Histone deacetylase 4 increases progressive epithelial ovarian cancer cells via repression of p21 on fibrillar collagen matrices

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Abstract. Histone deacetylase (HDAC) 4 is an emerging target in cancer therapeutics, but little is known about the function of HDAC4 in gynecologic malignancies. Therefore we investigated the mechanism of HDAC4 promoting the proliferation of epithelial ovarian cancer cells (OV). In this study, we observed that the proliferation of cells with HDAC4 inhibitor Trichostatin A (TSA) treatment was markedly decreased, Further, we showed that epithelial ovarian cancer tissues with stage III/IV had higher HDAC4 expression, compared to that with stage I/II. We examined first that the HDAC4 expression was increased in response to fibrillar collagen matrices. In addition, we found that HDAC4 was retained in the nucleus by regulation of PP1α, which regulated HDAC4 cellular fraction via phosphorylation of HDAC4. In addition, we found that HDAC4 bound to Sp1 in epithelial ovarian cancer cells. Finally, ovarian cancer cell line OVCAR3 was evaluated via gain/loss-of-function of HDAC4 by either overexpression of HDCA4 or knock-down of HDAC4 with shRNA. We examined both protein and mRNA of p21 by western blotting and qPCR. We performed analysis of colony formation in matrigel and migration by ECIS. Our results suggest that the accumulation of HDAC4 induced by fibrillar collagen matrices in the nucleus via co-localization of PP1α, leads to repression of the mRNA/protein of p21 and in turn promotes the proliferation and migration of epithelial ovarian cancer cells.

Introduction

Ovarian cancer is a significant cause of mortality in women around the world. Although ovarian cancer treatment has advanced in recent years, long-term survival remains stable (1,2). Evidence shows that the targeting of epigenetics including acetylation and deacetylation of the core nucleosome histones show great promise for improving the treatment of cancers (3-5).

Recent studies show that epigenetic disorder in cancer plays an important role in regulating cancer development (6-9). It is regarded as the most powerful regulator to mediate other transcription regulatory pathways which are related to transcription factors. It has been proven that the aberrant HDAC activity is linked to the development of cancers. Inhibition of HDACs leads to the restoration of transcriptionally silenced pathways or the repression of aberrantly expressed genes (10-16). Increasing evidence indicates that the causes of cancer are related not only to a variety of signaling pathways, but also to epigenetic modification, which allows adaptation to environmental alteration such as ECM remodeling, hypoxia stress and nutrient deprivation (17-19).

HDAC4 is a class II histone deacetylase, which modulates gene expression by undergoing nuclei-cytoplasmic shutting via its phosphorylation state. Nuclear HDAC4 represses transcription procedure when tethered to a promoter. Class II HDACs contain an N-terminal regulatory domain that is subject to phosphorylation. Nuclei-cytoplasmic shuttling of HDAC4 is controlled by its phosphorylation state. PP1α/PP2A is the critical regulator of HDAC4 function (20). Dephosphorylation of HDAC4 by PP1α/PP2A stabilizes and promotes its nuclear accumulation, whereas phosphorylated HDAC4 is located in the cytoplasm and is prone to degradation (20-23).

Previous studies showed that fibrillar collagen matrices enhanced the proliferation and invasion of epithelial ovarian cancer cells via activation of PTEN/Akt signaling (17). Therefore, we postulate that HDAC4 is associated with poor prognosis of ovarian cancer. We also investigated whether fibrillar collagen matrices influence the aberrant HDAC4 in epithelial ovarian cancer. Herein, we demonstrate that nuclear HDAC4 is a key regulator promoting the progressive epithelial ovarian cancer on fibrillar collagen matrices via co-localized PP1α, in which HDAC4 represses the mRNA/protein of p21.

Materials and methods

Epithelial ovarian cancer cell lines, OVCAR3 and SKOV3, were purchased from the American Type Culture Collection.

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Three-Dimensional fibrillar collagen (0.25 mg/ml final concentration) was as previously described (17). The antibodies of HDAC4, lamin-A, Sp1, p21 and Acetyl-histone-H3 were obtained from Cell Signaling Technology (Danvers, MA, USA). Both GAPDH and PP1α were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell and culture. All patients fulfilled the criteria for diagnosis of epithelial ovarian cancer with grades and stages as established by the International Federation of Gynecology and Obstetrics (FIGO) 2014. One hundred and two patients with epithelial ovarian cancer were confirmed by immunohistochemistry and/or western blotting. Less than 25% of nuclear HDAC4 expression was the lower HDCA4 patient group, and 25 to 100% the higher HDAC4 patient group according to evaluation of 200 cancer cells. Tumor tissues were collected from surgeries performed at Affiliated Nanjing Maternity and Child Health Care Hospital at Nanjing Medical University. Cells were cultured as previous described (17). We began our experiments by studying the proliferation of epithelial ovarian cancer cells which were treated with HDAC4 inhibitor, Trichostatin A (TSA). We observed the proliferation of cells with TSA treatment. To investigate the role of fibrillar collagen in regulating HDAC4 function, we examined HDAC4 location in the epithelial ovarian cancer cell line OVCAR3 in cells seeded with or without fibrillar collagen for 2 h.

Immunohistochemistry (IHC). We studied HDAC4 expression in epithelial ovarian cancer tissue specimens with stage I/II (n=36) or stage III/IV (n=66) by immunohistochemistry and western blot analysis (17). Immunohistochemistry studies were performed on formalin-fixed sections of tissue specimens. Sections were pretreated with trypsin (10 mg per 50 ml in Tris buffer, pH 8.1) for 10 min at 37˚C, followed by anti-HDAC4 antibody (1:1000 dilution in PBS) for 30 min. Slides were washed in PBS and incubated sequentially for 15 min with peroxidase-conjugated swine anti-mouse immunoglobulin G (1:50 dilution). Staining was performed using a Dako autostainer. Localization of reaction products was performed using diaminobenzidene reaction.

Immunofluorescence assay (IFA). PP1α location was examined by subcellular fractionation assay and immunofluorescence in epithelial ovarian cancer cells with or without fibrillar collagen. HDAC4 localization was analyzed also by immunofluorescence assay in epithelial ovarian cancer cells with or without the PP1α inhibitor calyculin A. Immunofluorescence was performed as previously described (17).

Immunoprecipitation assay (IP). We examined that HDAC4 interacted with Sp1 in nuclear fraction of OVCAR3 cells using immunoprecipitation assay by anti-HDAC4 antibody.

Western blot analysis. We examined HDAC4 expression by western blotting in OVCAR3 cells seeded with or without fibrillar collagen for 2 h and with overexpression of PP1α in adenoviral vector. In addition, protein of p21 was examined by western blotting in OVCAR3 cells performing gain/loss-
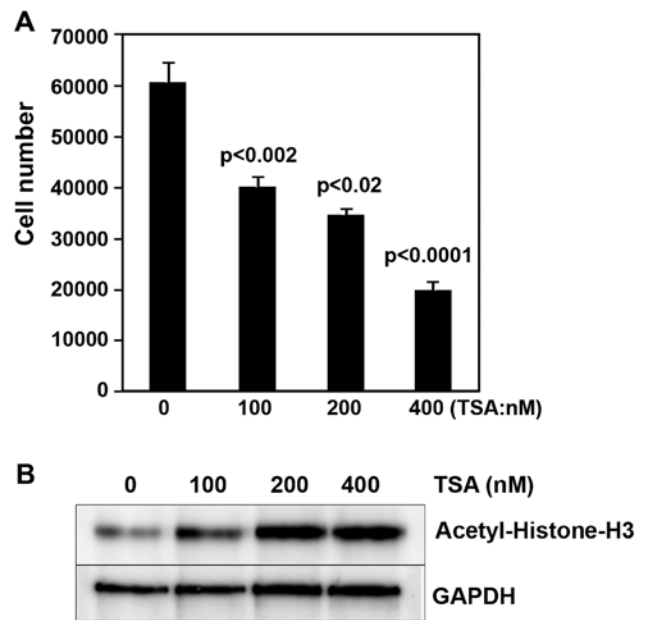

Figure 1. (A) HDACs inhibitor, TSA, suppresses the proliferation of epithelial ovarian cancer cells. (B) Western analysis of acetyl-histone-H3 in epithelial ovarian cancer cells treated with TSA.

of-function of HDAC4 by either overexpression of HDCA4 or knock-down of HDAC4 with shRNA. The cells were harvested in lysis buffer. The samples were centrifuged for 20 min at 13,000 x g. The protein concentration of the supernatant was determined by BCA assay. Sodium dodecylsulfate-polyacrylamide gel electrophoresis(SDS-PAGE) was carried out loading equal amount of proteins/lane. Gels were transferred to cellulose nitrate membranes and blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) buffer for 1 h. Then, the membranes were incubated with primary antibodies at a 1:5,000 dilution in 5% non-fat milk overnight at 4˚C, and washed five times with TBST for a total of 30 min. The membranes were then incubated which the secondary antibodies conjugated with horseradish peroxidase at a 1:5,000 dilution for 1 h at room temperature and then washed five times with TBST. The blots were visualized with Amersham ECL Plus Western Blotting Detection Reagents according to the manufacturer's instructions (17).

Cell migration assay. Cell migration was analyzed by Electric Cell-substrate Impedance Sensing (ECIS; Applied BioPhysics Troy, NY, USA), which is an impedance based method to study cell activities in tissue culture in real-time. We modified the protocol described by the company: 25,000 cells/cm^2 was seeded in ECIS arrays (8-well), and grown to about 100% confluency in approximately 24 h. The cells were then killed in a small active electrode. The additional fibrillar collagen was polymerized in each well at 0.25 mg/ml for 90 min. The migration was assessed by continuing impendent measurements for 20 h.

Quantitative qPCR. mRNA of p21 was examined by qPCR. cDNA was reverse transcribed using TaqMan reverse transcriptase kit (Roche) and qPCR was performed using the Roche Light-Cycler with SYBR Green dye (Roche) (24). The
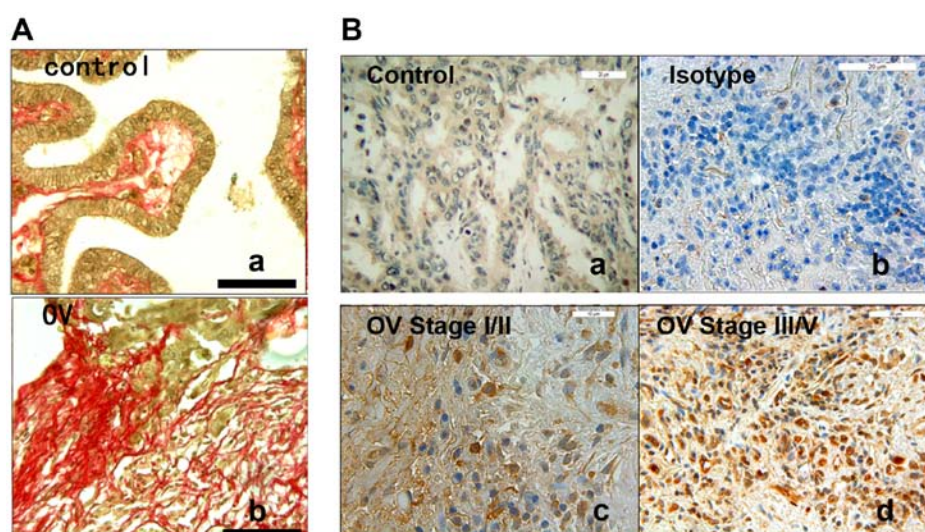
Figure 2. (A) Fibrillar collagen production and deposition in control and epithelial ovarian cancer (OV) was detected by picrosirius red. (B) IHC of HDAC4 immunoreactive cells in stage I/II; numerous HDAC4 immunoreactive cells in stage III/IV.
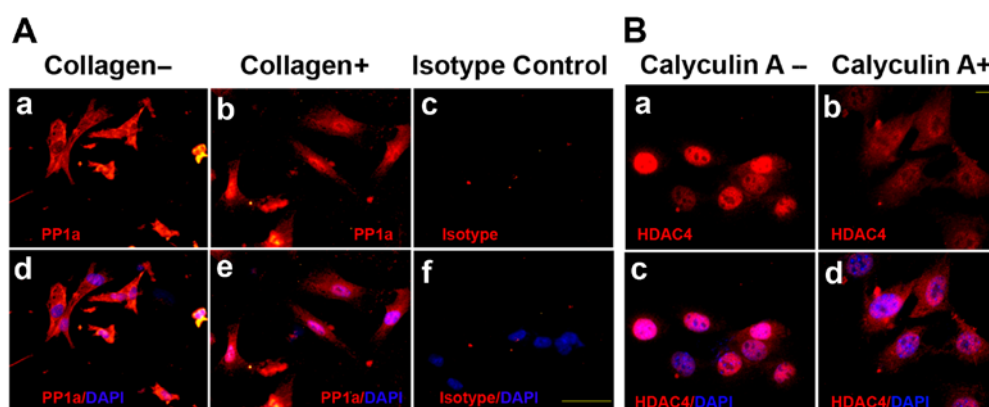

Figure 3. (A) PP1 localization was observed in both the nucleus and the cytoplasm in OV by IFA with or without fibrillar collagen. Red, Cy3 and blue, DAPI. (B) HDAC4 localization was analyzed in the nucleus and the cytoplasm by IFA in OV with or without calyculin.

following human primers were used: p21 FW: 5'-GAG GCCGGGATGAGTTGGGAGGAG, RV: 5'-CAGCCGG CGTTTGGAGTGGTAGAA; p53 FW: 5'-CCCCTCCT GGCCCCTGTCATCTTC, RV: 5'-GCAGCGCCTCAC AACCTCCGTCAT; GAPDH FW: 5'-AATCCCATCACC ATCTTCCA, RV: 5'-TGGACTCCACGACGTACTCA.

Statistics. Data are expressed as mean ± SD. The data in each experiment were performed with unpaired Student's t-tests using SPSS 13.0 software. The experiments were done a minimum of three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HDAC inhibitor attenuates the proliferation of epithelial ovarian cancer cells. An epigenetic disorder is one of the main factors leading to poor prognosis of cancer (18-20). Previous work indicated that fibrillar collagen matrices enhanced the proliferation and invasion in epithelial ovarian cancer cells (17). To directly examine the role of HDACs in contribu-

tion to modulation of epithelial ovarian cancer cells, we began our experiments by studying the proliferation of epithelial ovarian cancer cells in cells treated with the HDAC inhibitor, Trichostatin A (TSA), for 48 h in a dose-dependent manner. We observed that i) the proliferation of cells with TSA treatment was markedly decreased (Fig. 1A); ii) acetyl-histone-H3 expression in cells with TSA was increased (Fig. 1B). This is one of the reasons that HDAC4 promotes the proliferation of epithelial ovarian cancer cells. These data indicated that the epigenetic changes of HDACs might play a pivotal role in regulating epithelial ovarian cancer.

Higher HDAC4 expression was related to high stage of the epithelial ovarian cancer. HDACs are aberrant in many cancers including epithelial ovarian cancer (25-29). We postulated that aberrant HDAC4 expression was associated with fibrillar collagen matrices which were remodeled in the cancer microenvironment. To support this idea, we examined that synthesis and deposition of fibrillar collagen that was increased in epithelial ovarian cancer tissues specimens in comparison with controls by picrosirius red stain
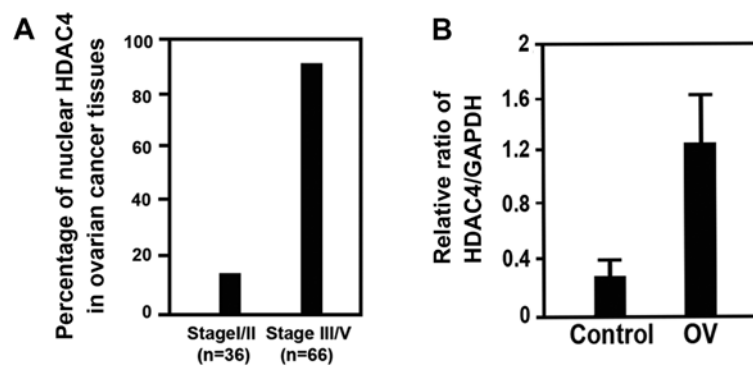
Figure 4. (A) The nuclear HDAC4 at 13.8 or 87.8% displayed in OV with stage I/II or stage III/IV, respectively. (B) The relative ratio of HDAC4 to GAPDH by quantitative densitometry. GAPDH is shown as a loading control.
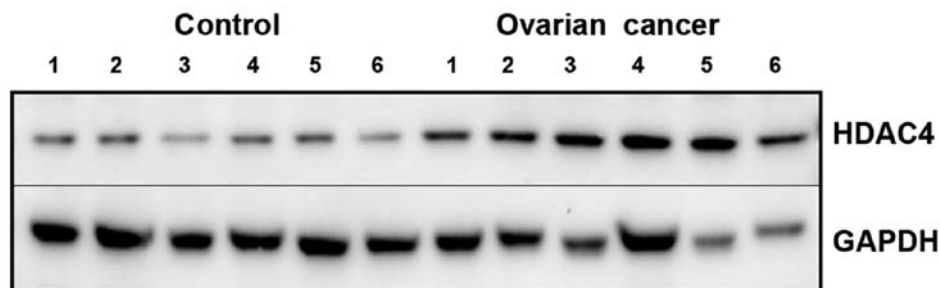

Figure 5. HDAC4 expression level in control (n=6) and epithelial ovarian cancer patients (n=6, top panel) was quantified by western blot analysis.
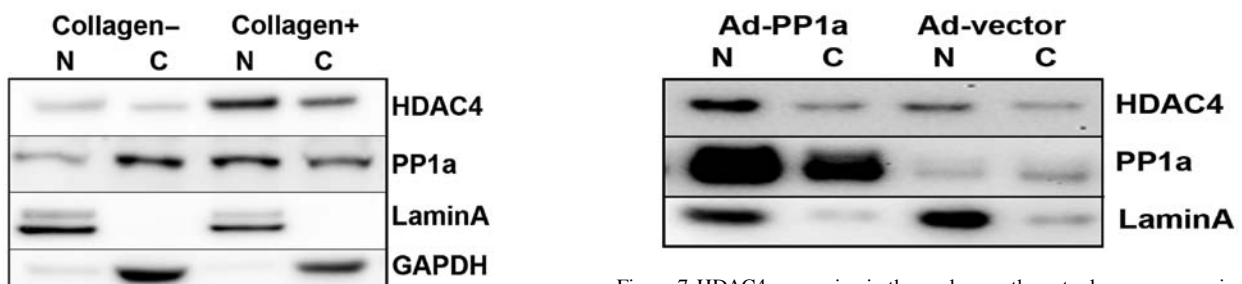

Figure 6. HDAC4, PP1. Lamin A and GAPDH was quantified in nuclear fraction (N) and cytoplasmic fraction (C) by western analysis in OV cells on fibrillar collagen. Lamin A or GAPDH is as a loading control of nuclear or cytoplasmic fraction.
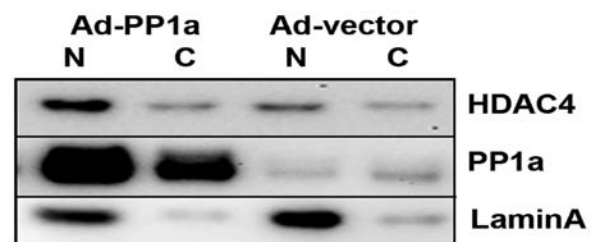

Figure 7. HDAC4 expression in the nucleus or the cytoplasm was examined in OV cells with adenoviral-PP1 (Ad-PP1) or adenoviral empty vector (Ad-vector) by western analysis using subcellular fractionation assay. Bar=10 μM.

(Fig. 2A). To explore *in vivo* the relevance of our findings, we studied HDAC4 expression in epithelial ovarian cancer tissue specimens with stage I/II (n=36) or stage III/IV (n=66) by immunohistochemistry (IHC). We found that 87.8% of epithelial ovarian cancer tissues with stage III/IV had higher HDAC4 expression, compared to 13.8% of that with stage I/II (Figs. 2B and 4A). Finally, we found that HDAC4 expression was increased in epithelial ovarian cancer tissues with stage III/IV (n=24), compared to control (n=18) by western blotting (Figs. 4B and 5). This shows that the expression rate of HDAC4 in epithelial ovarian cancer is related to the stage of the epithelial ovarian cancer, that is, later stage of epithelial ovarian cancer had higher expression rate of HDAC4. These data supported the concept of higher HDAC4 expression in epithelial ovarian cancer might be a biomarker, which was relevant to poor prognosis of epithelial ovarian cancer.

We also found that nuclear HDAC4 fibrillar collagen-induced was regulated via colocalization of PP1α. Previous data showed that fibrillar collagen matrices were abnormally deposited in epithelial ovarian cancer tissues (Fig. 2A), leading to enhanced proliferation and invasion of cancer cells (17). To further investigate the role of fibrillar collagen in regulating HDAC4 function, we examined HDAC4 location in the epithelial ovarian cancer cell line OVCAR3 in cells seeded with or without fibrillar collagen for 2 h. We found that HDAC4 expression was increased in response to fibrillar collagen matrices. Of note, we found that HDAC4 expression was relatively elevated in nuclear fraction in response to fibrillar collagen (Fig. 6). HDAC4, which plays a vital role in up- and down-regulation of the transcription factors, is one of the most frequent epigenetic changes.

Paroni and colleagues (20-22) reported that PP1/PP2A regulates HDAC4 cellular fraction via phosphorylation of HDAC4. To explore whether the HDAC4 was retained in the
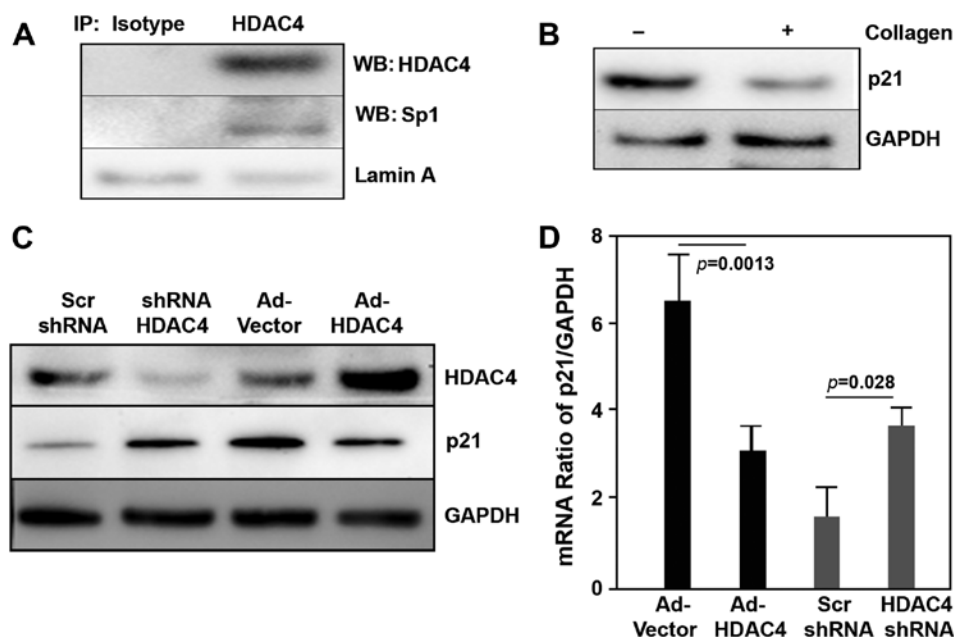
Figure 8. (A) HDAC4 interacts with Sp1 using IP. (B) Western analysis of p21 in OV. (C) Western analysis of HADC4 and p21 in OV with shRNA-HDAC4 or Ad-HDAC4. Scr-shRNA and Ad-vector are controls. (D) P21 mRNA in OV with either shRNA-HDAC4 or Ad-HDAC4.

nucleus by regulation of PP1α, PP1α location was examined by subcellular fractionation assay and immunofluorescence in epithelial ovarian cancer cells in response to fibrillar collagen. The results showed that PP1α expression was also retained and colocalized with HDAC4 in the nucleus on fibrillar collagen (Figs. 6 and 3A). To ascertain whether HDAC4 mainly remained in the nucleus via regulation of PP1α on fibrillar collagen, OVCAR3 cells were seeded on fibrillar collagen matrices for 2 h with or without PP1α pretreatment with 5 nM calyculin A. The images by immunofluorescence showed that HDAC4 expression in nuclear fraction was decreased in cells with calyculin A, compared to that without calyculin A on fibrillar collagen (Fig. 3B). We confirmed the HDAC4 in the nucleus was increased through regulation of PP1α by overexpression of PP1α in adenoviral vector in OVCAR3 cells. The results of western blotting showed that HDAC4 expression was increased in the nucleus fraction (Fig. 7).

HDAC4 promotes the proliferation via repression of p21 in epithelial ovarian cancer. Sp1 is involved in the regulation of many tumors. Prior studies showed that HDAC4 mediated p21 through either p53-dependent or p53-independent pathway (30,31). We investigated the HDAC4 interaction with Sp1 in the nuclear fraction of OVCAR3 cells using immunoprecipitation assay with anti-HDAC4 antibody. We found that HDAC4 bound to Sp1 in epithelial ovarian cancer cells (Fig. 8A). we also examined that p21 expression was suppressed when OVCAR3 cells were seeded on fibrillar collagen (Fig. 8B), whereas p53 did not change.

To directly test whether HDAC4/Sp1 complex regulated the downstream p21 signal, both protein and mRNA of p21 were examined by western blotting and qPCR when OVCAR3 cells were performed via gain/loss-of-function of HDAC4 by either overexpression of HDCA4 or knock-down of HDAC4 with shRNA. The results showed that overexpression of HDCA4

markedly decreased protein and mRNA level of p21 (Fig. 8C and D). In contrast, knock-down of HDAC4 significantly increased protein and mRNA of p21 (Fig. 8C and D). While we did not find that the gain/loss of HDAC4 regulated protein and mRNA of p53 in these experiments. These data supported a model of increased nuclear HDAC4/Sp1 complex, which in turn repressed p21, promoting the aggressive epithelial ovarian cancer in response to fibrillar collagen matrices.

To ascertain whether the nuclear HDAC4 operated the proliferation and migration of epithelial ovarian cancer cells through its effect on p21 signal on fibrillar collagen matrices, we knocked down HDAC4 by shRNA in epithelial ovarian cancer cells. We performed analysis of colony formation in matrigel and migration by ECIS. The knock-down results showed that i) ~60% of HDAC4 by shRNA inhibited the proliferation and colony formation (Fig. 9A and B); ii) knock-down of HDAC4 suppressed the migration of epithelial ovarian cancer cells (Fig. 9C). Together, these data indicated that increased nuclear HDAC4/Sp1 complex played a critical role in leading to enhancement of progressive epithelial ovarian cancer cells via repressing p21.

Discussion

Recent evidence of epigenetic disorders has led to the discovery of a potential approach to screen and treat epithelial ovarian cancer (2,3,5,6). Previous studies revealed that fibrillar collagen matrices promoted tumor progression (17,32,33). Our initial data indicate that the epithelial ovarian cancer cells with stage III/IV have higher HDAC4 expression than those with stage I/II. However, the mechanism of aberrant regulation of HDAC4 by fibrillar collagen in epithelial ovarian cancer remained unclear. In this study, we demonstrated that an aberrant nuclear HDAC4/Sp1 complex via co-localization of PP1α increases the aggressive epithelial ovarian cancer cells
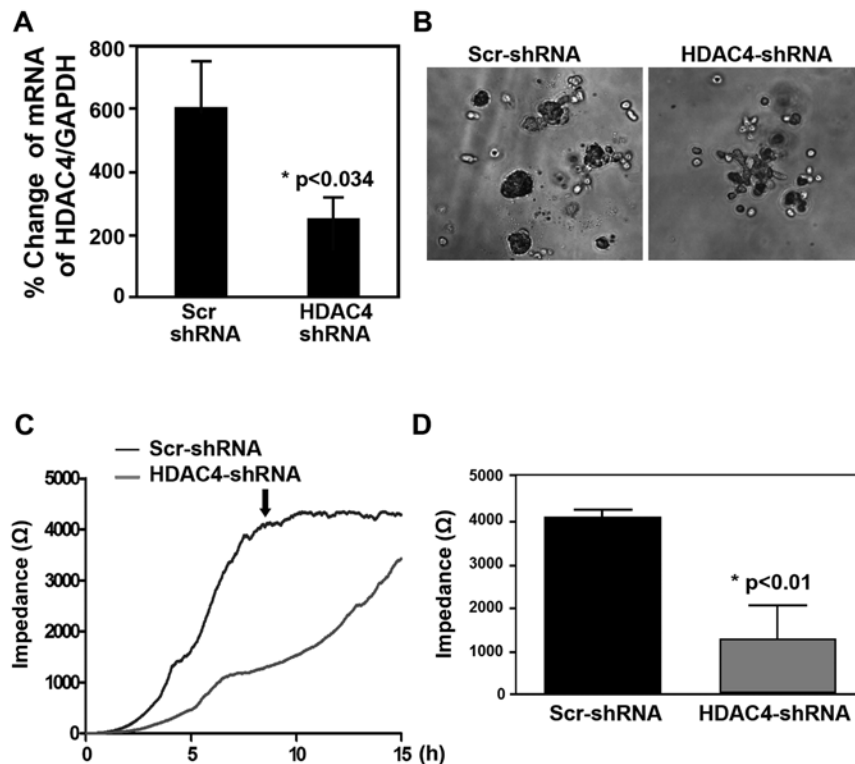
Figure 9. In OV with Scr-shRNA or HDAC4-shRNA, (A) the percentage of mRNA of HDAC4/GAPDH; (B) the colony formation of OV in matrigel. (C) The migration was decreased in OV with HDAC4-shRNA by analysis of ECIS; (D) impedance was decreased in OV with HDAC4-shRNA.

through repressing p21 genes when epithelial ovarian cancer cells interact with fibrillar collagen matrices. These data indicate an important role of HDAC4/Sp1/PP1α/p21 axis, which is a hallmark feature of epithelial ovarian cancer, in regulation of cancer cell behavior,

Studies in cancer indicate that cross-linked type I collagen matrices affect tumor progression. It is consistent with our finding that fibrillar collagen enhances proliferation and invasion of epithelial ovarian cancer. Of note, we demonstrate that increase of nuclear HDAC4 and PP1α co-localization is in response to fibrillar collagen. Changes in the pathologic ECM microenvironment can reprogram cells through epigenetic modifying mechanisms, which include alteration of histone modifications via HDACs activity. Deacetylation of histones by HDACs results in chromatin compaction, which represses gene transcription. HDAC4 shutting between cytoplasm and nucleus is controlled by its phosphorylation state. PP1 is a critical regulator of HDAC4 functions (21,22). As HDAC4 is phosphorylated by PP1, HDAC4 is exported and kept in the cytoplasm where it is susceptible to degradation by the proteasome. Tissue specimens *in vivo* showed that ~88% of patients with epithelial ovarian cancer with stage III/IV have higher nuclear expression, compared to ~14% of that with stage I/II.

In vitro, we discover that HDAC4 aberrantly remains in the nucleus of epithelial ovarian cancer cell via colocalization with PP1α on fibrillar collagen. We further confirmed that increase of HDAC4 in the nucleus of epithelial ovarian cancer cells is regulated by fibrillar collagen or overexpression of PP1α. In contrast, we showed that PP1α inhibitor leads to nuclear HDAC4 reduction. Although HDAC4 undergoes

nuclear import or retentive nucleus when HDAC4 is dephosphorylated by PP1α we cannot eliminate PP2A regulation. The PP2A function is regulated by expression, localization and holoenzyme composition (20). PP2A location might be a reason why PP2A plays a dual role in up or down-regulating signals in the cells.

It has been reported that microenvironmental changes play a role in regulating cell behavior, which eventually causes epigenetic disorders in cancer cells (34,35). Additionally, we found that both protein and mRNA of p21 are suppressed when epithelial ovarian cancer cells are seeded on fibrillar collagen. It is related to the increase of nuclear HDCA4, which represses p21 transcription and then leads to decreasing p21 protein. In this case, p53 does not changed significantly in epithelial ovarian cancer cells in response to fibrillar collagen (data not shown).

Upon nuclear HDAC4 accumulation, HDAC4 forms a complex with a variant of transcription factors, which repress their function and alter gene transcription (31). Prior studies indicated that HDCA4 might complex with Sp1, or Mef (36), which bound to transcription factors. Kang *et al* (31) showed that HADC4/Sp1 complex repressed p21 expression in cancer cells via Sp1 binding the promoter of p21. To verify this, we performed a series of experiments. We examined HDAC4 binding to Sp1 epithelial ovarian cancer cells by immunoprecipitation. Then, we demonstrated that protein and mRNA of p21 were negatively affected by know-down or overexpression of HDAC4. Our data indicate that the increase of HDCA4 collagen-matrices-induced in nucleus suppresses the mRNA/protein expression of p21, leading to promoting the proliferation of epithelial ovarian cancer cells. Noteworthy, change of

HDAC does not affect mRNA/protein of p53, which is usually considered as one of the regulators of p21. Finally, we demonstrated that loss-of-function of HDAC4 by shRNA can inhibit the colony formation and migration of epithelial ovarian cancer cells. These data are consistent with the previous findings (30). Despite this progress, we have not constructed the relationship between HDAC4 and survival; this work will be done in the future.

In summary, our data demonstrate that the accumulation of HDAC4 which was induced by fibrillar collagen matrices in the nucleus via co-localization of PP1α, leads to repressing the mRNA/protein of p21, and in turn promotes the proliferation and migration of epithelial ovarian cancer cells.

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