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HDAC1/DNMT3A-Containing Complex Is Associated with Suppression of Oct4 in Cervical Cancer Cells

Dongbo Liu[1,2#], Peng Zhou[1#], Li Zhang[1], Wei Gong[1], Gang Huang[1], Yingru Zheng[3]*, and Fengtian He[1]*

[1]*Department of Biochemistry and Molecular Biology, Third Military Medical University, Chongqing 400038, China; fax: +86-23-68752262; E-mail: hefengtian06@yahoo.com.cn; liu.db1980@yahoo.com.cn*
[2]*Depeartment 1 of State Key Laboratory of Trauma, Burns, and Combined Injury, Daping Hospital and Institute of Surgery Research, 10 Changjiang Road, Daping, Chongqing 400042, China*
[3]*Department of Obstetrics and Gynecology, Daping Hospital and Institute of Surgery Research, 10 Changjiang Road, Daping, Chongqing 400042, China; E-mail: zhengyingru65@tom.com*

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Abstract—Octamer-binding transcription factor 4 (Oct4), an important embryonic transcriptional factor, is highly expressed in several tumors and is considered as a hallmark of cancer stem cells. Knowledge about the expression and regulatory mechanisms of Oct4 can contribute to the treatment of cancers. As for cervical cancer, however, details remain obscure about Oct4 expression and its regulatory mechanism. In this study, we found that the level of Oct4 in human papillomavirus 16 (HPV16)- positive cervical cancer cells (CaSki cells) was higher than that in HPV-negative cervical cancer cells (C-33A cells), whereas both the level of histone deacetylase 1 (HDAC1) and DNA methyltransferase 3A (DNMT3A) were lower in CaSki cells than those in C-33A cells. Treatment with valproic acid, an HDAC inhibitor, could significantly increase the expression of Oct4 in C-33A cells, but only slightly increased Oct4 in CaSki cells. Co-immunoprecipitation assays showed that HDAC1 and DNMT3A existed in a common complex. The co-immunoprecipitated DNMT3A or HDAC1 was dose-dependently decreased with valproic acid treatment. These results indicated that HDAC1/DNMT3A-containing complex is associated with the suppression of Oct4 in cervical cancer cells, and the activity of HDAC1 is required in the repression of Oct4.

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Octamer-binding transcription factor 4 (Oct4) plays a critical role in maintaining pluripotency and the self-renewal of embryonic stem cells, and thus it is treated as a hallmark of the embryonic stem cell [1]. Normally, the level of Oct4 is slightly expressed in somatic cells with the exception of some cells in the germ line. However, the expression of Oct4 is upregulated in several tumors and tumor cell lines [2-4], which contributes to the malignant transformation of normal stem cells, proliferation, and therapeutic resistance of cancer cells [2, 4, 5]. Downregulation of Oct4 leads to the apoptosis of cancer cells [6]. Therefore, Oct4 is also considered as a hallmark of cancer stem cells (CSC). As for cervical cancer, although human papillomavirus type 16 (HPV16) is a key factor inducing carcinogenesis of cervical tissue by disturbing the expression or function of key proteins such as p53 or Rb [7-11], the multistep process of cervical carcinogenesis involves many other factors. A recent study confirmed that cervical cancers contain a subpopulation of stem-like cancer cells expressing Oct4 protein, which suggests that Oct4 may be associated with the initiation of cervical carcinogenesis [12].

Oct4 is regulated by many factors such as orphan receptor-regulated transcription [13, 14], miRNAs regulation [15, 16], and posttranslational modifications [17].

Abbreviations: CSC, cancer stem cells; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's media; DNMT, DNA methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPV, human papillomavirus; IP, immunoprecipitation; Oct4, Octamer-binding transcription factor 4; qPCR, quantitative real time polymerase chain reaction; RT-PCR, semiquantitative reverse transcriptional polymerase chain reaction; VPA, valproic acid.

[#] Contributed equally to this work.

* To whom correspondence should be addressed.

RT-PCR primers for DNMT3A, Oct4, HDAC, GAPDH, and qPCR primers for Oct4 and GAPDH

	Forward primer (5′→3′)	Reverse primer (5′→3′)
RT-PCR		
DNMT3A	GGATAGCCAAGTTCAGCAAAGT	TGGACTGGGAAACCAAATACC
Oct4	AGCCCTCATTTCACCAGGCC	CAAAACCCGGAGGAGTCCCA
HDAC1	TTTGCTGCTCAACTATGGTCTC	TCGTCATCAATCCCGTCTCG
GAPDH	CTCTCTGCTCCTCCTGTTCGAC	TGAGCGATGTGGCTCGGCT
qPCR		
Oct4	CGATCAAGCAGCGACTATG	GCCAGAGGAAAGGACACT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Recently, epigenetic regulation including DNA methylation [18, 19] and histone deacetylation [20] are emerging as possible underlying mechanisms of Oct4 expression. Several studies have shown that a high level of DNA methylation catalyzed by DNA methyltransferases (DNMTs) in the promoter or/and the first exon of Oct4 leads to the silencing of Oct4 expression, while demethylation in these regions can reverse the suppression of Oct4 expression [21]. On the other hand, histone deacetylation can also repress Oct4 by creating a compact chromatin state adjacent to the target gene and rendering it inaccessible for the binding of transcriptional factors [22]. However, our previous study confirmed that the degree of DNA methylation did not affect the transcription of Oct4 in HeLa (HPV18-positive), CaSki (HPV16-positive), and C-33A cells (HPV-negative) [23]. We still did not know the details of its epigenetic regulatory mechanism in cervical cancer cells.

In this study, we observed that there was a positive correlation between the expression of HDAC1 and the expression of DNMT3A in CaSki and C-33A cells. Moreover, the levels of these two factors were inversely correlated with the level of Oct4 in CaSki and C-33A cells. Furthermore, we demonstrated that HDAC1 and DNMT3A exist in a common complex, and the co-immunoprecipitated DNMT3A or HDAC1 is dose-dependently decreased with an inhibitor of HDAC (valproic acid, VPA) treatment. Meanwhile, the expression of Oct4 was upregulated in response to VPA treatment. These results indicate that a complex containing HDAC1/DNMT3A is associated with the suppression of Oct4 in cervical cancer cells, and the activity of HDAC1 is required in the repression of Oct4.

MATERIALS AND METHODS

Cell culture. CaSki and C-33A cells were cultured in high glucose Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM).

RNA extraction and RT-PCR. Total RNA was isolated from two cervical cancer cell lines in the log phase of growth with TRIzol reagent (Invitrogen, USA), and the first-strand cDNA was synthesized using Oligo $dT_{(18)}$ primer and reverse transcriptase M-MLV (TaKaRa, Japan). PCR of *Oct4* was performed as follows: 95°C for 2 min, (94°C for 30 sec, 56.8°C for 35 sec, 72°C for 50 sec) – 25 cycles. The amplification of *DNMT3A* and *HDAC1* were similar to the amplification of *Oct4* except for annealing temperature (56.9°C for *DNMT3A*, 60°C for *HDAC1*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (annealing temperature of 57°C). The primer pairs set for RT-PCR are listed in the table.

Preparation of nuclear extracts and Western blot. The preparation of nuclear extracts and Western blot were done according to protocols which are on the website of the Abcam company (http://www.abcam.com/index.html?pageconfig=protocols&intAbID=16632). The following primary antibodies were used: mouse anti-DNMT1 (1 : 1000; Abcam, USA); rabbit anti-DNMT3A, rabbit anti-DNMT3B (1 : 800; Santa Cruz, USA); rabbit anti-Oct4 (1 : 800; Abcam); rabbit anti-Lamin B1 (1 : 2000; Santa Cruz); rabbit anti-HDAC1 (1 : 800; Santa Cruz). Appropriate secondary antibodies were used to detect the target proteins.

VPA treatment and quantitative real time PCR (qPCR). To analyze the influence on the expression of Oct4 exerted by the inhibition of HDAC, C-33A cells were plated onto flat-bottomed 12-well plates (for qPCR) or 6-well plates (for IP), and then different concentrations of VPA (Sigma, USA) were added in culture medium of the corresponding wells. An equal volume of DMSO was added as the control. After 48 h, total RNA of all samples from the 12-well plates were separately extracted for the examination of transcripts of Oct4 by

qPCR. The qPCR was performed using an iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and associated software with the following cycling parameters: 30 sec at 98°C, followed by 40 cycles of 10 sec at 95°C, 10 sec at 60°C, and 20 sec at 72°C. GAPDH was used as the control. The primer pair set for real-time PCR are listed in the table.

Co-immunoprecipitation (Co-IP). Nuclear fractions were extracted as described above, and the protein content was normalized using a Bradford assay kit (Beyotime, China). We firstly added 2 μg of anti-HDAC1 antibody into 500 μg of the protein and gently rotated at 4°C overnight. The immunocomplex was captured by adding 30 μl protein A+G agarose beads (Beyotime) and gently rotating at 4°C for 4 h. Then the mixture was centrifuged at 1000*g* for 5 min at 4°C, and the supernatant was discarded. The precipitate was washed for five times with ice-cold RIPA buffer, resuspended in 1× SDS sample buffer, and boiled for 5 min to dissociate the immunocomplex from the beads. The supernatant was collected by centrifugation and subjected to Western blot with anti-DNMT3A antibody. Then we used anti-DNMT3A antibody to precipitate the protein complex, and examined the precipitated proteins with anti-HDAC1 antibody. Simultaneously, we used the same antibody to both precipitate and examined the immunocomplex to be sure that the Co-IP system was reliable.

Statistical analysis. Statistical analyses were performed using SPSS 13.0. All data are expressed as means ± SD unless otherwise stated and statistical differences between groups were determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The levels of HDAC1 and DNMT3A are inversely correlated with the level of Oct4 in both CaSki and C-33A cells. We first simultaneously detected the level of both mRNA and protein of Oct4, HDAC1, and DNMT3A by

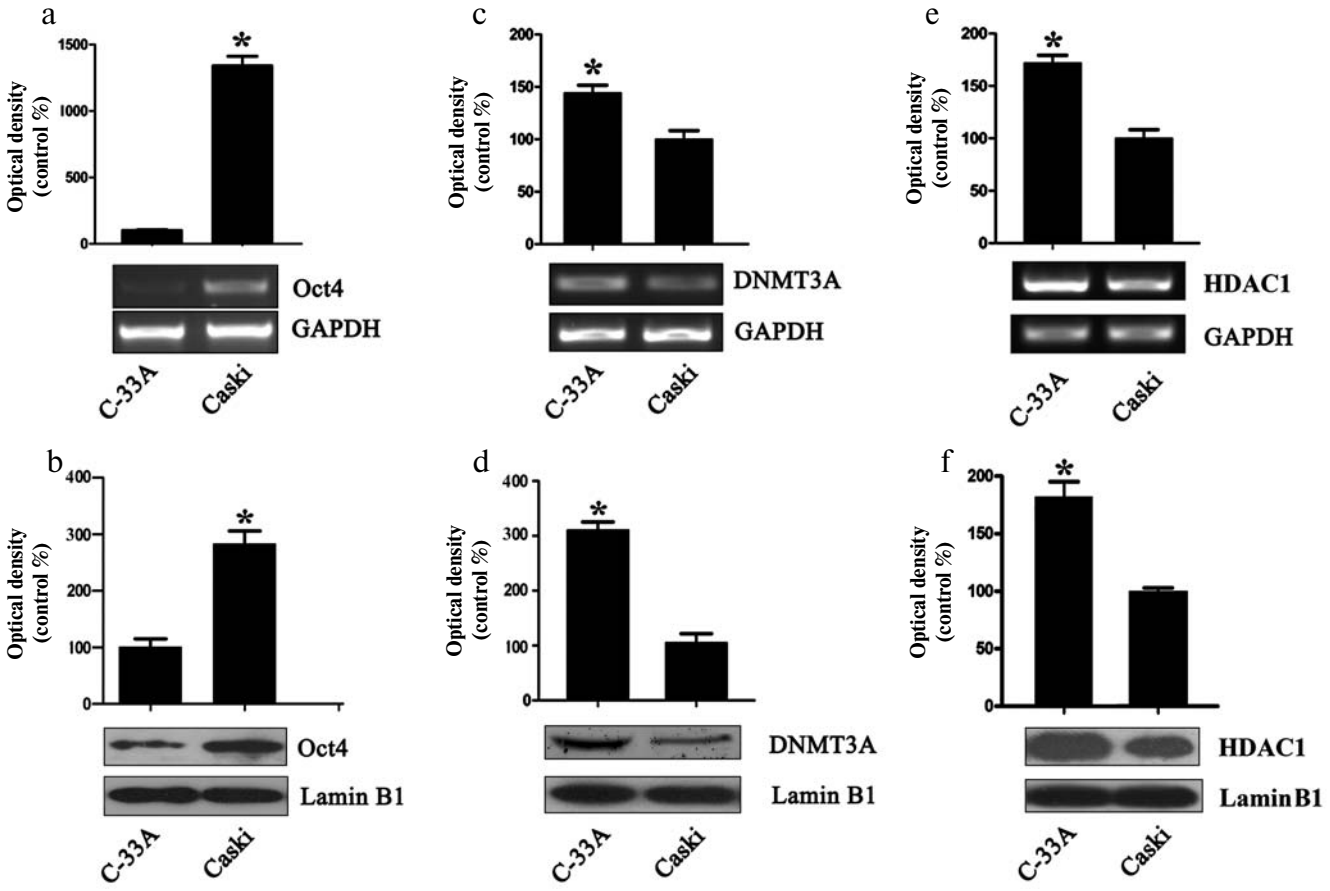

Fig. 1. There is a negative correlation between the level of Oct4 and the levels of DNMT3A and HDAC1 in CaSki cells and C-33A cells. a, b) Both the mRNA level and protein level of Oct4 in CaSki cells are higher than that of C-33A cells, which were analyzed by RT-PCR and Western blot. c, d) Both the mRNA level and protein level of DNMT3A negatively correlate with the level of Oct4 in C-33A cells and CaSki cells, which were identified by RT-PCR and Western blot. e, f) Analysis of RT-PCR and Western blot for HDAC1 in two cell lines, which also shows a negative correlation with the level of Oct4 in these two cell lines. GAPDH and Lamin B1 were used as control of RT-PCR and Western blot, respectively (*, significant difference).

RT-PCR and Western blot. The results showed that the level of Oct4 in CaSki cells was higher than that in C-33A cells (Figs. 1a and 1b), the level of DNMT3A in CaSki cells was lower than that in C-33A cells (Figs. 1c and 1d), and the HDAC1 level in CaSki cells was lower than that in C-33A cells (Figs. 1e and 1f). These results indicated that the levels of HDAC1 and DNMT3A were inversely correlated with the level of Oct4 in both CaSki and C-33A cells.

The activity of HDAC1 is required for repression of Oct4. As we had confirmed that DNA methylation was not associated with the regulation of Oct4 expression [23], we continued to explore in this study whether histone deacetylation could be a regulation mechanism for the expression of Oct4 in cervical cancer cells. As shown in Figs. 2a and 2b, exposure to increasing concentration of an inhibitor of HDAC (valproic acid, VPA) for 48 h significantly induced the expression of Oct4 in C-33A cells in a dose-dependent manner. This trend of dose dependence was also observed after 12, 24, and 96 h of treatment (data not shown). The expression of Oct4 in CaSki cells was also upregulated by VPA, but this was not as significant as that in C-33A cells (shown in Figs. 2c and 2d). These results confirm that the activity of HDAC1 is required in the suppression of Oct4 in cervical cancer cells.

HADC1 and DNMT3A exist in a common complex. As shown in Figs. 3a and 3b, the Co-IP experiment revealed that HDAC1 and DNMT3A exist in a common complex, and the level of the complex in C-33A cells is

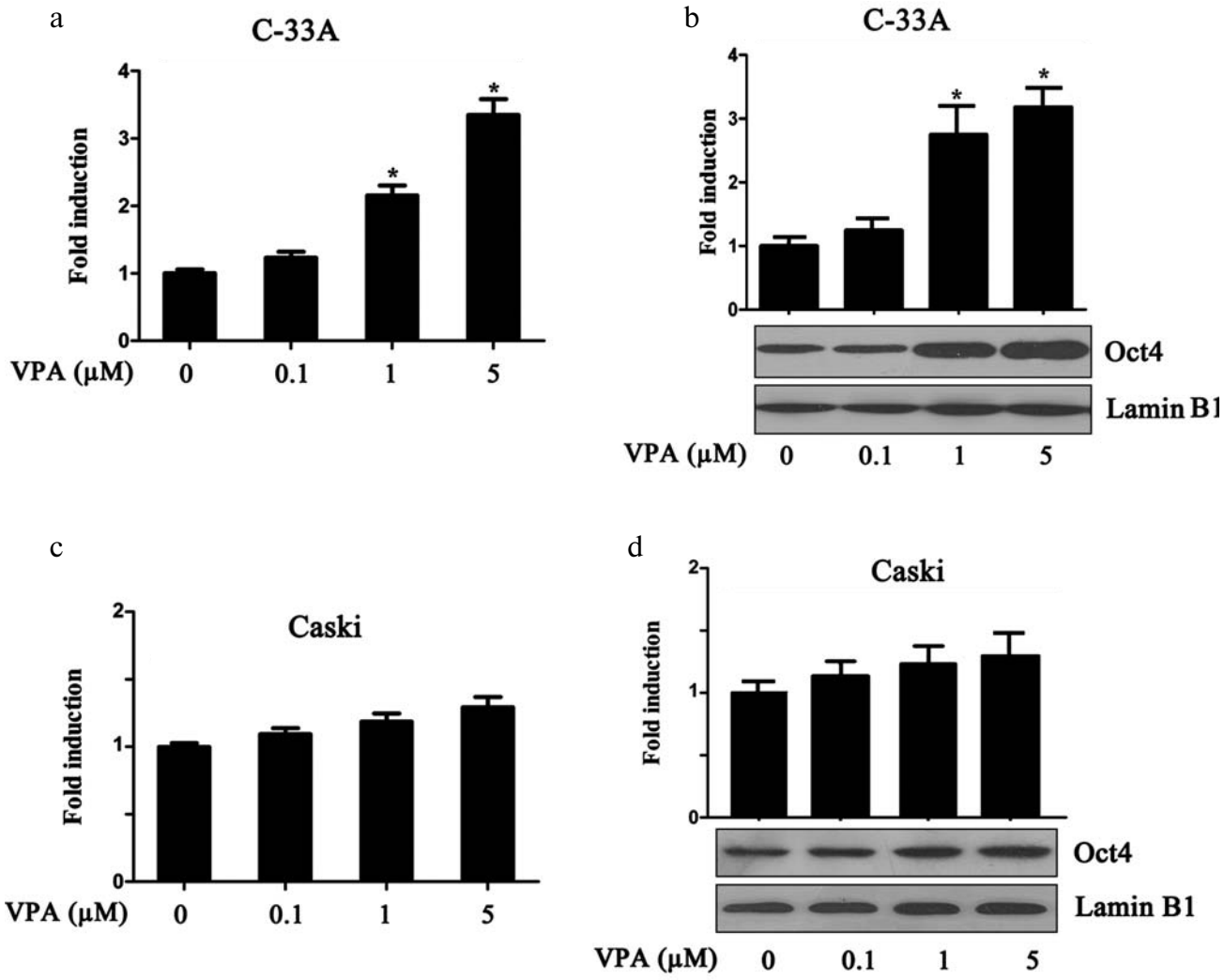

Fig. 2. The expression of Oct4 is upregulated by VPA treatment. a) Transcription of Oct4 was significantly induced in a dose-dependent manner after C-33A cells were treated with VPA for 48 h. b) The protein level of Oct4 was also upregulated in a dose-dependent manner after C-33A cells were treated with VPA for 48 h. GAPDH and Lamin B1 were used as control of qPCR and Western blot, respectively. c) The transcription of Oct4 was induced by VPA for 48 h in CaSki cells, which was examined by qPCR. d) The result of Western blot for Oct4 in CaSki cells is consistent with the results of qPCR (*, significant difference).

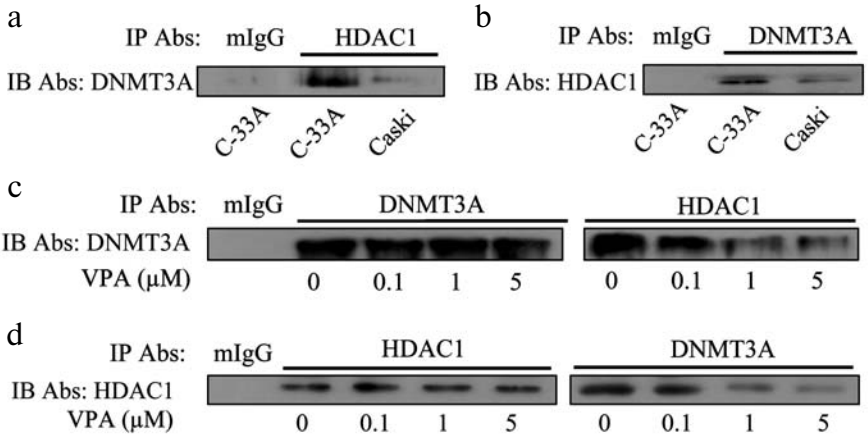

Fig. 3. HDAC1 and DNMT3A exist in a common complex, which is decreased by VPA treatment in a dose-dependent manner. a, b) The level of complex containing HDAC1 and DNMT3A in C-33A cells was higher than that in CaSki cells. Anti-HDAC1 antibody (a) or anti-DNMT3A (b) was used to precipitate the complex. The precipitated products were separated on 10% SDS-PAGE and blotted with anti-DNMT3A (a) or anti-HDAC1 (b). Mouse IgG was used as negative control (left). c, d) VPA decreased the level of HDAC1/DNMT3A-containing complex in a dose-dependent manner. C-33A cells were treated with increasing concentrations of VPA. The lysates were subjected to immunoprecipitation with mouse IgG, anti-HDAC1, or anti-DNMT3A antibodies. The immunoprecipitated proteins were separated with 10% SDS-PAGE gel and blotted with anti-DNMT3A (right panel of (c)) or anti-HDAC1 antibodies (right panel of (d)). At the same time, the same antibody (anti-DNMT3A or anti-HDAC1) was used in both immunoprecipitation and Western blot to ensure that this experimental system is reliable (left panels of (c) and (d)).

higher than that in CaSki cells. Moreover, this complex can be decreased by VPA treatment in a dose-dependent manner in C-33A cells (Figs. 3c and 3d).

DISCUSSION

Oct4 is responsible for the maintenance of pluripotency and self-renewal of stem cells, and its level decreases with the normal differentiation of stem cells. The theory of CSCs states that CSCs originate from the malignant transformation of normal stem cells in tissues. The abnormal upregulated Oct4 expression blocks the normal procedure of differentiation, followed by the malignant transformation from normal stem cells to CSCs. In addition to the functional Oct4 protein coded from full length mRNA, six Oct4 pseudogenes (Oct4-pg) [24-26] and three variants of Oct4 have also been reported in other cancer cells [27]. The molecular weight of Oct4-pg1 protein is near that of functional Oct4 protein, whereas the other five Oct4-pgs had significantly different molecular weight from the functional Oct4 protein. Therefore, Western blot could help us to identify the functional Oct4 protein. The antibody used in our study has not been reported to react with other Oct4-pgs proteins in cancer cells until now. Moreover, Oct4-pgs have not been found in CaSki and C-33A cells.

As for the variants of Oct4, only the full-length Oct4 variant could be translated into a functional Oct4 protein, whereas the other two truncated Oct4 variants were translated into a similar but non-functional Oct4 protein [28]. We used the primer pair designed by Liedtke et al., which had been confirmed to specially amplify the full length Oct4 variant [24], to detect the level of Oct4 mRNA. Therefore, we were sure that the mRNA and protein of Oct4 detected by us were true functional proteins.

We found that the expression of HDAC1 and DNMT3A are inversely correlated with the expression of Oct4 in CaSki and C-33A cells. However, our previous study had demonstrated that the level of DNMT3A was not consistent with the degree of DNA methylation, and the status of DNA methylation in regulatory regions of Oct4 was not associated with the expression of Oct4 [23]. These results indicated that DNMT3A did not regulate the expression of Oct4 via its methyltransferase activity. Besides DNA methylation, histone deacetylation could also inhibit the expression of Oct4. HDAC1 is an important member of the HDAC family, and several studies have confirmed that the inhibition of HDAC1 can promote the transcription of Oct4 [29, 30]. To ascertain whether HDAC1 can repress the expression of Oct4 in cervical cancer cells, CaSki and C-33A cells were treated with the HDAC inhibitor VPA, and the results showed that the expression of Oct4 in C-33A cells was significantly induced in a dose-dependent manner, whereas the upregulation of Oct4 in CaSki cells was not so significant. So we thought that the activity of HDAC1 was required in repressing the expression of Oct4 and higher level of HDAC1 in C-33A cells meant higher activity of HDAC1 worked on the inhibition of Oct4 expression. Therefore, the activity of HDAC1 was increasingly inhibited with increasing concentration of VPA, and the consequent

upregulation of Oct4 in C-33A cells appeared in a dose-dependent manner. However, the inherent level of HDAC1 in CaSki cells was so low that the inhibition of HDAC1 by VPA is not as obvious, and thus the following change in Oct4 expression is not as significant as that in C-33A cells.

Fuks et al. reported that HDAC1 represses RP58 via its association with DNMT3A in U2OS cells [31], and that this suppression did not require *de novo* methyltransferase activity but did require HDAC activity. In the present study, the Co-IP results also show that HDAC1 and DNMT3A exist in a common complex, and the level of this complex in C-33A is higher than that in CaSki cells. These results suggest that there might be a similar epigenetic regulatory mode of Oct4 in cervical cancer cells like the regulatory mode of RP58 in U2OS cells. Because both the levels of HDAC1 and DNMT3A were lower in CaSki cells, we chose C-33A cells as an object to observe whether this complex containing HDAC1 and DNMT3A would be affected by the inhibition of HDAC1 activity. As an inhibitor of HDAC1, VPA just inhibits HDAC1 activity but does not affects its expression, so after treatment with different concentrations of VPA, the HDAC1 levels have no significant changes in Western blot (as shown in left panel of Fig. 3d). Meanwhile, the results also showed the complex precipitated by anti-HDAC1 antibody or anti-DNMT3A antibody was decreased by VPA in a dose-dependent manner (as shown in right panels of Figs. 3c and 3d). These results indicate that the suppression of Oct4 in C-33A cells is at least partly due to the HDAC1/DNMT3A-containing complex, and the suppression could be attenuated by inhibiting the activity of HDAC1.

Previous studies confirmed that oncoprotein E7 of HPV16 can change the inherent functional pattern of HDAC1 through interacting with it, and induce the expression of Oct4 [32, 33]. Our results suggest that the interaction between oncoprotein E7 of HPV16 and HDAC1 might result in a dissociation of the complex consisting of HDAC1 and DNMT3A, which leads to the increase in Oct4 expression.

In summary, we demonstrated that the levels of HDAC1 and DNMT3A are inversely correlated with the level of Oct4 in both HPV16 positive and HPV negative cervical cancer cells. Treatment with HDAC inhibitor VPA increases the expression of Oct4 in C-33A cells. Moreover, HDAC1 and DNMT3A exist in a common complex, which is associated in the suppression of Oct4 in cervical cancer cells. More studies are warranted regarding the molecular mechanism by which HDAC1/DNMT3A-containing complex regulates Oct4 expression in cervical cancer.

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