific PCR product was verified by melting curve analysis and confirmed by running an agarose gel. The amplification curves were analyzed and fold changes were determined using the comparative threshold cycle (Ct) method to quantify Id-1 mRNA expression, with the average $2^{-\Delta\Delta Ct}$ values of normal H8 cells taken as one.[27]

Stable transducting H8 cells with viral vectors. The pWPI-hId1 (for Id-1 overexpression), the hId1-shRNA (for Id-1 silencing) and their respective control plasmids, pWPI and pGIPZ, were kindly provided by Dr Robert Benezra from the Department of Cancer Biology and Genetics, Memorial Sloan-Kettering Can-

cer Center, New York, USA. The validity of both hId1-shRNA and pWPI-hId1 was proved previously.[28] CaPO4 precipitation was used for lentiviral particle production. Briefly, 2×10^6 HEK293T cells were co-transfected with 9 μg of target plasmids and helper vectors (9 μg of pDelta8.9 and 6 μg of pVSVg) using 60 μL of $CaCl_2$ (2.5 M), 540 μL of H_2O (double distilled) and 600 μL of 2 × BBS (50 mM BES, Calbiochem cat. 391334), 280 mM NaCl, 1.5 mM Na_2HPO_4). Sixteen hours after transfection, the medium was refreshed with 15 mL of DMEM containing 10% of FBS, and the cells were cultured at 37°C with 10% CO_2. The first and second harvests of supernatant (viral preparation) were collected at 24 and 48 h, respectively, of incubation. Cell debris was removed by filtration with 0.45-μm filters. The viral preparation was added for 3 consecutive days to the H8 cell culture. As the transduced cells express GFP, the ratio of fluorescent versus nonfluorescent cells was calculated to determine transduction efficiency. As the pWPI-hId1 and controlled plasmid have no antibiotic selective marker, the stable transduced H8 cells were sorted and purified with a BD FACSAria Flow Cytometer (Becton-Dickinson Company, USA). To generate H8 cells with stable expression of shRNA, H8 cells transduced with either the pGIPZ vector (for generating a control) or the hId1-shRNA vector that were puromycin-resistant were selected by incubation with 5 μg/mL of puromycin for 15 days. The puromycin-resistant clones were pooled clones and the expression of Id-1 was determined by western blot to verify overexpression or silencing.

Statistics. All statistical analyses were carried out using the Student's *t*-test in the SPSS software package (version 13.0, SPSS Inc., Chicago, USA). Statistical significance was assumed at $P < 0.05$.

Results

Establishment of the N-nitrosopyrrolidine transformation model in H8 cells. The HPV-16 immortalized but not transformed H8 cells resemble normal human cervical epithelial cells but are not transformed by the HPV E6/E7 oncogene. We first confirmed that the H8 cells lack the capacities in anchorage-independent growth in soft agar and tumor-formation in nude mice, the malignant characteristics of cancer cells. The cervical cancer cell line SiHa was used as a positive control (Figs 1 and 2). After treatment with NYPR for 4 weeks, the cells became capable of forming colonies in soft agar (Fig. 1) and tumors in nude mice (Fig. 2a). The tumor growth curve also showed a parallel trend between SiHa and transformed H8 groups (Fig. 2b), suggesting that the cells were transformed by NYPR. Then the NYPR exposure protocol was used to assay the role of Id-1 in H8 transformation by NYPR.

Increased inhibitors of differentiation-1 expression during H8 transformation. We investigated Id-1 expression at both protein

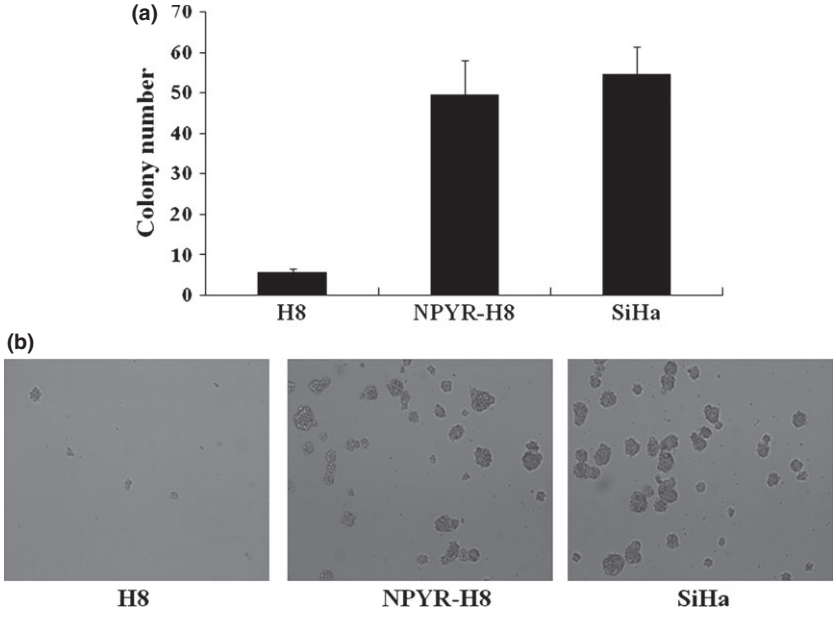

Fig. 1. Establishment of N-nitrosopyrrolidine (NPYR)-induced transformation in H8 cells. Cells treated with NPYR were seeded in soft agar, and colony numbers were counted 2 weeks after seeding. Untreated H8 cells and SiHa cells were seeded as negative and positive controls, respectively. (a) Representative images taken under a light microscope (40×). (b) Colony numbers in each group counted in five fields (mean ± SD).

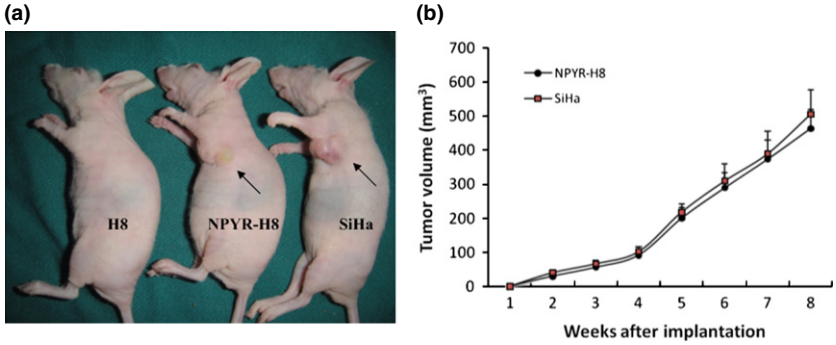

Fig. 2. Tumorigenicity of N-nitrosopyrrolidine (NPYR)-transformed H8 cells in nude mice. Nude mice were injected (s.c.) with H8 cells, NPYR transformed H8 (NPYR-H8) cells or SiHa cells, and were photographed 8 weeks after injection. Tumor size of each group is shown ($n = 10$). (a) One representative mouse from each group is shown. Injection sites are indicated by arrowheads. (b) Tumor growth curves of the NPYR-H8 and SiHa groups.

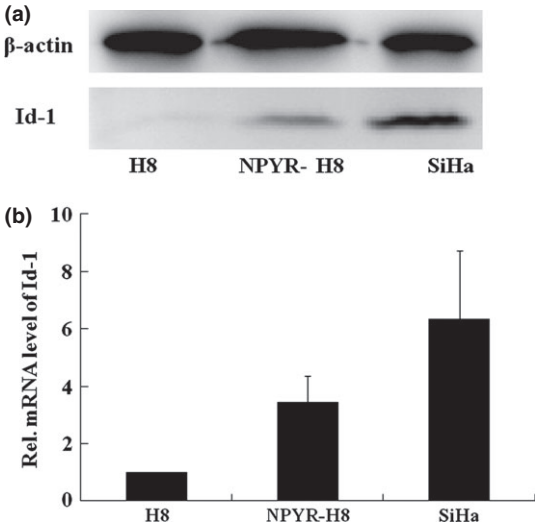

Fig. 3. Increased inhibitors of differentiation-1 (Id-1) expression in N-nitrosopyrrolidine (NPYR)-transformed H8 cells. (a) Total proteins extracted from H8, NPYR-H8 and SiHa cells were subjected to 10% SDS-PAGE gels and assayed by western blot. (b) Id-1 RNA levels in H8, NPYR-H8 and SiHa cells were detected by qRT-PCR. Expression of Id-1 gene, in each sample was normalized against the B2M endogenous control. The normalized values were then calibrated against the H8 cell values that were arbitrarily set as 1.

and RNA levels in parental and transformed H8 cells. SiHa cells were used as a positive control. A significant increase of Id-1 protein expression in transformed H8 cells compared to the parental H8 cells was detected by western blot (Fig. 3a). By quantitative RT-PCR, the Id-1 RNA level in transformed H8 cells was found to be 3.8-fold higher than that in the untransformed H8 cells (Fig. 3b, $P < 0.05$).

Increased inhibitors of differentiation-1 overexpression increases colony formation in soft agar and tumor growth in nude mice. To determine whether Id-1 expression is required for transformation of H8 cells, we ectopically expressed Id-1 in H8 cells through lentivirus transduction. The stable Id-1-overexpressed H8 cells were sorted and purified using the BD FACSAria Flow Cytometer. Effective expression of Id-1 in pWPI-hId1-transduced cells was confirmed by western blot (Fig. 4A). The cells were exposed to NPYR using our established protocol. As a result, H8 cells with a stable transduction of pWPI-hId1 showed an increased colony formation ability compared to the control plasmid pWPI (colony numbers: 75.4 ± 12.2 *vs* 48.4 ± 7.5, $P < 0.05$, Fig. 4B,C). The results strongly support a promoting role of Id-1 in cervical epithelial cell transformation. We further investigated the biological consequence of Id-1 activation in tumorigenesis with the nude mouse xenograft model. pWPI-hId1 H8 cells developed larger tumors compared to the pWPI controls at the end of experiments (tumor size for pWPI-hId1 group: 1044 ± 142 mm^3; control group: 427 ± 52 mm^3, $P < 0.05$, Fig. 5). Pathological analysis revealed that all the xenograft tumors were similar to human squamous cell carcinoma (SCC). However, tumors derived from Id1-overexpression cells (pWPI-hId-transfected) showed SCC with undifferentiated or low-grade differentiated types, while those from control cells (pWPI-transfected) revealed a better differentiation (Fig. 6).

Inhibition of Increased inhibitors of differentiation-1 expression via shRNA-mediated silencing inhibited colony formation in soft agar and tumor formation in nude mice. To further define

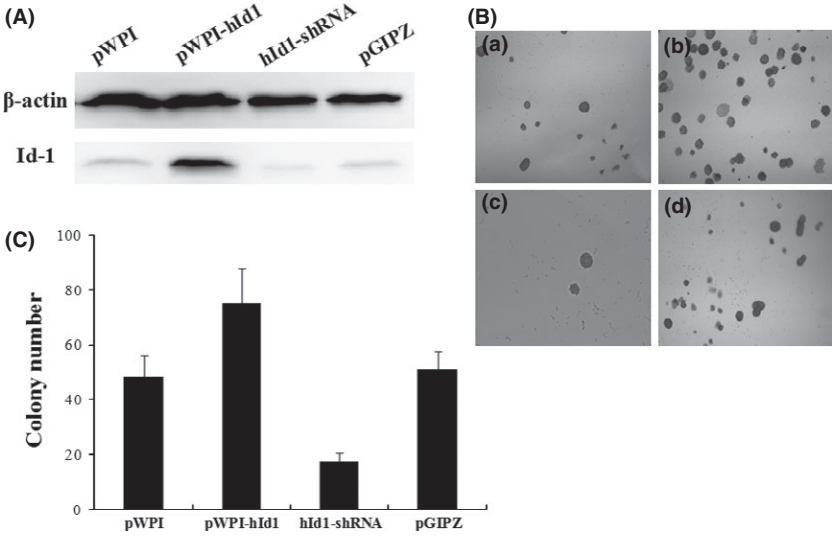

Fig. 4. N-nitrosopyrrolidine (z)-induced transformation of H8 cells with increased inhibitors of differentiation-1 (Id-1) overexpression or silencing. (A) Total proteins extracted from the indicated H8 cells were subjected to 10% SDS-PAGE gels and assayed by western blot. (B) Cells treated with NPYR were seeded in soft agar, and colony numbers were counted at 2 weeks. Representative images were taken under a light microscope (40×). (a) NPYR transformed pWPI H8 cells; (b) NPYR transformed pWPI-hId1 H8 cells; (c) NPYR transformed hId1-shRNA H8 cells; (d) NPYR transformed pGIPZ H8 cells. (C) Quantification of colony numbers in each group counted in five fields (mean ± SD).

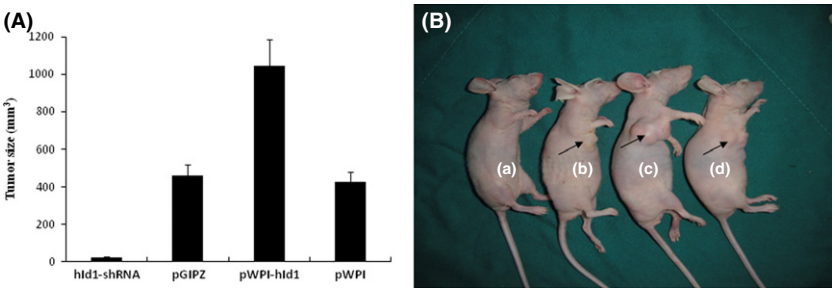

Fig. 5. Xenografted tumor formations in nude mice. Nude mice were injected (s.c.) with (a) N-nitrosopyrrolidine (NPYR) transformed hId1-shRNA H8 cells, (b) NPYR transformed pGIPZ H8 cells (as control), (c) NPYR transformed pWPI-hId1 H8 cells, (d) NPYR transformed pWPI H8 cells (as control), and were photographed 8 weeks after injection. There were 10 mice in each group. Tumor size was presented as means ± SD. One representative mouse from each group is shown. Injection sites are indicated by arrowheads.

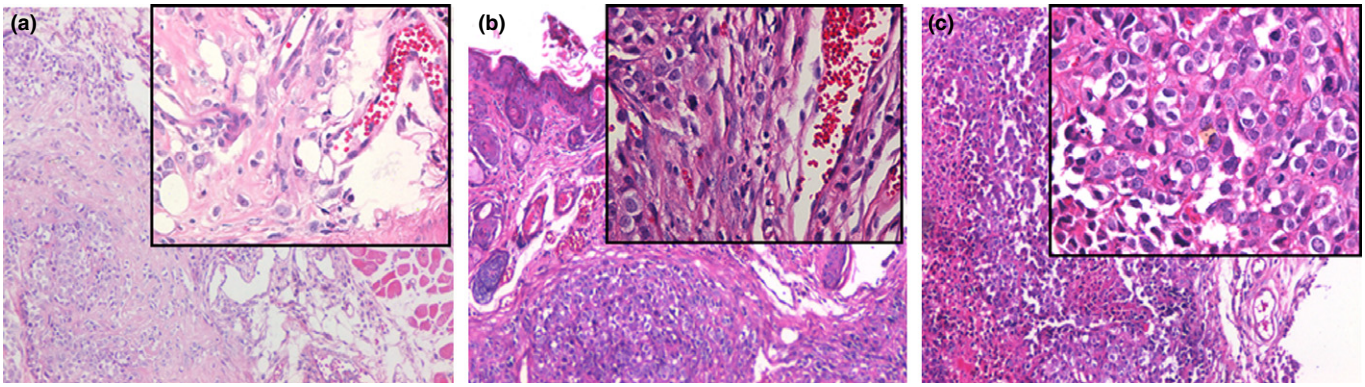

Fig. 6. HE staining xenografted tumors in nude mice. (a) Tumors from N-nitrosopyrrolidine (NPYR) transformed pWPI H8 cells showed squamous cell carcionoma (SCC) with a better differentiation. (b) Tumors from NPYR transformed pWPI-hId H8 cells showed SCC with undifferentiated or low-grade differentiated type. (c) Tumors formed by SiHa cells showed SCC with undifferentiated type. Representative images (100× and 400× for the inserts) are shown.

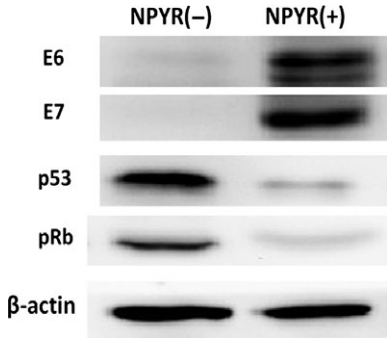

Fig. 7. Evaluations of human papillomavirus E6, E7 and p53, and pRb during N-nitrosopyrrolidine-induced cell transformation. Total proteins from NPYR transformed or untreated H8 cells were subjected to 10% SDS-PAGE gels and followed by western blot. β-actin served as an input control.

the function of Id-1 in cervical epithelial transformation, we generated stable Id-1 gene silencing in H8 cells with shRNA (Id-1-shRNA H8 cells). Then we performed NPYR-induced transformation under the same conditions as in the previous experiments. The results showed that Id-1 gene silencing significantly decreased cell colonies in soft agar assay (hId1-shRNA, 17.4 ± 3.2; pGIPZ, 51.2 ± 6.8, $P < 0.05$) (Fig. 4b,c). Id-1 gene silencing also decreased tumor sizes (at 8 weeks after injection, hId1-shRNA group: 23 ± 4 mm^3; pGIPZ group, 460 ± 59 mm^3, $P < 0.05$) (Fig. 5). These results strongly suggest that Id-1 plays an important role in NPYR-induced transformation of HPV-16 immortalized cervical epithelial cells.

Increased expression of human papillomavirus E6/E7 proteins and decreased expression of p53 and pRb in transformed H8 cells. To explore the possible mechanism of Id-1 upregulation in NPYR-treated H8 cells, we examined the expression of the HPV E6 and E7 oncoproteins and their target tumor suppressors p53 and pRb in transformed H8 cells induced by NPYR. The results show that the expression of HPV E6 and E7 proteins was significantly increased, while that of p53 and pRb protein was decreased in the transformed cells (Fig. 7).

Discussion

In our previous studies, we demonstrated a gradual increase of Id-1 expression that is associated with the progression of cervical carcinogenesis: cervical carcinoma (76%), high grade precancerous lesions (HSIL, 50%), low grade precancerous lesions (LSIL, 16%) and normal tissues (4%), which strongly suggest Id-1 to be a potential cervical cancer promoter.[16] Id-1 knockdown in cervical cancer cells reduced anchorage-independent growth *in vitro* (Fig. S1). Other studies have also shown that in cervical carcinoma, expression of Id-1 protein is correlated to invasive and metastatic behaviors.[29] This implies that Id-1 is a potential oncoprotein in HPV-related cervical cancer. However, the detailed mechanism by which Id-1 contributes to cervical cancer development is still elusive. We hypothesized that Id-1 could be involved in cancer development through promoting cervical epithelial transformation, the most important procedure in cancer development.

To test this hypothesis, we established an in vitro cervical epithelial transformation model. We transformed HPV-16 immortalized cervical H8 cells by NPYR, a metabolic product in the female genital tract with anaerobic vaginosis, which could be a potential carcinogen for cervical carcinogenesis.[20] With both soft agar assay and tumorigenesis in nude mice, we successfully established an effective transformation of H8 cells. With this model, we focused on the role of Id-1 in the transformation process, which converts normal cervical epithelial cells to malignant cells.

First, we found that Id-1 expression was increased in transformed H8 cells and cancer cells compared to untransformed H8 cells. This result was consistent with what we have found in human cervical tissues, suggesting a relationship between Id-1 expression and cervical cancer development. Then, we overexpressed Id-1 through ectopic expression or suppressed Id-1 expression with shRNA-mediated knockdown in H8 cells. Id-1 overexpression increased while the Id-1 silencing decreased H8 cell transformation by NPYR. These findings are supportive to our hypothesis that Id-1 plays an important role in promoting epithelial cell transformations and contributes to cervical cancer development. However, Id-1 overexpression could not increase significant colony formation in soft agar assay alone (Fig. S2), which suggests that although Id-1 potentiated carcinogen-induced cervical epithelial cell transformation, itself it is insufficient to cause cell transformation. Thus, Id-1 is most likely an endogenous tumor promoter for cervical cancer development. These results may add new insight into the etiology of cervical cancer and suggest a potential target for therapy.

Human papillomavirus infection, particularly high risk HPV infection, has already been proved to be the most important etiology in cervical cancer development.[8,9] Our previous study found that in carcinoma tissues, Id-1 expression was correlated to high risk HPV, especially HPV-16 infection.[17] In the present study, we found the HPV E6 and E7 oncoproteins were upregulated in the Id-1 mediated cell transformation. This is consistent with a previous study showing that Id-1 expression is correlated to HPV E6/E7 expression in breast carcinoma tissues, and E6 and E7 induce breast cancer cell invasion and metastasis through induction of Id-1 overexpression.[30] Because HPV E6 and E7 oncoproteins can promote cancer development through interacting with several tumor suppressors such as p53 and pRb, we hypothesize that Id-1 cooperates with HPV E6/E7 proteins, which involves inactivation of p53 and pRb in host cells, to promote cervical carcinogenesis. However, further studies are needed to verify this hypothesis.

In conclusion, our study suggests that Id-1 promotes NPYR-induced cervical epithelial transformation, which sheds light on the mechanism of cervical cancer development and implies that Id-1 is a potential target for cervical cancer prevention and therapy.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Soft agar assay of inhibitors of differentiation-1 knockdown in SiHa cells.

Fig. S2. Soft agar assay of H8 cells with or without Id-1 overexpression.