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Differentiation and upregulation of heat shock protein 70 induced by a subset of histone deacetylase inhibitors in mouse and human embryonic stem cells

Jeong-A Park[1,2], Young-Eun Kim[1,2], Hyun-Jeong Seok[1,2], Woo-Youn Park[3], Hyung-Joo Kwon[4,5] & Younghee Lee[1,2,]*

[1]Department of Biochemistry, College of Natural Sciences and [2]Biotechnology Research Institute, Chungbuk National University, [3]Department of Radiation Oncology, College of Medicine, Chungbuk National University, Cheongju 361-763, [4]Center for Medical Science Research and [5]Department of Microbiology, College of Medicine, Hallym University, Chuncheon 200-702, Korea

Inhibiting histone deacetylase (HDAC) activity modulates the epigenetic status of cells, resulting in an alteration of gene expression and cellular function. Here, we investigated the effects of HDAC inhibitors on mouse embryonic stem (ES) cells. The HDAC inhibitors trichostatin A, suberoylanilide hydroxamic acid, sodium butyrate, and valproic acid induced early differentiation of mouse ES cells and triggered induction of heat-shock protein (HSP)70. In contrast, class III HDAC inhibitors failed to induce differentiation or HSP70 expression. Transcriptional upregulation of HSP70 was confirmed by mRNA expression analysis, an inhibitor study, and chromatin immunoprecipitation. HSP70 induction was dependent on the SAPK/JNK, p38, and PI3K/Akt pathways. Differentiation and induction of HSP70 by a subset of HDAC inhibitors was also examined in human ES cells, which suggests that the phenomenon generally occurs in ES cells. A better understanding of the effects of HDAC inhibitors may give more insight into their application in stem cell biology. [BMB reports 2011; 44(3): 176-181]

INTRODUCTION

Modulation of gene expression by altering chromosomal structure, by modifying the acetyl groups in histone proteins through histone deacetylase (HDAC), and histone acetyltransferase activities has been implicated during development and in diverse human pathologies (1). The HDAC superfamily comprises three classes. The major groups are class I (HDACs 1, 2, 3, 8, and 11) and class II (HDACs 4, 5, 6, 7, 9, and 10). Sirtuins or sir2-related proteins (SIRT1, SIR2L1, or Sir2α) are the class III enzymes involved in the deacetylation of other non-histone proteins, such as p53 and nuclear factor-κB p65 (2).

HDAC inhibitors modulate the epigenetic status of cells and are a new class of chemotherapeutic agents against cancer (2-5). HDAC inhibitors also modulate pancreatic cell differentiation and the reprogramming of neurosphere cells (6, 7). According to a recent report on mouse embryonic stem (ES) cells, inhibiting HDACs with trichostatin A (TSA) accelerates the early events of stem cell differentiation based on transcriptomic and epigenetic analyses (8). Therefore, fine tuning of epigenetic regulation may be very important for cell fate control (8, 9).

Heat-shock proteins (HSPs) are a family of highly conserved proteins that are induced by various stimuli, such as infection, high temperature, free radicals, and mechanical stress (10). HSPs play essential roles as molecular chaperones in protein folding, protein trafficking, and cell signaling (10). To understand the effects of inhibiting HDACs in mouse and human ES cells, we used several different HDAC inhibitors and evaluated changes in ES cells. Our results showed that a subset of HDAC inhibitors induced early differentiation and HSP70 expression in mouse and human ES cells.

RESULTS

Differentiation of mouse ES cells induced by a subset of HDAC inhibitors

To examine the effects of HDAC inhibitors on mouse ES cell properties, we first treated ES cells with the HDAC inhibitors TSA, suberoylanilide hydroxamic acid (SAHA), and nicotinamide. Flow cytometry analysis revealed that the expression of stem cell marker genes, such as SOX-2, KLF-4, and NANOG, was greatly reduced after treatment with TSA and SAHA (Fig. 1). In contrast, nicotinamide did not change marker expression. A Western blot analysis of OCT4 protein levels revealed that OCT4 expression decreased markedly following treatment with the HDAC inhibitors TSA, SAHA, sodium butyrate, and valproic acid (Fig. 2A). These results agreed with pre-

*Corresponding author. Tel: 82-43-261-3387; Fax: 82-43-267-2306; E-mail: YHL4177@cbnu.ac.kr
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vious reports showing that HDAC inhibitors, including TSA, induce the early differentiation of mouse ES cells that accompanies downregulation of key regulatory genes such as NANOG and OCT4 (8, 11). Recently, it was reported that a repressor complex including mSin3A, HDAC1, and HDAC2 is involved in ES cell maintenance (11). Therefore, inhibiting this complex with a subset of HDAC inhibitors may cause a molecular change in ES cells. Differentiated cellular morphology was detected when we checked the morphology of mouse ES cells after treatment with TSA, SAHA, sodium butyrate, and valproic acid (Supplementary Fig. 1). This result was associated with a decrease in stem cell marker expression (Fig. 1 and 2). In contrast to the other inhibitors, nicotinamide failed to induce a morphological change in ES cells (Supplementary Fig. 1A). When we treated mouse ES cells with TSA at different concentrations for 12 h, the morphological change was evident even at a concentration of 5 nM (Supplementary Fig. 1B). All other HDAC inhibitors except nicotinamide also induced apoptosis in ES cells. Severe cell death was evident at 24 h after TSA stimulation (Supplementary Fig. 1C).

Induction of HSP70 by a subset of HDAC inhibitors in mouse ES cells by transcriptional regulation

The HDAC inhibitors SAHA and TSA induce HSP70 protein expression in neuronal cells (12-14). Here, we checked HSP70 expression and found that the HDAC inhibitors TSA, SAHA, sodium butyrate, and valproic acid induced HSP70 expression in mouse ES cells (Fig. 2A). In contrast with other HDAC in-

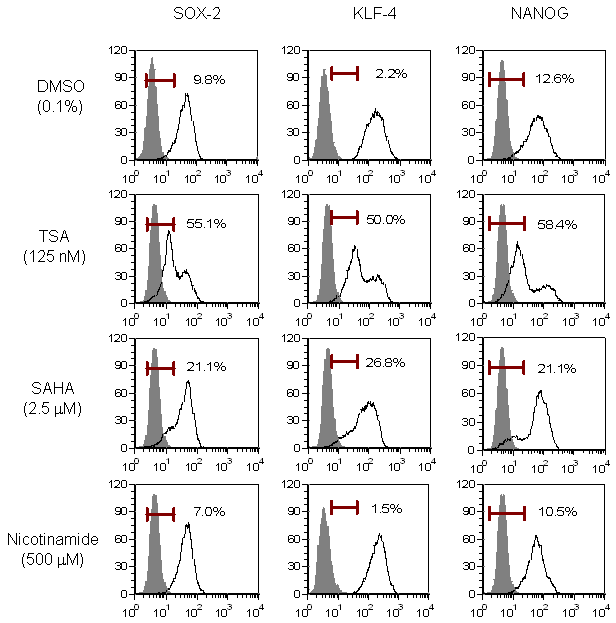

Fig. 1. Decrease of pluripotency marker gene expression by a subset of histone deacetylase (HDAC) inhibitors. Mouse embryonic stem (ES) cells were treated with DMSO, trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), or nicotinamide for 24 h, and SOX-2, KLF-4, and NANOG protein expression was analyzed by immunostaining and flow cytometry. The cell populations that expressed low levels of SOX-2, KLF-4, and NANOG are indicated. These results are representative of three experiments.

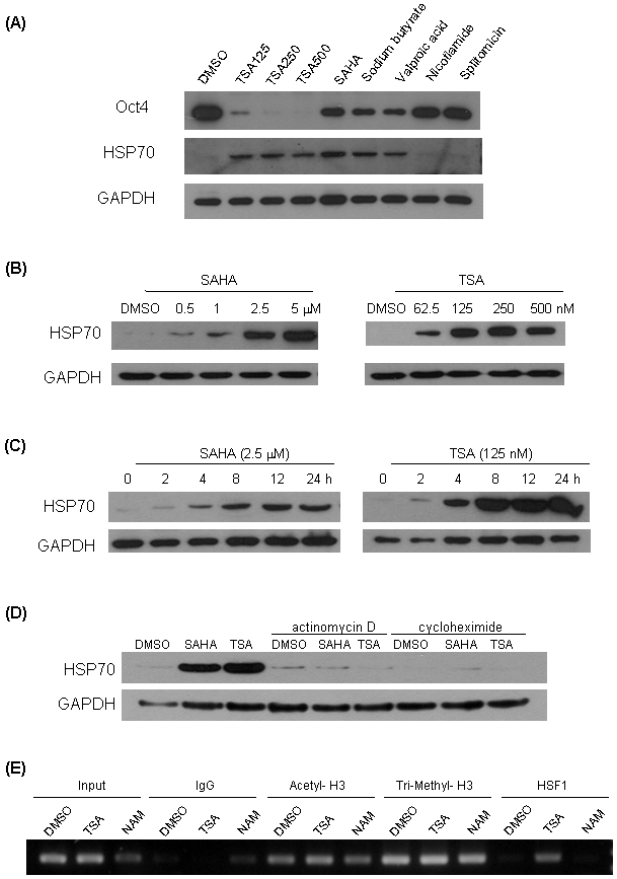

Fig. 2. Heat shock protein (HSP)70 expression by transcriptional upregulation in mouse ES cells treated with a subset of histone deacetylase (HDAC) inhibitors. (A) Expression of HSP70 and OCT4. Mouse embryonic stem (ES) cells were treated with trichostatin A (TSA) (125 nM, 250 nM, or 500 nM), suberoylanilide hydroxamic acid (SAHA) (2.5 μM), sodium butyrate (2 mM), valproic acid (2 mM), nicotinamide (500 μM) or splitomicin (120 μM) for 24 h. (B) Mouse ES cells were treated with SAHA or TSA at the indicated concentrations for 24 h. (C) ES cells were treated with SAHA or TSA for the indicated periods. (D) ES cells were cultured in the presence or absence of actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) for 1 h and then treated with SAHA (2.5 μM) or TSA (125 nM) for 6 h. HSP70 protein expression was determined by Western blotting (A-D). The amount of GAPDH protein was used as a loading control. These results are representative of three experiments that yielded similar results. (E) Mouse ES cells were treated with TSA (125 nM) or nicotinamide (500 μM) for 3 h. Chromatin was prepared and analyzed by chromatin immunoprecipitation assay with anti-acetyl-histone H3, anti-trimethyl-histone H3, and anti-HSF1 antibodies. The DNA purified from the sonicated nuclear lysates was used as an input control. These results are representative of three experiments.

hibitors, the SIRT1 inhibitors nicotinamide and splitomicin failed to induce HSP70 expression (Fig. 2A).

We conducted a detailed investigation of SAHA-induced and TSA-induced expression of HSP70 to understand the molecular mechanism involved in HSP70 expression. As shown in Fig. 2B and C, SAHA and TSA triggered the induction of HSP70 protein in a dose- and time-dependent manner. RT-PCR and real-time PCR analyses revealed that the expression level of HSP70 mRNA increased significantly in response to the HDAC inhibitors SAHA and TSA (Supplementary Fig. 2). Furthermore, neither SAHA nor TSA induced HSP70 protein in the presence of 5 μg/ml of actinomycin D (an RNA synthesis inhibitor) or 10 μg/ml of cycloheximide (a protein synthesis inhibitor) (Fig. 2D). Therefore, we deduced that HSP70 induction is regulated at the transcriptional level.

We performed a chromatin immunoprecipitation (ChIP) assay using antibodies specific to acetylated histone 3 (H3), tri-methylated H3, and HSF1 (15, 16) to check the epigenetic status of the HSP70 promoter and the involvement of HSF1, which is a transcriptional factor mediating HSP induction (17). As shown in Fig. 2E, the H3 in the HSP70 promoter was acetylated and tri-methylated independently of any treatment with HDAC inhibitors, suggesting that epigenetic status was unchanged by the HDAC inhibitors. However, a clear difference in the HSF1 binding patterns was observed in response to TSA and nicotinamide. TSA but not nicotinamide induced an association of HSF1 with the HSP70 promoter, indicating that HSF1 was activated and bound to the HSP70 promoter in response to TSA (Fig. 2E).

SAPK/JNK-dependent, p38 MAPK-dependent, and PI3K-dependent HSP70 expression induced by HDAC inhibitors

HSP70 induction is regulated through PI3K/Akt, p38 MAPK, and SAPK/JNK pathways (18, 19). We used the following signaling pathway inhibitors to check the involvement of these

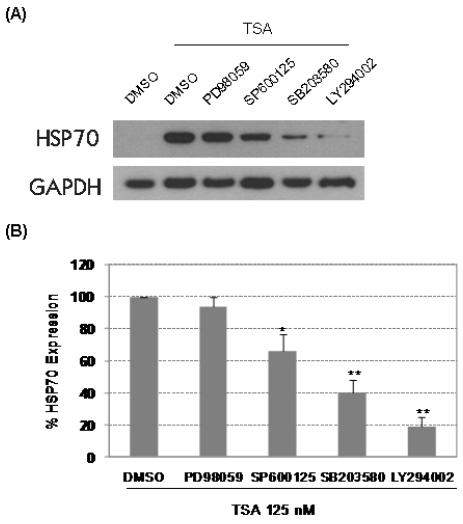

Fig. 3. Heat shock protein (HSP)70 expression is dependent on the SAPK/JNK, p38, and PI3K pathways. (A) Mouse embryonic stem (ES) cells were preincubated for 1 h in the presence of SP600125 (20 μM), PD98059 (50 μM), SB203580 (10 μM), or LY294002 (30 μM). After treatment with trichostatin A (TSA) (125 nM) for 6 h, HSP70 expression levels were determined by Western blotting. The amount of GAPDH was used as a loading control. The results are representative of three experiments. (B) Statistical analysis of HSP70 expression induced by TSA in the presence of signaling pathway inhibitors. Analysis of the percent HSP70 expression relative to the DMSO vehicle control is based on a data set obtained from three independent experiments (*P < 0.05, **P < 0.01).

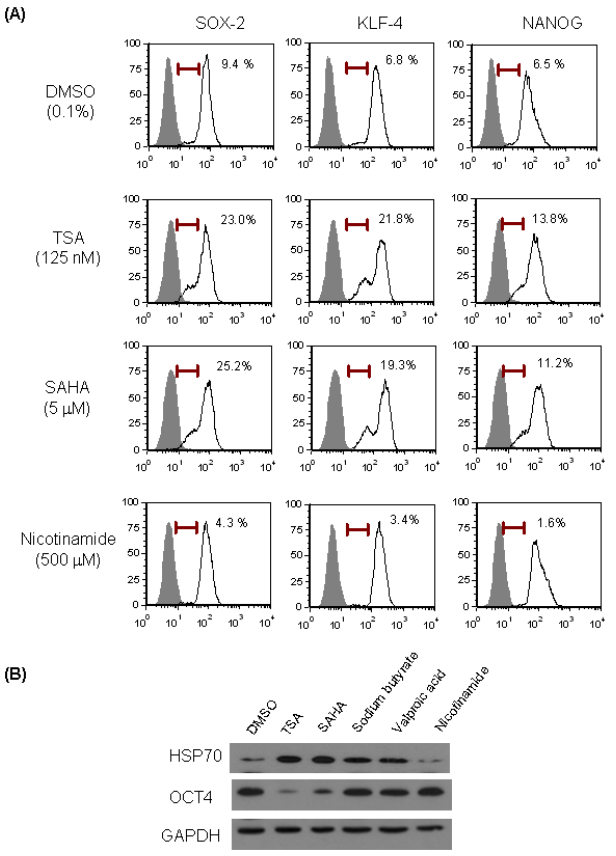

Fig. 4. Differentiation and heat shock protein (HSP)70 expression induced by histone deacetylase (HDAC) inhibitors in human ES cells. (A) Decrease of pluripotency marker gene expression. H9 cells were treated with DMSO or the indicated HDAC inhibitors for 24 h, and SOX-2, KLF-4, and NANOG protein expression was analyzed by immunostaining and flow cytometry. The cell populations that expressed low levels of SOX-2, KLF-4, and NANOG are indicated. (B) H9 cells were treated with TSA (125 nM), suberoylanilide hydroxamic acid (SAHA) (2.5 μM), sodium butyrate (2 mM), valproic acid (2 mM), or nicotinamide (500 μM) for 24 h. The HSP70 expression level was determined by Western blotting. The amount of GAPDH was used as a loading control. These results are representative of three experiments.

pathways: the SAPK/JNK inhibitor SP600125, the MEK1/ERK pathway inhibitor PD98059, the p38 pathway inhibitor SB203580, and the PI3K inhibitor LY294002. As shown in Fig. 3, the SAPK/JNK, p38 and PI3K pathway inhibitors reduced HSP70 expression, whereas the ERK pathway inhibitor, which was used as a control, had no effect on HSP70 induction. Therefore, we concluded that the PI3K/Akt, p38 MAPK, and SAPK/JNK pathways contribute to the induction of HSP70 in mouse ES cells.

HSP70 expression and differentiation induced by a subset of HDAC inhibitors in human ES cells

Although mouse and human ES cells have some differences, they share OCT4 and NANOG expression and high levels of alkaline phosphatase and telomerase activities (20). We checked whether the HDAC inhibitors also induced early differentiation and HSP70 expression in human ES cells. SOX-2, KLF-4, and NANOG expression decreased significantly with TSA and SAHA treatment but not by nicotinamide (Fig. 4A). A morphological examination revealed that the HDAC inhibitors, except nicotinamide, induced early differentiation of human ES cells (Supplementary Fig. 3). Furthermore, we found that the inhibitors TSA, SAHA, sodium butyrate, and valproic acid but not nicotinamide induced HSP70 expression in human ES cells (Fig. 4B). This result is similar to the results of mouse ES cells (Fig. 1 and 2). Thus, a subset of HDAC inhibitors induced early differentiation and HSP70 expression in ES cells from different species.

DISCUSSION

Because epigenetic regulation is essential for maintaining cell specificity, inhibiting the epigenetic process can cause changes in cellular properties, including the stemness of pluripotent cells (8, 9, 21, 22). We used different classes of HDAC inhibitors to examine their effects on ES cell properties. The short-chain fatty acids sodium butyrate and valproic acid and the hydroxamic acid derivatives SAHA and TSA seemed to inhibit class I and class II HDAC activity by binding to the active sites and the essential zinc ions (23). Nicotinamide and splitomicin inhibit NAD-dependent class III HDACs (24).

In ES cells, the stem-cell regulatory genes OCT4 and NANOG induce the expression of genes involved in the maintenance of stemness and suppress the expression of genes involved in differentiation (22). Treating ES cells with the HDAC inhibitors TSA and SAHA decreased the expression levels of the stem cell regulatory genes (Fig. 1 and 4) and can cause a change in global gene expression profiles leading to early differentiation of ES cells (8). Here, we also found that the HDAC inhibitors TSA, SAHA, sodium butyrate, and valproic acid, which induced differentiation in ES cells, increased HSP70 expression in mouse and human ES cells (Fig. 2 and 4). In contrast, the HDAC inhibitors nicotinamide and splitomicin, which target class III HDACs, failed to induce differentiation or HSP70 expression (Fig. 1, 2, and 4). To explain this result, we postulated that the HSP70 gene promoter was suppressed by HDAC activity in ES cells and that the promoter is sensitively activated in response to HDAC inhibitors targeting class I or class II HDACs. However, the class III HDAC inhibitors have no effect on promoter activity, as they modify the acetylation level of non-histone proteins (25). To check this possibility, we performed a ChIP assay and found that the epigenetic status of the HSP70 promoter was unchanged by treatment with any HDAC inhibitor (Fig. 2E). Therefore, treating class I or class II HDACs with HDAC inhibitors leading to differentiation of ES cells is likely to induce HSP70 as a stress response protein. Inhibiting Sirt1 activity, which is possibly not stressful to ES cells, did not induce HSP70 expression. This outcome is in agreement with a report stating that Sirt1 knockout mouse ES cells show no phenotypic abnormalities under normal conditions (25). HSP expression is enhanced by various types of stress through transcription factor HSF1 (17). As shown in Fig. 2E, TSA but not nicotinamide induced an association of HSF1 with the HSP70 promoter. The results of the inhibitor assays showed that the PI3K/Akt, p38 MAPK, and SAPK/JNK pathways contributed to the induction of HSP70, as previously reported for other cells (Fig. 3). Therefore, we consider that the HSP70 expression induced by the HDAC inhibitors was a stress response in ES cells.

Molecular chaperones have multiple functions, and the potential of using chaperones in treatment is a new frontier of recent therapies against cancer, cardiovascular disease, and neurodegeneration (10, 12-14). Treatment with SAHA reduces ischemic injury in mouse brain and increases the expression of the neuroprotective proteins HSP70 and BCL-2 (13). Valproic acid induces functional HSP70 through class I HDAC inhibition in rat cortical neurons and this type of induction may contribute to the neuroprotective and therapeutic effects of valproic acid (12, 14). Thus, the functional role of HSP70 might be the protection of cells against HDAC inhibitor-induced apoptosis.

A recent study has shown that the HDAC inhibitors valproic acid and sodium butyrate improve the reprogramming efficiency of induced pluripotent stem (iPS) cell generation (26). However, the potent HDAC inhibitor TSA does not increase the efficiency as much as expected. ES cells are very sensitive to TSA. For example, they show a decrease in stem-cell marker expression, apoptosis, and robust expression of HSP70. Accordingly, our results may explain why TSA is not so helpful for iPS generation. The results also suggest that fine tuning of HDAC inhibitor activity and the treatment protocol may improve the efficiency of reprogramming with HDAC inhibitors. Thus, a better understanding of the functional effects of HDAC inhibitors in ES and somatic cells may shed more light on the application of HDAC inhibitors.

This study has shown that a subset of HDAC inhibitors targeting class I and class II HDACs induced early differentiation in ES cells and simultaneously induced HSP70 expression in

mouse and human cells. Our results confirm that HDACs help maintain ES cell properties; they also show that HDAC inhibitors have differential effects on ES cells, particularly for differentiation and the stress response.

MATERIALS AND METHODS

Maintenance of mouse ES cells

The mouse ES cell line TC-1 was maintained on mouse embryonic fibroblast (MEF) cells in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (Hyclone Inc., Logan, UT, USA) and 0.1 mM β-mercaptoethanol, as described previously (27).

Maintenance of human ES cells

The human ES cell line H9 (WiCell Research Institute, Madison, WI, USA) was maintained on MEF cells in a human ES medium, which included DMEM/F12, 20% KnockOut SR medium (Invitrogen, Carlsbad, CA, USA), 0.1 mM β-mercaptoethanol, and 4 ng/ml human basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA). The cultures were passaged after being treated with collagenase IV (Invitrogen) and subjected to physical dissociation using pipettes, as described previously (28).

Treatment with HDAC inhibitors

A sample of $5 \times 10^5 - 1 \times 10^6$ mouse ES cells was placed in gelatin-coated six-well plates and grown overnight in an MEF-conditioned medium prepared as described previously (27). Additionally, a sample of $2-3 \times 10^5$ human ES cells obtained by physical dissociation was placed in six-well plates coated with Matrigel (BD Biosciences, Bedford, MA, USA) and grown overnight in an MEF-conditioned human ES medium supplemented with bFGF. The HDAC inhibitors (Sigma-Aldrich, St. Louis, MO, USA) used in this study are as follows: TSA, SAHA, sodium butyrate, valproic acid, nicotinamide, and splitomicin.

Western blotting

Protein lysates were analyzed by Western blotting as previously described (28). Antibodies to HSP70 (W27) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (6C5) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody to OCT3/4 was obtained from BD Biosciences.

Detection of pluripotent stem cell markers by flow cytometry analysis

ES cells treated with a vehicle control or HDAC inhibitors were stained with one of the following antibodies: anti-SOX-2 (H65, Santa Cruz Biotechnology), anti-KLF-4 (H180, Santa Cruz Biotechnology), or anti-NANOG (Millipore Corp, Bedford, MA, USA). After being treated with the FITC-conjugated secondary antibody, the cells were analyzed with a Becton Dickinson FACSCalibur cytometer (Heidelberg, Germany). The data were analyzed with FCS Express 3 software (De Novo Software, Los Angeles, CA, USA).

ChIP assay

Chromatin from mouse ES cells treated with HDAC inhibitors was prepared as described previously (29). A ChIP assay was performed with 5 μg of the following antibodies: a control serum, anti-acetyl-histone H3 (Lys9) (Cell Signaling Technology, Beverly, MA, USA), anti-tri-methyl-histone H3 (Lys4) (C42D8, Cell Signaling Technology), or anti-HSF1 (E-4, Santa Cruz Biotechnology). The immune complexes were eluted, and the DNA was analyzed by PCR with the following primer pair specific for the mouse HSP70 promoter: 5'-CCTGGTCTGAGTCC CACACT-3' and 5'-CGCTAGAGAGTACGGATTCCTG-3' (product size, 163 bp). The DNA that was purified from the sonicated nuclear lysates was directly analyzed by PCR with the same primer set, and the result was used as an input control.

Statistical analysis

The data were evaluated with a Student's *t*-test. A $P < 0.05$ was considered significant.

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