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The Expression of p16 is Regulated by The Reversible Histone Acetylation*

WANG Xiu-Li[1], FENG Yun-Peng[1], ZHAO Jing[1], ZHANG Guo-Ping[2], PAN Hong[1], HUANG Bai-Qu[1], LU Jun[1]**

([1] *Institute of Genetics and Cytology, Northeast Normal University, Changchun* 130024, China;
[2] *Biology Staff Room of Guangdong Medical College, Dongguan* 523808, China)

Abstract $p16^{INK4a}$ plays a key role in control of cell cycle progression by negatively regulating the CDK4/6 activity. It was shown that histone acetyltransferase p300 had a positive effect on the activation of $p16^{INK4a}$ promoter, whereas, histone deacetylases HDAC3/4 counteracted the p300-mediated activation of $p16^{INK4a}$ promoter, and decreased the $p16^{INK4a}$ mRNA and protein levels. Chromatin immunoprecipitation (ChIP) tests revealed that the transfection of p300 reversed the hypoacetylation status of histones at the $p16^{INK4a}$ promoter mediated by HDAC3/4. Moreover, the immunofluorescence study showed that the nucleo-cytoplasmic shuttling of HDAC4 may play an important role. Furthermore, Western blot and ChIP assays demonstrated that the HDAC inhibitor sodium butyrate (NaBu) enhanced $p16^{INK4a}$ expression through inducing histone hyperacetylation. Based on these data, a hypothetical model was proposed for the involvement of reversible histone acetylation in transcriptional regulation of the $p16^{INK4a}$ gene.

Key words p16, p300, HDAC3, HDAC4, reversible acetylation
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As a tumor suppressor, the $p16^{INK4a}$ (hereafter p16) functions as a cell cycle inhibitor that negatively regulates the cell cycle kinases CDK4 and CDK6, which controls cell cycle progression in G1 phase through phosphorylation of the retinoblastoma protein (pRb) [1-2]. Overexpression of p16 causes a G1-phase cell cycle arrest[3].

The histone acetylation/deacetylation modification has emerged as a major form of epigenetic mechanism that regulates the expression of genes in eukaryotic cells[4]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) constitute the two distinct families of enzymes, which were originally characterized as nuclear enzymes modifying histones acetylation/deacetylation[5]. HATs such as p300/CBP are important transcriptional co-activators involved in regulation of many genes [6]. HDACs have been categorized into three classes based on their sequence homology and domain organization. Class Ⅰ HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) are similar to the yeast deacetylase Rpd3 [7], and are expressed primarily in the nucleus participating in silencing of both specific genes and entire chromosomal domains. Class Ⅱ HDACs (HDAC4, HDAC5, HDAC6, and HDAC7) contain catalytic domains homologous to that of yeast Hda1 [8], and possess the capability of active nucleo-cytoplasmic shuttling, which is suggested to be regulated by CaMK (Ca^{2+}/calmodulin-dependent protein kinase)-dependent phosphorylation [9]. Proteins similar to the yeast NAD1-dependent deacetylase Sir2 [10] compose the third class of HDACs. Class Ⅰ and Ⅱ HDACs have been found to function as corepressors for transcriptional repression, whereas the class Ⅲ HDACs are important for gene silencing at telomeres and HM (mating type) loci in yeast[11].

Our previous work revealed that p300 was involved in activation of p16 expression through

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**Corresponding author.
Tel: 86-431-85099362, E-mail: ycsuo@nenu.edu.cn
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recruitment by Sp1 [12], while HDAC3/4 inhibited the p16 promoter activity *via* YY1 and ZBP-89 [13-14]. ZBP-89 may compete with Sp1 for binding of the same or overlapping GC-rich sequences [15], and ZBP-89 physically associates with YY-1 [16]. In this study, we present experimental data showing that the reversible acetylation catalyzed by p300 and HDAC4 affected the p16 promoter activity as well as the p16 mRNA and protein levels, and this was accompanied by alterations in acetylation status of histones at the p16 promoter. Moreover, the nucleo-cytoplasmic shuttling of HDAC4 may play an important role.

1 Materials

1.1 Cell culture, transfection and luciferase reporter assay

293T cells were maintained in IMDM medium supplemented with 10% FBS, 100 g/L penicillin and 100 g/L streptomycin in a humidified atmosphere containing 5% CO_2 at 37℃. Transient transfection of 293T cells was performed using the conventional calcium phosphate-DNA precipitation method. 24 h after the transfection of the expression vectors, cells were harvested for luciferase reporter activity, RT-PCR, Western blot or ChIP assays. Transfected cells were analyzed for luciferase reporter activity using a Promega dual-luciferase reporter assay system. The Renilla luciferase control plasmid pREP7-RLuc was co-transfected in each experiment for normalization [17]. All the results represent the $\bar{x} \pm s$. based on at least three independent experiments.

1.2 Plasmid constructs

The p16 promoter reporter (–869 to +1 bp from the cap site) ligated to the luciferase gene (pGL2 basic, Promega) was provided by Dr. Eiji Hara (Imperial Cancer Research Fund Laboratories, London, U.K.). A series of deletion mutants of p16 promoter and the site mutations were generated as described previously[12, 18]. The Flag-ZBP-89-myc plasmid was provided by Dr J. L. Merchant (Department of Internal Medicine and Physiology, University of Michigan, USA). The plasmid expressing human HDAC4 (fused to the GFP-epitope) was a generous gift from Dr. R. Bassel-Duby (Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA). YY1 was kindly supplied by Dr E. Bonneloy (Transcriptional regulation and genetic diseases, CNRS UPR 2228, University Paris 5, France). The expression constructs of Sp1 were the gifts from Dr. Robert Tjian (Department of Molecular and Cell Biology, University of California, Berkeley).

2 Methods

2.1 RNA extraction and RT-PCR

Total cellular RNA was extracted from the 293T cells according to the Promega Total RNA Isolation System manual following a previous procedure [19]. RNA was re-suspended in RNase-free water and quantitated by spectrophotometry before being reverse transcribed. PCR products were resolved in 2% agarose gel. The p16 primer pairs were: sense 5′ ttcctggacacgctggt 3′ and antisense 5′ caatcggggatgtctgag 3′. The β-actin primer pairs were: sense 5′ tcgtgcgtgacattaaggag 3′ and antisense 5′ atgccagggtacatggtggt 3′. The number of cycles of PCR amplification was 25 for the β-actin, and 30 for the p16 gene.

2.2 Chromatin immunoprecipitation (ChIP)

The protocol for ChIP was described previously[20]. Briefly, 293T cells were transfected with the HDAC3 and HDAC4 or/and p300 expression vector or treatment with sodium butyrate (NaBu). At 24 h after treatments, cells were processed. The experiments were performed using anti-acetyl-histone H3 (Upstate Biotechnology), anti-acetyl-histone H4 (Upstate Biotechnology), or no antibody as the control. Samples were analyzed by PCR. The sequences of the primers used were: P1 sense 5′ agtttcgctcttgtctcccag 3′, antisense 5′ atggcgaaaccctgtctctac 3′; P2 sense 5′ agacagccgttttacacgcag 3′, antisense 5′ caccgagaaatcgaaatcacc 3′; and P3 sense 5′ taggaaggttgtatcgcggagg 3′, antisense 5′ caaggaaggaggactgggctc 3′ [21].

2.3 Western blotting

293T cells were harvested after transfection. 1×10^6 cells were digested and lysed in the lysis buffer for 30 min at 4℃. Total cell extracts were separated in 12% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with anti-p16 (Santa Cruz, sc-468), or anti-β-actin (Sigma, A1978) antibodies, and visualized by using the Chemiluminescent Substrate method with the SuperSignal West Pico kit provided by Pierce Co. β-actin was used as an internal control for normalizing the loading materials.

2.4 Immunofluorescence

Immunofluorescence was performed in 293T cells

as described elsewhere[18]. The treated 293T cells were washed twice in PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 at room temperature and then quenched in ice-cold PBS. After blocking with 5% BSA, collected cells were incubated with rabbit anti-YY1 (Santa Cruz, sc-1703) antibodies for 1h and stained with TRITC-conjugated goat anti-rabbit secondary antibody (Zhongshan, China) for 45 min at 4℃. Cells were examined under a fluorescence microscope (Nikon, Japan) and the images were collected at ×20 magnification with appropriate filters.

3 Results and discussion

3.1 Reversible histone acetylation was involved in transcriptional regulation of p16 gene

Our previous work revealed that p300 was an activator of p16 promoter[12] and HDAC3/4 inhibited the p16 promoter activity[13]. Whether the expression of p16 is regulated by the reversible histone acetylation mediated by p300 and HDAC3/4 has not been investigated. To test this, we co-transfected increasing amounts of HDAC3/4 with p16-luc reporter constructs and p300 expression vector into 293T cell for luciferase reporter assays. The results indicated that overexpression of p300 increased the p16 promoter activity in 293T cells, whereas the enhancement of p16 transcriptional activity by p300 was counteracted by co-transfection with HDAC3 and HDAC4 in a dose-dependent manner (Figure 1a and 1b). RT-PCR and Western blotting assays showed that the p16 mRNA and protein levels were elevated by p300 transfection, whereas moderated by co-transfection of HDAC3 and HDAC4 (Figure 1c and 1d), indicating the antagonistic effect between HDAC3/4 and p300 in control of p16 gene expression. The ChIP assays revealed that the acetylation level of histone H4 was markedly influenced by p300 and HDAC3. Specifically, in cells transfected with p300, elevated accumulations of acetyl-H4 were observed in the regions of p16 promoter examined. In contrast, transfection of HDAC3 led to a decrease of the

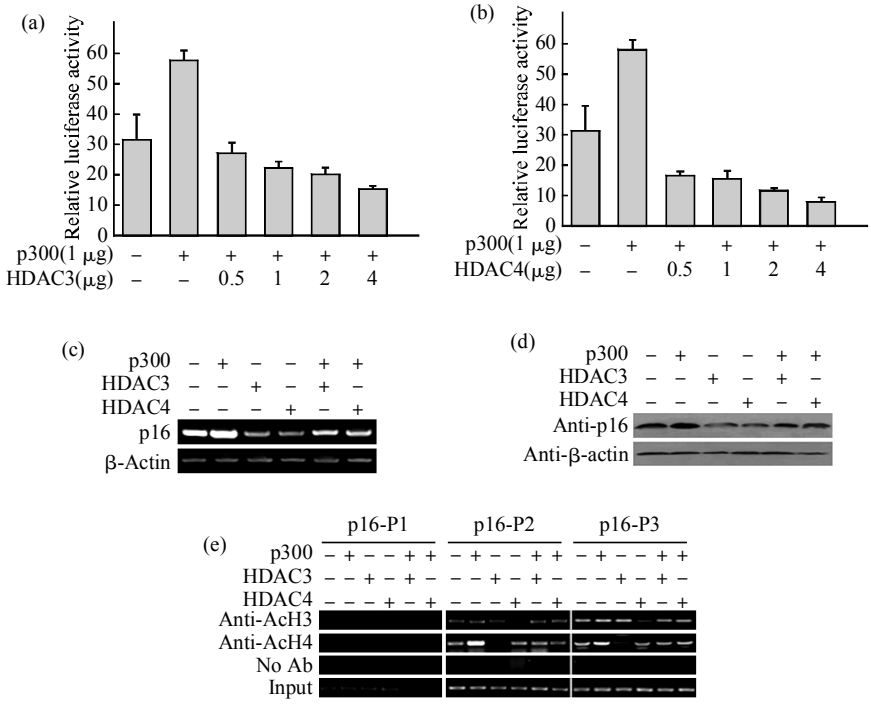

Fig. 1 The reversible histone acetylation regulated the expression of p16

(a), (b) HDAC3 and HDAC4 inhibited the p300-mediated transactivation of p16 gene. 293T cells were transiently transfected with 1 μg of constructs expressing p300 together with different amounts of HDAC3 (a) or HDAC4 (b). Luciferase activities were determined and normalized to Renilla activity 24 h after transfection. (c) Quantitative estimation of p16 mRNA level in 293T cells. Cells were transfected with p300 expression vector and HDAC3 or HDAC4 expression vector for 24 h. Total RNA was isolated, reverse transcribed and the level of p16 mRNA was measured by PCR. β-Actin was used as an internal control. (d) Western blot analysis of the p16 protein in 293T cells transfected with p300 expression vector and the HDAC3 or HDAC4 vector. (e) ChIP assays for the detection of the presence of acetylated histone H3 and H4 on p16 promoter. 293T cells were transfected with HDAC3, HDAC4 or p300 vector. Cells were harvested, DNA was sheared and immunoprecipitated with anti-acetylated histone H3 antibody and anti-acetylated histone H4 antibody. PCR products were resolved on a 1.5% agarose gel. Input indicates the DNA prior to immunoprecipitation.

acetylation level of H4 at the p16 promoter. Meanwhile, cells co-transfected with both HDAC3 and p300 exhibited higher levels of acetyl-H4 compared with HDAC3-transfected cells but lower levels of acetyl-H4 compared with p300-transfected cells (Figure 1e). Moreover, the acetylation level of histone H3 was decreased by HDAC4, while overexpression of p300 counteracted this effect (Figure 1e). These results manifested the antagonistic roles between HDAC3/4 and p300 in modifying the status of histone acetylation at the p16 promoter, and demonstrated that the reversible acetylation catalyzed by HDAC3/4 and p300 exerted critical functions in the transcriptional regulation of p16 gene.

3.2 The nucleo-cytoplasm shuttling of HDAC4 was important to inhibit the p16 promoter activity

As a member of the Class Ⅱ HDACs, HDAC4 possesses the capability of nucleo-cytoplasm shuttling. The subcellular localization of HDAC4 is suggested to be regulated by CaMK Ⅳ. Through phosphorylation, HDAC4 binds to a partner protein 14-3-3 and this leads to efficient nuclear export[9]. In order to further confirm the direct repressive role of HDAC4, we examined the influence of overexpression of pCaMK Ⅳ on the activity of the p16 promoter. We showed that CaMK Ⅳ increased the p16 promoter activity, and this elevation was moderated by transfection of pCaMK Ⅳ-ΔdCT, an inactive mutant of pCaMK Ⅳ (Figure 2a). Furthermore, pCaMK Ⅳ enhanced p16 promoter activity even when co-transfected with HDAC4, whereas the mutant pCaMK Ⅳ-ΔdCT did not (Figure 2a). While overexpression of pCaMK Ⅳ failed to counteract the repression of p16 promoter mediated by HDAC3, a member of the Class Ⅰ HDACs (Figure 2a). Additionally, this process was further verified by immunofluorescence study in 293T cells co-transfected with HDAC4/YY1, plus pCaMK Ⅳ or pCaMK Ⅳ - ΔdCT expression vectors. The results implicated that HDAC4 and YY1 co-existed in the nuclei (Figure 2b). Co-transfection of pCaMK Ⅳ caused the transport of HDAC4 out of the nuclei, which consequently prevented YY1 from interacting with HDAC4 in the nucleus (Figure 2b). These data demonstrated that HDAC4 suppressed the transcription of p16, and this process was related to nucleo-cytoplasm shuttling of HDAC4.

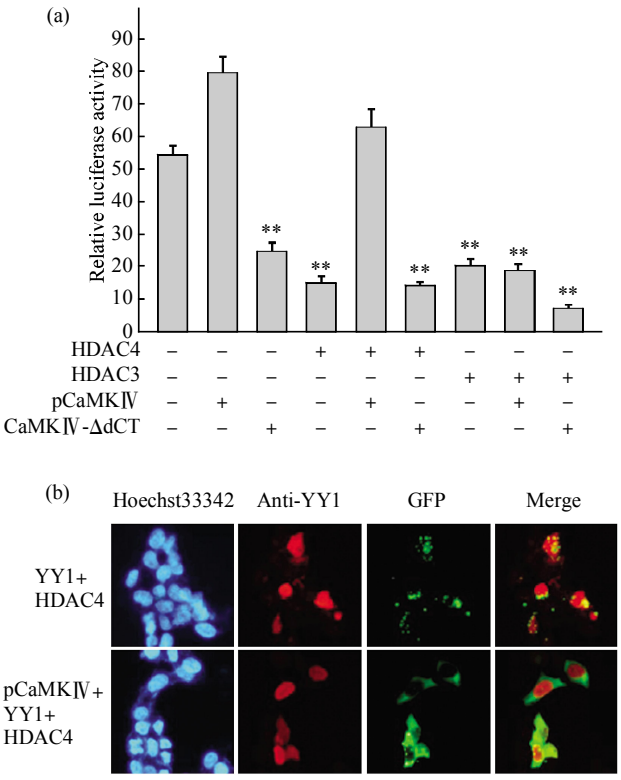

Fig. 2 The nucleo-cytoplasm shuttling of HDAC4 was important to inhibit the p16 promoter activity

(a) 293T cells were transfected with HDAC4 or HDAC3, co-transfected with expression plasmids encoding CaMK Ⅳ or CaMK Ⅳ-ΔdCT (inactive mutant), as indicated, along with pGL3-p16-luc reporter plasmid. $^{**}P < 0.01$, $^{*}P < 0.05$, n=3. (b) The co-localization of YY1 and HDAC4 in 293T cells. 293T cells were plated on to glass slides and transfected with HDAC4-GFP plus YY1 and pCaMK Ⅳ, or HDAC4- GFP plus YY1. Cells were fixed in formaldehyde and stained with anti-YY1 antibody followed by a TRITC secondary antibody, and visualized under a fluorescence microscope. YY1 was immunostained in red and HDAC4 in green. The nuclei were counterstained with Hoechst33342 (blue). The merged images were created with Simple PCI software (Cimaging).

3.3 Identification of the binding sites for transcription factors within the p16 promoter

The transcription factors ZBP-89, YY1 and Sp1 were found to participate in expression regulation of p16 through HDAC3/4 and p300 in our previous work[12–14]. To identify the promoter regions responsible for p16 transcription regulation by ZBP-89, YY1 and Sp1, 293T cells were transfected with a series of p16 promoter truncation mutants linked to a luciferase reporter, together with ZBP-89, YY1 and Sp1. The luciferase reporter assay results demonstrated that the T0 promoter region(–630～+1 bp) displayed a decrease

of the basal reporter activity compared with the WT promoter, whereas the basal activity of T1 (–479 ~ +1 bp) showed an increase compared with WT and T0 (Figure 3a). Meanwhile, the other two truncation mutants T2 and T3 had roughly the same basal activity as the wild-type and T0 promoter (Figure 3a). Moreover, these promoter regions T2 and T3 exhibited increased reporter activity when transfected with Sp1, while they exhibited decreased reporter activity when transfected with ZBP-89 or YY1, compared with the basal promoter activity (Figure 3a). In cells co-transfected with Sp1 and ZBP-89, we found that Sp1 was able to counteract the ZBP-89 action of decreasing the promoter activity. (Figure 3a). These data implicate that an inhibitory region may exist between –630 and –479 bp, and an activation region may be present between –479 and –449 bp. These results may probably be attributed to the existence of an INK4a transcription silence element (ITSE) between –491 and –485 bp of the p16 promoter[22].

We then generated p16 promoter constructs containing point mutations of the CG-rich region. The construct Ma contains a putative YY1 binding site (CCAT) (between –630 and –479 bp) mutation, Mb contains two overlapping Sp1 binding sites (between –479 and –449 bp) mutation, and Mc contains a Sp1 binding site (between –449 and –62 bp) mutation. We discovered that Mb mutation caused higher basal activity than the wild-type promoter, and it displayed higher induced activity in response to Sp1 and ZBP-89 than the basal activity (Figure 3b), implying that the Mb site was ZBP-89 binding site, in which process, ZBP-89 inhibited the activity of the Mb promoter. Moreover, the Ma mutation broughtar about 1.5-fold increase of basal activity, and transfection of YY1 expression vector inhibited the activity (Figure 3b), indicating that there are other YY1 binding sites besides the Ma site at the p16 promoter. Furthermore, the Mc construct showed a lower basal activity than WT promoter, and transfection of YY1 expression vector increased the activity (Figure 3b), implying the importance of Mc site in the regulation of p16 promoter by YY1. The interaction among Sp1, ZBP-89 and YY1 was reported previously [12–14], and ZBP-89 shares with Sp1 and other Sp like factors the ability to recognize GC-rich sequences in target genes. This overlapping DNA recognition has been shown to lead to a competitive model of inhibition in which ZBP-89 represses gene transcription by displacing factors such as Sp1 and Sp3 [15, 18]. Additionally, an analysis of the proximal promoter of ornithine decarboxylase (ODC) gene revealed that Sp1 and ZBP-89 bound to the GC elements in a mutually exclusive manner [23]. In other cases, ZBP-89 appeared to inhibit gene activity by binding to DNA independent of Sp1 [24]. An inspection of a number of promoters of sterol-regulated genes revealed that several of these promoters contained potential YY1 binding sites (CCAT or ACAT), either overlapping or adjacent to binding sites for NF-Y, Sp1, or SREBP [25], and YY1 could interact with Sp1 [16]. There have been indications that YY1 can physically interact with ZBP-89 *via* its third and fourth zinc fingers to suppress COX Vb gene expression[16]. These data have led us to speculate that ZBP-89, Sp1 and YY1 work coordinately to contribute to the regulation of p16 expression.

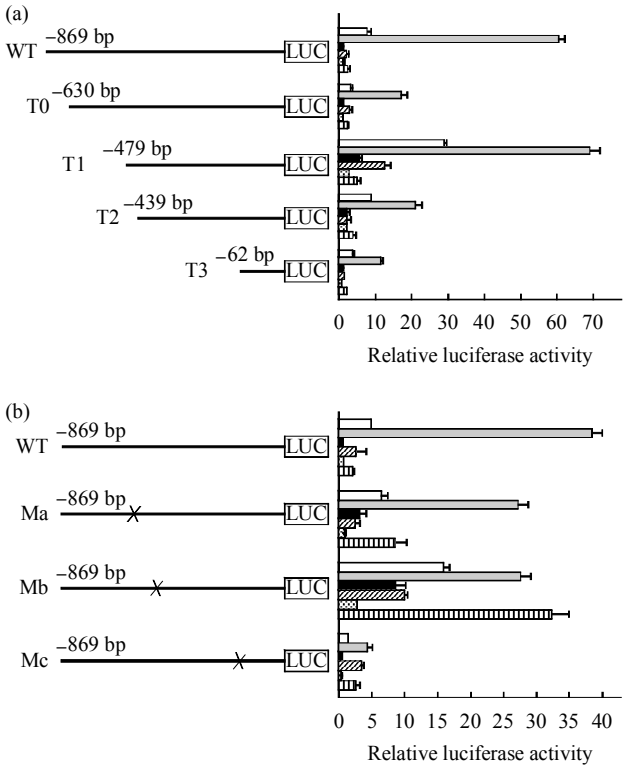

Fig. 3 Identification of the transcription factors binding sites within the p16 promoter

(a) Luciferase reporter assays using a series of deletion constructs of the p16 promoter. One μg of the WT, T1, T2 and T3 reporter vectors, together with 1 μg ZBP-89 or/and Sp1, YY1 vector were transfected into 293T cells, respectively. Luciferase activities were determined and normalized to Renilla activity 24 h after transfection. (b) Site-directed mutation studies of p16 promoter. Wild-type p16 promoter reporter plasmid and its mutants were co-transfected with ZBP-89, Sp1 and YY1 in different combinations as indicated. Relative luciferase activities were normalized against Renilla activity. Data represent averages of at least three independent experiments with standard deviations. □: Control; ▩: Sp1; ■: ZBP-89; ▨: YY1; ▦: ZBP-89+YY1; ▥: ZBP-89+Sp1.

3.4 The HDAC inhibitor sodium butyrate (NaBu) upregulated p16 expression

Finally, since HDAC inhibitors are known to influence the expression of some genes through induction of histone hyperacetylation at the promoters[26], we wanted to find out whether HDAC inhibitors participate in the expression of p16. To test this, we examined the effect of sodium butyrate (NaBu), an inhibitor of HDACs, on the p16 expression. Western blotting showed that treatment of NaBu increased the p16 protein level in 293T cells (Figure 4a). To look further into the roles of NaBu in regulation of p16 expression, we used chromatin immunoprecipitation (ChIP) assays to test whether NaBu affects the histone acetylation level at p16 promoter. The ChIP results revealed that the acetylation level of histone H4 and H3 at P2 and P3 promoter regions was indeed enhanced by treatment of NaBu (Figure 4b). Clearly, NaBu increased the expression of p16 through inducing histone hyperacetylation, further implicating the participation of the reversible histone acetylation in the transcriptional regulation of p16 gene.

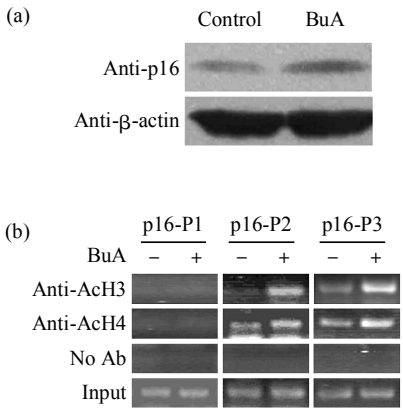

Fig. 4 NaBu upregulated p16 expression

(a) NaBu potentiated p16 expression. 293T cells were treated with 0.5 mg/L NaBu. The presence of p16 protein was determined by Western blotting. β-Actin was used as an internal control. (b) 293T cells were treated with 0.5 mg/L NaBu. Anti-acetyl-H4 and anti-acetyl-H3 antibodies were used for immunoprecipitation. The presence of acetyl-H4 and acetyl-H3 in each region were measured by PCR. PCR products were resolved in 1.5% agarose gel.

Data presented above indicate that the HAT/HDAC-mediated histone modification is one of the epigenetic mechanisms that regulates p16 expression. Although HDAC inhibitors may promote global elevation of histone acetylation level through reducing the activity of HDACs, the expression of only a small fraction of cellular genes is changed in response to histone hyperacetylation[26]. To date, two contradictory viewpoints can be found in the literature regarding the effects of NaBu in regulating the transcription of p16. Matheu *et al.*[27] showed that the level of p16 protein was decreased in human and murine fibroblasts upon exposure to high concentrations of NaBu (10 mmol/L). On the contrary, other workers described that the p27 and p16 protein levels were markedly increased following NaBu treatment in NCI-H460(0.5 mmol/L)[28]. Presumably, this discrepancy can be attributed to the different cell lines and concentrations of NaBu used.

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可逆的组蛋白乙酰化调控 p16 的表达 *

王秀莉[1)] 冯云鹏[1)] 赵 静[1)] 张国平[2)] 潘 虹[1)] 黄百渠[1)] 陆 军[1)**]

([1)] 东北师范大学遗传与细胞研究所，长春 130024；[2)] 广东医学院生物学教研室，东莞 523808)

摘要 $p16^{INK4a}$ 通过抑制 CDK4/6 的活性而在细胞周期进行中发挥重要的作用，研究发现，组蛋白乙酰转移酶 p300 能促进 $p16^{INK4a}$ 启动子活性，而组蛋白去乙酰化酶 HDAC3/4 能够逆转由 p300 介导的 $p16^{INK4a}$ 启动子活性的增加，HDAC3/4 能够降低 $p16^{INK4a}$ mRNA 和蛋白质的水平．染色质免疫沉淀(ChIP)实验结果表明转染 p300 表达质粒能够逆转由 HDAC3/4 介导的 $p16^{INK4a}$ 启动子组蛋白的低乙酰化状态．此外，免疫荧光实验结果表明 HDAC4 的核质穿梭起着重要的作用．免疫印迹和染色质免疫沉淀实验证明 HDAC 的抑制剂丁酸钠盐(NaBu)能通过诱导组蛋白的高乙酰化而促进 $p16^{INK4a}$ 的表达．基于这些实验结果，推测出可逆的组蛋白乙酰化参与 $p16^{INK4a}$ 基因转录调控的模型．

关键词 p16，p300，HDAC3，HDAC4，可逆的乙酰化

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** 通讯联系人．

Tel: 0431-85099362，E-mail: ycsuo@nenu.edu.cn

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