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# HDAC3 regulates stability of estrogen receptor α mRNA

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ABSTRACT

Estrogen receptor alpha (ERα) expression is a risk factor for breast cancer. HDAC inhibitors have been demonstrated to down-regulate ERα expression in ERα-positive breast cancer cell lines, but the molecular mechanisms are poorly understood.

Here, we showed that HDAC inhibitors decrease the stability of ERα mRNA, and that knockdown of HDAC3 decreases the stability of ERα mRNA and suppresses estrogen-dependent proliferation of ERα-positive MCF-7 breast cancer cells. In the Oncomine database, expression levels of HDAC3 in ERα-positive tumors are higher than those in ERα-negative tumors, thus suggesting that HDAC3 is necessary for ERα mRNA stability, and is involved in the estrogen-dependent proliferation of ERα-positive tumors.



## 1. Introduction

Breast cancer is the most common malignancy and the second most common cause of cancer-related death among women. It has been established that estrogens are important for the initiation and progression of breast cancer. The effects of estrogen on breast tumorigenesis are mainly mediated through estrogen receptor alpha (ERα). Therefore, activation of ERα is considered to be a risk factor for the development of breast cancer. In treatment of ERα-positive breast cancer, modulation of estrogen signaling pathways using anti-estrogens was one of the first recognized targeted therapies and is currently the first-line treatment for ERα-positive cancers. Therefore, the underlying mechanisms of ERα gene transcription in breast cancer are an area of active investigation with potential clinical significance [1].

In recent years, chromatin regulation mediated by histone deacetylases (HDACs) has been a critical component of ERα gene transcriptional regulation in breast cancer [2–4]. To date, eighteen human HDACs have been identified. These are divided into four families according to sequence homologies: class I (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9 and 10), class III (sirtuins) and class IV (HDAC11) [5,6]. Recently, various HDAC inhibitors have been shown to down-regulate ERα expression in ERα-positive breast cancer cell lines [7–11]. However, the HDACs involved in ERα repression are unknown.

Here, we showed that HDAC inhibitors (TSA, MS-275 and Apicidin) decreased ERα mRNA stability in MCF-7 cells. In addition, we found that knockdown of HDAC3 decreases stability of ERα mRNA and suppresses estrogen-dependent proliferation in ERα-positive MCF-7 cells. In the Oncomine database, expression levels of HDAC3 in ERα-positive tumors are higher than those in ERα-negative tumors. Taken together, these results suggest that HDAC3 is necessary for maintaining ERα mRNA stability, and is involved in the estrogen-dependent proliferation of ERα-positive tumors.

## 2. Materials and methods

### 2.1. Cell culture and treatment

MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin mixed solution (Nacalai tesque). Cells were maintained at 37 °C in an atmosphere containing 5% $CO_2$ and 100% humidity. Treatments with trichostatin A (Sigma), MS-275 (Wako) or Apicidin (BioVision Inc.) were performed at the indicated concentrations and times.

### 2.2. RNA interference

For transfection of siRNAs, MCF-7 cells ($1.5 \times 10^5$ cells) were transfected with 20 nM of siRNA using Lipofectamine RNAi max (Invitrogen) according to the manufacture's protocol. The siRNA reagents specific for HDAC1 (Sigma), HDAC2 (Invitrogen) or HDAC3 (Santa Cruz biotechnology) were commercially obtained. Stealth RNAi™ Luciferase reporter control duplex (Invitrogen) was used

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as a negative control. Protein and RNA were extracted at 60 h after transfection of siRNA.

### 2.3. Western blotting

Cells were lysed in TNE buffer [10 mM Tris–HCl (pH 7.8), 1% Nonidet P-40 (NP-40), 0.15 M NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)], and then immunoblotted with the appropriate antibodies. The antibodies used in this study were: anti-ERα and anti-β-actin antibodies were purchased from Santa Cruz biotechnology, Anti-HuR antibody was from Abcam, and Anti-HDAC3 antibody was from Cell signaling. Specific proteins were visualized using an enhanced chemiluminescence (ECL) Western blot detection system (Millipore).

### 2.4. RNA purification and RT-qPCR

Total RNA was isolated from cultured cells with Sepasol RNA I Super reagent (Nacalai tesque) according to the manufacturer's instruction. Total RNA (1 μg) was reverse transcribed using random hexamers and RevertraAce transcriptase (TOYOBO) according to the manufacturer's protocol. Real-time quantitative PCR analysis was performed in 20 μL reactions using the Thermal Cycler Dice™ TP800 (Takara) and SYBR Premix Ex Taq™ (TaKaRa). Primers for human cyclophilin, ERα, HDAC1, HDAC2, HDAC3, cyclin D1 mRNA were designed to amplify 100–300-bp amplicons. All the primer sets used gave no signal in the control reactions lacking template. Dissociation–curve analysis showed that single products with the expected $T_m$ values were generated by each primer set. Relative gene expression was determined by $\Delta\Delta C_T$ method. To normalize values obtained in the samples, control cyclophilin $C_T$ values were subtracted from ERα the $C_T$ values for each sample ($\Delta C_T$). Then, $\Delta C_T$ of the unstimulated sample was subtracted from $\Delta C_T$ of the stimulated sample ($\Delta\Delta C_T$). The relative levels of ERα were calculated as $2\Delta\Delta^{CT}$. For PCR amplification, the specific primers, 5′-ACGTGGTATAAAAGGGGCGGGAG-3′ and 5′-TCACCACCCTGACAC ATAAACCCTG-3′ for cyclophilin, 5′-ACAGACACTTTGATCCACCTGAT-3′, and 5′-TACAGATGCTCCATGCCTTTGTT-3′ for ERα, 5′-ACAGCGACTGTTTGAGA ACCTTAGA-3′ and 5′-CATCCTCTGTTTTGACTCTCTTGCC-3′ for HDAC1, 5′-C ACAGGAGACTTGAGGGATA-3′ and 5′-CACCTTTGAGATAATAGGC-3′ for HDAC2, 5′-TATCTGGACCAGATCCGCCAGCAA-3′ and 5′-CACAGCATCCCAA GCCACTCTTAAA-3′ for HDAC3, 5′-GTGCCACAGATGTGAAGTTC-3′, 5′-GTC CGGGTCACACTTGAT-3′ for cyclin D1 were used.

### 2.5. Luciferase assay

For luciferase assays, ERα promoters A, B, C and F containing Luc plasmids were transfected into MCF-7 cells. We cotransfected the ph-RL-TK vectors into MCF-7 cells as a reference plasmid. Twenty-four hours after transfection, we added DMSO or TSA (500 nM) to the cells for an additional 12 h incubation. Luciferase assays were performed using cell extracts according to the manufacturer's protocol (Promega).

### 2.6. mRNA stability determination

MCF-7 cells ($1.5 \times 10^5$ cells) were seeded prior to treatment with or without trichostatin A (500 nM) and actinomycin D (1 μM). RNA was isolated after 0 or 3 h treatment, and gene expression changes were quantified by RT-qPCR.

### 2.7. Nuclear/cytoplasmic fractionation

MCF-7 cells were homogenized in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA,1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). The homogenates were swollen on ice for 15 min, after which 10% NP-40 was added to a final concentration of 0.5%. The homogenate was vortexed for 10 s and centrifuged for 5 min at 5000 rpm. The nuclear pellets were lysed in TNE buffer [10 mM Tris–HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, and 1 mM EDTA].

### 2.8. Immunoprecipitation

MCF-7 cells were lysed in lysis buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 2 mM EDTA and 0.1% NP-40) at 4 °C for 30 min. The cleared lysate was incubated for 4 h with 2 μg of antibodies against the indicated proteins, added 10 μl of protein G Sepharose, and rotated for 1 h. After washing 3 times with the same buffer, immunoprecipitates were separated on 12% SDS–polyacrylamide gels, and analyzed by immunoblotting with the indicated antibodies.

### 2.9. Proliferation assay

MCF7 cells ($2 \times 10^4$ cells) transfected with HDAC3-specific siRNA or control siRNA, were seeded in DMEM containing 4% charcoal-stripped FBS. After 24 h (day 0), cells were added with vehicle (DMSO) or estrogen ($10^{-8}$ M). At 0, 24, 48 and 72 h, cells were trypsinized and treated with 0.2% trypan blue. Viable cells were counted using Countess Automated Cell Counter (Invitrogen).

### 2.10. Oncomine database searches

Microarray data set of Lu [12] was assessed using the Oncomine Cancer Profiling Database (www.oncomine.org). Expression of HDAC3 in estrogen receptor positive or negative breast tumour tissues and statistics were obtained directly through the Oncomine 3.0 software.

### 2.11. Statistical analysis

Significance of differences was determined by Student *t*-test analysis.

## 3. Results

### 3.1. HDAC inhibitors decreased stability of ERα mRNA in breast cancer cells

Immunoblot analysis using total cell lysates showed decreases in ERα protein levels on treatment with several histone deacetylase (HDAC) inhibitors (Hydroxamate Trichostatin A (TSA), Benzamide MS-275 and Cyclic tetrapeptide Apicidin) in MCF-7 cells (Fig. 1A). Several studies have reported a decrease in ERα mRNA levels on treatment with various HDAC inhibitors [1,13]. Consistent with previous reports, reverse transcription-quantitative PCR (RT-qPCR) analysis revealed that TSA, MS-275 and Apicidin decreased ERα mRNA levels in MCF-7 cells (Fig. 1B). We next examined the effects of TSA on transcription from ERα gene promoters using transient transcription assay. Transcription of the ERα gene is driven by four different promoters which span over 300 kb [14]. In MCF-7 cells, levels of transcripts originating from promoters A, B, C and F were not decreased on TSA treatment (Fig. 1C). These results suggested that the decrease in ERα mRNA levels by HDAC inhibitors is not due to the transcriptional regulation of the ERα gene promoter.

Therefore, we next tested the effects of HDAC inhibitors on ERα mRNA stability. We blocked de novo mRNA transcription using actinomycin D, an inhibitor of mRNA transcription. MCF-7 cells

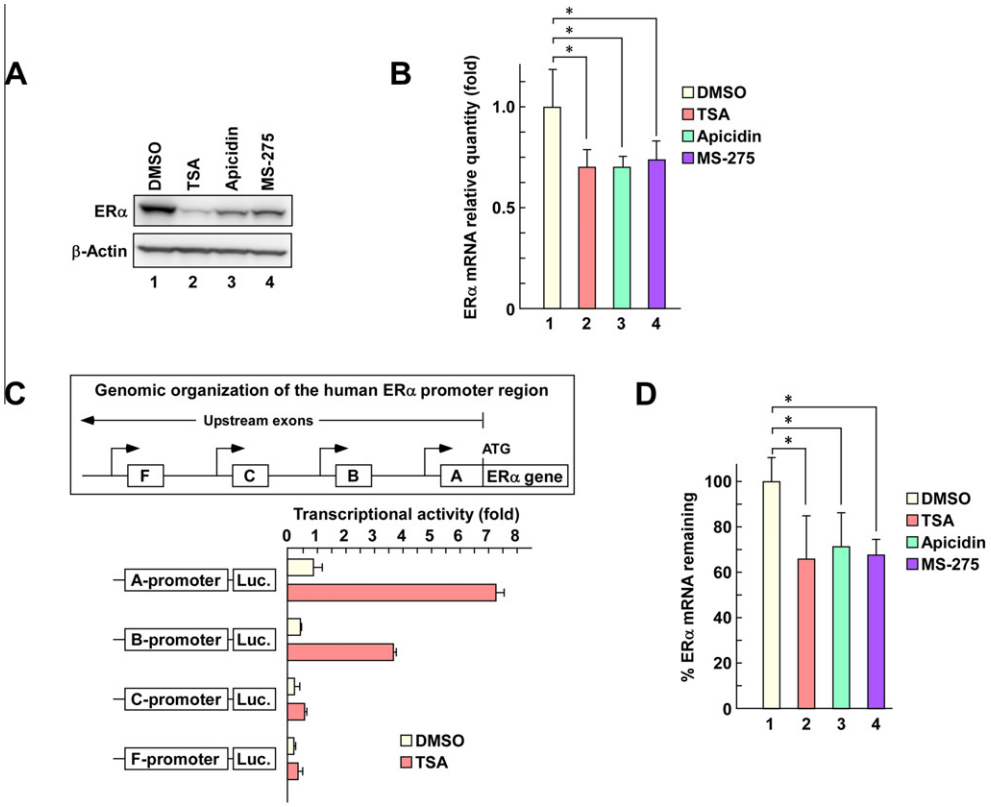


**Fig. 1.** HDAC inhibitors decreased stability of ERα mRNA in breast cancer cells. (A) HDAC inhibitors treatment reduced ERα protein levels in MCF-7 cells. MCF-7 cells were treated with 500 nM TSA, 50 nM Apicidin, 1 mM MS-275 or DMSO for 12 h. Protein levels of ERα were analyzed by Western blotting using anti-ERα or anti-β-actin antibodies. (B) HDAC inhibitors treatment reduced ERα mRNA levels in MCF-7 cells. MCF-7 cells were treated with 500 nM TSA, 50 nM Apicidin, 1 mM MS-275 or DMSO for 3 h. Expression of ERα was analyzed by reverse transcription-quantitative PCR (RT-qPCR) and was normalized to expression of the housekeeping gene, cyclophilin. (C) TSA increased the transcriptional activity of ERα gene promoters. The location of multiple promoters and corresponding upstream exons of the human ERα gene is shown. Upstream exons are represented by boxes and their promoters shown as arrows (Upper panel). MCF-7 cells were transfected with human ERα promoters A, B, C and F containing Luc plasmids as indicated. Twenty-four hours after transfection, cells were treated with the presence or the absence of TSA for 12 h before the luciferase assays were performed (Lower panel). (D) HDAC inhibitors treatment with actinomycin D decreased ERα mRNA levels. After treatment with the combined actinomycin D and 500 nM TSA, 50 nM Apicidin, 1 mM MS-275 or DMSO for 3 h in MCF-7 cells, RNA was harvested. Expression of ERα was analyzed by RT-qPCR and was normalized to expression of the housekeeping gene, cyclophilin. Each bar represented the mean ± s.e.m. of three experiments. $^{*}P < 0.05$ versus respective control values.

were treated with actinomycin D in the presence of TSA, MS-275, Apicidin or DMSO, and mRNA expression levels were determined using RT-qPCR. As shown in Fig. 1D, ERα mRNA levels on treatment with TSA, MS-275 or Apicidin with actinomycin D were significantly lower when compared with those on the treatment with DMSO and actinomycin D. These results suggested that HDAC inhibitors decrease the stability of ERα mRNA in MCF-7 cells.

It has been reported that TSA shows strong inhibitor potency against HDAC1, 2, 3, 4, 6, 7, and 9 [5,15]. MS-275 and Apicidin efficiently inhibit HDAC1, 2, and 3 [15]. Taken together, it has been suggested that HDAC1, 2 or 3 is involved in the stability of ERα mRNA in MCF-7 cells.

### 3.2. Inhibition of HDAC3 decreased stability of ERα mRNA

In order to determine the role of HDACs in HDAC inhibitors-dependent ERα mRNA instability, we generated siRNA against HDAC1, 2 and 3. We confirmed that mRNA levels of each HDAC were effectively decreased by siRNA for HDAC1, 2 and 3 using RT-qPCR (Fig. 2A). Levels of ERα mRNA and protein were reduced by knockdown of HDAC3 (Fig. 2B and C). However, knockdown of HDAC 1 and 2 did not affect ERα mRNA and ERα protein levels (Fig. 2B and C). Furthermore, we confirmed that knockdown of HDAC3 reduced the stability of ERα mRNA in MCF-7 cells (Fig. 2D). These results suggested that HDAC3 is necessary for maintaining ERα mRNA stability.

It has previously been reported that the RNA-binding protein, HuR binds to the 3′-UTR of ERα mRNA and maintains stability [13]. HuR protein is a chaperone protein that shuttles mRNA molecules between the nuclear and cytoplasmic compartments [16–18]. Within the cytoplasmic compartment, HuR stabilizes mRNA and increases its half-life. Thus, we examined the effects of HDAC3 knockdown on HuR levels in MCF-7 cells. Knockdown of HDACs in MCF-7 cells did not affect either the nuclear or cytoplasmic level of HuR protein (Fig. 2E). We next examined the interaction between HuR and HDAC3 by the co-immunoprecipitation method. As shown in Fig. 2F, HDAC3 was not associated with HuR. These results suggested that the quantity or localization of HuR is not involved in the HDAC3-dependent maintenance of ERα mRNA stability.

### 3.3. Correlations between HDAC3 and ERα expression in breast cancer

Estrogen increases proliferation of ERα-positive breast cancer cells through the increase of cyclin D1 gene transcription [19]. Therefore, we examined the effect of HDAC3 knockdown of estrogen-dependent cyclin D1 expression in MCF-7 cells. As shown in Fig. 3A, estrogen treatment increased cyclin D1 mRNA levels in MCF-7 cells treated with control or HDAC1 siRNA. In contrast, estrogen-dependent cyclin D1 expression was not observed in HDAC3 knockdown MCF-7 cells. Next, we examined the estrogen-dependent proliferation under knockdown of HDAC3 in MCF-

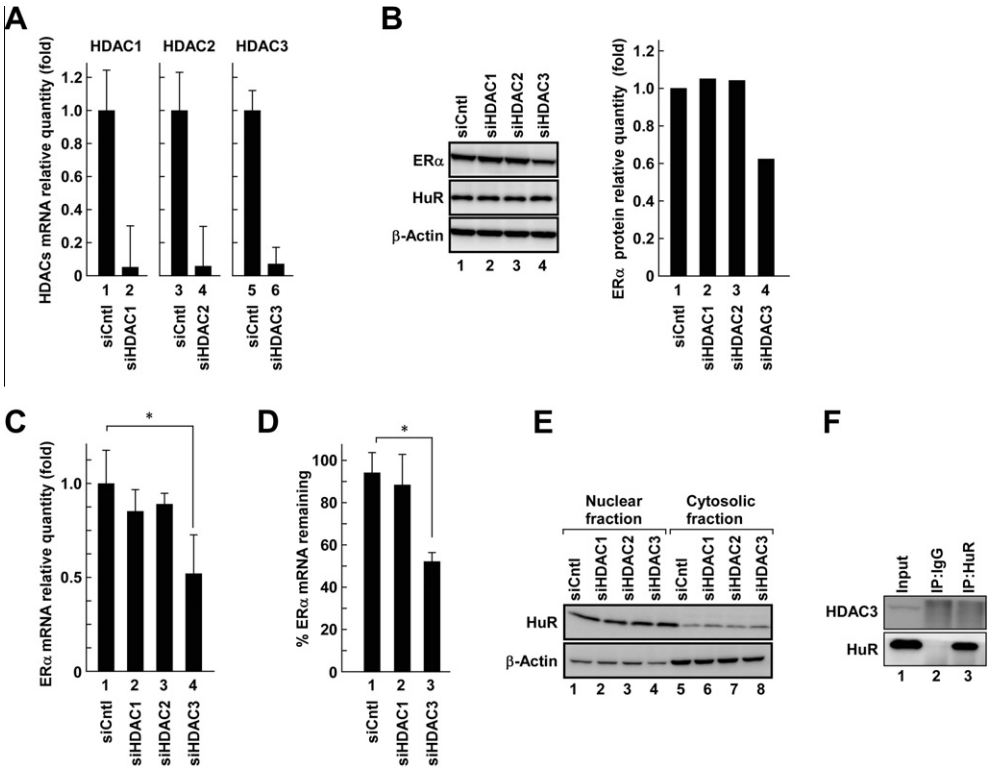


**Fig. 2.** Knockdown of HDAC3 decreased stability of ERα mRNA. (A) Knockdown by siRNAs for HDACs significantly reduced each target mRNA levels. After treatment of each HDAC1, 2, 3 or control siRNAs for 60 h in MCF-7 cells, mRNA levels of HDAC1, 2 or 3 were analyzed by RT-qPCR and were normalized to expression of the housekeeping gene, cyclophilin. (B) Knockdown of HDAC3 decreased ERα protein levels. After treatment of each HDAC1, 2, 3 or control siRNAs for 60 h in MCF-7 cells, protein levels of ERα and HuR were analyzed by Western blotting using anti-ERα, anti-HuR or anti-β-Actin antibodies (left panel). The intensities of the ERα proteins were corrected using β-Actin protein level. The intensity of the control siRNA treated cells was normalized to 1.0 (right panel). (C) Knockdown of HDAC3 decreased ERα mRNA levels. After treatment of each HDAC1, 2, 3 or control siRNAs for 60 h in MCF-7 cells, mRNA levels of ERα were analyzed by RT-qPCR and were normalized to expression of the housekeeping gene, cyclophilin. (D) Knockdown of HDAC3 decreased ERα mRNA stability. After treatment of HDAC1, 3 or control siRNAs for 60 h in MCF-7 cells, nascent RNA transcription was stopped using actinomycin D. RNA was harvested after actinomycin D treatment for 3 h. Expression of ERα was analyzed by RT-qPCR and was normalized to expression of the housekeeping gene, cyclophilin. (E) Cytoplasmic and nuclear expression of HuR did not change by knockdown of HDAC3 in MCF-7 cells. After treatment of HDAC1, 3 or control siRNAs for 60 h in MCF-7 cells, either cytoplasmic or nucleus extracts were isolated and used to analyze HuR protein levels by Western blotting using anti-HuR or anti-β-Actin antibodies. (F) HuR did not associate with HDAC3 in MCF-7 cells. MCF-7 cells lysates were immunoprecipitated using anti-HuR or normal IgG antibodies, and analyzed by immunoblotting with anti-HDAC3 or anti-HuR antibodies. Each bar represented the mean ± s.e.m. of three experiments. $^{*}P < 0.05$ versus respective control values.

7 cells (Fig. 3B). Consistent with cyclin D1, treatment of MCF-7 cells with control siRNA induced estrogen-dependent cell growth; however, treatment of MCF-7 cells with HDAC3 siRNA significantly reduced estrogen-dependent cell growth. These results suggested that ERα mRNA instability by knockdown of HDAC3 reduces the estrogen-dependent proliferation of ERα-positive MCF-7 cells.

Next, to investigate the relationship between HDAC3 mRNA levels and ERα positivity, we used the Oncomine cancer profiling database (http://www.oncomine.org), which provides publicly available datasets on cancer gene expression (Fig. 3C). Representative results of the datasets containing gene chip profiles classified by ERα positivity (Xuesong Lu et al., 2008) [12] characterized by large population sizes are shown. This dataset demonstrated a significant correlation between HDAC3 expression levels and ERα positivity. In addition, these results raise the possibility that HDAC3 and ERα coordinately regulate breast cancer progression.

## 4. Discussion

In our study, we initially demonstrated that HDAC3 functions to maintain ERα mRNA stability. It is known that ERα mRNA has a long 3′-UTR encoding several RNA binding protein sites that can regulate mRNA stability of ERα [20]. Previously, it is reported that HDAC inhibitors induced ERα mRNA instability is required for the subcellular localization changes of the ERα 3′-UTR binding protein, HuR [13]. However, in our study (Fig. 2E), knockdown of HDAC3 did not change the localization and the quantities of HuR. Because HDAC3 is known as one of the deacetylases, HDAC3 may regulate the activity of HuR through the acetylation of it. Further study is required to determine the molecular mechanism of HDAC3 for the regulation of stability of ERα mRNA.

Our data suggested that the reduction of ERα mRNA induced by the knockdown of HDAC3 may be effective for the suppression of estrogen-dependent growth in ERα-positive breast cancer. It is previously reported that knockdown of HDAC3 stimulates the expression of p21 and suppresses the proliferation of several cancer cells [21–23]. In this study, knockdown of HDAC3 suppresses the estrogen-dependent expression of cyclin D1 and cell growth in MCF-7 cells. In treatment of ERα-positive breast cancer, modulation of estrogen signaling pathways using anti-estrogens was indeed one of the first recognized targeted therapies. However, during the hormone therapy, the breast cancers develop resistance to anti-hormone therapy, and it will be become more aggressive and correlate with poor prognosis. From our study, it is suggested that the inhibition of HDAC3 is one of the alternative targets for the suppression of estrogen signaling. Therefore, HDAC3 inhibitor will have effect in hormone resistance of ERα-positive breast cancer.

Overall, in this report, we show a novel role of HDAC3 in the regulation of ERα expression in breast cancer cells through the regulation of ERα mRNA stability. In addition, our findings suggested that HDAC3 are involved in the estrogen-dependent proliferation

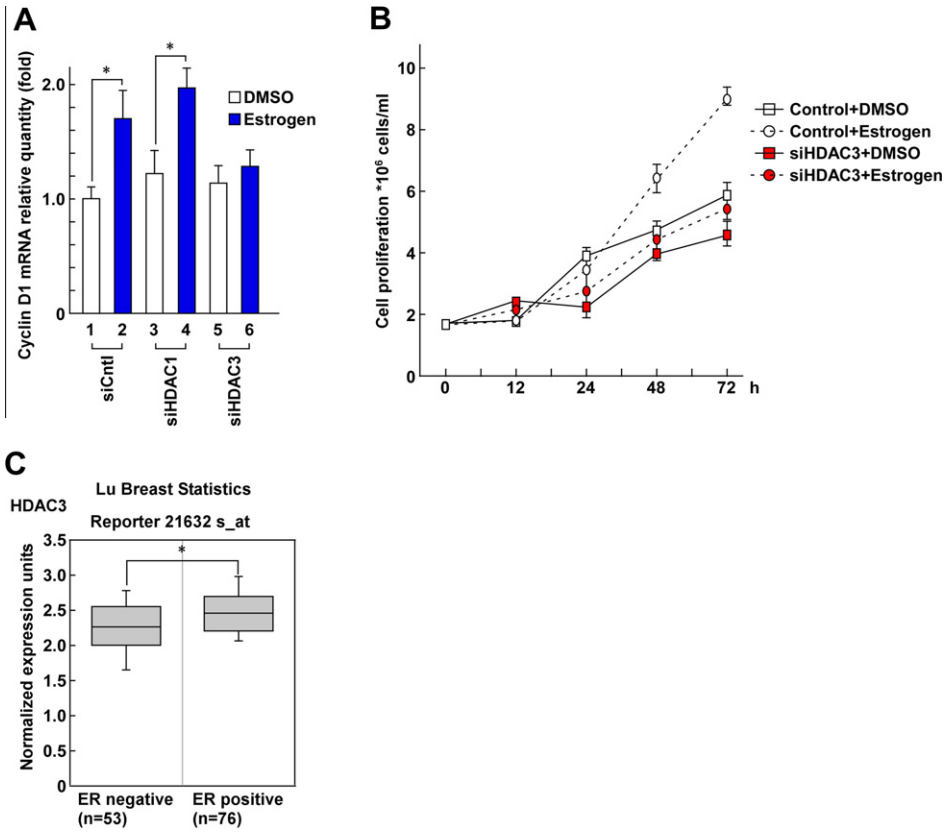


**Fig. 3.** Correlations between HDAC3 and ERα expression in ERα-positive breast cancer. (A) Knockdown of HDAC3 inhibited estrogen-induced cyclin D1 expression in MCF-7 cells. After treatment of HDAC1, 3 or control siRNAs for 60 h in MCF-7 cells, expression of cyclin D1 was analyzed by RT-qPCR and was normalized to expression of the housekeeping gene, cyclophilin. (B) Knockdown of HDAC3 inhibited estrogen-dependent proliferation in MCF-7 cells. After treatment of HDAC3 or control siRNAs for 24 h in MCF-7 cells, cells were added with DMSO or estrogen ($10^{-8}$ M). At 0, 24, 48 and 72 h, cell proliferation was measured by Trypan blue exclusion assay at the indicated times with or without estrogen ($10^{-8}$ M). Each bar represented the mean ± s.e.m. of three experiments. $^{*}P < 0.05$ versus respective control values. (C) Expression of HDAC3 was positively related with ERα expression in ERα-positive breast cancer. HDAC3 mRNA expression in clinical breast cancer samples registered in the ONCOMINE database. Left bars, ERα mRNA in ERα-negative samples; right bars, ERα mRNA in ERα-positive samples. Data were analyzed using ONCOMINE algorithms. The mRNA expression levels were represented by normalized units. The line within each colored box represented the median value for each group, and the upper and lower edges of each box indicated the 75th and 25th percentiles, respectively. $^{*}P < 0.05$ versus respective control values.

of ERα-positive tumors. Further investigations are needed to clearly understand the detail of HDAC3 effects on ERα regulation and are currently underway. Findings from our studies provide the necessary insight into the mechanism of anti-cancer effects as well as to improve the therapeutic efficacy of HDAC inhibitors.

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