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Inhibition of SIRT1 deacetylase suppresses estrogen receptor signaling

Yuan Yao, Hongzhe Li[1], Yansong Gu[1], Nancy E.Davidson[2] and Qun Zhou*

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, USA, [1]Department of Radiation Oncology, University of Washington School of Medicine, Seattle, WA 98195, USA and [2]University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232, USA

*To whom correspondence should be addressed. Tel: +1 410 706 1615; Fax: +1 410 706 8297;
Email: qzhou@som.umaryland.edu

Estrogen receptor α (ERα) mediates estrogen-dependent gene transcription, which plays a critical role in mammary gland development, reproduction and homeostasis. Histone acetyltransferases and class I and class II histone deacetylases (HDACs) cause posttranscriptional modification of histone proteins that participate in ERα signaling. Here, we report that human SIRT1, a class III HDAC, regulates ERα expression. Inhibition of SIRT1 activity by sirtinol suppresses ERα expression through disruption of basal transcriptional complexes at the ERα promoter. This effect leads to inhibition of estrogen-responsive gene expression. Our *in vitro* observations were further extended that SIRT1 knockout reduces ERα protein in mouse mammary gland. Finally, ERα-mediated estrogen response genes are also decreased in mouse embryonic fibroblasts derived from SIRT1-knockout mice. These results suggest that inhibition of SIRT1 deacetylase activity by either pharmacological inhibitors or genetic depletion impairs ERα-mediated signaling pathways.

Introduction

Estrogen, such as 17β-estradiol (E2), is synthesized locally or peripherally via aromatization (1). Compelling evidence demonstrates that estrogen is essential for mammary gland development as well as breast carcinogenesis (1,2). The biological functions of estrogen are elicited through estrogen receptor α (ERα)-mediated signaling pathways. This process involves ligand binding, followed by ERα dimerization and receptor binding to estrogen response elements at the promoter of estrogen-responsive genes such as pS2 and progesterone receptor (PR) (2). ERα acts in conjunction with coactivators important for stimulation of gene expression (3). It has been known that members of the steroid receptor coactivator (SRC) family (SRC-1, SRC-2 and SRC-3/AIB1) participate in the regulation of ERα-dependent gene expression (4). Studies of estrogen action have demonstrated that SRC family proteins are associated with histone acetyltransferases such as p300/CBP, which create histone acetylation affecting the accessibility of the promoter chromatin. This active chromatin subsequently recruits additional nuclear receptor coactivators and transcription factors at the ERα target gene promoters and ultimately leads to activation of gene transcription (5).

Mammalian histone deacetylases (HDACs) can be classified as class I (HDAC1–3 and 8), class II (HDAC4–7 and HDAC9–10), class III (SIRT1–7) or class IV (HDAC11) based on their protein structure and enzymatic activity. Class I, II and IV HDACs use zinc as a cofactor for their enzyme activity. In contrast, class III HDACs require nicotinamide adenosine dinucleotide (NAD+) as their cofactor and are insensitive to class I, II and IV HDAC inhibitors (6). HDAC1 can act as a corepressor at the ERα promoter and silences ERα gene as shown in an ERα-negative breast cancer cell culture model (7). In addition, HDACs can directly interact with ERα protein and regulate its downstream gene transcription (8,9). Class I and II HDACs can reverse p300-mediated acetylation in ERα, thereby inhibiting ERα-dependent gene transcription (10). Several specific class I and II HDAC family members have been shown to modulate ERα function. For example, inhibition of HDAC2 by small interfering RNA (siRNA) downregulates ERα expression, which attenuates estrogen response and potentiates anti-estrogen therapy (11). HDAC4 interacts with the N-terminus of ERα and stimulates its binding to estrogen-responsive gene promoters leading to suppression of ERα transcription (12). HDAC6 is also capable of a direct interaction with ERα in the cytoplasm and facilitates the non-genomic action of estrogens (13). Moreover, inhibition of HDAC6 depletes ERα and downregulates estrogen-induced gene transcription (14).

Among the class III HDACs, SIRT1 deacetylase modulates the activity of histone proteins as well as a number of transcription factors, including p53, FOXO1, nuclear factor kappa B and p300 (15,16). However, the function of SIRT1 remains controversial. For example, studies show that SIRT1 may function as a tumor suppressor gene because SIRT1-deficient mice develop tumors in multiple tissues, whereas SIRT1 overexpression inhibits intestinal tumorigenisis in SIRT1 transgenic mice (17,18). Several studies support the notion that SIRT1 acts as an oncogene since SIRT1 inhibitors reduce tumor cell growth (19–21). SIRT2 predominantly localizes in the cytoplasm and deacetylates α-tubulin (22). The targets of other sirtuin family members are not clear. While much progress has been made in understanding the role of specific class I or class II HDAC family members in ERα-mediated signaling, it remains unclear whether class III HDACs play a key role in regulation of ERα function. We have previously found that SIRT1-deficient female mice display lactation failure due to a development defect in mammary gland development (23). In the present study, we found that inhibition of the SIRT1 deacetylase activity suppresses ERα expression and attenuates estrogen-dependent gene transcription in breast cancer cell lines. These results demonstrate that the enzymatic activity of SIRT1 deacetylase affects the efficacy of ERα-mediated signaling pathways in differentiated epithelial cells.

Abbreviations: ERα, estrogen receptor α; E2, 17β-estradiol; HDAC, histone deacetylase; MEF, murine embryonic fibroblast; mRNA, messenger RNA; PCR, polymerase chain reaction; PR, progesterone receptor; siRNA, small interfering RNA; SRC, steroid receptor coactivator.

Materials and methods

Cell culture

MCF-7, T47D and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 1% glutamine (Invitrogen, Carlsbad, CA). Cells were grown at 37°C in an atmosphere containing 5% CO2. Primary murine embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% minimum essential medium (MEM), 1% *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, 50 mM β-mercaptoethanol, 1% penicillin–streptomycin and 1% L-glutamine (Invitrogen).

siRNA transfection

Cells were plated in six-well plates at a density of 5×10^5 cells per well. A final concentration of 100 nmol/l siRNA against SIRT1 (Dharmacon, Lafayette, CO) was transfected into the cells with Oligofectamine 2000 (Invitrogen). Seventy-two hours after transfection, cells were then treated with or without the designated drugs.

Luciferase reporter assays

MCF-7 cells were seeded in six-well plates (5×10^5 cells per well) and transfected with ERP-Luc (24) and SIRT1 constructs (25) by Lipofectamine 2000 (Invitrogen). Forty hours after transfection, cell lysates were prepared in 500 μl 1× passive lysis buffer (Promega, Madison, WI), and luciferase activity was determined using the dual luciferase assay system (Promega). Luciferase activity was normalized to *Renilla* luciferase activity.

Immunoblotting

Whole cell lysates were prepared by lysing the cells with 1% sodium dodecyl sulfate and 10 mM Tris–HCl (pH 7.4). The supernatants were collected with

microcentrifugation. Equal amounts of protein were denatured in sodium dodecyl sulfate sample buffer and separated on 10% polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membrane and probed with antibodies (SIRT1, cyclin D1, pS2 and ERα) from Santa Cruz Biotechnology (Santa Cruz, CA). Protein expression was detected by chemiluminescence (ECL, Amersham, Piscataway, NJ). The expression of β-actin was used as a loading control.

Messenger RNA isolation and quantitative real-time polymerase chain reaction

Messenger RNA (mRNA) was collected and complementary DNA was synthesized as described previously (24). Levels of ERα, c-Myc, pS2 and PR mRNA were measured by reverse transcription–polymerase chain reaction (PCR) or quantitative real-time PCR. Complementary DNA and primers were added into SYBR Green PCR Master Mix (Bio-Rad Laboratories, Los Angeles, CA) and measured using Lightcycler 480II (Roche Applied Science, Indianapolis, IN). Data were analyzed and normalized to the GAPDH housekeeping gene.

Chromatin immunoprecipitation followed by quantitative PCR

Briefly, cells were cross-linked with 1% formaldehyde at 37°C for 10 min and then sonicated with a microtip ultransonicator on ice three times for 15 s each. Soluble chromatin was collected and incubated on a rotating platform with anti-RNAPII and TATA-binding protein antibodies (Santa Cruz Biotechnology) overnight at 4°C. Following immunoprecipitation and reversal of cross-linking, DNA was extracted in phenol–chloroform–isoamyl alcohol, precipitated in ethanol, resuspended in H_2O and diluted before quantitative real-time PCR analysis. The PCR primers were specific for ERα promoter (forward 5′-TGAACCGTCCGCAGCTCAAGATC-3′ and reverse 5′-GTCTGACCG-TAGACCTGCGCGTTG-3′). PCR was performed and results were quantified.

Murine embryonic fibroblasts

Timed pregnancy was terminated on embryonic day 13.5, and littermate embryos were isolated in cold phosphate-buffered saline. For each embryo, the head was separated and stored in 10% formalin solution (Fisher Scientific, Pittsburgh, PA) for histology analysis. The torso was used to isolate MEFs, and the tail was digested with proteinase K (Sigma, St. Louis, MO) to extract DNA for genotyping. MEFs were obtained through three cycles of trypsin treatment.

Immunofluorescence imaging

Paraffin-embedded sections from mammary fat pads were prepared and incubated with anti-ERα antibody (Santa Cruz Biotechnology). Following washing, the slides were incubated with the secondary antibody, Texas Red (Molecular Probes, Invitrogen) and then washed with fetal bovine serum washing buffer. Finally, the slides were counterstained with 4′,6-diamidino-2-phenylindole (Prolong Gold, Molecular Probes) and mounted with a glass coverslip. Microscopy was carried out on an AxioVert 200M microscope with AxioVision 4.5 software (Carl Zeiss).

SIRT1-knockout mouse development

SIRT1-knockout mice were generated as described previously (23). Briefly, the breeding of $SIRT1^{co/co}$ mice and CMV-Cre transgenic mice results in mice harboring a germ line-transmitted deletion of exon 4 of the SIRT1 gene ($SIRT1^{+/ko}$ mice). Both $SIRT1^{co/co}$ mice and $SIRT1^{ko/ko}$ mice were in a mixed 129SvJ/C57B6 background. Mice were housed in a specific pathogen-free facility and all procedures were approved by the University Animal Care and Use Committee. A PCR-based genotyping method was established to identify wild-type, co and ko loci of the SIRT1 gene.

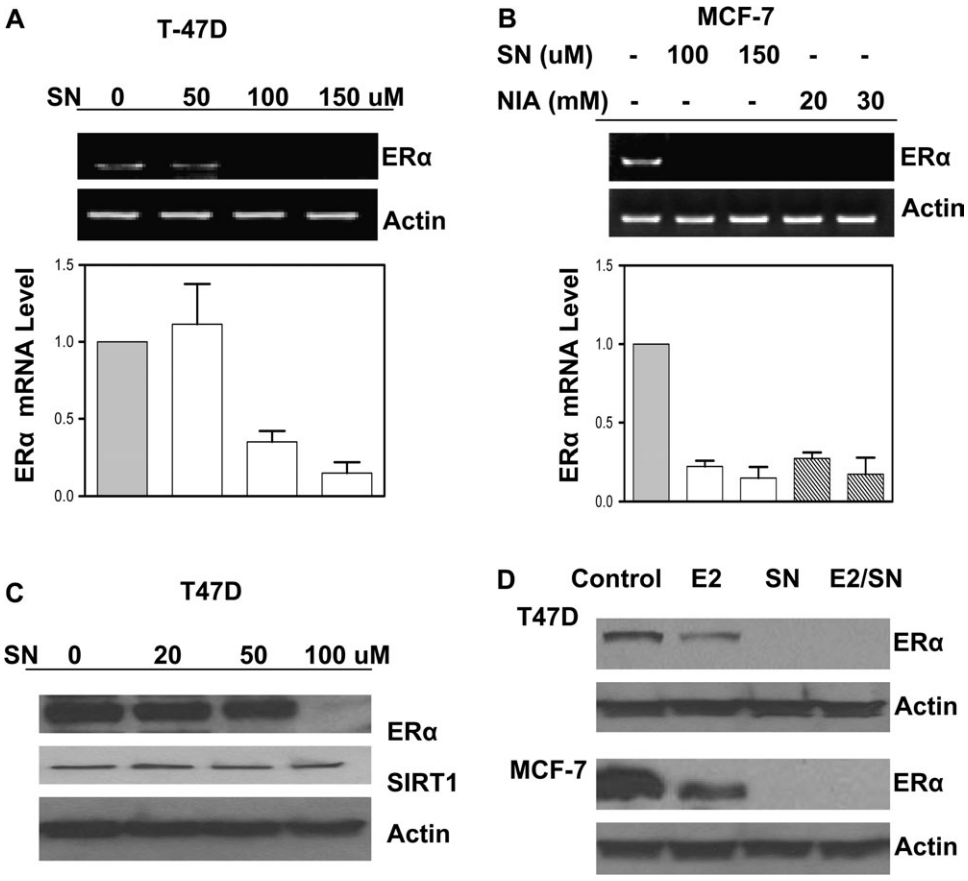

Fig. 1. Effect of class III HDAC inhibitors on ERα expression. (**A** and **B**) Class III HDAC inhibitors suppress ERα expression. T47D and MCF-7 cells were treated with SN or nicotinamide (NIA) for 48 h, and mRNA levels of ERα were measured by reverse transcription–PCR and quantitative real-time PCR analysis. Columns represent mean real-time PCR results of four independent experiments. Error bars represent SEM. (**C**) SN downregulates protein levels of ERα. T47D cells were treated with SN for 48 h, and whole cell lysates were immunoblotted using anti-ERα antibodies. (**D**) SN inhibits ERα expression in estrogen-stimulated cells. T47D cells were treated with 10 nM E2, 100 μM SN or combination both for 48 h. Whole cell lysates were immunoblotted using anti-ERα antibodies. Three experiments showed similar results.

Statistical analysis

Statistical analyses were performed with one-way analysis of variance followed by Bonferroni's *t*-test for independent samples, and the data are expressed as mean ± SE.

Results

Inhibition of SIRT1 activity suppresses ERα transcription

To address the role of SIRT1 in ERα-mediated transcription, the ERα-positive breast cancer cells MCF-7 and T47D were used as cell culture models to monitor effect of class III HDACs on ERα expression. Cells were treated with nicotinamide, a non-specific sirtuin inhibitor for class III HDAC enzymatic activity (26,27), or sirtinol (SN), a specific inhibitor for SIRT1 without affecting the other sirtuin members (28). Reverse transcription–PCR analysis showed that the level of ERα mRNA in T47D cells was reduced after 48 h of 100 μM SN treatment (Figure 1A). The level of ERα mRNA in MCF-7 cells was also reduced after treatment with SN (100 μM) and nicotinamide (20 mM) (Figure 1B). A quantitative real-time PCR analysis confirmed the reduction of ERα mRNA. These results demonstrated that inhibition of SIRT1 deacetylase activity reduces the steady-state level of ERα mRNA. To examine if downregulation of ERα is correlated with its protein level, western blot analysis showed that the ERα protein level was completely abolished while the level of SIRT1 protein remains unchanged in T47D cells after 48 h of treatment with 100 μM SN (Figure 1C). To further investigate the effect of nicotinamide and SN treatments on ERα, MCF-7 and T47D cells were grown in the same media but in the absence of estrogen for 72 h followed by the treatment with 10 nM E2, 100 μM SN or a combination of both for 48 h (Figure 1D). Although E2 stimulation slightly reduced ERα expression, treatment with either SN alone or SN with E2 caused a significant reduction of ERα protein in both cell lines tested. These data suggest that inhibition of SIRT1, a class III HDAC, suppresses ERα expression at both mRNA and protein levels.

SIRT1 regulates ERα promoter activity

To determine how the activity of SIRT1 affects ERα promoter activity, MCF-7 cells were transfected with ERα promoter reporter gene construct (1.0 kb upstream of the start site) that has been used to characterize effects of transcription regulators on ERα promoter activity (29). In the present study, the ERα promoter report construct was co-transfected into MCF-7 cell with either wild-type or mutant SIRT1 constructs. As shown in Figure 2A, co-transfection with wild-type SIRT1 increased the ERα promoter luciferase activity, whereas co-transfection with a catalytically inactive SIRT1 (SIRT1H355A that contains a mutation of histidine to arginine at position 355 in the SIRT1 protein) failed to alter the basal ERα promoter activity (Figure 2A). These findings demonstrated that SIRT1 deacetylase activity could enhance ERα promoter activity.

Inhibition of SIRT1 by SN disrupts transcription complexes at the ERα promoter

To investigate the effect of SIRT1 on chromatin organization at the ERα promoter, we performed chromatin immunoprecipitation assay and found that there is no association of SIRT1 with the ERα promoter (data not shown). To determine the possibility of SIRT1-mediated deacetylation of histone proteins at the promoter, we measured the acetylated H3-K9 and H4-K16 using chromatin immunoprecipitation assay assay since these two sites are known to be direct targets of SIRT1 in human cells (30,31) (Figure 2B). We found that inhibition of SIRT1 after SN treatment did not change the levels of acetylated H3-K9 and H4-K16 at the promoter in MCF-7 cells. However, treatment with SN depleted the occupancy of RNAPII and TBP (TATA-binding protein) at the ERα promoter (Figure 2C). These results indicate that,

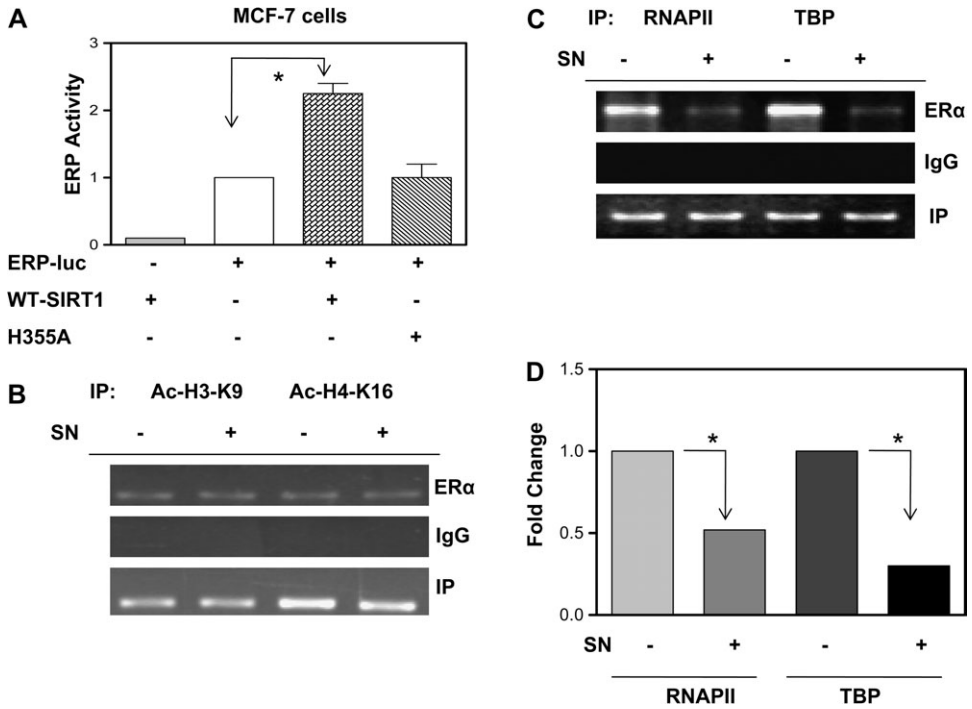

Fig. 2. SIRT1 deacetylase regulates ERα transcription. (**A**) Effect of SIRT1 on ERα promoter reporter gene activity. MCF-7 cells (5×10^5 cells per well in six-well plates) were transfected with ERP and SIRT1 (wild-type or mutant SIRT1) constructs. After 40 h, the ERP luciferase activity was normalized renila luciferase activity and is shown as the mean value of three experiments; $^*P < 0.05$. (**B**–**D**) Recruitment of basal transcription factors at the ERα promoter can be disrupted by SN treatment. Formaldehyde cross-linked chromatin from MCF-7 cells treated with 100 μM SN for 48 h was immunoprecipitated with antibodies specific for acetylated H3-K9, acetylated H4-K16, RNAPII and TATA-binding protein (TBP). The immune complexes were pulled down with protein A agarose/salmon sperm DNA beads and washed extensively as described in Materials and Methods, and cross-linking was reversed. The purified DNA was analyzed by PCR using specific primers spanning ERα promoter (B and C). IP, input. Mean values from quantitative chromatin immunoprecipitation assay from three independent experiments is shown (D); $^*P < 0.05$.

while SIRT1 may not directly participate in the modification of histone protein at the promoter, inhibition of SIRT1 by treatment with SN disrupts the formation of RNAPII transcription complexes at the promoter and attenuates ERα transcription.

Treatment with SN suppresses ERα-mediated gene expression

To determine if depletion of ERα by SN alters estrogen-dependent gene expression, reverse transcription–PCR analysis of estrogen-responsive genes was performed. As shown in Figure 3A, treatment with E2 increased the mRNA levels of estrogen-responsive genes, PR, c-Myc and pS2, whereas treatment with SN alone reduced the basal levels of these genes. We observed that co-treatment with SN decreased the expression of estrogen-responsive genes. Consistent with the changes of gene expression at mRNA levels, treatment with SN alone markedly reduced protein levels of ERα and estrogen-responsive genes including pS2 and cyclin D1 (Figure 3B). The effect of SN on estrogen-responsive genes was further examined in MCF-7 cells (Figure 3C). Co-treatment with SN attenuated E2-stimulated expression of pS2 and cyclinD1, and the SN inhibitory effect was not rescued by increased E2 concentration, suggesting that reduction of ERα expression leads to insensitivity of cells to estrogen stimulation.

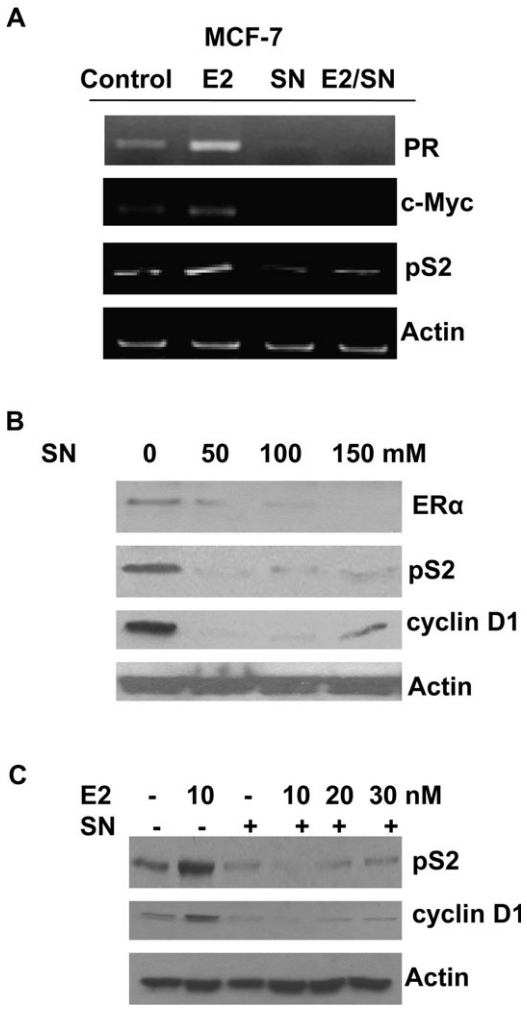

Fig. 3. SK downregulates estrogen receptor signaling. (**A**) SN inhibits ERα-mediated gene expression. MCF-7 cells were treated as indicated for 48 h and mRNA levels of PR, c-Myc and pS2 were measured by reverse transcription–PCR. (**B**) SN inhibits protein levels of pS2 and cyclin D1. MCF-7 cells were treated as indicated for 48 h and whole cell lysates were immunoblotted with anti-ERα, pS2 or cyclin D1 antibodies. β-Actin was used as a loading control. (**C**) Effect of SN on pS2 and cyclinD1 in E2-treated cells. MCF-7 cells were treated with 10 nM E2, 100 μM SN or combination of SN with increased concentrations of E2 (10–30 nM) for 48 h. Western blot analysis was performed using antibodies against cyclin D1, c-myc and pS2. Shown are representative blots from three independent experiments that gave similar results.

SIRT1 deficiency leads to suppression of ERα expression in mouse mammary gland and MEF cells

To extend our *in vitro* observations into an *in vivo* system, we examined the effect of SIRT1 on ERα expression in SIRT1-deficient mice that harbor an in-frame mutation at the exon 4 encoding a part of SIRT1 catalytic domain. Similar to SIRT1-null mutant mice, this strain of SIRT1-deficient mice displays several characteristic phenotypes, such as increased perinatal death (about two-third of newborns) and growth retardation in surviving mice (23,32). However, it is not clear if ERα expression is altered in SIRT1-deficient mice. We examined ERα expression by staining mammary gland with anti-ERα antibody. We found that SIRT1-knockout mice show reduction of ERα expression in mammary gland (Figure 4A), suggesting that ERα was downregulated, which may relate to growth retardation in SIRT1-deficient mice.

To further evaluate the effect of SIRT1 deficiency on ERα signaling, we treated MEFs derived from SIRT1-deficient littermate embryos with E2. Treatment with E2 enhanced cyclin D1 expression in wild-type MEF cells, suggesting that ERα is functional and responsive to E2 (Figure 4B). We found that the ERα expression was markedly decreased as determined by western blot analysis in two independently derived SIRT1$^{-/-}$ MEF cell lines, and induction of cyclin D1 by E2 was impaired in SIRT1$^{-/-}$ MEFs (Figure 4B). While the wild-type MEF cells were sensitive to estrogen stimulation, SIRT1$^{-/-}$ MEF cells showed less sensitivity to estrogen stimulation in 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay (Figure 4C). These data again demonstrated that SIRT1 plays a critical role for ERα-mediated estrogen response in ERα-expressing cells.

Discussion

In this study, we explored the role of SIRT1 in ERα-mediated signaling in human breast cancer cells, as well as in wild-type and SIRT1-deficient mice. The results of our studies indicate that the pharmacological inhibition of SIRT1 leads to suppression of ERα expression by disrupting the basal transcription factor complex at the ERα promoter. As a result, the expression of estrogen-responsive genes including PR, cyclinD1 and pS2 is reduced. Supporting these observations, mice harboring an in-frame mutation in the SIRT1 gene have reduced levels of ERα expression in the mammary gland. SIRT1$^{-/-}$ MEF cells derived from this line of SIRT1-deficient mice are less sensitive to estrogen stimulation as compared with wild-type MEF cells. In mouse models, the altered ERα signaling may partially contribute to the SIRT1-deficient phenotypes of blunt mammary ductal morphogenesis (23). The results from our current studies provide evidence that inhibition of SIRT1 activity suppresses ERα expression and its mediated gene expression.

The role of SIRT1 in hormone receptor-mediated gene transcription is controversial. SIRT1 can act as a corepressor or coactivator to regulate androgen receptor nuclear signaling in prostate cancer cells (21,33–35). We observed that treatment with SN suppresses activation of estrogen-dependent genes. However, increased concentrations of estrogens are unable to overcome the SN action since treatment with SN depletes ERα expression and subsequently leads to insensitivity of cells to estrogen stimulation. Consistent with these observations, SIRT1−/− MEF cells derived from SIRT1-knockout mice show suppression of ERα-mediated estrogen response. Our studies suggest that the presence of SIRT1 deacetylase activity is necessary for ERα-mediated signaling and support finding from other laboratories showing a positive role of SIRT1 in ERα signaling (10,20,36).

ERα interacts with several coactivators and many nuclear proteins in response to estrogen stimulation. Our studies demonstrate that

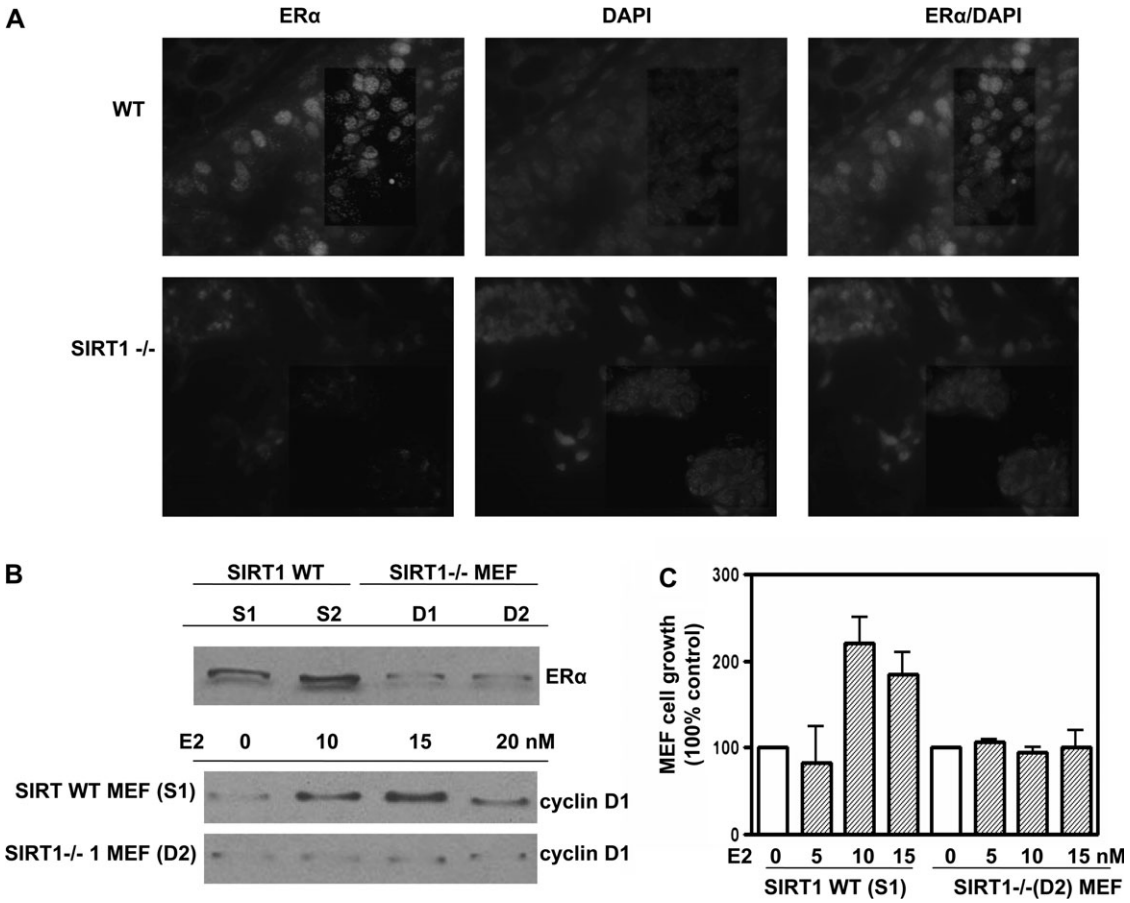

Fig. 4. (**A**) Knockdown of SIRT1 decreases ERα expression in mammary gland. Mammary fat pads were fixed and stained with anti-ERα antibodies. The slides were incubated with the secondary antibody and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescent staining ERα was visualized by an AxioVert 200M microscope (Carl Zeiss); magnification ×60. (**B**) SIRT1 deficiency inhibits estrogen-induced cyclin D1 in MEF cells. Protein levels of ERα were measured in two wild-type MEF cell lines (S1 and S2) and two SIRT1−/−MEF cell lines (D1 and D2) derived from SIRT1−/− animals (top panel). Wild-type and SIRT1−/− MEF cells were treated with increased concentrations of E2 for 48 h, and cyclin D1 from whole cell lysates was measured by western blot analysis. (**C**) SIRT1 deficiency impairs estrogen response in MEF cells. Wild-type and SIRT1−/− MEF cells (3×10^4 cells per well in 96-well plates) were grown in Dulbecco's modified Eagle's medium supplemented with 5% charcoal–dextran-stripped fetal bovine serum for 72 h prior to start of the experiment. Cells were treated with E2 for 72 h and analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. Bars (mean ± SEM) indicate the average of three independent experiments.

SIRT1 is not directly associated with the ERα promoter, and inhibition of SIRT1 deacetylase activity is unable to alter H3-K9 and H4-K16, two specific markers modified by SIRT1 in humans. We also confirmed that inhibition of SIRT1 activity disrupts the association of RNAPII with the ERα promoter. Our data suggest that SIRT1 may not participate in histone modification but is involved in the formation of basal transcription complexes at the ERα promoter, which contributes to ERα downstream gene transcription (36).

It is important to note that our findings do not exclude the possibility of a SIRT1 effect on other components of ERα signaling that could also lead to estrogen-dependent gene activation, such as PR, pS2 and cyclin D1. We observed that a downregulation of ERα protein expression occurs in SIRT1 inhibitor-treated cells and SIRT1−/− mice. However, knockdown of SIRT1 by siRNA does not alter ERα expression in MCF-7 and T47D cells (data not shown), suggesting that other members of SIRT family may compensate SIRT1 depletion in a cell culture model. This compensatory effect may be sufficient to maintain ERα expression. Of note, knockdown of SIRT1 by siRNA does impair estrogen response (data not shown), suggesting it may have effects on other aspects of ERα signaling.

In conclusion, the results of our present study clearly show that SIRT1, a member of class III HDACs, is required for modulation of ERα-signaling pathways. Inhibition of SIRT1 activity leads to suppression of ERα and its downstream gene transcription.

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