Application of the histone deacetylase inhibitors for the treatment of endometriosis: histone modifications as pathogenesis and novel therapeutic target

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**BACKGROUND:** Accumulating evidence suggests that various epigenetic aberrations play definite roles in the pathogenesis of endometriosis. We investigated the histone acetylation status in endometriosis and the application of the histone deacetylase inhibitors (HDACIs) for the treatment of endometriosis.

**METHODS:** The levels of acetylated histones in the endometriotic cyst stromal cells (ECSCs) and normal endometrial stromal cells (NESCs) were evaluated. The effects of the HDACIs on cell proliferation, the cell cycle, apoptosis of ECSCs and NESCs, and the expression of genes related to these cellular events were investigated. The effects of HDACIs on histone acetylation in chromatin of the promoter region of the cell cycle regulatory genes in ECSCs were also investigated.

**RESULTS:** The acetylated histone levels were significantly lower in ECSCs than in NESCs ($P < 0.025$). HDACIs inhibited cell proliferation and induced cell cycle arrest and apoptosis of ECSCs. The effects of HDACIs on NESCs were marginal or weak. These HDACIs induced an accumulation of acetylated histones in total cellular chromatin and in the promoter regions of the p16$\text{INK4a}$, p21$\text{Waf1/Cip1}$, p27$\text{Kip1}$ and cycle checkpoint kinase 2 genes in ECSCs. HDACIs induced the protein expression of these cell cycle regulators and suppressed the protein expression of Bcl-2 and Bcl-X$_L$ in ECSCs.

**CONCLUSIONS:** The present findings demonstrated that aberrant histone modifications are present in endometriosis and that HDACIs reactivated epigenetically silenced genes, resulting in the suppression of cell proliferation, induction of cell cycle arrest and apoptosis of ECSCs. HDACIs are therefore promising agents for the treatment of endometriosis.

**Key words:** endometriosis / epigenetics / histone modification / histone deacetylase inhibitor

**Introduction**

Endometriosis, a common, benign, estrogen-dependent tumor-like disease affecting 3–10% of women of reproductive age, is characterized by the ectopic growth of endometrial tissue, which is found primarily in the peritoneum, ovaries and rectovaginal septum (Giudice and Kao, 2004). Women suffering from endometriosis may present with chronic pelvic pain, dysmenorrhea, dyspareunia and/or subfertility (Giudice and Kao, 2004).

Various therapies have been used to treat endometriosis, including both surgical and medical strategies. Current medical treatments aim to inhibit the growth of endometriotic implants by suppressing ovarian steroids and inducing a hypoestrogenic state, and are effective in relieving endometriosis-associated pain (Practice Committee of the American Society for Reproductive Medicine, 2004; Nasu et al., 2009). However, treatments for endometriosis that aim to lower circulating estradiol concentrations can be used only for a limited time because of unacceptable side effects (Lessey, 2000; Bulun et al., 2005).
In addition, high recurrence rates of up to 45% after the completion of medical treatments remain a significant problem (Bergqvist, 2000). Therefore, novel therapeutic strategies are necessary to improve the clinical management of patients with endometriosis.

Epigenetics refers to the stable inheritance of phenotypes of cells and organisms without changes in DNA sequence or DNA content. The epigenetic phenotypes are conferred via nuclear processes, such as DNA methylation and chromatin modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, biotinylation, ribosylation and isomerization of histones), and underlie the regulation of all genome functions, including gene expression, DNA replication and genome stability (Turner, 2002; Jaenisch and Bird, 2003). Epigenetic processes are known to be involved in development, homeostasis, disease and aging, and are responsible for phenomena such as X chromosome inactivation and genomic imprinting (Robertson and Wolffe, 2000; Rodenhiser and Mann, 2006). Epigenetic alterations reported to date in endometriotic cells include the gene methylation of progesterone receptor (PR)-B (Wu et al., 2006), steroidogenic factor-1 (SF-1) (Xue et al., 2007b) and estrogen receptor (ER)-β (Xue et al., 2007a).

Acetylation levels of histones are controlled by a balance between histone acetyltransferases and histone deacetylases (HDACs). Histone acetyltransferases transfer acetyl groups from acetyl-CoA to lysine residues on the aminoterminal region of histones and activate gene transcription. Conversely, HDACs restore the positive charge on lysine residues by removing the acetyl groups and prevent transcription. HDACs comprise large multiprotein complexes that target promoter sites through their interaction with sequence-specific transcription sites (Takai and Narahara, 2007). HDAC inhibitors (HDACIs) can inhibit cell proliferation, induce cell differentiation and cell cycle arrest, and stimulate apoptosis of various cell types (Takai and Narahara, 2007). Several classes of HDACs have been identified, including (a) organic hydroxamic acids [e.g. Trichostatin A and suberoyl anilide bishydroximine (SAHA)], (b) short-chain fatty acids [e.g. butyrates and valproic acid (VPA)], (c) benzamides (e.g. MS-275), (d) cyclic tetrapeptides (e.g. trichopin) and (e) sulfonamide anilides (Takai and Narahara, 2007). Recently, Guo and his colleagues demonstrated the effects of Trichostatin A on proliferation, invasiveness and nuclear factor-κB activation in endometriotic cells in vitro (Wu et al., 2007a, 2010) and in vivo (Lu et al., 2010). However, the mechanisms of action of HDACIs on endometriotic cells have yet to be elucidated.

The current study was designed to define the epigenetics involved in the pathogenesis of endometriosis and the biologic and therapeutic effects of HDACIs in the treatment of endometriosis. At the start of this research, we evaluated the levels of acetylated histones in human endometriotic cyst stromal cells (ECSCs) in primary culture in comparison with those in human normal endometrial stromal cells (NESC). We focused particularly on VPA, SAHA and apicidin, which are recognized as the least toxic HDACIs. We examined whether VPA, SAHA and apicidin were able to mediate cell growth inhibition, cell cycle arrest, apoptosis and the expression of genes related to these cellular events in ECSCs and NESC. In addition, we examined whether HDACIs were able to induce the accumulation of acetylated histones in the chromatin of the promoter region of the p16INK4a, p21Waf1/Cip1, p27Kip1 and cell cycle checkpoint kinase 2 (chk2) genes in ECSCs.

Materials and Methods

Isolation procedure for ECSC and NESC and cell culture conditions

Endometriotic tissues were obtained from premenopausal patients who had undergone salpingo-oophorectomy or ovariectomy for ovarian endometriotic cysts (n = 23, aged 28–40 years). NESC were isolated from premenopausal patients who had undergone hysterectomies for subserous leiomyoma and had no evidence of endometriosis (n = 11, aged 37–43 years). None of the patients had received any hormonal treatments for at least 2 years prior to operation. All the specimens were diagnosed as being in the mid- to late-proliferative phases according to the pathological observation and/or the menstrual cycles. This study was approved by the institutional review board of the Faculty of Medicine, Oita University, and written informed consent was obtained from all patients.

ECSCs were isolated from ovarian endometriotic tissues by enzymatic digestion with collagenase as previously described (Nishida et al., 2004). Briefly, the tissues were minced in Hank’s balanced salt solution (Gibco-BRL, Gaithersburg, MD, USA) and digested with 0.5% collagenase (Gibco-BRL) in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL) at 37°C for 40 min. The dispersed cells were filtered through a 70-μm nylon mesh to remove undigested tissue pieces. The filtrated fraction was then further separated from the epithelial cell clumps by differential sedimentation at unit gravity, as follows. The cells were resuspended in 2 ml DMEM and layered slowly over 10 ml of the medium in a centrifuge tube. Sealed tubes were placed in an upright position at 37°C in 5% CO2 in air for 30 min. After sedimentation, the top 8 ml of the medium was collected. Finally, the medium containing stromal cells was filtered through a 40-μm nylon mesh. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to the culture dishes for 30 min at 37°C, followed by the removal of non-anchoring epithelial cells. NESC were also isolated by digesting the endometrial tissue fragments with 0.5% collagenase as previously described (Nishida et al., 2004). Isolated ECSCs and NESC were cultured in DMEM supplemented with 100 IU/ml of penicillin, 50 mg/ml of streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (all obtained from Gibco-BRL) at 37°C in 5% CO2 in air.

ECSCs and NESC in the monolayer culture after the third passage were >99% pure, as determined by immunocytochemical staining with antibodies to vimentin, CD10, cytokeratin, factor VIII and leukocyte common antigen (Nishida et al., 2004), and were used for the following experiments. Each experiment was performed in triplicate and repeated at least five times.

Assessment of the effects of HDACIs on proliferation of ECSCs and NESCs

Cell proliferation of ECSCs and NESCs after HDACI treatment was determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation using an enzyme-linked immunosorbent assay (Cell Proliferation ELISA, Roche Diagnostics GmbH, Penzberg, Germany) as previously described (Nishida et al., 2005). We placed 1 × 104 cells in DMEM supplemented with 10% FBS into each well of a 96-well flat-bottomed microplate (Corning, New York, NY, USA), which was incubated overnight at 37°C. The medium was then removed, and the cells were incubated for 72 h with 100 μM of experimental medium containing various concentrations of VPA (1–16 mM), SAHA (1.25–20 μM) or apicidin (0.1–100 μM). We then added 10 μl of BrdU (10 mM) to each well and incubated the samples for 2 h. BrdU incorporation was then evaluated according to the manufacturer’s protocols. Cell proliferation was evaluated by measuring the absorbance at 450 nm. Data were calculated as the ratio
of the values obtained for the HDACI-treated cells to those for the corresponding untreated controls.

Assessment of the effects of HDACIs on the cell cycle of ECSCs and NESCs by flow cytometry

The effects of HDACIs on the cell cycle of ECSCs and NESCs were analyzed by flow cytometry after 72 h of culture with or without VPA, SAHA or apicidin, as previously described (Nishida et al., 2005). Briefly, ECSCs and NESCs were plated at a density of 1 × 10^4 cells per 10 cm dish and incubated overnight at 37°C. The cells were further cultured for 8 h in the presence of VPA (8 mM), SAHA (5 μM) or apicidin (10 μM). Formaldehyde was then added to a final concentration of 1% to the cells, which were incubated at room temperature for 10 min. Unreacted formaldehyde was quenched by the addition of 10× glycine. The medium was removed, and the cells were washed twice with ice-cold PBS containing protease inhibitor cocktails, resuspended in 0.5 ml of cell lysis buffer and incubated on ice for 15 min. The supernatant was removed and the cell pellet was resuspended in nuclear lysis buffer. Nuclear lysates were sonicated to shear DNA. Debris was removed from the samples by centrifugation for 10 min at 12,000 g at 4°C. An aliquot of the shear-extracted cell cycle analysis was then subjected to PCR for the promoter region of the p16INK4a, p21Waf1/Cip1, p27Kip1, and chk2 genes (these cell cycle regulators were chosen as representative targets of HDACIs). The sequences of the primers and PCR conditions are shown in Table I. PCR products were analyzed by electrophoresis on a 1.2% agarose/ethidium bromide gel.

Western blot analysis

The levels of histone acetylation in ECSCs and NESCs, and the effects of HDACIs on the histone acetylation and expression of apoptosis-related proteins [B-cell lymphoma/leukemia (Bcl)-2, Bcl-XL, and cleaved caspase-9] and cell-cycle-related proteins (p16INK4a, p21Waf1/Cip1, p27Kip1, and chk2) in ECSCs were investigated by Western blot analysis as previously described (Takai et al., 2004a; Nishida et al., 2005). Subconfluent ECSCs were cultured for 24–72 h with or without the addition of VPA (8–16 mM), SAHA (5–10 μM) and apicidin (1–10 μM). The cells were then washed with PBS and whole cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris–HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, and 0.1% phenylmethylsulfonyl fluoride). The samples were centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was collected. The cellular protein was also extracted from untreated NESCs. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL, USA). The whole cell protein extract was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis using 20% acrylamide gel under reducing conditions. After transfer to an Immobilon-P transfer membrane (Millipore), the protein was stained with Ponceau S (Sigma) to verify uniform loading and transfer. The membranes were blocked overnight with 5% skim milk (Becton-Dickinson, Franklin Lakes, NJ, USA) in Tris-buffered saline with Tween 20 (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (TBS-T) and subsequently incubated with primary antibodies [acetylated histone H3 (Lys9) (Cell Signaling, Beverly, MA, USA), acetylated histone H4 (Lys12) (Millipore), Bcl-2 (E17; Epitomics, Inc.,
Histone H3 and acetylated histone H4 proteins in NESCs were analyzed using the public domain Image program ImageJ. As shown in Fig. 1A, lower levels of acetylated histone H3 and acetylated histone H4 proteins in ECSCs and NESCs were evaluated by Western blot analysis. As shown in Fig. 2, these HDACIs significantly inhibited BrdU incorporation into ECSCs in a dose-dependent manner. BrdU incorporation decreased to 28.7 ± 1.2% of the initial value after treatment with VPA at 8 mM and further decreased to 15.4 ± 1.6% after treatment with VPA at 16 mM. VPA showed the strongest effect among these three HDACIs. BrdU incorporation decreased to 67.9 ± 4.7% of the initial value after treatment with SAHA at 1.25 μM and further decreased to 47.8 ± 1.2% after treatment with SAHA at 20 μM. SAHA showed the weakest effect among these three HDACIs. BrdU incorporation decreased to 70.2 ± 4.1% of the initial value after treatment with apicidin at 0.01 mM and further decreased to 29.5 ± 3.0% after treatment with apicidin at 100 μM. The effects of VPA and SAHA on proliferation of NESCs were marginal, whereas BrdU incorporation of NESCs decreased to 77.1 ± 4.1% of the initial value after treatment with apicidin at 0.1 mM and further decreased to 40.8 ± 4.1% after treatment with apicidin at 100 μM. The inhibitory effect of apicidin on proliferation of NESCs was weak in comparison with that of ECSCs.

### Statistical analysis

Data were calculated as percentages relative to the corresponding untreated controls, presented as the mean ± SD, and appropriately analyzed by the Mann–Whitney U-test and the t-test with Bonferroni correction with Sigmaplot 11.2 (Systat Software, Inc., San Jose, CA, USA). Values of *P* < 0.05 were considered to be statistically significant.

### Results

#### Decreased expression of acetylated histones in ECSCs

As an initial step in the present study, the protein levels of acetylated histone H3 and acetylated histone H4 in untreated ECSCs and untreated NESCs were evaluated by Western blot analysis. As shown in Fig. 1A, lower levels of acetylated histone H3 and acetylated histone H4 protein were detected in untreated ECSCs in comparison with untreated NESCs. When the relative expression of acetylated histone H3 and acetylated histone H4 proteins in NESCs were defined as 100%, the relative expression of these acetylated histones in ECSCs was 53.4 ± 5.8 and 48.3 ± 3.9%, respectively (Fig. 1B and C). These findings prompted us to examine whether epigenetic alterations with histone modifications might occur in endometriosis. To this end, we conducted the following experiments.

#### Inhibition of the cell proliferation of ECSCs and NESCs by HDACIs

The effects of VPA, SAHA and apicidin on the cell proliferation of ECSCs and NESCs were investigated by a BrdU incorporation assay. As shown in Fig. 2, these HDACIs significantly inhibited BrdU incorporation into ECSCs in a dose-dependent manner. BrdU incorporation decreased to 28.7 ± 1.2% of the initial value after treatment with VPA at 8 mM and further decreased to 15.4 ± 1.6% after treatment with VPA at 16 mM. VPA showed the strongest effect among these three HDACIs. BrdU incorporation decreased to 67.9 ± 4.7% of the initial value after treatment with SAHA at 1.25 μM and further decreased to 47.8 ± 1.2% after treatment with SAHA at 20 μM. SAHA showed the weakest effect among these three HDACIs. BrdU incorporation decreased to 70.2 ± 4.1% of the initial value after treatment with apicidin at 0.01 mM and further decreased to 29.5 ± 3.0% after treatment with apicidin at 100 μM. The inhibitory effect of apicidin on proliferation of NESCs was weak in comparison with that of ECSCs.

#### Induction of cell cycle arrest of ECSCs and NESCs by HDACIs

The effects of VPA, SAHA and apicidin on the cell cycle of ECSCs and NESCs were determined by flow cytometry. As shown in Fig. 3A–D, culturing of ECSCs for 72 h in the presence of VPA (8 mM) resulted in an accumulation of these cells in the G0/G1 phase of the cell cycle (67.8 ± 1.1 to 78.4 ± 0.9%, *P < 0.0005*), with a concomitant decrease in the proportion of ECSCs in the S phase (13.3 ± 1.4 to 2.4 ± 0.9, *P < 0.0005*). Treatment of ECSCs with apicidin (1 μM) also resulted in an accumulation of these cells in the G0/G1 phase (64.5 ± 1.9 to 69.9 ± 2.2%, *P < 0.001*), with a concomitant decrease in the proportion of ECSCs in the S phase (5.8 ± 0.7 to 3.9 ± 0.6, *P < 0.0005*), with a concomitant decrease in the proportion of ECSCs in the S phase (15.7 ± 0.4 to 11.9 ± 1.9%, *P < 0.0005*), with a concomitant decrease in the proportion of ECSCs in the S phase (7.1 ± 1.5 to 1.3 ± 0.9, *P < 0.001*).

As shown in Fig. 3E–H, culturing of NESCs for 72 h in the presence of VPA (8 mM) resulted in an accumulation of these cells in the G0/G1 phase of the cell cycle (79.8 ± 0.7 to 87.2 ± 0.3%, *P < 0.0005*), with a concomitant decrease in the proportion of these cells in the S phase (10.4 ± 0.6 to 1.4 ± 0.2%, *P < 0.001*). On the other hand, treatment of NESCs with SAHA (5 μM) resulted in an accumulation of these cells in the G2/M phase (15.3 ± 0.9 to 32.3 ± 1.3%,
P < 0.0005), with a concomitant decrease in the proportion of NESCs in the S phase (25.8 ± 1.1 to 4.8 ± 1.5%, P < 0.0005). Interestingly, treatment of NESCs with apicidin (1 μM) also resulted in an accumulation of these cells in the G2/M phase (14.2 ± 0.7 to 30.7 ± 2.4%, P < 0.0005), with a concomitant decrease in the proportion of NESCs in the S phase (25.5 ± 0.7 to 5.5 ± 0.5%, P < 0.0005).

Enhanced expression of cell-cycle-related proteins in ECSCs by HDACIs

To analyze the underlying mechanisms of the HDACI-induced cell cycle arrest of ECSCs, the expression of cell-cycle-related proteins in these cells were evaluated. As shown in Fig. 4A.

Enhanced expression of cell-cycle-related proteins in ECSCs by HDACIs

To analyze the underlying mechanisms of the HDACI-induced cell cycle arrest of ECSCs, the expression of cell-cycle-related proteins in these cells were evaluated. As shown in Fig. 4A.
Figure 3  The effects of VPA, SAHA and apicidin on the cell cycle of ECSCs (A–D) and NESC (E–H). ECSCs and NESC were cultured for 72 h in the presence of VPA, SAHA and apicidin. VPA and apicidin induced the G0/G1 phase cell cycle arrest of ECSCs, with a concomitant decrease in the proportion of ECSCs in the S phase. In contrast, stimulation of ECSCs with SAHA resulted in the accumulation of these cells in the G2/M phase of the cell cycle, with a concomitant decrease in the proportion of ECSCs in the S phase. VPA induced the G0/G1 phase cell cycle arrest of NESCs, with a concomitant decrease in the proportion of NESCs in the S phase. In contrast, stimulation of NESCs with SAHA and apicidin resulted in the accumulation of these cells in the G2/M phase of the cell cycle, with a concomitant decrease in the proportion of NESCs in the S phase. *P < 0.001, **P < 0.0005 versus untreated controls (Bonferroni test).
Figure 3  Continued
VPA (8 mM), SAHA (5 μM) and apicidin (1 μM) induced the protein expression of p16INK4a, p21Waf1/Cip1, p27Kip1 and chk2.

### Induction of apoptosis of ECSCs and NESCs by HDACIs

The apoptotic effects of VPA, SAHA and apicidin on ECSCs and NESCs were assessed by evaluating the presence of internucleosomal DNA fragmentation with a Cell Death Detection ELISA assay. As shown in Fig. 5, apoptosis of ECSCs and NESCs was significantly induced by the addition of increasing amounts of VPA, SAHA and apicidin. The proportion of apoptotic cells in ECSCs and NESCs increased to 569.6 ± 4.6 and 305.7 ± 27.0% of the baseline value after treatment with VPA at 16 mM, respectively (P < 0.0005). The proportion of apoptotic cells in ECSCs and NESCs increased to 1201.0 ± 18.3 and 293.4 ± 39.7% after treatment with SAHA at 20 μM, respectively (P < 0.0005). The proportion of apoptotic cells in ECSCs and NESCs increased to 461.1 ± 3.5 and 231.4 ± 13.7% after treatment with apicidin at 100 μM, respectively (P < 0.0005).

SAHA showed the strongest effect on ECSCs among these three HDACIs. These three HDACIs showed mild effects on NESCs in comparison with ECSCs.

To assess the effects of HDACIs on the cell death (apoptosis and necrosis) of ECSCs and NESCs, VPA-, SAHA- and apicidin-treated ECSCs and NESCs were double-stained with annexin V and PI, and were analyzed using flow cytometry. As shown in Fig. 6A–D, VPA (8 mM), SAHA (10 μM) and apicidin (10 μM) induced a simultaneous...
increase in both the annexin V+/PI− fraction (early apoptotic) and the annexin V+/PI+ fraction (regarded as necrotic) in ECSCs. As shown in Fig. 6E–H, VPA (8 mM) induced an increase in the annexin V+/PI− fraction (early apoptotic) in NESCs, whereas SAHA (10 mM) and apicidin (10 μM) showed marginal effects on NESCs.

Enhanced expression of apoptosis-related proteins in ECSCs by HDACIs

To analyze the underlying mechanisms of the HDACI-induced apoptosis of ECSCs, the expression of apoptosis-related proteins in these cells was evaluated. As shown in Fig. 4B, VPA, SAHA and apicidin...
down-regulated the expression of Bcl-XL protein, an anti-apoptotic factor that shows enhanced expression in ECSCs (Nishida et al., 2005). VPA, SAHA and apicidin up-regulated the expression of the cleaved caspase-9 protein in ECSCs. SAHA and apicidin showed stronger effects than VPA. Interestingly, SAHA and apicidin also suppressed the expression of Bcl-2, an anti-apoptotic factor that shows enhanced expression in ECSCs (Nishida et al., 2005), whereas the levels of Bcl-2 protein in ECSCs remained unchanged after VPA treatment. Taken together, SAHA- and apicidin-induced apoptosis may be mediated by both Bcl-2 and Bcl-XL, whereas
VPA-induced apoptosis may be mediated by Bcl-X<sub>L</sub>, but not by Bcl-2. These findings suggest that different mechanisms of inducing apoptosis may be involved for these three HDACIs.

**Induction of histone acetylation in ECSCs by HDACIs**

To examine whether HDACIs modulate the acetylation of histones in ECSCs, we evaluated the global acetylation of histones H3 and H4 in these cells by western blot analysis. As shown in Fig. 4B, marginal levels of acetylated histone H3 and acetylated histone H4 were detected in unstimulated ECSCs. In contrast, the levels of acetylated histone H3 and acetylated histone H4 in ECSCs were markedly increased by the addition of VPA, SAHA and apicidin.

**Enhanced acetylation of promoter regions of the p16<sup>INK4a</sup>, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and chk2 genes in ECSCs by HDACIs**

To examine the effects of HDACIs on the acetylation of the promoter regions of the p16<sup>INK4a</sup>, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and chk2 genes in ECSCs, ChIP assays with antibodies against acetylated histone H3 and acetylated histone H4 were performed. ECSCs were cultured for 8 h in the presence of VPA (8 mM), SAHA (5 μM) and apicidin (10 μM). The immunoprecipitated DNAs were subjected to PCR using primers for the promoter regions of the p16<sup>INK4a</sup>, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and chk2 genes. VPA, SAHA and apicidin markedly increased the amounts of PCR products, suggesting that the acetylation of histones H3 and H4 was enhanced in the promoter region of the p16<sup>INK4a</sup>, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and chk2 genes (Fig. 7).

**Discussion**

In the present study, we demonstrated for the first time that the levels of acetylated histones H3 and H4 were significantly lower in unstimulated ECSCs in comparison with NESCs, suggesting that aberrant histone modifications are present in endometriosis. This initial finding encouraged us to evaluate the efficacy of HDACIs for the treatment of endometriosis. Our subsequent experiments demonstrated that HDACIs significantly inhibited the proliferation of ECSCs, and also induced significant levels of cell cycle arrest at the G0/G1 or G2/M phases and significant apoptosis of these cells. Interestingly, HDACIs showed marginal to weak effects on NESCs in comparison with ECSCs. Moreover, HDACI treatment significantly inhibited HDAC activity and resulted in the accumulation of acetylated histones H3 and H4 in total cellular chromatin and in the promoter regions of the p16<sup>INK4a</sup>, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and chk2 genes in these cells. Western blot analysis revealed increased protein levels of p16<sup>INK4a</sup>, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and chk2, suppression of Bcl-2 and Bcl-X<sub>L</sub>, protein levels, and activation of caspase-3 and caspase-9 in ECSCs after treatment with HDACIs.

Epigenetic changes appear to be a common denominator for hormonal and immunological aberrations in endometriosis. Xue et al. (2007b) identified a classical Cpg island at the promoter and exon I region of the SF-1 gene and presented evidence that promoter methylation is a major mechanism of SF-1 silencing in normal endometrial cells and its aberrant expression in endometriotic cells. This mechanism may be, in part, the basis for activation of steroidogenic acute regulatory protein (StAR), aromatase and other genes critical for estrogen biosynthesis in endometriosis.

It is well known that there is a general tendency towards progesterone resistance in endometriosis (Giudice and Kao, 2004). The down-regulation of PR isoform B (PR-B), but not PR isoform A (PR-A), in endometriosis has been noted. Wu et al. (2006) found that the promoter region of PR-B, but not PR-A, is hypermethylated in endometriosis, resulting in reduced PR-B expression in the endometriotic tissue. PR-B promoter hypermethylation thus provides a plausible explanation as to why PR-B is persistently down-regulated in endometriosis.

Among ERs, ER-β levels in endometriotic tissue are extremely high in comparison to those in the eutopic endometrium. Xue et al. (2007a) demonstrated that hypomethylation of a CpG island at the promoter region of the ER-β gene causes high levels of expression of the gene in endometriotic stromal cells, and hypermethylation silences it in endometrial stromal cells. It is suggested that the high ER-β levels in endometriotic stromal cells lead to increased ER-β binding to the PR promoter and mediate the down-regulation of expression of PRs.

In addition, Wu et al. (2007b) found that DNMT1, 3A and 3B (the genes coding for DNA methyltransferases that are responsible for DNA methylation) are over-expressed in endometriotic lesions, suggesting that aberrant methylation may be widespread in endometriosis. This also provides a strong piece of evidence that endometriosis ultimately may be an epigenetic disease. Our present findings...
suggested that the histone modification controlled by HDACs is aberrant in endometriosis.

HDACs play an important role in the regulation of gene transcription and oncogenesis through remodeling of chromatin structure and dynamic changes in nucleosomal packaging of DNA (Marks et al., 2001). Hyperacetylation of histones H3 and H4 is often associated with activated transcription and hypoacetylation of histones H3 and H4 correlates with transcriptional silencing or repression (Norton et al., 1989). Aberrant recruitment of HDAC activity has been associated with the occurrence and development of certain human malignancies (Lemercier et al., 2003; Verdin et al., 2003). In addition, recent findings suggest that the substrates of HDACs are not restricted to histones but include transcriptional regulators, such as p53, EZF-I, Mad-I, BCL-6 and ETO (Choi et al., 2004). In this regard, global gene expression analyses have shown that HDACs affect the expression levels of 2–20% of genes in the genome, of which about half are up-regulated and half down-regulated (Joseph et al., 2004).

The molecular events that mediate the biological effects of HDACs are incompletely understood. Inhibition of HDAC by HDACIs increases histone acetylation and maintains chromatin structure in a more open conformation, resulting in the reactivation of transcriptionally silenced pathways or suppression of aberrantly expressed genes through recruitment of repressors (Richon and O’Brien, 2002; Verdin et al., 2003). HDACIs can reactivate genes silenced by promoter hypermethylation as well as the demethylation agents (Cameron et al., 1999). HDACIs also cause mitotic defects through non-transcriptional mechanisms (Warrener et al., 2003). HDACIs can reverse the changes in oncogene-transformed cell morphology, prevent proliferation and induce differentiation, cell cycle arrest at the G1 and/or G2/M phases, and apoptosis of numerous tumor cell types, including neuroblastomas, acute myelogenous leukemia and carcinomas of the skin, breast, prostate, bladder, lung, colon, cervix, endometrium and ovary (Takai and Narahara, 2007). It has been suggested that decreased apoptosis in endometriotic cells plays the essential role in the development and progression of endometriosis (Nishida et al., 2005). Therefore, our findings suggest that HDACIs are promising agents for the treatment of endometriosis.

It has been demonstrated that HDACIs (such as VPA and Trichostatin A) can suppress proliferation, induce cell cycle arrest, inhibit inter leukin-1β-induced cyclo-oxygenase-2 expression and nuclear factor-κB activation, up-regulate peroxisome proliferator-activated receptor γ, p21 and PR-B expression, attenuate cell invasiveness and reactivate silenced E-cadherin gene expression of endometriotic cells (Wu and Guo, 2007, 2008, 2009; Wu et al., 2007a, 2008, 2010). Treatment with Trichostatin A has been shown to significantly reduce the growth of endometriotic lesions in a murine model (Lu et al., 2010).

In the present study, we demonstrated that regulatory factors of the cell cycle, including p16INK4a, p21Waf1/Cip1, p27kip1 and chk2, and anti-apoptotic factors Bcl-2 and Bcl-XL, are involved in the mechanisms of action of HDACIs in ECSCs. The p16INK4a protein controls cell cycle proliferation during G1 by inhibiting the ability of cyclin D/CDK4 and cyclin D/CDK6 complexes to phosphorylate retinoblastoma protein (pRb) (Lowe and Sherr, 2003). p21Waf1/Cip1 and p27kip1 are cyclin-dependent kinase (Cdk) inhibitors that bind to cyclin-Cdk complexes and decrease kinase activity, and may block cell cycle progression at the G0/G1 phase (Takai et al., 2004b). chk2 encodes a nuclear serine/threonine kinase that plays a crucial role in the DNA damage response and helps guard the integrity of the genome by regulating cell-cycle checkpoints, DNA repair and apoptosis (Kato et al., 2004). HDACIs have been reported to regulate the expression of p16INK4a, p21Waf1/Cip1 and chk2 in other cell types (Gui et al., 2004; Kato et al., 2004; Matheu et al., 2005). Our results of the ChIP assay and western blot analysis suggested that the expression of cell cycle regulatory factors were epigenetically induced by HDACIs.

In summary, we demonstrated that the levels of acetylated histone H3 and H4 were significantly lower in unstimulated ECSCs in comparison with NESCs, suggesting that endometriosis is an epigenetic disorder. HDACIs reactivated epigenetically silenced genes, including p16INK4a, p21Waf1/Cip1, p27kip1 and chk2, resulting in the suppression of cell proliferation and induction of cell cycle arrest and apoptosis of ECSCs. Some HDACIs, including VPA and SAHA, are currently being examined in clinical trials for the treatment of adenomyosis (Liu and Guo, 2007) as well as cancer (Takai and Narahara, 2007). Our results suggest that HDACIs would be promising agents for the treatment of endometriosis. Further research with endometriotic cells treated with a DNA methylation inhibitor and/or an HDAC inhibitor in conjunction with cDNA microarray analysis may elucidate other candidate genes that are epigenetically silenced in endometriosis.

**Author’s roles**

K.N. and H.N. took part in study design, supervision, coordination and drafting of manuscript. Y.K., H.L., A.T., W.A., and N.T. took part in study execution, and data collection and discussion.

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