

Histone Deacetylase 5 Is Not a p53 Target Gene, But Its Overexpression Inhibits Tumor Cell Growth and Induces Apoptosis

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ABSTRACT

p53 tumor suppressor is activated by phosphorylation and acetylation on DNA damage. One of unknown p53 early transcripts was identified to be histone deacetylase-5 (HDAC5). We tested a hypothesis that HDAC5 is a p53 down-stream target gene that on induction by p53 inactivates p53 by removal of acetyl group in p53 molecule, thus functioning as an auto-regulatory negative feedback loop in analogue to p53-murine double minute 2 interaction. Six p53 binding consensus sites were identified in the promoter of HDAC5. p53 binds to one of the sites weakly. However, luciferase constructs driven by the HDAC5 promoter containing three to six potential binding sites were not activated by p53, nor was the expression of HDAC5 mRNA induced by p53-activating agents. Furthermore, HDAC5 does not bind to p53 nor reduces etoposide-induced p53 acetylation. Thus, HDAC5 is not a p53 target gene and may act in a p53-independent manner. We next studied the effect of HDAC5 on tumor cell growth and apoptosis. Transfection of HDAC5 inhibited growth of multiple tumor cell lines including U2OS osteogenic sarcoma cells, SY5Y neuroblastoma cells, and MCF breast carcinoma cells. The growth suppression seen in HDAC5-overexpressing cells appears to be attributable partly to a reduced growth rate as revealed by cell growth assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and mainly to spontaneous apoptosis as shown by DNA fragmentation ELISA and morphological appearance. Mechanistically, repression of three cell proliferation genes in mitogen-activated protein kinase pathway and induction of seven apoptosis-related genes were identified by microarray profiling in HDAC5-overexpressed cells. Among induced genes, four (*TNFR1*, *TNFSF7*, caspase-8, and *DAPK1*) were associated with the tumor necrosis factor ligand-receptor death pathway. Induction of *TNFR1*, *TNFSF7*, and caspase-8 were confirmed by Northern and Western analyses. Thus, activation of tumor necrosis factor death receptor pathway appears to be associated with HDAC5-induced spontaneous apoptosis.

INTRODUCTION

Gene transcription is controlled in part by the dynamic acetylation and deacetylation of histone proteins (1). In general, histone acetylation catalyzed by histone acetyl-transferases increases gene expression by altering nucleosomal conformation and making chromatin templates accessible by transcription factors. On the other hand, histone deacetylation catalyzed by HDACs⁴ would reverse this process to shut down gene expression (1, 2). In addition to histone protein, increasing numbers of nonhistone proteins, such as transcription factors, were also subject to regu-

lation by acetylation/deacetylation as a post-translational modification (3, 4). Thus, precise balance between protein acetylation and deacetylation controls a variety of intracellular activities involving gene expression and protein activation, thereby playing an important role in cell growth, differentiation, and apoptosis.

HDACs are a family of enzymes that catalyze the removal of acetyl group from post-translationally acetylated proteins, which include histones, transcription factors, and architectural proteins (5). Currently, the genes for 15 different known and putative HDACs have been identified in humans (5). They can be divided into three different classes according to homology to prototypical HDACs identified in yeast that are generally involved in repression of transcription. The class I includes the HDACs 1, 2, 3, and 8, which share significant similarity in amino acid sequence and domain organization with yeast RPD3. The class II includes HDACs 4, 5, 6, 7, and 9. Their catalytic domains are more similar to that of yeast HDAC1 than that of RPD3 (6, 7). In addition, the sequences of the class II enzymes are roughly twice as long as the class I proteins and have a different domain organization. The recently discovered class III is yeast protein SIR2-related proteins that possess NAD-dependent HDAC activity *in vitro*, termed human SIRT1-7 (5). A growing body of literature has established the role of HDACs in chromatin remodeling and gene expression. In general, HDACs are associated with gene silencing because they form multisubunit complexes with other proteins and function as transcriptional corepressors. Inhibition of HDAC activity by HDAC inhibitors leads to activation of expression for a variety of genes, notably *p21*^{WAF1/Cip1} (8), *p57* (9), and *Bcl-2* (10).

HDACs have been implicated in the development of cancer (11). The activity of several tumor suppressors is regulated in part by HDACs. For example, the retinoblastoma tumor suppressor protein Rb represses transcription by recruiting HDAC1 (12). The p53 tumor suppressor protein physically interacts with sin3-HDAC complex, which mediates its transcriptional repression activity (13). The p53-dependent gene activation is specifically inhibited by HDAC1-3 (14). Of particular interest are recent findings that human SIR2 functions as an NAD-dependent p53 deacetylase and represses p53-dependent apoptosis induced by DNA damage and oxidative stress (15, 16). In addition, the COOH-terminal domain of the breast cancer susceptibility protein BRCA1 associates with the HDACs HDAC1 and HDAC2 (17). Furthermore, HDAC inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects (18). Thus, inhibition of HDACs was considered as a new strategy for anticancer treatment through targeting epigenetic modifications (19).

The tumor suppressor protein p53 plays an important role in cell growth and apoptosis (20, 21). In response to various stress conditions such as DNA damage, p53 is activated to induce growth arrest to allow cells to repair damaged DNA or apoptosis to eliminate damaged cells, if the damage is severe and repair becomes impossible (22, 23). p53-induced growth arrest and apoptosis are mainly mediated through its transcription activity by transactivating or transrepressing a number of down-stream target genes (20, 21). Importantly, the DNA binding and transcriptional activities of p53 require a post-translational modification by acety-

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⁴ The abbreviations used are: HDAC, histone deacetylase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PET, p53 early transcript; SAGE, serial analysis of gene expression; SIR2, silent information regulator 2; TNF, tumor necrosis factor; TSA, trichostatin A; MAP, mitogen-activated protein; nt, nucleotide; β -gal, β -galactosidase; MEF2, myocyte enhancer factor-2; mdm-2, murine double minute 2; DAPK, death-associated protein kinase; NAD, nicotinamide adenine dinucleotide.

lation (3, 24). The acetylation of p53 can be induced by a variety of p53-activating agents, which include agents that induce DNA damage, hypoxia, and oxidative stress (25, 26). On the other hand, removal of acetyl group from p53 by HDACs, namely HDAC1-3, inhibits p53-dependent gene activation, whereas inhibition of HDAC activity by HDAC inhibitor increases p53 stability (14).

Through the use of SAGE profiling, Yu *et al.* (27) has identified recently 34 PETs that were induced >10-fold soon after p53 activation. We have performed cluster analysis of all of the unknown PETs and identified PET18 as *HDAC5*. Additional computer data analysis identified six putative p53-binding sites in the promoter region of the *HDAC5*. Thus, *HDAC5* could be a p53 downstream target. Our working hypothesis is that in response to DNA damage, p53 is activated by phosphorylation and acetylation. Activated p53 then transactivates and induces *HDAC5*, which in turn deactivates p53 by removal of its acetyl group. This negative auto-regulatory feedback loop is analogous to p53-Mdm2 interaction to keep p53 activity in check (28).

Here we report our characterization of *HDAC5* as a potential p53 downstream target gene by assays for DNA binding, transcriptional activation, and endogenous gene induction. Because p53 bound weakly to the *HDAC5* promoter, p53 did not transactivate *HDAC5* promoter and endogenous *HDAC5* mRNA was not induced by p53 activating agent, we concluded that the *HDAC5* is not a p53 target gene. Additional characterization of the role of *HDAC5* in cell growth control revealed that forced expression of *HDAC5* inhibits tumor cell growth and induces spontaneous apoptosis. Mechanistically, it appears to operate through the repression of MAP kinase pathway and activation of TNF ligand-receptor death pathway, as suggested by microarray profiling.

MATERIALS AND METHODS

Cell Cultures. Human osteogenic sarcoma U2OS cells, neuroblastoma SY5Y cells, and breast carcinoma MCF7 cells were obtained from American Type Culture Collection and maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma). Cells were grown at 37°C with 5% CO₂ in air and were routinely subcultured with trypsin (0.25%/EDTA (1 mM) when they reached 80–90% confluence.

Computer Database Search and Analysis. A SAGE tag, PET-18 (27), 5'-CATGGCAGTGGGCT-3' was compared with a database of predicted mRNA transcripts. The database was generated by clustering the publicly available Expression Sequence Tag and mRNA sequences from GenBank using LEADS clustering software (Compugen Ltd., Tel-Aviv, Israel).⁵ Comparisons were made using the blastn option of the National Center for Biotechnology Information Basic Local Alignment Search Tool application (29) with default parameters except filtering was turned off and the expected value cutoff was set to 100. Transcripts containing perfect matches to the tags were examined for tag orientation and the presence of 3' *Nla*III sites. Transcript identities were determined from the mRNA sequences contributing to the transcript cluster and by comparison to the nonredundant sequence databases from National Center for Biotechnology Information. PET-18 sequence was identified at the nts 2406–2419 of *HDAC5* open reading frame (GenBank accession no. AF132608 or NM_005474; Ref. 30).

Gel Shift Assay. Six potential p53-binding sites were identified in the promoter of the *HDAC5* gene (GenBank accession no. AC004150; nts 1–2590), allowing up to four mismatches (Fig. 1). All six of these sites were ³²P end labeled and subjected to p53 binding gel shift assay as detailed previously (31).

Promoter Cloning and Luciferase Reporter Assay. The promoter fragments containing a variety of putative p53 binding sites were PCR amplified using human placenta DNA (Oncor) as the template and cloned into pGL-Basic-3 luciferase reporter construct (Promega). The primers used for *HDAC5*

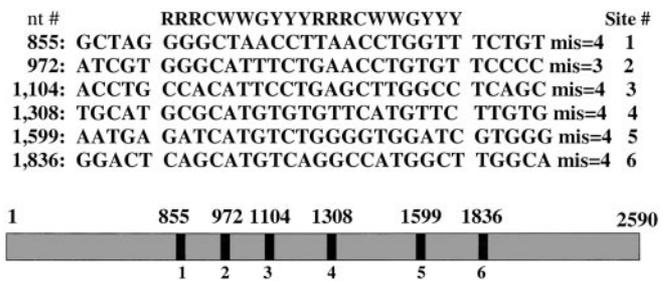


Fig. 1. Detection of six putative p53-binding sites in the promoter of *HDAC5* promoter: The *HDAC5* promoter was searched against p53 binding site consensus sequence shown on the top of figure, allowing four mismatches. Six matches were identified, and their relative location in the promoter was shown in a bar graph at the bottom.

with six p53BS (nts 855–2590; containing all six sites) were *HDAC5*-P01, 5'-GGGCTAACCTTAACCTGGTT-3' and *HDAC5*-P02, 5'-CCCTGGGG-AGAGATGGAGCA-3'. For *HDAC5* with five p53BS (nt 972–2590; containing five sites), the primers were *HDAC5*-P03, 5'-GGGCATTCTGAACCTGTGT-3' and *HDAC5*-P02. For *HDAC5* with four p53BS (nts 1308–2590; containing three sites with binding site 4 included), the primers were *HDAC5*-P05, 5'-GCGCATGTGTTCATGTTC-3' and *HDAC5*-P02 and for *HDAC5* without p53BS (nts 1328–2590; containing two sites with binding site 4 deleted), the primers were *HDAC5*-P07, 5'-TTGTGTTTCTGCCATAGCTG-3' and *HDAC5*-P02. All of the PCR fragments were verified by DNA sequencing after cloning. DNA transfection and luciferase assay were performed as detailed previously (32).

***HDAC5* Cloning and DNA Transfection.** The cDNA encoding the entire *HDAC5* open reading frame with a FLAG-tag attached at the NH₂ terminus was cloned into pcDNA3 (In Vitrogen) at XbaI/KpnI sites by reverse transcription-PCR followed by DNA sequencing confirmation. pcDNA3-*HDAC5* and pcDNA3 vector plasmids were prepared using Wizard Plus Miniprep DNA Purification System (Promega) following the supplier's protocol. To examine the effect of *HDAC5* in monolayer cell growth, pcDNA3-*HDAC5* and pcDNA3 vector plasmids from three independent preparations were transfected into three human tumor cell lines, U2OS, SY5Y, and MCF7 using LipofectAMINE reagent (Life Technologies, Inc.). pcDNA3 vector or pcDNA3-*HDAC5* (2 μg) plus 0.5 μg of pCMV β-gal were used in the transfection, as described previously (33). The concentration of G418 used for selection is 600 μg/ml for U2OS and SY5Y, and 800 μg/ml for MCF 7 cells. The resulted monolayer colonies were stained with Coomassie blue solution (0.25% Coomassie blue in 10% acetic acid, 40% methanol, and 50% water). The total number of colonies of each plate were counted and normalized according to β-gal activity of each transfected cell population. Statistical analysis was performed with Student's *t* test using Microsoft Excel analysis tools (*n* = 3).

Establishment of Stable Cell Lines. To generate cell lines that stably express *HDAC5* protein, U2OS cells were transfected with pcDNA3-*HDAC5* construct with a FLAG-tag at the NH₂ terminus using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instruction. Control stable cell lines were also generated by transfection of the pcDNA3 vector. Stable clones were selected in culture medium containing 600 μg/ml G418 for 3 weeks after transfection. Single clones were isolated by ring cloning and subcultured in a selection medium (34). A total of 18 clones were tested for *HDAC5* expression by Western analysis using antibodies against FLAG-tag or *HDAC5*.

Western Analysis and Immunoprecipitation. Protein extraction and Western analyses were performed as described previously (35). Briefly, protein samples (50 μg) were resolved on a SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots were probed using either antihuman *HDAC5* (Santa Cruz), anti-FLAG (Sigma), or antihuman p53 (Ab-6; Oncogene Science), or anticaspase 8 (Upstate) at a dilution suggested by the supplier and developed using enhanced chemiluminescence reagent (Amersham). For detection of *HDAC5*-p53 binding or p53 acetylation, immunoprecipitation-coupled Western blot analysis was performed. Human kidney 293 cells were cotransfected with *HDAC5* and *p53* (for binding assay), or U2OS cells were treated with etoposide for 24 h (for acetylation assay) in 100-mm culture plates

⁵ Internet address: <http://www.cgen.com>.

followed by cell lysis. Cell lysates were immunoprecipitated by either anti-FLAG antibody or anti-acetyl-p53 (lys373 and 382; Upstate) at a dilution of 1:250 at 4°C for 4 h, followed by addition of 20 μ l of protein G-plus-agarose (Santa Cruz) and incubated for 2 h. The immunoprecipitates were then collected, washed, and analyzed by Western blotting as described above.

HDAC Activity Assay. HDAC activity assay was performed using HDAC Colorimetric Activity Assay kit (Biomol) following the protocol provided by the supplier. The assay is based on cleavage by HDAC of an acetylated lysine side chain in the Colorimetric Histone deAcetylase Lysyl Substrate. This cleavage sensitizes the substrate to react with a developer to produce a yellow color being captured by an absorbance at 405 nm. Briefly, 10 μ g of total cell lysate were incubated with 0.5 mM Colorimetric Histone deAcetylase Lysyl Substrate for 10 h at 37°C with or without the presence of 1 μ M TSA in a total volume of 50 μ l. After the incubation, 50 μ l of 1 \times developer were then added to the sample and incubated at 37°C for 15 min. Absorbance was measured at 405 nm using a colorimetric plate reader.

Cell Growth MTT Assay. The effect of HDAC5 overexpression on cell growth was studied using a cell growth proliferation kit I (Roche), a MTT-based colorimetric assay. Briefly, cells were seeded at a density of 2×10^3 cells/well in 100- μ l culture medium into 96-well microtiter plates and grown for 24 h to 5 days. After the incubation period, 10 μ l of the MTT labeling reagent were added into each well and incubated for 4 h in a tissue culture incubator. Solubilization solution (100 μ l) was then added into each well and incubated overnight in the incubator. After incubation, the spectrophotometrical absorbance of the samples was measured using a microtiter plate reader.

Drug Treatment and DNA Fragmentation ELISA. Four U2OS stable cell lines (two HDAC5 overexpressing lines and two vector controls) were subcultured into a 96-well plate at a density of 1×10^4 cells/well and grown for 24 h. Cell culture medium were then replaced with fresh medium either with DMSO (control) or 25 μ M etoposide (Sigma) and incubated for an additional 24 h. Apoptosis characterized by DNA fragmentation was assayed using the Cell Death Detection ELISA kit (Boehringer Mannheim). Briefly, after 24 h of drug treatment, U2OS cells were lysed by incubation with 200 μ l of cell lysis buffer for 2 h at room temperature. The lysate were then centrifuged at $200 \times g$ for 10 min. Supernatant (20 μ l) was transferred into the streptavidin-coated microtiter plate for analysis using the reagents provided within the assay kit. Statistical analyses were performed with Student's *t* test using Microsoft Excel analysis tools. Fold induction of etoposide-induced apoptosis was calculated by dividing absorbance values from etoposide treatment group with those from the DMSO control.

RNA Isolation, Preparation, cDNA Expression Array, and Northern Analysis. Total cellular RNA was isolated with TRIzol Reagent (Life Technologies, Inc.) following the protocol specified by the supplier. The RNAs were then prepared using the atlas pure total RNA labeling system (Clontech). Briefly, 50 μ g of total RNA were digested with 50 units of DNase I at 37°C for 30 min, and extracted with phenol and chloroform. Total RNAs (2 μ g) were analyzed for quality and purity by gel electrophoresis. RNA (30 μ g) was then used for cDNA probe synthesis in a thermal cycler in the presence of [α - 32 P]dATP (Amersham), deoxynucleotide triphosphate (minus dATP), and Moloney murine leukemia virus reverse transcriptase following the instruction of the supplier. The radiolabeled cDNA probes were then hybridized to the atlas human apoptosis array (Clontech) containing 205 apoptosis and cell cycle-related genes. After 18 h hybridization, the array was washed and exposed to a phosphorimage screen for 48 h, and analyzed using the phosphorimage software. The individual gene signals were normalized against the signals of three housekeeping genes in the same array. Northern blot analysis using 15 μ g of total RNA prepared from each transfectant was carried out as described previously (36). Probes were generated by reverse transcription-PCR amplification using gene-specific primers. The primer sequences for TNF receptor (GenBank accession no. M33294) are: (a) the upstream, 5'-CTGTAC-CAAGTGCCACAAAG-3'; and (b) the downstream, 5'-GTAGAGCTTG-GACTTCACC-3' to yield a 559-bp fragment. The primers for TNFSF7 (GenBank accession no. L08096) are: (a) the upstream, 5'-GTGATCTGC-CTCGTGGTGTG-3'; and (b) the downstream, 5'-CCACTGCACTCCAAA-GAAGG-3' to yield a fragment of 480 bp. The PCR fragments were then gel purified and labeled with [α - 32 P]dCTP and used as the probes.

RESULTS

Identification of PET-18 as HDAC5. Through SAGE profiling several unknown PETs have been identified recently that are induced at least 10-fold by p53 (27). To identify their identities, we have performed computer data analysis by comparing these tag sequences to a database of predicted mRNA transcripts, generated by clustering the publicly available EST and mRNA sequences from GenBank using LEADS clustering software from Compugen. The transcripts containing perfect matches to the tags were examined for tag orientation and the presence of 3' *Nla*III sites. PET-18 sequence was identified at the nts 2406–2419 of HDAC5 open reading frame (GenBank accession no. AF132608 or NM_005474; Ref. 30).

Weak Binding of p53 Protein to One of Six Putative Binding Sites Identified in the Promoter of the HDAC5 Gene. p53 is activated via phosphorylation and acetylation in response to DNA damage (24, 25). We hypothesized that HDAC5 could be a p53 downstream target that on induction by p53, deacetylated and inactivated p53, acting in an auto-regulatory feedback loop in analogue to Mdm2 (28). To characterize whether HDAC5 is a p53 target, we first identified the promoter sequence of the HDAC5 gene by blasting genomic database with HDAC5 cDNA sequence. A 2590-bp fragment upstream potential transcription site was identified (GenBank accession no. AC004150; nts 1–2590). The nucleotides 1746–2590 correspond to the first 845 nts identified recently as the 5' upstream promoter region of the HDAC5 gene (37). The entire 2590 bp fragment was searched for potential p53 binding sites, allowing four mismatches without space between two motifs. As shown in Fig. 1, six putative sites were identified, and all of them were subjected to gel shift assay for p53 binding. Only binding site 4, but none of other five sites binds weakly to purified p53 protein (38), as compared with a positive control (Fig. 2, compare Lanes 2 to 4; data not shown). The same gel shift assay was also performed using binding site 4 as the probe to incubate with nuclear extract prepared from U2OS cells after etoposide treatment to activate p53 (39). Again, a weak binding was observed (data not shown). The results indicate that the p53 binds weakly, if any, to one of six putative binding sites.

Lack of Transactivation of the HDAC5 Promoter by p53. We next examined whether p53 transactivates HDAC5 promoter by a luciferase reporter assay. A fragment containing the binding site 4 (nts

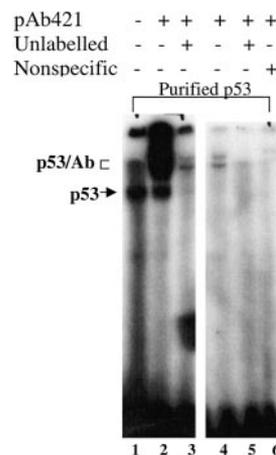


Fig. 2. p53 weakly binds to the site 4 in the promoter of the HDAC5 gene. The site 4 (5'-GCGCATGTGTGTCATGTTTC-3') consisting of the p53-binding consensus sequences (Lanes 4–6), alone with a positive control (5'-AGACATGCCCTAGACATGCC T-3'; Lanes 1–3) were labeled with [γ - 32 P]ATP and used as probes in gel shift assays. Partially purified p53 protein (3 μ g) was used with or without pAb421 antibody as indicated. Competition was performed with a $\times 100$ excess of unlabelled specific oligonucleotides, or nonspecific oligonucleotide mT3SF (5'-GGGGTTGCTTGAAGA GCGTC-3'; Ref. 72). The p53-Ab supershifted bands are indicated.

1308–2590, designated as HDAC5 with four p53BS) and one having this site deleted (nts 1328–2590, designated as HDAC5 without p53BS) were cloned into pGL-basic-3 luciferase reporter. The reporter constructs, along with the vector control, were transfected into U2-OS cells (p53+/+), followed by etoposide treatment to activate p53. As shown in Fig. 3 (top), activation of luciferase activity is minimal and independent of the binding site 4. Lack of p53-dependent luciferase activation was also seen when HCT116 colon carcinoma cells, another wild-type p53 line, was used in the assay (data not shown). To test whether other putative binding sites may confer p53 responsiveness, we also made luciferase reporters driven by the promoter fragments containing five (nts 972–2590, designated as HDAC5 with five p53BS) or six (nts 855–2590, HDAC5 with six p53BS) binding sites, respectively. These constructs were tested for their potential p53 activation using a p53-temperature-sensitive H1299 cell model at which p53 converts from a mutant to a wild-type conformation after temperature shift from 39°C to 32°C (40). As shown in Fig. 3 (bottom), temperature shift did not cause any induction of luciferase activity in any one of constructs driven by the HDAC5 promoter sequences. Furthermore, we have cotransfected these constructs with wild-type p53 into Saos-2 p53-null cells and found no induction of luciferase activity (data not shown). Thus, the promoter of the HDAC5 is not subject to p53 regulation.

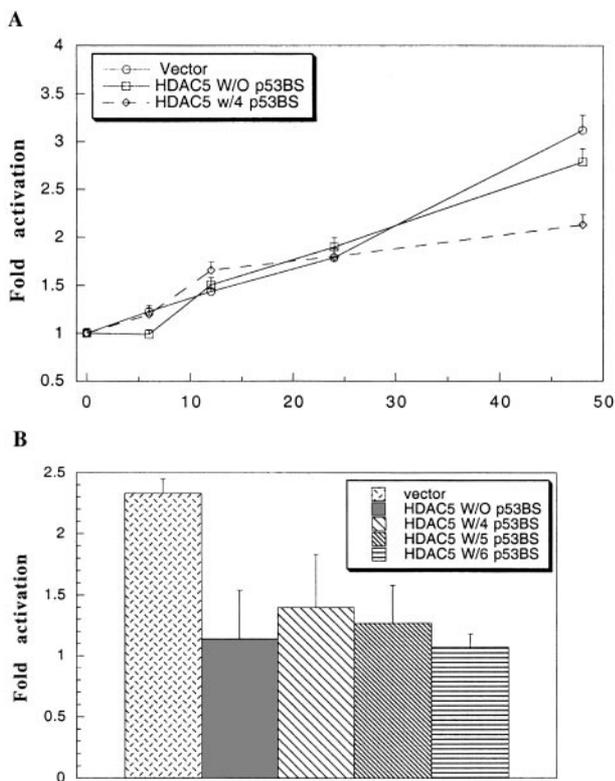


Fig. 3. Lack of transactivation of HDAC5 promoter by p53. A, U2OS cells were transfected with luciferase reporters driven by HDAC5 promoter fragment containing two (HDAC5 w/o p53BS, with binding site 4 deleted) or three binding sites (HDAC5 w/4 p53BS, including binding site 4). Cells were treated 24 h after transfection with 25 μ M etoposide for the indicated periods of time, followed by luciferase assay. B, p53 temperature-sensitive H1299 cells were transfected with luciferase reporters driven by the HDAC5 promoter sequences containing various p53-binding sites as indicated. After transfection (24 h), one set of cells was grown at 39°C for additional 24 h, whereas the other set was shifted to 32°C and grown for 24 h to activate p53. Cells were then lysed, and lysates were assayed for luciferase activity. The fold induction was calculated by dividing luciferase reading at 32°C by that at 39°C. The results are presented as fold activation derived from three independent transfections, each run in duplicate, after normalization with β -gal activity for transfection efficiency; bars, \pm SE.

Lack of HDAC5 mRNA Induction by p53-inducing Agent.

Finally, we examined whether endogenous HDAC5 would be induced by etoposide, a p53-inducing agent (39). Two p53-positive cell lines (U2-OS and H460) and two p53-negative cell lines (Saos-2 and H1299) were subjected to etoposide treatment for 6 and 24 h, followed by Northern analysis. We were unable to detect any expression of HDAC5 under nontreated or treated condition (data not shown), indicating that endogenous HDAC5 mRNA is expressed at a very low level in these cells and that it is not subject to etoposide induction. Overall, from all of these experiments, we concluded that HDAC5 is not a p53 target gene. We have also performed Northern analysis using cDNAs encoding seven other known HDACs (HDAC1-4 and 6-8) as probes for their potential induction by etoposide in these four cell lines. Although their endogenous expression level varies in cell lines, none of HDACs was induced by etoposide, regardless of p53 status (data not shown). Thus, it is possible that none of HDACs is directly subject to p53 regulation.

p53 Is Not an HDAC5 Targeting Protein. It has been known that class I HDAC1 and class III hSIR2 binds to p53 and regulates p53 activity via deacetylation (14–16, 24, 25). We then determined whether HDAC5, a class II HDAC, interacts with p53. Two HDAC5-overexpressing cells and two vector control cells were treated with etoposide, and subjected to an immunoprecipitation-coupled Western analysis to determine the level of total p53 and acetylated p53. Etoposide treatment remarkably increased p53 protein level, as shown previously (39), as well as p53 acetylation. However, overexpression of HDAC5 did not decrease, but slightly increased the amount of acetylated p53 in HDAC5 highly expressed U2H15 cells (data not shown). We also determined whether two proteins bind together *in vivo*. Two constructs expressing p53 and FLAG-tag HDAC5 were cotransfected into 293 cells. Cell lysates were immunoprecipitated either by anti-p53 antibody or anti-FLAG antibody. Western blot showed no HDAC5 protein in p53 immunoprecipitant nor p53 protein in HDAC-5 immunoprecipitant, indicating two proteins did not bind to each other even under overexpressed conditions *in vivo* (data not shown). These results indicate that in contrast to HDAC1, 2, and 3, and hSIR2, p53 is not a target directly subjected to post-translational modification by HDAC5.

HDAC5 Transfection Inhibits Tumor Cell Growth. To examine the potential role of HDAC5 in tumor cell growth, we transfected U2OS cells with HDAC5-expressing construct or the vector control, along with β -gal expression vector, followed by G418 selection. The transfection efficiency of HDAC5 construct and the vector control were normalized according to the β -gal activity of each sample measured 2 days after transfection. Three independent transfections with plasmids prepared independently were conducted. The stable clones derived from 3 weeks of G418 selection were stained with Coomassie Blue. Fig. 4A showed a representative plate of HDAC5 transfection compared with the vector control. Indeed, HDAC5 transfection significantly reduced colony numbers. To quantitate the difference, the colonies of each plate were counted, and the average number of three plates after normalization is presented as bar graph in Fig. 4B. HDAC5 transfection led to 5-fold reduction of colony numbers as compared with the vector control. The difference is statistically significant ($P < 0.01$), indicating that HDAC5 transfection inhibited tumor cell growth. To determine whether this observation can be extended to other human tumor cell lines, we performed the same experiment with two additional tumor lines, SY5Y and MCF7 cells. As shown in Fig. 4, C and D, HDAC5 transfection caused up to 3-fold reduction in colony numbers, which is statistically significant with a $P < 0.01$ and 0.05 in SY5Y and MCF7 cells, respectively. The results strongly suggested a general role of HDAC5 expression in suppression of tumor cell growth.

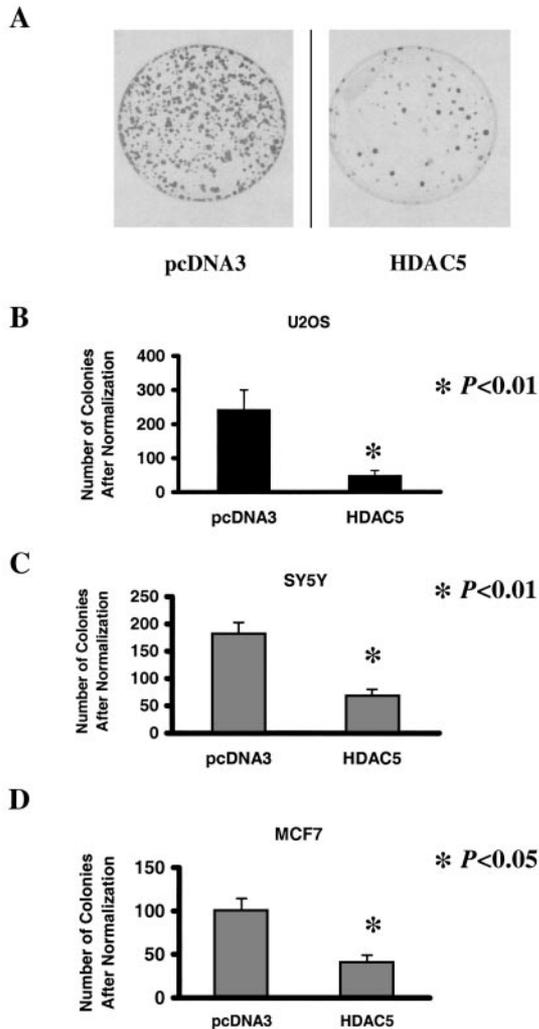


Fig. 4. Inhibition of tumor cell growth by HDAC5 transfection. U2OS (A and B), SY5Y (C), and MCF-7 (D) cells were co-transfected with plasmid expressing HDAC5 and β -gal along with the vector control (pcDNA3). Transfectants were selected by G418 (600 μ g/ml for U2OS and SY5Y, 800 μ g/ml for MCF7) for 2–3 weeks and stained by Coomassie Blue. Total number of the colonies in each plate from three independent transfections were counted, normalized against their β -gal activities, and plotted as number of colonies per plate. A statistically significant difference at the $P < 0.01$ or $P < 0.05$ level was indicated between pcDNA3 and HDAC5 transfectants. A pair of representative plate was shown (A); bars, \pm SE.

Overexpression of HDAC5 Increases Total Cellular HDAC Activity. To further examine HDAC5-induced growth inhibition and its potential mechanism of action, we cloned 18 stable lines from HDAC5-transfected U2OS cells and measured HDAC5 protein level by Western blotting using anti-HDAC5 antibodies. As shown in Fig. 5A, the vector control cells showed no detectable endogenous HDAC5 expression (Fig. 5, Lanes 1 and 2), whereas 2 of 18 HDAC5 transfectants showed moderate (U2H7) to high (U2H15) levels of HDAC5 expression (Fig. 5, Lanes 3 and 4). Differential HDAC5 expression among these transfectants provides a tool to study the dosage effect of HDAC5.

We next examined whether HDAC5 overexpression increased total cellular HDAC activity or, in other words, whether overexpressed HDAC5 is enzymatic active. Total cell extract was prepared from all four of the transfectants and subjected to HDAC activity assay in the absence and presence of HDAC inhibitor, TSA. As shown in Fig. 5B, although there is a clonal variation in HDAC activity between two vector lines, U2V1 and U2V2, two HDAC5 expressing lines, U2H7 and U2H15 do have a higher HDAC activity with the high expresser,

U2H15 the highest. The assay is rather specific for HDACs, because the enzymatic activity, as reflected by an absorbance value, can be completely inhibited by a HDAC-specific inhibitor, TSA. The results suggest that overexpressed HDAC5 is enzymatic active.

Overexpression of HDAC5 Reduces Cell Growth Rate and Induces Spontaneous Apoptosis. Growth suppression can result from growth arrest, apoptosis, or both. We then examined the cell growth rate of the U2OS stable transfectants, as compared with the vector control, using cell growth MTT assay. The cell growth was measured daily up to 5 days. The results were plotted as a function of the absorbance (reflection of cell numbers) versus culture time (Fig. 6A). U2H15, which expressed the highest level of HDAC5, revealed the slowest growth rate as compared with two other clones (U2V1 and U2V2). U2H7, which expressed HDAC5 at much less magnitude, showed no difference from the vector controls. This suggests that the effect of HDAC5 on cell growth is dose dependent and was only seen when HDAC5 level is above a certain threshold. We also performed fluorescence-activated cell sorter analysis (41) in these cells and did not observe any significant G₁ or S phase delay resulting from HDAC overexpression (data not shown). Thus, HDAC5-induced growth inhibition is not mainly because of growth arrest or delay.

During normal subcultivation, we noticed that HDAC5 overexpressing cells, particularly U2H15, underwent cell round up and detachment, the characteristic signs of apoptosis. To accurately measure the level of spontaneous apoptosis induced by HDAC5 overexpression, we performed DNA fragmentation ELISA assay in four U2OS stable transfectants (two vector controls and two HDAC5 clones) under normal and etoposide-treated conditions. The results are shown in Fig. 6, B and C. HDAC5 overexpression induced spontaneous apoptosis, which was reflected by an increased DNA fragmentation, in a dose-dependent manner. Both HDAC5 stable transfectants show significant induction of apoptosis as compared with the vector controls, with U2H15 showing a highest level among all four of the transfectants (Fig. 6B). Treatment with etoposide induced apoptosis in all four of the transfectants.

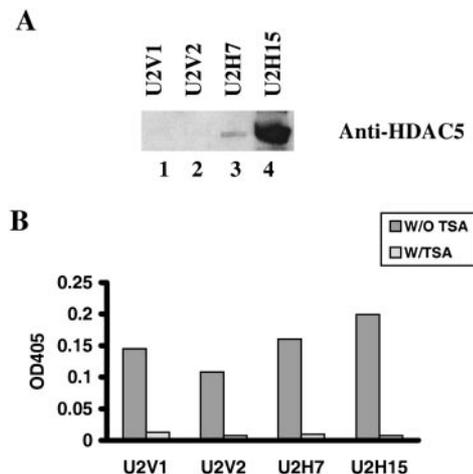


Fig. 5. Establishment of HDAC5 overexpressing lines that showed an increased total cellular HDAC activity. A, HDAC5 expression in stable cell lines: U2OS cells were transfected with HDAC5, along with the vector control. Stable clones were isolated after G418 selection and subjected to Western analysis for HDAC5 expression. Lanes 1 and 2, pcDNA3 stable transfectants as the vector controls (U2V1 and U2V2) and Lanes 3 and 4, HDAC5 transfectants (U2H7 and U2H15). Total cell lysates (50 μ g) were loaded onto each lane, and antihuman HDAC5 antibody was used. B, total cellular HDAC activity: 10 μ g cell lysate from all four transfectants were subjected to HDAC activity assay in the absence or presence of HDAC inhibitor, TSA as detailed in "Materials and Methods." The absorbance value reflects enzymatic activity that is completely inhibited by TSA. One representative experiment is shown.

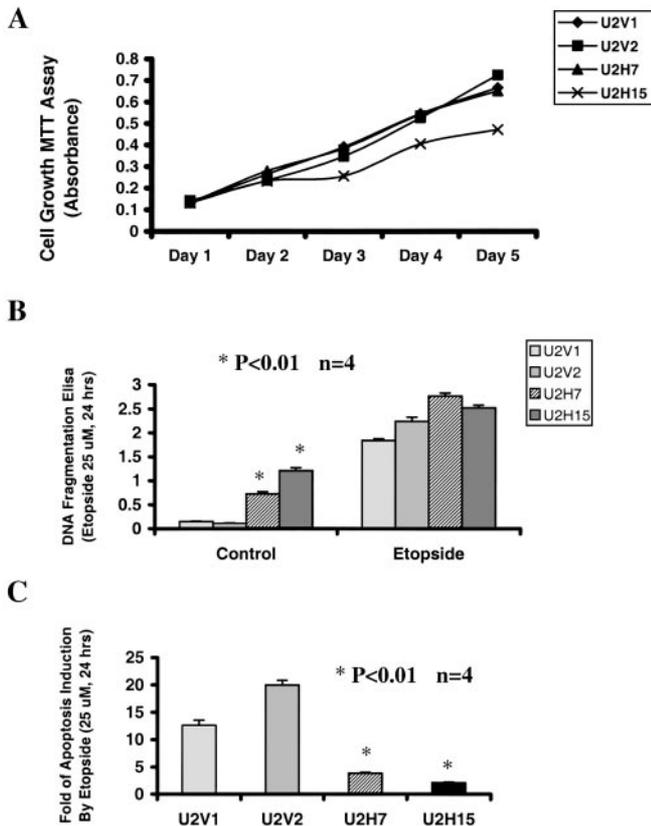


Fig. 6. HDAC5 overexpression decreases growth rate and induces apoptosis. *A*, inhibition of cell growth rate: the cell growth rate of four stable transfectants was monitored daily over a period of 5 days using MTT-based colorimetric assay. The data were presented as absorbance (reflecting cell numbers) versus time (days). *B*, induction of spontaneous apoptosis: four stable cell lines were seeded into 96-well plates and treated with DMSO (*Control*) or etoposide as detailed in "Materials and Methods." Absorbance value that is directly correlated to the degree of DNA fragmentation was plotted. Results were presented as mean from four independent experiments, each run quadruplicate. * indicated a statistical difference as compared with U2V1 cells; *bars*, \pm SE. *C*, fold induction of apoptosis by etoposide: the data presented in *B* is replotted as fold induction of apoptosis by etoposide by comparing absorbance values with or without etoposide treatment in each line. Again, * indicated a statistical difference, as compared with U2V1 cells; *bars*, \pm SE.

tants. However, the fold induction of apoptosis after etoposide was significantly lower in HDAC5 transfectants than that in the vector controls, which is in a HDAC5 dose-dependent manner with the lowest value in U2H15 (Fig. 6C). The results suggest either that overexpression of HDAC5 hindered the etoposide-initiated apoptosis signal pathway or that spontaneous apoptosis induced by HDAC overexpression was so dramatic that etoposide treatment cannot further significantly potentiate it.

We have also documented the morphological appearance of these four stable transfectants in both untreated and etoposide-treated conditions. Under the normal culture, both HDAC5 transfectants (U2H7 and U2H15) showed that a high percentage of cell population underwent cell round up and detachment, a sign of spontaneous apoptosis, which is readily seen particularly in U2H15 cells. Cell density and confluence were significantly lower in HDAC5-expressing cells as compared with the vector controls even although an equal number of cells were seeded in each line (Fig. 7, *top four panels*). Treatment with etoposide increased apoptotic cell populations in all of the transfectants, regardless of HDAC5 expression. However, the number of etoposide-induced apoptotic cells was significantly higher in the vector transfectants than in the HDAC5 transfectants (Fig. 7, compare *bottom four panels* with the *top four*), consistent with the observation made by DNA fragmentation assay.

Overexpression of HDAC5 Alters Expression of the Genes Involved in TNF Receptor Death Pathway and MAP Kinase Pathway. We next investigated potential mechanism by which HDAC5 overexpression induces growth suppression and apoptosis. Microarray filters spotted with 205 cell cycle- and apoptosis-related cDNAs were probed separately with radiolabeled U2H15 and U2V1 mRNA, and analyzed using PhosphorImager techniques. The microarray profiling results were shown in Fig. 8. Arrows indicated the genes of which the expression was either induced by 4-fold or repressed by 3-fold. The detailed description of each individual gene and their locations in the filters was presented in Table 1. Compared with U2V1, 3 cell proliferation genes, *CDK7*, *MEK* kinase 3, and *MAPK7* were down-regulated at least 3-fold, whereas 7 apoptosis-associated genes, including *TNFR1*, *TNFSF7*, caspase-8, death-associated protein kinase (*DAPK*), *RTS1*, *RBQ1* retinoblastoma bind-

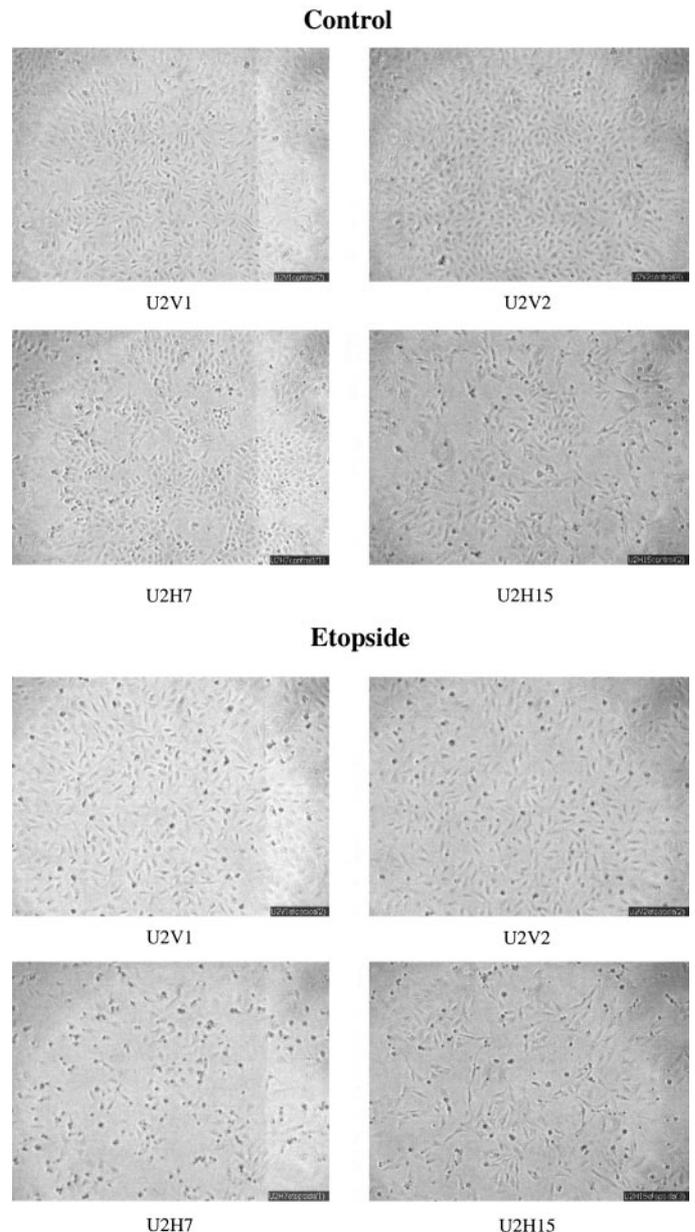


Fig. 7. Morphological appearance of four transfectants under untreated and etoposide-treated conditions. All four transfectants were seeded at a density of 1×10^4 in a 96-well plate and grown for 48 h before photograph. The *top four panels* are control when cells were grown under normal condition, whereas in the *bottom four panels*, cells were treated with 25 μ M etoposide for 24 h.

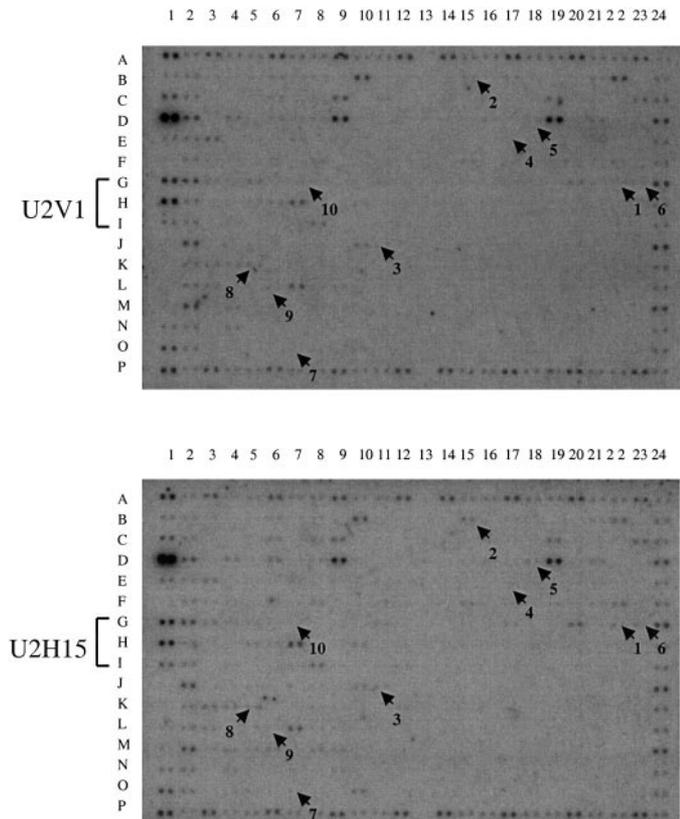


Fig. 8. Microarray profiling of cell cycle- and apoptosis-related genes in HDAC5 overexpressing cells. Total RNA was isolated and subjected to microarray as detailed in "Materials and Methods." Each blot was normalized against the average density of three housekeeping genes dotted in positions of G1, H1, and I1, respectively. The relative density of each of 205 genes was compared between vector U2V1 cells and HDAC5-overexpressing cells (U2H15). Arrows indicated genes of which the expression was either increased 4-fold or decreased 3-fold. The numbers and coordinates corresponding to each individual gene was listed in Table 1.

Table 1 Alteration of apoptosis- and cell cycle-related gene expression by HDAC5 overexpression

Accession no.	Genes	Fold of change ^a (U2H15 vs U2V1)	Coordinate
L08096	Tumor necrosis factor superfamily member 7 (TNFSF7)	+4	22G (1)
M33294	Tumor necrosis factor receptor (TNFR1)	+4	15B (2)
U60520	Caspase-8	+5	11J (3)
X76104	Death-associated protein kinase 1 (DAPK1)	+7	17E (4)
U37688	RATS1	+6	18D (5)
D21090	UV excision repair protein RAD23 homolog B (hHR23B)	+60	23G (6)
X85133	RBQ1 retinoblastoma binding protein	+24	7O (7)
X92669	CDK-activating kinase assembly factor (CAK assembly factor)	-8	5K (8)
U25278	Mitogen-activated protein kinase 7 (MAPK7)	-138	6L (9)
U78876	MEK kinase 3	-3	7G (10)

^a Genes that were up-regulated (+) for at least 4 folds or down-regulated (-) for at least 3 folds were included.

ing protein, and *hHR23B* were up-regulated at least 4-fold. Thus, growth suppression and apoptosis induction caused by HDAC5 overexpression could be mediated by altered expression of these genes. To further validate that these gene changes are specific for apoptosis induction, we next performed microarray analysis with the same two transfectants treated with etoposide when they underwent apoptosis to

a similar extent (Figs. 6B and Fig. 7). None of above genes was either induced or repressed (data not shown), suggesting that altered expression of these genes does correlate with apoptosis induction.

Because 4 of 7 induced genes (*TNFSF7*, *TNFR1*, caspase 8, and *DAPK1*) are involved in the TNF death receptor pathway, we went on to confirm induction of *TNFSF7*, *TNFR1*, and caspase 8 by either Northern or Western analysis. As shown in Fig. 9A, increased *TNFR* expression was seen in U2H15 but not U2H7 cells, whereas increased *TNFSF* expression was seen in both HDAC5-expressing lines, largely confirming the microarray results. We also performed Western blot analysis using antibody against caspase 8, and as shown in Fig. 9B, the level of caspase 8 protein was indeed higher in HDAC5-expressing lines U2H7 and U2H15 than the vector controls, indicating that TNF ligand-receptor death pathway was indeed induced on HDAC5 overexpression.

DISCUSSION

In response to DNA damage, p53 is activated through phosphorylation by a number of protein kinases such as ataxia-telangiectasia mutated (ATM) and ATM and Rad 3-related (ATR) (23), and acetylation by p300 and p300/CBP-associated factor (PCAF) (24), leading to its dissociation with Mdm2 ubiquitin ligase and increased half-life (42). Activated p53 transactivates a list of downstream target genes to induced cell growth arrest and apoptosis (21, 43). One of p53 downstream target is *Mdm2*, which on activated by p53 binds to and degrades p53 to keep p53 level in check (28). Another way to regulate p53 activity is through the deacetylation of p53 by the members of class I HDACs, including HDAC1-3 (14) and class III SIR2 NAD-dependent HDAC (15, 16). Because none of the HDACs has been found previously to be a direct target of p53, an auto-regulatory negative feedback loop was not established. Here we tested our hypothesis that *HDAC5*, a gene of which the expression was induced 10-fold by p53 in a SAGE profiling experiment (27), is a p53 target. On induction by p53, HDAC5 would in turn inactivate p53 by deacetylation to keep p53 activity in check. Although we identified six putative p53-binding sites in the promoter of the *HDAC5* gene, only one showed weak binding to p53. As tested in multiple cell models, p53 does not transacti-

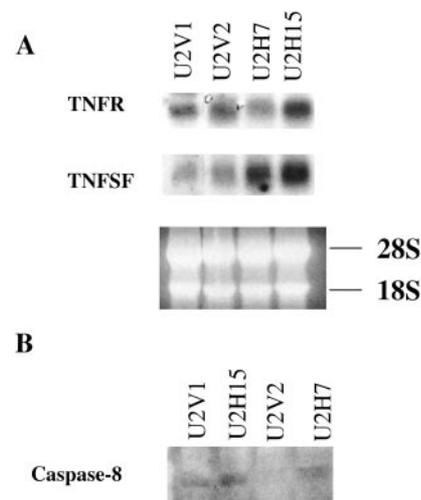


Fig. 9. Confirmation of microarray data by Northern and Western blot. A, total cellular RNA was isolated from all four transfectants and subjected to Northern analysis using probes hybridizing TNF-7 and TNFR as detailed in "Materials and Methods." The ribosomal 28S and 18S were shown as the loading controls. B, total cell lysates were prepared from all four transfectants. Equal amount of cellular protein (50 μ g) was loaded and subjected to Western blot analysis using anticaspase 8 antibody (Upstate).

vate the luciferase reporters driven by the HDAC5 promoter. Furthermore, p53-activating agent, etoposide, does not induce endogenous HDAC5 expression. Finally, HDAC5 neither binds to p53 nor decreases etoposide-induced p53 acetylation. We conclude from this study that: (a) *HDAC5* is not a p53 target gene; and (b) unlike HDAC1-3 and hSIR2, HDAC5 does not regulate p53 activity. We have additionally investigated the possibility that any other known HDACs (HDACs1-4 and 6-8) could be a p53 target by Northern analysis of their potential induction by p53-activating agent, etoposide in p53-positive (U2-OS and NCI-H460) and p53 negative (Saos-2 and H1299) cells. None of *HDACs* was inducible by etoposide in either of cell lines tested. Thus, although class I HDAC1-3 and class III hSIR2 have been shown to bind to p53, deacetylate p53, and inhibit p53 activity (14–16), class II HDACs are probably not direct p53 targets, and an auto-regulatory feedback mechanism, as seen in p53-mdm2 regulation (28), cannot be established.

Normal cell growth requires a precise balance of activities between acetylase and deacetylase, because it maintains specific gene expression patterns. Disruption of this balance may lead to an altered expression of a variety of cellular genes, which may lead to morphological and functional abnormality of cells. During our study we found that tumor cells derived from osteogenic sarcoma, neuroblastoma, and breast carcinoma, when transfected with *HDAC5* expressing vector, formed less monolayer colonies as compared with the vector control, suggesting that forced *HDAC5* expression in general inhibited tumor cell growth. Additional studies using HDAC5 stable transfectants revealed that overexpression of HDAC5 induced growth delay and spontaneous apoptosis in a HDAC5 dose-dependent manner, suggesting both growth arrest and apoptosis account for HDAC5-induced tumor cell inhibition. Interestingly, under etoposide-treated condition, HDAC5 transfectants revealed less apoptosis induction as compared with the vector controls, suggesting that HDAC5 overexpression might partially inhibit etoposide-mediated apoptosis pathway. A recent report indirectly supported this notion. It was found that sodium butyrate, a HDAC inhibitor, sensitizes human leukemic cells to etoposide-induced apoptosis. Although sodium butyrate appeared to induce DNA topoisomerase II α expression as a major mechanism, inhibition of HDAC activity could also play a role (44). However, we cannot excluded the possibility that decreased response to etoposide-induced apoptosis merely reflected a high level of spontaneous apoptosis by HDAC5 overexpression. It is noteworthy that we managed to clone HDAC5-overexpressing U2H15 cells during stable selection and clonal expansion, although they grew relatively slower than the other stable clones because of spontaneous apoptosis. For growth suppression and apoptosis induction experiments conducted later, we only used the few early passages of U2H15 cells in which a high level of HDAC5 protein was detected (Fig. 5A).

HDAC5 belongs to the class II HDACs and contains a COOH-terminal catalytic domain and an noncatalytic NH₂-terminal domain. It has been found recently that HDAC5 regulates muscle differentiation by binding to MEF2 via its NH₂-terminal domain and repressing expression of MEF2-dependent genes (45, 46). It was additionally demonstrated that HDAC5 also binds to 14-3-3 protein (47) in a manner largely dependent on calcium/calmodulin-dependent protein kinase signaling (48). When myoblasts are triggered to differentiate, HDAC5 was phosphorylated at serines –259 and –498 by calcium/calmodulin-dependent protein kinase and phosphorylated HDAC5 bound to 14-3-3. The binding to 14-3-3 resulted in a dissociation of HDAC5 from MEF2 and led to HDAC5 nuclear export via a signal-responsive nuclear export sequences and MEF2 activation (47–49). Furthermore, HDAC5 was found to bind to nuclear receptor corepressor silencing mediator for retinoid and thyroid receptor and nuclear

receptor corepressor (50–52), and this binding played an important role in preventing muscle cell differentiation (53).

It has been shown recently that both class I (HDAC1-3) and class III (hSIR2) HDACs bind to p53, deacetylate p53, and inhibit p53-induced apoptosis (14–16). We showed here for the first time that forced expression of HDAC5, a class II HDAC, suppresses growth of several tumor cell lines mainly by induction of spontaneous apoptosis (Figs. 6 and 7), partly by inhibition of growth rate (Fig. 6) in a p53-independent manner. What is then the mechanism of action? Because HDAC5 is in general a transcription repressor, we reasoned that the effect of HDAC5 on cell growth and apoptosis occurred most likely at the transcriptional level. Therefore, we screened cDNA expression arrays consisting of 205 genes known to be involved in cell growth and apoptosis for their potential changes after HDAC5 overexpression. We arbitrarily set up our threshold to include genes of which the expression was increased for at least 4-fold or was repressed for at least 3-fold. Seven apoptosis-related genes were induced, whereas 3 cell cycle-related genes were repressed (see Table 1). Among those up-regulated apoptosis-related genes, 4 are involved in TNF ligand-receptor death pathway, one of two major apoptosis signal pathways (54). The first gene product is TNFSF7 (TNF superfamily 7), a ligand for CD27, with homology to TNF (55). It has been shown that in combination with several of the TNF family ligands, TSNSF7/CD27L induces apoptosis in tumor cells (56). The second identified gene product is TNFR, a well-known receptor mediating TNF-induced apoptosis (57, 58). The third is caspase 8/Mch5, a caspase that mediates TNF/Fas ligand-receptor death pathway, and its level determines cellular sensitivity to apoptosis (59, 60). Indeed, an increased protein level of caspase 8 was confirmed in HDAC5-overexpressed U2H15 cells (Fig. 9), which could account for its high level of spontaneous apoptosis. And the final one is DAPK-1, a member of a serine/threonine kinase family that mediates the γ -IFN-induced cell death (61), and in some cases, it also mediates apoptosis induced by Fas/APO-1 and TNF- α (62, 63). Furthermore, DAP kinase inhibits oncogene-induced transformation by activating a p19/p53-dependent apoptotic checkpoint to eliminate premalignant cells (64), and hypermethylation of DAP kinase is a common abnormality in early stage of non-small cell lung cancers, which correlated with a poor survival of patients (65).

Accordingly, expression of three growth-promoting genes was repressed. Two of them, MAP/extracellular signal-regulated kinase kinase 3 (66) and *MAPK7* (67) are superfamily members of MAP kinases, the essential components of signal pathway leading to cell proliferation (68). The third repressed gene identified is a cyclin-dependent kinase-activating kinase assembly factor, *MAT1* (69). It has been shown that MAT1 interacts with retinoblastoma protein to regulate cell cycle G₁ exit (70), and abrogation of MAT1 induces G₁ arrest (71). Thus, at least in part, induction of TNF/TNFR death receptor pathway appears to play a role in HDAC5-induced apoptosis, whereas repression of MAP kinase pathway and MAT1 could be associated with growth delay. Future experiments are directed to understand the precise role of these genes in HDAC5-induced apoptosis and growth arrest. Finally, because our study was limited to commercially available known genes, additional studies are necessary on a more complete gene expression profiling to examine potential mechanisms in a broader scope. It will be also of interest and importance to investigate how HDAC5 overexpression changes expression of these genes, particularly how it induces TNF receptor death pathway, because HDACs in general silence gene expression.

In summary, we have shown here that *HDAC5* is not a direct p53 downstream target nor is p53 a HDAC5 target. Our data disfavor the hypothesis that a p53-HDAC auto-regulatory feedback loop in analogue to p53-Mdm2 exists in human cells. We also showed here that

forced expression of HDAC5 inhibits tumor cell growth via inducing spontaneous apoptosis and reducing growth rate. Up-regulation of TNF death receptor pathway and down-regulation of MAP kinase pathway appears to play a mechanistic role. It is of great interest and significance to link HDAC5 to these two well-known signal pathways in regulation of cell proliferation and apoptosis.

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