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Histone deacetylase 3 represses p15^{INK4b} and p21$^{WAF1/cip1}$ transcription by interacting with Sp1 ☆

Weifeng Huang, Dapeng Tan, Xiuli Wang, Songyan Han, Jiang Tan, Yanmei Zhao, Jun Lu *, Baiqu Huang

Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China

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Abstract

Histone deacetylase 3 (HDAC3) has been implicated to play roles in governing cell proliferation. Here we demonstrated that the overexpression of HDAC3 repressed transcription of p15^{INK4b} and p21$^{WAF1/cip1}$ genes in 293T cells, and that the recruitment of HDAC3 to the promoter regions of these genes was critical to this repression. We also showed that HDAC3 repressed GAL4-Sp1 transcriptional activity, and that Sp1 was co-immunoprecipitated with FLAG-tagged HDAC3. We conclude that HDAC3 can repress p15^{INK4b} and p21$^{WAF1/cip1}$ transcription by interacting with Sp1. Furthermore, knockdown of HDAC3 by RNAi up-regulated the transcriptional expression of p15^{INK4b}, but not that of p21$^{WAF1/cip1}$, implicating the different roles of HDAC3 in repression of p15^{INK4b} and p21$^{WAF1/cip1}$ transcription. Data from this study indicate that the inhibition of p15^{INK4b} and p21$^{WAF1/cip1}$ may be one of the mechanisms by which HDAC3 participates in cell cycle regulation and oncogenesis.

Keywords: HDAC3; Sp1; Repression; p15^{INK4b}; p21$^{WAF1/cip1}$; Cell cycle

Introduction

Both p15^{INK4b} and p21$^{WAF1/cip1}$ are the cyclin kinase inhibitors (CKIs), which can bind to and inhibit the cyclin-dependent kinases (CDKs) [1]. They play significant roles in checkpoint transition of cell cycle progression and cellular differentiation, and therefore subject to different levels of regulation in different cellular settings and biological phenomena [2]. Both histone acetylases (HATs) and histone deacetylases (HDACs) are the key enzymes that catalyze the reversible acetylation/deacetylation of core histone tails, which is an essential mechanism of the epigenetic control of gene expression [3]. Recent evidence implicated that some histone deacetylase inhibitors may arrest human tumor cells at the G1 phase of the cell cycle by increasing the mRNA and protein levels of p21$^{WAF1/cip1}$ or p15^{INK4b} [4,5]. These studies highlight the importance of the HDACs in control of p15^{INK4b} and p21$^{WAF1/cip1}$ transcription in normal cell cycle regulation.

The mammalian HDACs so far identified fall into three classes based on their structural and biochemical characteristics [6]. The most extensively studied HDACs are those of the mammalian Class I HDAC enzymes that are highly homologous to yeast Rpd3, comprising HDAC1, HDAC2, HDAC3, HDAC8, and HDAC11. Although there is clear evidence for the role of HDAC3 in the regulation of cell cycle progression [7], the molecular mechanisms of the involvement of HDAC3 in these processes have not been fully elucidated.

In this study, we investigated the functional effect of HDAC3 on p15^{INK4b} and p21$^{WAF1/cip1}$ transcriptional regulation. We show that HDAC3 was recruited to p15^{INK4b} and p21$^{WAF1/cip1}$ promoter regions to repress p15^{INK4b} and p21$^{WAF1/cip1}$ transcription, and this repression was

☆ *Abbreviations:* HDAC, histone deacetylase; HAT, histone acetylases; CKI, cyclin kinase inhibitor; CDKs, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; RNAi, RNA interference.

* Corresponding author.
E-mail addresses: ycsuo@nenu.edu.cn, luj809@nenu.edu.cn (J. Lu).

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mediated, at least in part, through the interaction of HDAC3 with Sp1, a transcription factor of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoters. Also, knockdown of HDAC3 expression by siRNA targeting HDAC3 up-regulated the transcriptional expression of $p15^{INK4b}$, but not that of $p21^{WAF1/cip1}$. Data presented in this report implicate that the down-regulation of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ may contribute to the potential of HDAC3 to promote cell proliferation and oncogenesis.

Materials and methods

Plasmids and cell culture. Plasmids of CMV-Sp1 and pSG4+Sp1 were kindly provided by Dr. Robert Tjian (University of California at Berkeley). Plasmids of PCMx-GAL4DBD, PMH100-TK-luc, p15(−2500/+160), p15(−1040/+160), p15(−750/+160), p15-luc(−113/+160), and p21-luc(−297/+8) were described previously [8–10]. The plasmids of FLAG-tagged HDAC1 and HDAC3 were gifts from Dr. Wolfgang Fischle (Gladstone institute of Virology and Immunology). The plasmid of p21-luc(−2400/+11) was a gift from Dr. Bert Vogelstein (The Johns Hopkins University School of Medicine at Maryland).

293T human embryonic kidney epithelial cells were maintained in IMDM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator under 5% CO_2.

Transient transfection and luciferase assay. 293T cells were transfected using a conventional calcium phosphate co-precipitation technique. At 27 h after transfection, cells were analyzed for luciferase activities using a Promega Dual-Luciferase Reporter Assay System. Luciferase activities were normalized with co-transfected *Renilla* luciferase control. All the results represent means ± SD of three independent experiments.

mRNA isolation and real-time quantitative PCR. Total RNA isolation and the reverse transcription reaction were performed by using the RNA extraction and RT Systems supplied by Promega. Quantification of mRNA was done using an ABI PRISM 7100 Sequence Detection System and SYBR Green (TaKaRa) as a double-stranded DNA-specific fluorescent dye. β-Actin was used as a housekeeping gene for standardizing $p15^{INK4b}$ and $p21^{WAF1/cip1}$ mRNA expression. Amplification primers were: for $p15^{INK4b}$ gene, 5′-CGGAGTCAACCGTTTCGG-3′ and 5′-GGTGAGAGTGGCA GGGTCTG-3′ [11]; for $p21^{WAF1/cip1}$ gene, 5′-TGGAGACTCTCAGGGT CGAAA and 5′-GGCGTTTGGAGTGGTAGAAATC-3′ [11]; and for *β*-actin gene, 5′-TCGTGCGTGACATTAAGGAG-3′ and 5′-ATGCC AGG GTACATGGTGGT-3′, respectively. Data were analyzed by using the $2^{-\Delta\Delta C_t}$ method [12]. All the results represent means ± SD of three independent experiments.

Chromatin immunoprecipitation. 293T cells were transfected with the FLAG-tagged HDAC3 expression vector. At 24 h after transfection, cells were processed using a chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology). The experiments were performed using anti-FLAG polyclonal antibody (Sigma, F7425) or no antibody as the control. Immunoprecipitated chromatin was assayed by semi-quantitative PCR using the following primers: for $p15^{INK4b}$ (−163/+220) gene, 5′-TGCGTC CTAGCATCTTTGG-3′ and 5′-ACCCTCCCGTCGTCCTT-3′; and for $p21^{WAF1/cip1}$ (−194/+88) gene, 5′-ACCGGCTGGCCTGCTGGAACT-3′ and 5′-TCTGCCGCCGCTCTCTCACCT-3′ [13], respectively. PCR products were separated by agarose gel electrophoresis.

Co-immunoprecipitation (Co-IP). 2.7×10^7 cells from two 6-well dishes were rinsed in phosphated-buffered saline (PBS), lysed in 1.5 ml buffer I (20 mM Tris–HCl, pH 7.5, 120 mM KCl, 0.1% NP40, 1 mM EDTA, 10% glycerol, 50 mM NaF, 10 mM Na-pyrophosphate, 1 mM phenylmethylsulfonyl, and protease inhibitor cocktail), spun at 13,000 rpm for 10 min at 4°C, and pre-cleared with 40 μl of protein A–Sepharose beads. The anti-FLAG polyclonal antibody (5 μg) was added and the mixture was incubated with gentle shaking for 8 h at 4°C. Then, 40 μl of protein A–Sepharose beads was added. The mixture was further incubated with gentle shaking for 3 h at 4°C. After three washes with buffer II (20 mM Tris–HCl, pH 7.5, 150 mM KCl, 0.5% NP40, 1 mM EDTA, 10% glycerol, 50 mM NaF, 10 mM Na-pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail), the beads were resuspended in 25 μl sample buffer and boiled for 8 min, and 20 μl of supernatant was applied to 12% SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membrane and immunoblotted with anti-Sp1 antibody (Santa Cruz, H-225).

RNA interference (RNAi). The HDAC3 siRNA plasmid was constructed by inserting a pair of annealed DNA oligonucleotides into pSliencer2.0-U6 vector (Ambion) between *Bam*HI and *Hin*dIII restriction sites. The target sequence for HDAC3 was 5′-AAGATGCTGAACCA TGCACCT-3′ [14]. Cells in 6-well dishes were transfected with 4 μg HDAC3 siRNA vector per well, and at 48 h after transfection cells in each well were divided into 6-well. After additional incubation for 24 h, a second transfection was performed with 4 μg HDAC3 siRNA vector for RNA extraction or 4 μg HDAC3 siRNA vector, 0.5 μg HDAC3 expression vector, and 0.5 μg GFP expression vector (HDAC3 transfection was normalized to GFP expression) for immunoblotting analysis. Cells were incubated for additional 48 h. The mRNA expression level of HDAC3 was then assayed by semi-quantitative PCR. β-Actin was used as a housekeeping gene for standardizing HDAC3 mRNA expression. Amplification primers were: for HDAC3 gene, 5′-GTGGTTATACTGTCCGAAA TGTTG-3′ and 5′-AGTCCTGTCATAGGTCAGGAGGT-3′; and for β-actin gene, 5′-ACACTGTGCCCATCTACG-3′ and 5′-CTCGTCA TACTCCTGCTTG-3′, respectively. PCR products were separated by agarose gel electrophoresis. Western blot was done with the anti-FLAG antibody or anti-GFP antibody (Roche, 1814460). $p15^{INK4b}$ and $p21^{WAF1/cip1}$ mRNA expression was detected as described above.

Results

HDAC3 inhibited the $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter activity

It has been implicated that HDACs contribute to the regulation of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription. We used both $p15^{INK4b}$ (−2500/+160) and $p21^{WAF1/cip1}$ (−2400/+11) reporter constructs to investigate the effect of HDAC1/3 on these promoters. The results of luciferase activity analyses indicated that HDAC3 repressed both $p15^{INK4b}$ (−2500/+160) and $p21^{WAF1/cip1}$ (−2400/+11) promoter-driven luciferase activities by 46% and 56%, respectively (Fig. 1A). Meanwhile, HDAC1 significantly inhibited $p21^{WAF1/cip1}$ (−2400/+11) promoter activity by 64% but it had little effect on $p15^{INK4b}$ (−2500/+160) promoter activity (Fig. 1A). In the following experiments, we focused our interest on the functions of HDAC3 in $p15^{INK4b}$ and $p21^{WAF1/cip1}$ regulation.

Histone deacetylase inhibitors activate $p21^{WAF1/cip1}$ through Sp1 sites at the promoter [3]. To define promoter regions that are responsible for the HDAC3-mediated repression of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ genes, we co-transfected 293T cells with plasmids containing $p15^{INK4b}$ promoter of progressive deletions, or plasmid of p21-luc (−297/+8, containing 6 Sp1 binding sites) and HDAC3 expression vector, and the results of transient transfection assays showed that HDAC3 was able to repress $p15^{INK4b}$ (−113/+160) and $p21^{WAF1/cip1}$ (−297/+8) promoter constructs as efficiently as the long $p15^{INK4b}$ (−2500/+160) and $p21^{WAF1/cip1}$ (−2400/+11) promoter constructs (Fig. 1B). As shown in Fig. 1B, HDAC3 caused the inhabitation of $p15^{INK4b}$ (−113/+160) and $p21^{WAF1/cip1}$ (−297/

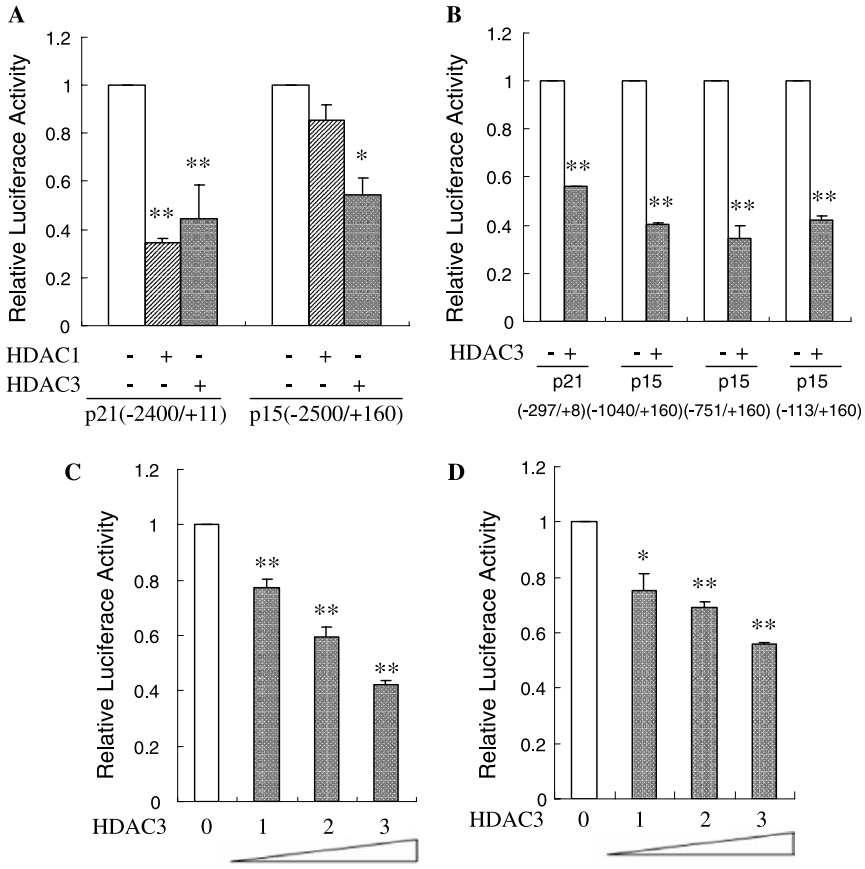

Fig. 1. HDAC3 inhibited $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter activity. (A) HDAC3 repressed both $p15^{INK4b}$ (−2500/+160) and $p21^{WAF1/cip1}$ (−2400/+11) promoter activity and HDAC1 significantly inhibited $p21^{WAF1/cip1}$ (−2400/+11) promoter activity, but it had little effect on $p15^{INK4b}$ (−2500/+160) promoter activity. (B) p15-luc (−113/+160) and p21-luc (−297/+8) were sufficient for the repression by HDAC3. HDAC3 inhibited $p15^{INK4b}$ (−113/+160) (C) and $p21^{WAF1/cip1}$ (−297/+8) (D) promoter activities in a dose-dependent manner. 293T cells were transfected with the p15-luc or p21-luc reporter constructs and HDAC1 or HDAC3 expression vector. Total DNA of the plasmid was adjusted to the same amount by transfecting pcDNA3.1 empty vector (in micrograms). All the results represent means ± SD of three independent experiments. *Significant difference ($p < 0.05$); **highly significant ($p < 0.01$).

+8) promoter activities by 58% and 44%, respectively. Co-transfection of 293T cells with p15-luc (−113/+160) (Fig. 1C) or p21-luc (−297/+8) (Fig. 1D) plasmid, together with the increasing amounts of the HDAC3 expression vector, revealed a dose-dependent inhibition of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter activities by HDAC3.

$p15^{INK4b}$ and $p21^{WAF1/cip1}$ mRNAs were down-regulated by HDAC3 and the recruitment of HDAC3 to their promoter regions accounted for the repression

To determine whether the endogenous mRNA levels of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ can be affected by HDAC3, 293T cells were transfected either with HDAC3 expression vector or with empty vector (pcDNA3.1). $p15^{INK4b}$ and $p21^{WAF1/cip1}$ mRNA levels were monitored by real-time quantitative PCR. The results showed that HDAC3 repressed the endogenous $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription by 51% and 30% compared with the control (Figs. 2A and B). Thus, $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription was repressed by overexpression of HDAC3.

In order to understand the mechanism of the repression of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription by HDAC3, we carried out chromatin immunoprecipitation (ChIP) assays. Since the proximal regions of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoters were sufficient for the repression by HDAC3 (see above), primers amplifying the $p15^{INK4b}$ (−163/+220) and $p21^{WAF1/cip1}$ (−194/+88) promoter regions were used. It is clear from Fig. 2C that the $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter regions were precipitated with anti-FLAG antibody, while only faint background bands were visible with no antibody group. HDACs repress transcription but do not bind DNA directly. Thus, this experiment provided evidence that HDAC3 was recruited by transcriptional factors to suppress $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription.

HDAC3 inhibited the transcriptional activity of GAL4-Sp1 and interacted with Sp1 in vivo

One of the common features of $p15^{INK4b}$ (−113/+160) and $p21^{WAF1/cip1}$ (−297/+8) promoter regions is that both

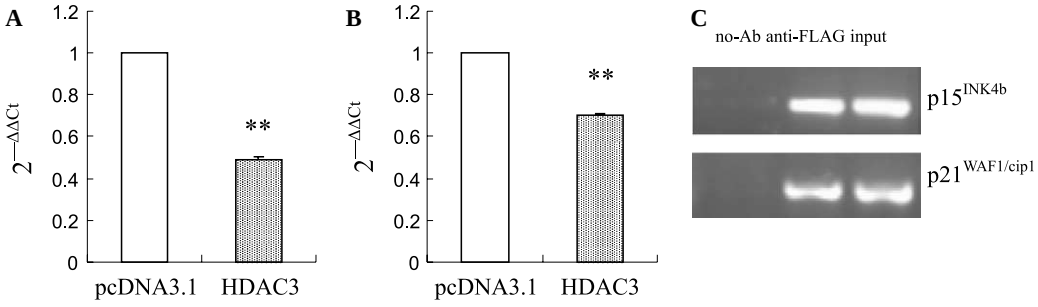

Fig. 2. Endogenous $p15^{INK4b}$ (A) and $p21^{WAF1/cip1}$ (B) mRNA levels were down-regulated by HDAC3. 293T cells were transfected with 4 μg HDAC3 or pcDNA3.1 empty vector as a control. Real-time Quantitative PCR was performed to determine the mRNA expression levels of $p15^{INK4b}$ and $p21^{WAF1/cip1}$. All the results represent means ± SD of three independent experiments. **Highly significant ($p < 0.01$) compared with the control. (C) HDAC3 was recruited to $p15^{INK4b}$ and $p21^{WAF1/cip1}$ proximal promoters. ChIP assay was performed using antibody against FLAG or no-antibody, followed by PCR with primers amplifying the $p15^{INK4b}$ (−163 to +220) and $p21^{WAF1/cip1}$ (−194 to +88) promoter regions. PCR products were separated by agarose gel electrophoresis.

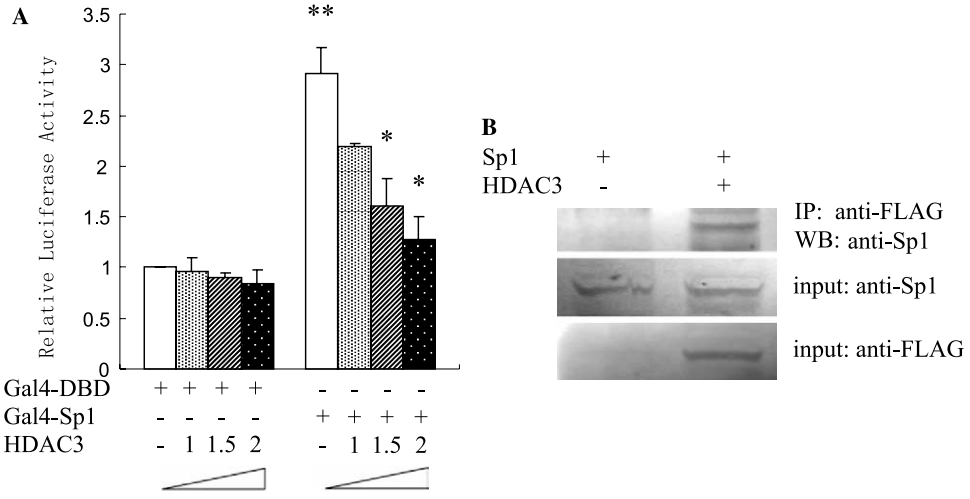

Fig. 3. HDAC3 interacted with Sp1. (A) HDAC3 repressed Sp1 transcriptional activity in a dose-dependent manner. 293T cells were transfected with PMH100-TK-luc reporter genes, GAL4 DNA-binding domain or GAL4-Sp1, and increasing amounts of HDAC3 expression plasmid. GAL-Sp1 increased the reporter gene activity by 3-fold compared with GAL4 DNA-binding domain. Total DNA of the plasmid was adjusted to the same amount by transfecting pcDNA3.1 empty vector (in micrograms). All the results represent means ± SD of three independent experiments. *Significant difference ($p < 0.05$); **highly significant ($p < 0.01$). (B) Sp1 was co-immunoprecipitated with FLAG-tagged HDAC3. 293T cells were co-transfected with Sp1 and FLAG-tagged HDAC3 expression vectors or pcDNA3.1 empty vector as a control. Whole-cell extracts were immunoprecipitated (IP) with anti-FLAG antibody, and Western blot (WB) was performed with the anti-Sp1 antibody.

contain the Sp1-binding sites. To testify whether Sp1 participates in the repression, we used GAL4 transactivation assays by fusing Sp1 to the GAL4 DNA-binding domain. As shown in Fig. 3A, GAL4-Sp1 activated reporter genes by 3-fold compared with GAL4 DNA-binding domain only. Transfection with increasing amounts of HDAC3 inhibited the transcriptional activity of GAL4-Sp1 in a dose-dependent manner, whereas it had a little effect on the GAL4 DNA-binding domain alone (Fig. 3A). These results imply that HDAC3 may inhibit Sp1 transcriptional activity by interacting with Sp1 in vivo.

Co-IP assays confirmed this assumption. In this experiment, 293T cells were co-transfected with Sp1 expression vector and FLAG-tagged HDAC3 expression vector or the empty control vector. The immunoprecipitation was done with anti-FLAG antibody and the precipitated proteins were assayed by Western blot with the antibody against Sp1. As can be seen in Fig. 3B, when Sp1 and HDAC3 were co-expressed in cells, an interaction between the two proteins was observed. Thus, we figure that the inhibition of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription activity by HDAC3 was mediated, at least in part, by binding to Sp1.

HDAC3 reversed the Sp1-mediated activation of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoters in a dose-dependent manner

It has been previously established that Sp1 is a strong activator of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoters [15]. HDAC3 would presumably inhibit the activation effect of Sp1 on $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoters, if Sp1 recruits HDAC3 to repress the expression of $p15^{INK4b}$

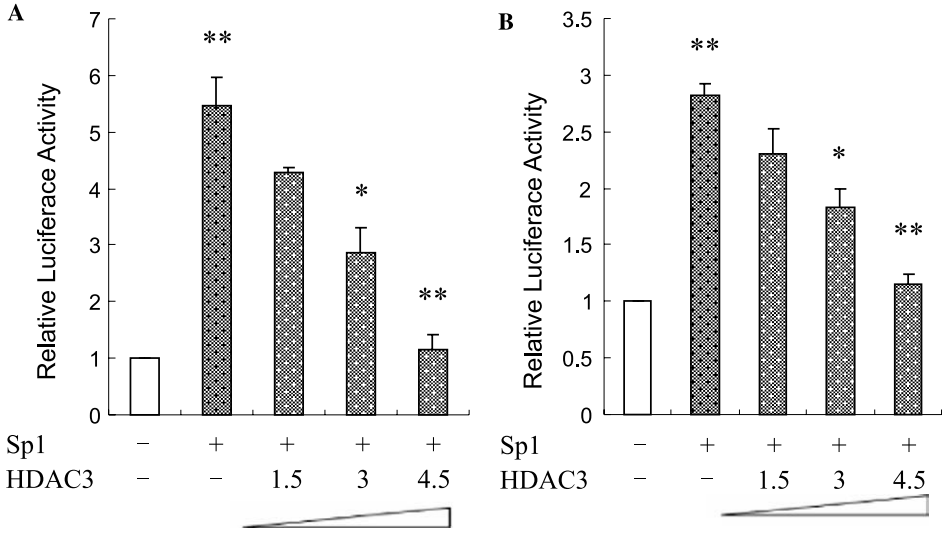

Fig. 4. HDAC3 reversed the Sp1-mediated activation of $p15^{INK4b}$ (−113/+160) and $p21^{WAF1/cip1}$ (−297/+8) promoters in a dose-dependent manner. Increasing amounts of HDAC3 were co-transfected with the p15-luc (−113/+160) (A) or p21-luc (−297/+8) (B) reporter constructs and Sp1 expression vector, as indicated. Sp1 significantly activated p15 (−113/+160) and p21 (−297/+8) promoter constructs compared with the control. The total plasmid DNAs were adjusted to the same amount by transfecting pcDNA3.1 empty vector (in micrograms). All the results represent means ± SD of three independent experiments. *Significant difference ($p < 0.05$); **highly significant ($p < 0.01$).

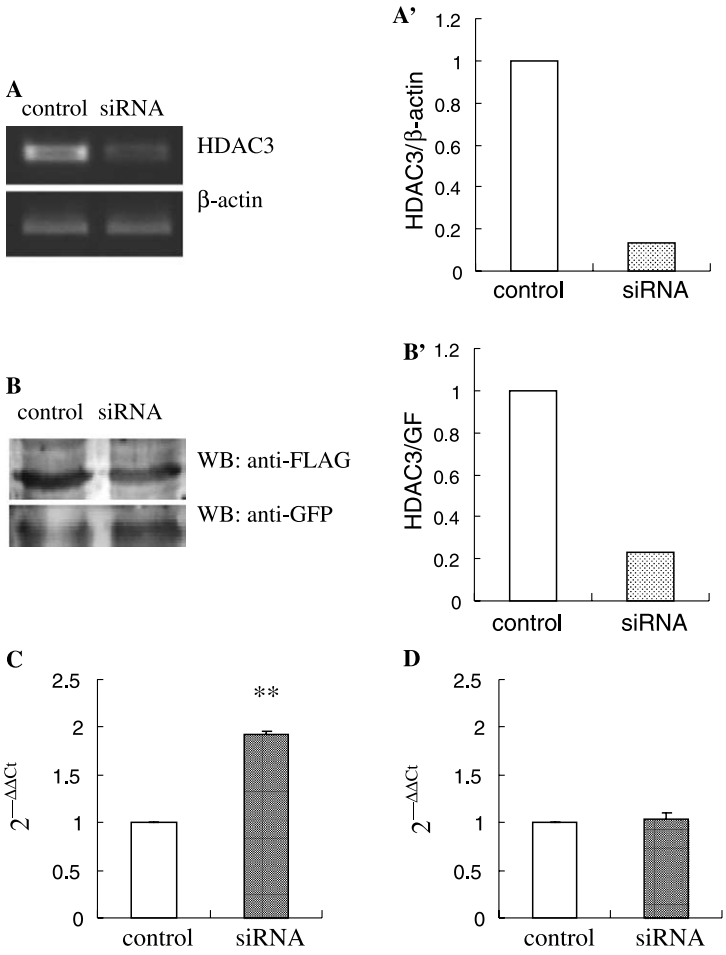

Fig. 5. Knockdown of HDAC3 up-regulated the transcription of $p15^{INK4b}$, but not that of $p21^{WAF1/cip1}$. (A) Endogenous HDAC3 mRNA was silenced by HDAC3 siRNA. (A′) Photodensitometric analysis of the semi-quantitative PCR products in (A). (B) Ectopic HDAC3 expression was knocked down by HDAC3 siRNA. (B′) Results of photodensitometric analysis of (B). (C) $p15^{INK4b}$ mRNA was up-regulated when endogenous HDAC3 was knocked down. (D) siRNA targeting HDAC3 gene had no effect on the transcription of $p21^{WAF1/cip1}$. All the results represent means ± SD of three independent experiments. **Highly significant ($p < 0.01$) compared with control.

and $p21^{WAF1/cip1}$. We then carried out co-transfection reporter assays to testify this. As shown in Fig. 4, Sp1 activated p15 (−113/+160) and p21 (−297/+8) promoters by 5.4- and 2.8-fold, respectively, and HDAC3 repressed the Sp1-mediated activation of $p15^{INK4b}$ (Fig. 4A) and $p21^{WAF1/cip1}$ (Fig. 4B) promoters in a dose-dependent manner. These results support our assumption that HDAC3 can repress the $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter activity by the interaction with Sp1.

Expression of endogenous $p15^{INK4b}$ mRNA, but not that of $p21^{WAF1/cip1}$, was enhanced by knockdown of HDAC3

As the transcription of both $p15^{INK4b}$ and $p21^{WAF1/cip1}$ was repressed by ectopic expression of HDAC3, we wanted to examine how $p15^{INK4b}$ and $p21^{WAF1/cip1}$ were regulated by HDAC3 in vivo. To achieve this, we employed RNAi protocol to suppress the HDAC3 expression in vivo [14]. First, we tested and confirmed that this RNAi protocol practically worked well in our system. As shown in Fig. 5A, endogenous mRNA of HDAC3 was knocked down by transfection of siRNA targeting HDAC3 gene. Since the anti-HDAC3 antibody was not available, we tested the effect of siRNA on ectopic expression level of HDAC3 by Western blotting using the anti-FLAG antibody. As can be seen from Fig. 5B, siRNA effectively decreased the ectopic expression level of HDAC3. Next, we showed that after knockdown of HDAC3 expression, the $p15^{INK4b}$ mRNA level was up-regulated by about 1.9-fold (Fig. 5C), whereas, interestingly, we did not detect any change in $p21^{WAF1/cip1}$ expression (Fig. 5D).

Discussion

Previous studies demonstrated that histone deacetylase inhibitors could induce the arrest of cells at the G1/G2 phase, implicating the specific roles of HDACs in control of cell cycle progression and cell differentiation [16]. One of the important molecular bases underlying these effects is the induction and activation of CKI proteins such as $p15^{INK4b}$ and $p21^{WAF1/cip1}$. HDAC3 has been demonstrated to contribute to the control of cell cycle. In this report, we showed that overexpression of HDAC3 repressed $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter activity (Fig. 1) as well as the endogenous $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription (Figs. 2A and B). ChIP experiments revealed that HDAC3 could be recruited to the proximal promoter regions. Our results indicated that HDAC3 played a role in the repression of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription. Interestingly, transfection of siRNA targeting HDAC3 gene increased the mRNA level of $p15^{INK4b}$ (Fig. 5C), while that of $p21^{WAF1/cip1}$ remained unaffected (Fig. 5D). We propose that other HDACs, such as HDAC1, may compensate for the loss of the function of HDAC3 on $p21^{WAF1/cip1}$ promoter when it was knocked down. HDAC1 had little inhabitation on $p15^{INK4b}$ (−2500/+160) promoter activity in our experimental system.

Sp1 is a general transcription factor, which binds and interacts with the GC-boxes in the promoter regions of target genes. It has been generally accepted that this protein is an extremely versatile factor involved in the regulation of expression of a wide range of genes, especially growth-regulated genes. By using the S-phase-specific mouse thymidine kinase (TK) promoter as a model system, Doetzlhofer et al. [17] show that HDAC1 can mediate transcriptional repression via the Sp1 binding site and HDAC1 is associated with Sp1. In this study, we found that HDAC3 inhibited Sp1 transcriptional activity, and HDAC3 and Sp1 can be part of the same complex (Fig. 3). Moreover, $p15^{INK4b}$ (−113/+160) and $p21^{WAF1/cip1}$ (−297/+8) promoter regions containing Sp1 binding sites were sufficient to mediate the repression by HDAC3 (Fig. 1). Considering the fact that HDAC3 was recruited to $p15^{INK4b}$ and $p21^{WAF1/cip1}$ promoter regions to inhibit $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription, we conclude that the HDAC3-induced inhibition of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ transcription was mediated, at least in part, by binding to Sp1.

Up-regulation of $p15^{INK4b}$ generally contributes to cell cycle arrest and cell differentiation. Our result is compatible with the observation that the cytokine GMCSF causes a large decrease in HDAC3 expression in GMCSF-induced differentiation of immune cells [18]. In addition, knockdown of HDAC3 gives rise to a concentration-dependent inhibition of cell proliferation [7]. In this report, we found that the reduction of HDAC3 augmented the $p15^{INK4b}$ expression (Fig. 5C), and this may result in cell cycle arrest and cell differentiation.

Perturbation of cell cycle control is a common biological feature in human cancer. The progression of cell cycle is driven by the activation of defined CDKs. The CDK activities that govern cell cycle progression require a coordinated regulation. The activities of CDK complexes are tightly regulated by a variety of mechanisms, such as the periodic cyclin accumulation and degradation, nuclear localization, phosphorylation of CDKs, and association with a number of different CKIs [19]. Both $p15^{INK4b}$ and $p21^{WAF1/cip1}$ are among the major regulators controlling the G1/S transition. Moreover, $p21^{WAF1/cip1}$ also plays a key role during the G2/M transition [20]. HDAC3 is one of the genes commonly up-regulated in many tumors [21]. Immunoblot analysis revealed that HDAC3 level was elevated in 92% of lung carcinomas [22]. Overexpression of HDAC3 down-regulates both $p15^{INK4b}$ and $p21^{WAF1/cip1}$ expression (Figs. 2A and B), which in turn will disrupt the tightly regulated and subtly balanced regulatory network of CDK activities. Collectively, our results indicate that the down-regulation of $p15^{INK4b}$ and $p21^{WAF1/cip1}$ may be one of the mechanisms by which HDAC3 regulates cell proliferation and oncogenesis.

Acknowledgments

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