Reversible Histone Acetylation Involved in Transcriptional Regulation of WT1 Gene

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Abstract To validate the involvement of reversible histone acetylation in the transcriptional regulation of human Wilms’ tumor 1 gene (WT1), we analyzed the roles of histone deacetylases (HDACs) and histone acetyltransferase in this epigenetic process. Of the six HDACs (HDAC1−6) examined, HDAC4 and HDAC5 were found to have significant repressing effects on the activity of the WT1 reporter gene, as revealed by luciferase reporter assays and quantitative real-time reverse transcription-polymerase chain reaction assays. Luciferase reporter assays showed that the histone acetyltransferase p300 was able to counteract the HDAC4/HDAC5-mediated repression and that p300/CBP synergized with transcription factors Sp1, c-Myb, and Ets-1 in activation of the WT1 reporter. Chromatin immunoprecipitation experiments showed that p300 promotes the acetylation level of histone H3 at the WT1 intronic enhancer. Based on these data, we proposed a hypothetical model for the involvement of reversible histone acetylation in transcriptional regulation of the WT1 gene. This study provides further insight into the mechanisms of transcriptional regulation of the WT1 gene and WT1-associated diseases treatment.

Keywords histone acetylation; WT1; HDAC; p300/CBP; transcriptional regulation

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The Wilms’ tumor suppressor protein, WT1, was identified as a candidate factor in paediatric malignancy of the kidneys that affects approximately 1 in 10,000 children [1]. The human WT1 gene has been identified as a major player in the development of Wilms’ tumor [2]. In order to understand how the expression of WT1 is temporally and spatially restricted, a number of research groups have characterized the cis-acting regulatory elements and the trans-acting factors of the WT1 gene. The WT1 promoter is highly GC-rich and contains neither a CCAAT box nor a TATA box [3–5]. DNase I footprinting analysis showed that the WT1 promoter was bound by the transcription factor Sp1 at numerous sites [5]. Studies by Cohen et al. showed that Sp1 is a critical regulator of the WT1 gene [6]. Zhang et al. identified a 258 bp enhancer in intron 3 of the WT1 gene, approximately 11 kb downstream of the promoter [7]. Sequence analysis showed that this 258 bp fragment contains many potential binding sites for transcription factors, including Ets-1, GATAs, and c-Myb. Cotransfection and chloramphenicol acetyl transferase assays showed that GATA-1 and c-Myb were responsible for the activity of this intronic enhancer of the WT1 gene [7].

The reversible acetylation of histones has long been linked to the transcriptional activity of genes in eukaryotic cells. Over the last few years, several reports have described the purification and identification of a large number of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Many of these enzymes had previously been described as transcriptional coactivators or corepressors. Coactivators with HAT activity stimulate transcription, whereas corepressors with HDAC activity repress transcription [8,9]. p300 and CBP are highly

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homologous global transcriptional coactivators that have been shown to acetylate nucleosomal histones [10]. Apart from their ability to acetylate histones, p300/CBP are known to acetylate and regulate various transcription factors, including p53, GATA-1, c-Myb, and Sp1 [11–13]. Various cellular and viral factors target at p300/CBP to modulate transcription and/or cell cycle progression [14]. The HDAC family members can be primarily cataloged into two classes. Four Class I (HDAC1, 2, 3, and 8) and six Class II (HDAC4, 5, 6, 7, 9, and 10) HDACs have been identified and partially characterized in human [15,16], and there are potentially more deacetylases in this family according to the analysis of the genome sequence [17]. Among the Class II HDACs, HDAC4 and 5 have been shown to be involved in myogenesis and T cell receptor-mediated apoptosis of thymocytes because of their roles in regulating factors of the myocyte enhancer factor 2 (MEF2) family [18].

In a previous study, we described that the HAT p300 and its activity were involved in regulation of WT1 transcription [19]. However, the mechanisms of WT1 gene transcriptional regulation, especially the roles of HDACs in this process, have not been elucidated. In this study, we examined the effects of various HDACs and HATs on WT1 gene transcription.

Materials and Methods

Plasmid construction

The expression vectors of p300, CBP, C/EBPβ, c-Myb, and Ets-1 were kindly provided by Dr. Joan BOYES (Institute of Cancer Research, London, UK), Dr. Paul M. LIEBERMAN (The Wistar Institute, Philadelphia, USA), Dr. Richard M. POPE (Northwestern University Medical School, Chicago, USA), Dr. Odd S. GABRIELESEN (University of Oslo, Oslo, Norway), and Dr. Thierry ROGER (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), respectively. The vector of pGL3-Basic was a gift from Dr. Maty TZUKERMAN (Technion-Israel Institute of Technology, Haifa, Israel). Vectors of HDACs were provided by Dr. Edward SETO (H. Lee Moffitt Cancer Center and Research Institute, Tampa, USA).

For the construction of the −480/+251 WT1 promoter-driven luciferase plasmid containing the 258 bp WT1 intronic enhancer (pGL3-P-E-Luc), the WT1 promoter fragment was amplified by polymerase chain reaction (PCR) using the primers 5′-GGGTGCAAAGCGAAGGTA-3′(sense) and 5′-GGGACGAATGCCTGACG-3′(antisense). The fragment was cloned into the pUCm-T vector (Sangon, Shanghai, China) and, after cutting with NotI and XhoI, inserted into pREP4-Luc vector (Promega, Madison, USA). The WT1 promoter fragment was then cut from the vector with NotI and XhoI, and inserted into pGL3-Basic, creating the WT1 promoter/luciferase plasmid (pGL3-P-Luc). The WT1 intronic enhancer fragment was amplified with the primers 5′-GTGCGACAAGCTTTCCGCCCTCCG-3′(sense) and 5′-GTGCAGGATCCCGAGCACCCAG-3′(antisense). The fragment was also cloned into pUCm-T vector, and after cutting with SalI, inserted into pGL3-P-Luc, generating the plasmid pGL3-P-E-Luc containing both the WT1 promoter and intronic enhancer. The correct orientation of the insertion was confirmed by restriction mapping. The plasmid pGL3-P-E-Luc was used in all the transfection experiments unless indicated otherwise. The mutant vector pGL3-P-E-delta-Ets-1-Luc was constructed by PCR. To amplify the WT1 intronic enhancer fragment in which the Ets-1 binding site was deleted, the primers 5′-AAGCTTTTCCCCGTCGGCTTCTCGCTCTCGCTCA-3′(sense) and 5′-GTCGACGGATCCCGAGCACCCAG-3′(antisense) were used. The fragment was cloned into the pUC19 vector (Sangon) digested by SmaI and, after cutting with SalI, inserted into pGL3-P-Luc, generating the plasmid pGL3-P-E-delta-Ets-1-Luc. The correct orientation of the insertion was confirmed by restriction mapping.

Cell culture and transfection

The 293T human embryonic kidney epithelial cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using a standard calcium phosphate method with 2.5×10⁵ cells and 1 μg of DNA unless indicated otherwise.

Luciferase reporter gene assays

After transfection, 293T cells were analyzed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega, Madison, USA). As a transfection control for the luciferase assays, the Renilla luciferase control plasmid was cotransfected in all the experiments. Data were normalized to Renilla luciferase as indicated in the figure legends. Relative luciferase activity was calculated using the luciferase activity of cells transfected with the reporter DNA alone as 1 unless noted.

Quantitative real-time reverse transcription-PCR assay of endogenous WT1 mRNA

PCR primers for the WT1 gene were 5′-TCAAGGAC-
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TGTGAACGAA-3' (sense) and 5'-TTGTGATGGCGGA-CTAA-3' (antisense). PCR primers for β-actin (as an internal control) were 5'-TCGTCGTGACATTAAGGAG-3' (sense) and 5'-ATGCCAGGGTACATGGTGTT-3' (antisense).

293T cells were transfected with 4 μg HDAC4 or HDAC5 expression vectors, or pcDNA3.1 empty vector as the control. Twenty-four hours after transfection, total RNA was extracted from cells with the Total RNA Isolation System (Promega), and RNA was converted to cDNA by the reverse transcriptase enzyme reaction (AMV transcriptase-reverse; Promega), followed by quantitative real-time PCR with either WT1-specific or β-actin-specific primers. The length of the resulting fragment was 281 bp or 304 bp.

Chromatin immunoprecipitation (ChIP)

For cross-linking, formaldehyde was added at a final concentration of 2% to cell culture plates 24 h after transfection, and the plates were placed on a rocker for 10 min at room temperature. Cross-linking was terminated by several washes in phosphate-buffered saline and cells were stored in sodium dodecyl sulfate lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Tris, pH 8.1) with protease inhibitors. After sonication, samples were processed using a ChIP assay kit, essentially as described by the manufacturer (Upstate Biotechnology, Lake Placid, USA). ChIP experiments were carried out using polyclonal anti-acetyl-histone H3 antibody, obtained from Upstate Biotechnology. Immunoprecipitated chromatin was then assayed by PCR using primers specific to sequences at the WT1 intronic enhancer and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter.

The primers used to detect WT1 intronic enhancer were 5'-GTCGACAAGCTTTTCCCCGCTCCG-3' (sense) and 5'-GTCGACGGATCCCCGAGCACCAG-3' (antisense), which amplified a fragment covering 258 bp WT1 intronic enhancer. The primers used to detect GAPDH promoter were 5'-TACTAGCGGTTTTACGGGCGG-3' (sense) and 5'-TCGACAGGAGGAGCGAGGAGA-3' (antisense).

Statistical analysis

Experiments were repeated three times. Data are presented as the mean±SD. Student’s t-test in two groups and one-way ANOVA in multiple groups were used to analyze the statistical significance of differences. P<0.05 was considered statistically significant, P<0.01 was considered statistically highly significant.

Results

Overexpression of HDAC4 and HDAC5 significantly repressed WT1 reporter activity

In eukaryotic cells, there is a kinetic equilibrium between acetylation and deacetylation modifications of core histones, which are accomplished by two categories of enzymes, HATs and HDACs. In a previous study, we reported that HAT p300 promoted the activation of human WT1 promoter and intronic enhancer and the HAT activity of p300 was important to its function in the regulation of WT1 gene expression [19]. Conceivably, HDACs would also participate in the transcriptional regulation of the WT1 gene. In order to test this, we examined the effects of HDACs on WT1 transcription. The reporter construct pGL3-P-E-Luc was cotransfected with expression vectors for human HDAC1–6 into 293T cells. As shown in Fig. 1, among the six HDACs tested, HDAC4 and HDAC5 significantly repressed the reporter gene expression, whereas the influence of HDAC1–3 and HDAC6 were not significant. It has been reported that HDAC4 and HDAC5 can interact specifically with and repress the myogenic transcription factor MEF2, and MEF2 plays an essential role in muscle differentiation [20]. WT1 is also a transcription factor that plays a critical role.

Statistical analysis

Experiments were repeated three times. Data are presented as the mean±SD. Student’s t-test in two groups and one-way ANOVA in multiple groups were used to analyze the statistical significance of differences. P<0.05 was considered statistically significant, P<0.01 was considered statistically highly significant.

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in growth and differentiation of several organs, including kidneys, gonads, and spleen. HDAC4 and HDAC5 might regulate the growth and differentiation of these organs by repressing WT1 gene expression. Experimental data presented here provide preliminary evidence that histone deacetylation participates in the transcriptional regulation of the WT1 gene.

p300 Counteracted HDAC-mediated repression of WT1 reporter activity

Histone acetylation modification is a reversible process that involves the catalytic activities of both HDACs and HATs. In order to address whether p300, an important coactivator with intrinsic HAT activity, can counteract the inhibition of the WT1 gene mediated by HDAC4 and HDAC5, we carried out transient expression assays. We cotransfected the WT1 luciferase reporter construct pGL3-P-E-Luc with expression vectors of HDAC4 or HDAC5 and p300 or p300 Δ HAT (p300 mutant deficient in acetyltransferase activity). It was reported that p300 played important roles in maintaining the genome integrity and was the molecular target for HDAC inhibitors [21]. Data presented in Fig. 2(A,B) clearly show that both HDAC4 and HDAC5 strongly inhibited the transcription stimulating effect of p300 and p300 Δ HAT. These results support our assumption that reversible acetylation is involved in the transcriptional regulation of the WT1 gene.

As shown in Fig. 2(A,B), apart from p300, HDAC4 and HDAC5 can also inhibit the transcription stimulating effect of p300 Δ HAT. p300 Δ HAT is a mutant deficient in acetyltransferase activity, but it can stimulate reporter gene transcription as a protein bridge connecting transcription factors and the basal transcription machinery, or as a molecular scaffold mediating the assembly of multiprotein complexes. HDACs can deacetylate both the histones and the transcription factors; therefore, HDAC4 and HDAC5 can also inhibit the transcriptional activity of p300 Δ HAT.

Overexpression of HDAC4 and HDAC5 decreased endogenous WT1 mRNA level

To examine whether HDAC4 and HDAC5 are also able to affect the endogenous transcription of the WT1 gene, we carried out quantitative real-time PCR analysis of the WT1 mRNA level. 293T cells were transfected with plasmids expressing HDAC4 or HDAC5, and as the control, the empty vector (pcDNA3.1) was also transfected. Total mRNA was isolated and equal amounts of mRNA were subjected to real-time reverse transcription-PCR using primers specific for WT1, as well as for the β-actin gene (a housekeeping gene used as internal reference). It is clear from Fig. 3 that the expression of WT1 mRNA was significantly decreased on the ectopic expression of HDAC4 [Fig. 3(A)] and HDAC5 [Fig. 3(B)]. Thus, both HDAC4 and HDAC5 were able to decrease the endogenous expression of WT1 in 293T cells.

p300/CBP synergized with transcription factors in activation of WT1 reporter

Sequence analysis showed that the 652 bp WT1 proximal promoter region contained the binding sites for transcription factors C/EBP (NF-IL6), Ets-1, and Sp1,
and it was reported that C/EBP, Ets-1, and Sp1 can recruit transcriptional coactivators p300/CBP and promote the activation of target genes through their interactions [5, 22]. Therefore, we were interested in finding out whether p300/CBP can promote the transcription factor-mediated activation of WT1 promoter. In order to address this question, we carried out cotransfection experiments with pGL3-P-Luc, C/EBP, Ets-1, Sp1, and p300/CBP vectors. Among these transcription factors, only Sp1 acted synergistically with p300/CBP. From Fig. 4(A), it can be seen that p300 and CBP alone caused an approximately 2-fold increase and Sp1 alone resulted in a 2.2-fold increase in activation, whereas combinations of p300/Sp1 and CBP/Sp1 brought about an 8.4-fold and an 8-fold enhancement, respectively. This indicated that p300/CBP worked synergically with Sp1 in the activation of the WT1 promoter reporter gene. Cohen et al. reported that Sp1 is a critical regulator of the WT1 gene [6], and our results were consistent with theirs.

Wu et al. reported that the WT1 promoter functioned in all cell lines tested, thus the tissue-specific expression of this gene must rely on additional regulatory elements [23]. Moreover, a 258 bp intronic enhancer located in the third intron of the WT1 gene was identified [7]. This intronic enhancer exerted important functions in the tissue-specific expression of the WT1 gene. The 258 bp WT1 intronic enhancer fragment contained the binding sites for transcription factors c-Myb and Ets-1, and it was reported

**Fig. 3** Endogenous human Wilms’ tumor 1 gene (WT1) mRNA level was down-regulated by histone deacetylases (HDAC)4 (A) and HDAC5 (B)
Human embryonic kidney epithelial 293T cells were transfected with 4 μg HDAC4 (A) or HDAC5 (B) expression vectors, or pcDNA3.1 empty vector as a control. Total RNA (1 μg) was reverse transcribed with an oligo(dT) primer, followed by quantitative real-time polymerase chain reaction with either WT1-specific or β-actin-specific primers. **P<0.01 compared with the control.

**Fig. 4** Synergistic action of histone acetyltransferases p300/CBP and transcription factors in the activation of human Wilms’ tumor 1 gene (WT1) reporter
p300 or CBP expression vector was cotransfected with pGL3-P-Luc and Sp1 (A) expression vectors as indicated. p300 or CBP expression vector was cotransfected with pGL3-P-E-Luc and c-Myb (B) or Ets-1 (C, left bars) expression vectors as indicated. In panel (C), the right bars represent the same cotransfection experiments as the left bars, except the mutant vector pGL3-P-EΔEts-1-Luc was used. +, transfected; −, untransfected.

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that c-Myb transactivated the WT1 intronic enhancer. It has been shown that both c-Myb and Ets-1 increased the activation of their target genes through interaction with p300/CBP. These data intrigued us and prompted us to investigate whether p300/CBP play a role in the transcription factor-mediated activation of the WT1 gene. We carried out transient reporter assays by cotransfecting pGL3-P-E-Luc and p300/CBP and/or the two transcription factors c-Myb and Ets-1. The results clearly showed that p300/CBP synergized with both c-Myb [Fig. 4(B)] and Ets-1 [Fig. 4(C), left bars] in activation of the WT1 reporter.

Both the WT1 promoter and the intronic enhancer contain the binding sites for transcription factor Ets-1; we were then interested in finding out which binding site was important in p300/CBP-mediated activation of WT1 reporter. We therefore carried out the same cotransfection experiments using the mutant vector pGL3-P-E-Δ-Ets-1-Luc. As shown in Fig. 4(C) (right bars), when the binding site for Ets-1 of WT1 intronic enhancer was deleted, the activation of WT1 reporter by Ets-1 and the synergism of p300/CBP and Ets-1 were abrogated, whereas the promotive role of p300/CBP on WT1 reporter activity remained unaffected. This result indicated that the binding site for Ets-1 on WT1 intronic enhancer was important in activation of the WT1 gene by p300/CBP. Because of the function of c-Myb, the stimulating role of p300/CBP was not affected when the binding site for Ets-1 on WT1 intronic enhancer was deleted [Fig. 4(C), right bars].

p300 Increased the acetylation level of histone H3 at WT1 intronic enhancer

We have previously shown that the HAT activity of p300 was essential in WT1 transcriptional regulation [19]. We then wanted to test whether the transactivation function of p300 was achieved through the acetylation of histones at the WT1 gene. We carried out ChIP experiments in 293T cells using polyclonal anti-acetyl-histone H3 antibody after transfection with the p300 expression vector, and the precipitated DNA was assayed by PCR using primers specific to sequences at the WT1 intronic enhancer and the GAPDH promoter region. (A, B) PCR products separated by agarose gel electrophoresis. (C) Photodensitometric analysis of PCR products in (A) and (B). 1, the α-Ac-H3/Input of control in (A); 2, the α-Ac-H3/Input of cells transfected with p300 expression vector in (A); 3, the α-Ac-H3/Input of control in (B); 4, the α-Ac-H3/Input of cells transfected with p300 expression vector in (B). +, transfected; −, untransfected.

Fig. 5 Effect of histone acetyltransferase p300 on the acetylation level of histone H3 at the human Wilms' tumor 1 gene (WT1) intronic enhancer

Human embryonic kidney epithelial 293T cells were transfected with 3 μg p300 expression vector or pcDNA3.1 empty vector as the control. Chromatin immunoprecipitation was carried out using antibody against acetylated H3 (α-Ac-H3), followed by polymerase chain reaction (PCR) with primers amplifying the WT1 intronic enhancer region and the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) promoter region. (A, B) PCR products separated by agarose gel electrophoresis. (C) Photodensitometric analysis of PCR products in (A) and (B). 1, the α-Ac-H3/Input of control in (A); 2, the α-Ac-H3/Input of cells transfected with p300 expression vector in (A); 3, the α-Ac-H3/Input of control in (B); 4, the α-Ac-H3/Input of cells transfected with p300 expression vector in (B). +, transfected; −, untransfected.

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To summarize, we have reached the conclusion that reversible acetylation is involved in the activation of the WT1 gene and we propose a hypothetical model for the reversible acetylation-mediated regulation of the WT1 gene (Fig. 6). Transcription factors Sp1, Ets-1, and c-Myb recruit p300/CBP to the WT1 promoter and intronic enhancer, and they form a transcriptional coactivator complex. p300/CBP cause the acetylation of histones at the WT1 intronic enhancer, leading to an open chromatin structure and facilitating the function of RNA polymerase II. p300/CBP acetylate one or several transcription factors, increasing their activities, and thus enhances the transcription of the WT1 gene. However, HDAC4 and/or HDAC5 work as counteracting enzymes of HAT to antagonize p300/CBP function, resulting in the deactivation of the WT1 gene. These results will be helpful in establishing the theoretical basis for the cure of diseases associated with WT1.

**Conclusion**

In conclusion, we have shown that both HDAC4/HDAC5 and p300/CBP were involved in transcriptional regulation of the WT1 gene. p300/CBP synergized with transcription factors Sp1, c-Myb, and Ets-1 in the activation of WT1 reporter. p300 promoted the acetylation level of histone H3 at the WT1 intronic enhancer. This work provides further insight into the mechanisms of WT1 gene control and WT1-related diseases.

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