

Plakoglobin is a new target gene of histone deacetylase in human fibrosarcoma HT1080 cells

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Histone deacetylase (HDAC) plays a key role in gene expression, by suppressing the transcription of a number of target genes. Identification of such genes is important for deciphering the functional role of HDAC. Here, using cancer gene-focused DNA microarray analysis, we identified plakoglobin as a new target gene of HDAC. Functional inhibition of HDAC by its specific inhibitors induced the expression of plakoglobin by eight-fold in human fibrosarcoma HT1080 cells. However, the expression of β -catenin, which is closely related to plakoglobin, was not altered, implying the specific function of HDAC in plakoglobin expression. Using antiacetyl-H4 antibody, chromatin immunoprecipitation analysis revealed that the distal region (–945 ~ –646) of the promoter of plakoglobin is responsible for the HDAC-mediated repression of the gene. Moreover, the induced expression of plakoglobin by the inhibition of HDAC activated the Tcf/Lef-dependent luciferase reporter gene, a well-known downstream effector of the *Wnt* signaling pathway. Furthermore, transient transfection of plakoglobin also activated Tcf/Lef reporter gene expression. Taken together, our results demonstrate that plakoglobin is a new target gene governed by HDAC, and that it acts as an oncogene in HT1080 cells.

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Introduction

Histone deacetylase (HDAC) is a component of a multiprotein complex which modifies chromatin, resulting in the silencing of a number of genes (Taunton *et al.*, 1996; Hassig *et al.*, 1997). Aberrant recruiting of HDAC to its target site can cause abnormal expression of several genes, including tumor suppressors and tumor activators (Lin *et al.*, 1998; Kim *et al.*, 2001). Elevated expression of HDACs often occurs in malignant tumors

and HDAC-dependent aberrant transcription is implicated as one of the main mechanisms for oncogenesis (Park *et al.*, 2000; Choi *et al.*, 2001). Thus, inhibition of HDAC function has been recognized as a potent strategy for cancer therapy (Richon and O'Brien, 2002; Kim *et al.*, 2003).

Well-known tumor-suppressor genes targeted by HDAC are p21^{WAF1}, p53, gelsolin, von Hippel Lindau (VHL), and semaphorin III. p21^{WAF1}, an inhibitor of cyclin-CDK, is required for the HDAC inhibitor-induced G₁ cell cycle arrest in a variety of tumor cells (Richon *et al.*, 2000). The fact that re-expression of p21^{WAF1} in tumor cells by an HDAC inhibitor is p53-independent provides an alternative way to overcome drug-resistant tumors in which p53 is mutated (Sambucetti *et al.*, 1999). Gelsolin is an actin-binding protein believed to be involved in HDAC inhibitor-induced morphological changes in tumor cells (Hoshikawa *et al.*, 1994). p53, VHL, and semaphorin III were identified as critical target genes of HDAC-mediated tumor angiogenesis (Kim *et al.*, 2001; Deroanne *et al.*, 2002). In addition to these tumor-suppressor genes, about 2% of human genes are transcriptionally regulated by HDACs (Van Lint *et al.*, 1996). Therefore, identification of new target genes of HDAC has provided significant clues for the functional role of HDAC in cellular phenotypes, and highlighted the clinical significance of HDAC inhibitors in cancer therapy.

Plakoglobin (also known as γ -catenin) is a common plaque component present in both types of cell–cell junctions, adherens junctions, and desmosomes (Karnovsky and Klymkowsky, 1995). Plakoglobin is highly homologous to the *Drosophila* armadillo protein and to *Xenopus* β -catenin, and belongs to the armadillo family (Peifer and Wieschaus, 1990; Peifer *et al.*, 1994). The armadillo family of proteins has been shown to play a central role in *Wnt* signaling pathways in which the Tcf/Lef transcription factor is activated (Behrens *et al.*, 1996; Brunner *et al.*, 1997). β -Catenin can form a complex with Tcf/Lef via its arm repeats, and in turn activates several oncogenic target genes, such as c-Myc and cyclin D1 (He *et al.*, 1998; Tetsu and McCormick, 1999). Plakoglobin also consists of 12–13 arm repeats that serve as binding sites for various cellular proteins, including Tcf/Lef transcription factor, though its binding pattern is distinct from that of β -catenin (Simcha

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et al., 1998). Thus, plakoglobin can activate Tcf/Lef target genes and promote neoplastic transformation of immortalized epithelial cells (Kolligs *et al.*, 2000). In contrast, several reports have argued that plakoglobin has tumor-suppressive effects, that is, the levels of plakoglobin are often reduced in cancer cells (Pantel *et al.*, 1998), and overexpression of plakoglobin was shown to suppress tumorigenicity (Simcha *et al.*, 1996; Winn *et al.*, 2002). It is now acceptable that plakoglobin has both-sided functions, that is, oncogenic and tumor suppressive, which is dependent on both the cellular context and its capability to activate the downstream signaling pathway. Therefore, the expressional change of plakoglobin in certain cells can be an important factor which governs cell fates including transformation and differentiation.

In this study, we performed a large-scale analysis of gene-expression changes in human fibrosarcoma HT1080 cells after the inhibition of HDAC activity, and identified plakoglobin as one of the target genes repressed in the cells by HDAC. In contrast to β -catenin, the expression of plakoglobin was transcriptionally regulated by HDAC and the inhibition of HDAC activity resulted in overexpression of plakoglobin in HT1080 cells. Although the biological significance of the upregulation of plakoglobin after HDAC inhibition has not been fully understood, overexpression of plakoglobin appeared to exert oncogenic properties in the fibrosarcoma cells.

Results

DNA microarray analysis of gene-expression changes in HT1080 cells in response to an HDAC inhibitor

To identify genes regulated by HDAC, we performed cDNA microarray analysis of transcriptional changes in HT1080 cells treated with trichostatin A (TSA), a well-known specific inhibitor of HDAC. A number of genes were up- or downregulated in response to TSA, as shown in the scatter plot of expression (Figure 1). Among the 1024 human cancer-focused genes arrayed, eight were augmented in their expression more than four-fold (Table 1). Since HDAC is a transcriptional repressor, direct target genes of HDAC may be upregulated by its specific inhibitors. Among the upregulated genes, junction plakoglobin showed the highest increase of gene-expression level after the inhibition of HDAC in HT1080 cells, and was selected as a candidate for further study.

Plakoglobin, but not β -catenin, is upregulated by TSA and 5-aza-2'-deoxycytidine treatment

We next conducted reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to validate the results from the cDNA microarray analysis. Figure 2a shows a time-dependent increase of plakoglobin mRNA by TSA treatment. The increase of plakoglobin by TSA occurred over the first 12 h, with a decrease observed at 24 h. In

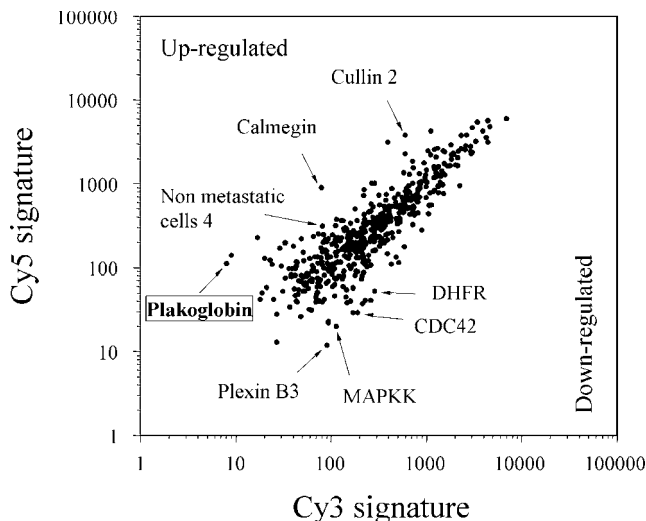


Figure 1 Gene-expression profile of HT1080 cells analysed with cDNA microarray. The scatter plot of expression in TSA-treated HT1080 cells (Cy5) versus that in control ones (Cy3) is shown. Cells were treated for 12 h with TSA prior to analysis. Representative genes, that are up- or downregulated, are presented. DHFR, dihydrofolate reductase; CDC42, cell division cycle 42; MAPKK, mitogen-activated protein kinase kinase

Table 1 Identified genes showing expressional changes greater than fourfold by TSA

	Gene ID	Log ₂ of Cy5/Cy3 ratio	Description
Downregulated genes	R46653	-2.91	Plexin B3
	AA668681	-2.71	Cell division cycle 42
	H07920	-2.48	Mitogen-activated protein kinase kinase
	R00884	-2.43	Dihydrofolate reductase
Upregulated genes	AA035637	3.79	Junction plakoglobin
	AA778675	3.51	Calmegin
	AA454094	2.67	Cullin 2
	H54417	1.95	Nonmetastatic cells 4

contrast, the expression of β -catenin, a closely related protein to plakoglobin, was not increased by TSA. Previously, Potter *et al.* (2001) showed that the promoter region of plakoglobin contains several CpG islands and treatment with 5-aza-2'-deoxycytidine (5Aza-dC), an upstream inhibitor of methyl-CpG-binding protein (MeCP2), causes the re-expression of plakoglobin in human thyroid carcinoma cells. Thus, we investigated the effect of 5Aza-dC on the expression of plakoglobin in HT1080 cells. 5Aza-dC weakly, but reproducibly, increased plakoglobin expression in HT1080 cells (Figure 2b). The expression of β -catenin was not changed by 5Aza-dC treatment. Treatment with both TSA and 5Aza-dC synergistically increased the expression of plakoglobin in the cells (Figure 2c). These data suggest that plakoglobin is transcriptionally

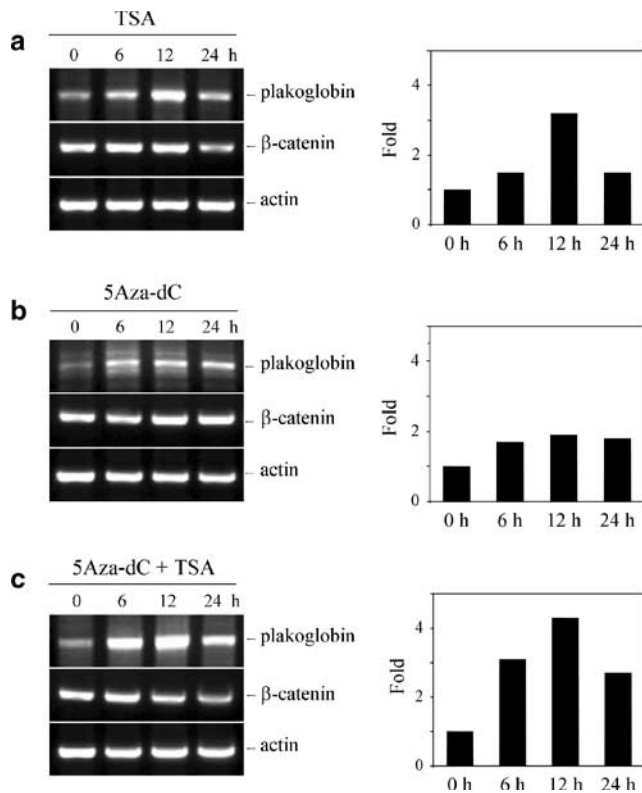


Figure 2 RT-PCR analysis of mRNA expression in HT1080 cells. Time-course analysis of mRNA expression in HT1080 cells after treatment with TSA (a), 5Aza-dC (b), and TSA + 5Aza-dC (c) is shown. Bar graphs in the right panel of each figure represent plakoglobin content after normalization by actin mRNA content. The fold increases of plakoglobin mRNA relative to that obtained in 0 h are shown. Data are representative results from at least three independent experiments

governed by HDAC, and that epigenetic alteration, that is, CpG methylation in the promoter of plakoglobin, may be present in HT1080 cells.

TSA increases the protein level of plakoglobin

We next examined changes in the protein level of plakoglobin and other related genes in response to an HDAC inhibitor. Western blot analysis showed that TSA causes a rapid induction of histone acetylation, with a decrease observed at 12 h (Figure 3a). A CDK inhibitor p21^{WAF1} was also induced by TSA in an early time period, with the highest increase at 6 h. Interestingly, the protein level of plakoglobin increased gradually for a relatively long time and showed the highest increase at 12 h after treatment with TSA. β -Catenin did not show any change in protein level in response to TSA. These data are in consistent with those in RT-PCR experiments, and show that the expressional change of plakoglobin by HDAC is different from that of p21^{WAF1} with respect to time and duration. To investigate the universality of plakoglobin induction by HDAC inhibitor in cells having different tissue origins, three cancer cell lines, including Hs578T (breast carcinoma), HT29 (colorectal carcinoma), and AGS

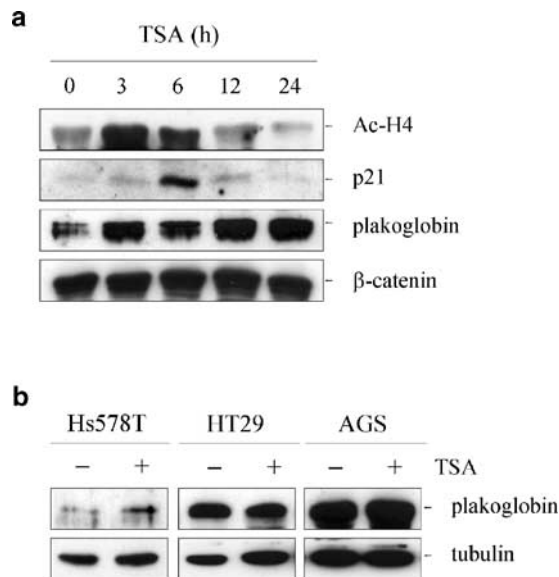


Figure 3 Western blot analysis of plakoglobin expression in HT1080 cells. (a) Time course analysis of each protein expression in HT1080 cells after treatment with TSA is shown. (b) Expression of plakoglobin in three different cell lines is shown. Cells were treated with TSA for 12 h, and the experiment was performed as described in 'Materials and methods'. Data are representative results from at least three independent experiments

(gastric cancer), were examined. Hs578T cells showed an increased expression of plakoglobin after treatment with TSA (Figure 3b). However, HT29 and AGS cells did not show any increase in plakoglobin expression in response to TSA. These results suggest that the induction of plakoglobin expression after the HDAC inhibition is not universal among the cell lines.

The increase of plakoglobin protein by HDAC inhibitors was further confirmed by immunofluorescence microscope analysis in HT1080 cells. The immunostaining was performed with monoclonal antibodies against plakoglobin or β -catenin, and subsequently with FITC-conjugated anti-mouse IgG. In control cells, plakoglobin mainly localized at the cellular junctions with weak staining observed (Figure 4a). TSA treatment highly increased the plakoglobin levels in the cells, as shown in Figure 4b. In addition, the localization of plakoglobin changed from the cell junctions to the nuclei of the cells with TSA treatment. In contrast, the strong fluorescence intensity was observed in β -catenin-stained HT1080 cells (Figure 4c) and TSA did not affect the fluorescence level of β -catenin in the cells (Figure 4d). These data are in consistent with RT-PCR and Western blot experiments, and demonstrate that the inhibition of HDAC activity increases the transcript and the protein levels of plakoglobin in HT1080 cells.

Chromatin modification by HDAC occurs at the distal promoter region of plakoglobin

HDAC-mediated local chromatin modification can occur after recruiting HDAC to a specific promoter target site. To investigate the site of chromatin modifica-

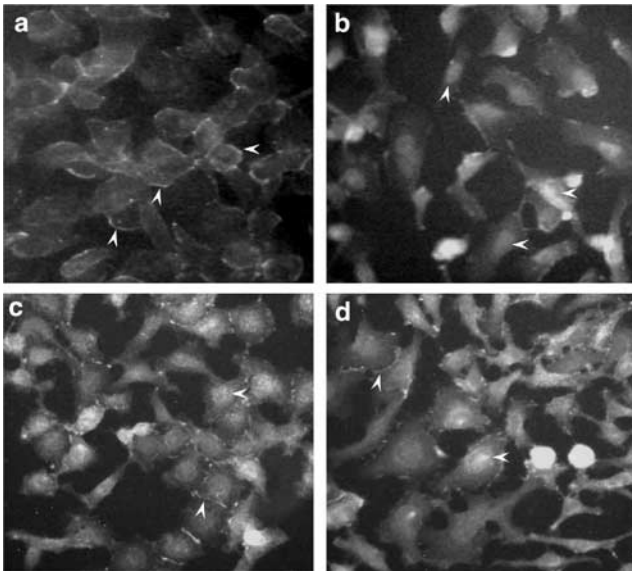


Figure 4 Immunofluorescence microscope analyses of plakoglobin and β -catenin in HT1080 cells. Control (a) and TSA-treated (b) cells were labeled with plakoglobin. Control (c) and TSA-treated (d) cells labeled with β -catenin are shown. Arrowheads indicate the plakoglobin or β -catenin fluorescence in the cells. Representative immunofluorescence data of at least three independent experiments are shown

tion, we used the chromatin immunoprecipitation (ChIP) assay using antiacetyl-histone (H4) antibody. Inhibition of HDAC activity causes hyperacetylation of local histones, and immunoprecipitation of these acetylated histones co-precipitates DNA sequences bound to the acetylated histones. The co-precipitated DNA sequences can be analysed by PCR using specific primer pairs corresponding to the specific promoter region. We generated three sets of primers (P1, P2, and P3) based on the sequence of the plakoglobin promoter, as shown in Figure 5a. HT1080 cell lysates were prepared as described in 'Materials and methods'. The lysates were immunocaptured by antiacetyl-H4 antibody and subsequently precipitated using protein A-agarose beads. As a negative control, the lysates were precipitated by protein A-agarose beads without antiacetyl-H4 antibody. The precipitates were used as templates for PCR analysis. In the ChIP assay, control cells did not show any amplified DNA sequences with all the three primer sets used. However, TSA treatment caused the precipitation of specific DNA sequences that can be amplified by P1 and P2 (Figure 5b). P3, a primer pair that corresponds to the proximal region ($-341 \sim +14$) of the plakoglobin promoter, could not amplify the precipitated DNA in both control and TSA-treated HT1080 cell lysates (Figure 5b). These data indicate that the promoter regions recognized by both P1 and P2 primer sets are hyperacetylated by TSA. We further examined whether the same amount of input DNA was used in the ChIP assay. Among the primer sets, P2, which generated the most specific product of amplified DNA (Figure 5b), and actin primers were used to determine the amount of input DNA used in the assay. P2 and actin DNA were

amplified in both control and TSA-treated cell lysates when PCR was performed before immunoprecipitation (Pre-IP) by antiacetyl-H4 antibody (Figure 5c). The extent of the DNA amplification was relatively the same between control and TSA-treated samples. In contrast, P2 DNA was amplified only in TSA-treated cell lysates, but not in control cell lysates, when PCR was performed after immunoprecipitation (Post-IP). This result is in consistent with the data shown in Figure 5b.

To evaluate the relationship between HDAC inhibitor-induced increase in acetylation of the distal region (P2) of the promoter of plakoglobin gene and its expression, we conducted a time-course experiment of ChIP analysis. As shown in *Pre-IP panel*, the amount of input P2 DNA was relatively same in all time conditions (Figure 5d). Interestingly, TSA increased the amount of amplified P2 DNA in a time-dependent manner (Post-IP panel in Figure 5d). The time course of the accumulation of histone acetylation in the plakoglobin promoter is exactly correlated with the increased expression of plakoglobin gene, which showed the highest increase at 12 h. These data demonstrate that local chromatin modification by HDAC takes place in the plakoglobin promoter, and suggest that the distal region of the promoter ($-945 \sim -646$) is responsible for the HDAC-induced transcriptional repression of the plakoglobin gene.

Inhibition of HDAC activity induces activation of the Tcf/Lef promoter

We next investigated the biological significance of the overexpression of plakoglobin by HDAC inhibitors in HT1080 cells. As described above, plakoglobin can bind to the Tcf/Lef transcription factor and activate its target genes in some types of tumor cells. To investigate the effect of HDAC inhibitors on the activity of Tcf/Lef target genes, two luciferase reporter constructs that contain either three wild-type TCF-binding sites (TOPFLASH) or three mutant TCF-binding sites (FOPFLASH) were used. HT1080 cells were transfected with either TOPFLASH or FOPFLASH. β -Gal reporter construct was cotransfected with each luciferase gene to control the transfection efficiency. After 24 h, cells were treated with various concentrations of TSA for 24 h and the luciferase assay was performed. TOPFLASH exhibited twofold higher luciferase activity compared to FOPFLASH (Figure 6a). The treatment with TSA dose-dependently increased the luciferase activity in the TOPFLASH-transfected cells, but not in the FOPFLASH-transfected cells. The TOPFLASH reporter activity peaked at 200 ng/ml of TSA. A high concentration of TSA (400 ng/ml) did not further increase the reporter activity. Other HDAC inhibitors also activated TOPFLASH reporter activity, though their potencies were different (Figure 6b). These data indicate that inhibition of HDAC activity activates Tcf/Lef target promoters.

It is not clear whether overexpression of plakoglobin by HDAC inhibitor is responsible for the activation of Tcf/Lef target promoter. Since TCF transcription factor is known to interact with Groucho, which can recruit

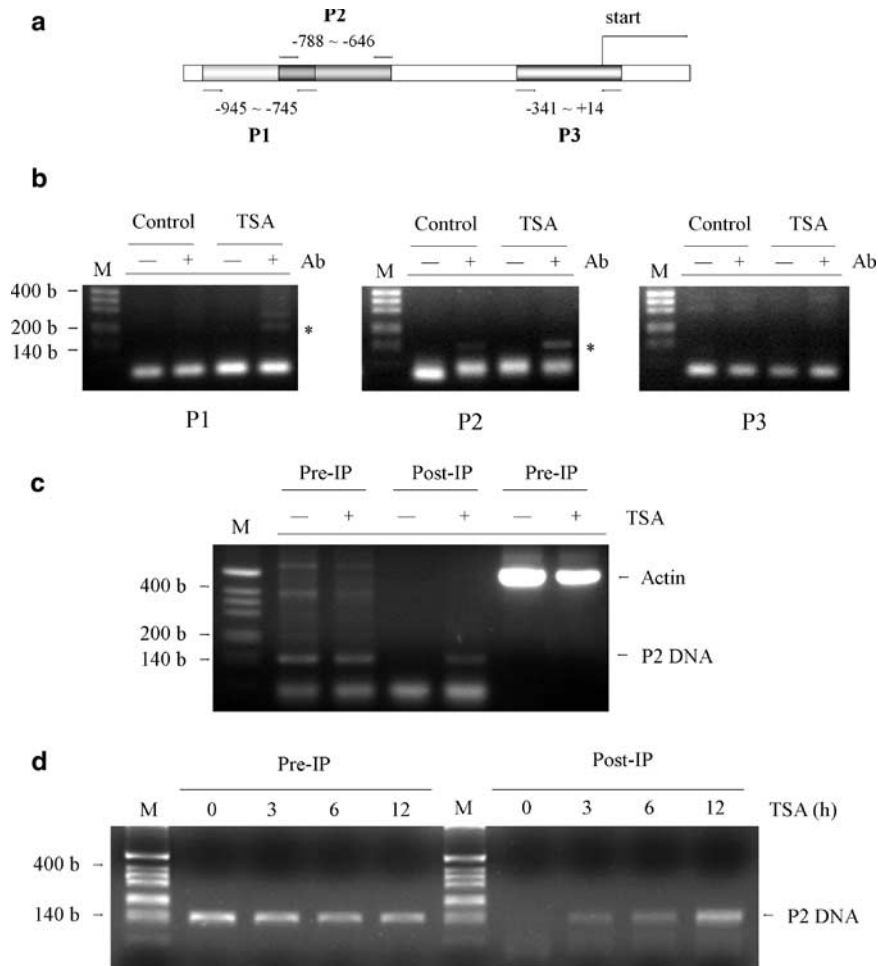


Figure 5 ChIP analysis of the plakoglobin promoter in HT1080 cells. **(a)** Graphical representation of the plakoglobin promoter and primer sets (P1, P2, and P3) used in ChIP analysis. The numbers indicate promoter regions that can be amplified by each primer set. Start represents the transcriptional start site in the plakoglobin gene. **(b)** ChIP analysis of the plakoglobin promoter in control and TSA-treated HT1080 cells using the indicated primer sets. Ab (+) indicates that antiacetyl-H4 antibody was used in the immunoprecipitation, while Ab (-) represents no antibody control. M denotes DNA size marker. Asterisks indicate specific DNA fragments amplified by specific primer sets. **(c)** ChIP analysis of plakoglobin promoter from the chromatin solution prepared prior to immunoprecipitation (Pre-IP) or after immunoprecipitation (Post-IP) by antiacetyl-H4 antibody. P2 and actin primer sets were used in this experiment. P2 DNA represents specific DNA fragments amplified by P2 primer sets. **(d)** Time-course experiments of ChIP analysis after treated with TSA. Data are representative results from at least three independent experiments

HDAC to repress the transcription of target genes (Cavallo *et al.*, 1998), it cannot be excluded that the activation of Tcf/Lef target promoter by TSA may result from the direct inhibition of HDAC activity in the promoter. Thus, we examined the effect of plakoglobin alone on the Tcf/Lef target promoter activity in HT1080 cells. HT1080 cells were cotransfected with a mammalian expressing vector pcDNA3 containing either plakoglobin, or β -catenin full-length cDNA, and TOPFLASH reporter plasmid. Transient transfection of plakoglobin in HT1080 cells highly activated Tcf/Lef target promoter, as shown in Figure 6c. β -catenin as well activated the reporter activity. The inhibition of HDAC activity additively increased both plakoglobin- and β -catenin-induced activation of Tcf/Lef target promoter. These data demonstrate that plakoglobin alone can activate the Tcf/Lef target promoter in HT1080 cells,

and suggest that the inhibition of HDAC activity results in the activation of the Tcf/Lef target promoter, at least in part, via the overexpression of plakoglobin.

Discussion

The present study demonstrates that plakoglobin is upregulated upon inhibition of HDAC activity in HT1080 cells. Through the use of cancer-focused DNA microarray analysis, plakoglobin was identified as one of the target genes of HDAC. Other genes listed in Table 1 are also of interest as possible target genes of HDAC, and experiments are currently ongoing to validate their transcriptional regulation by HDAC. Upregulation of plakoglobin by TSA was further confirmed by mRNA, protein, and cell analyses. Other

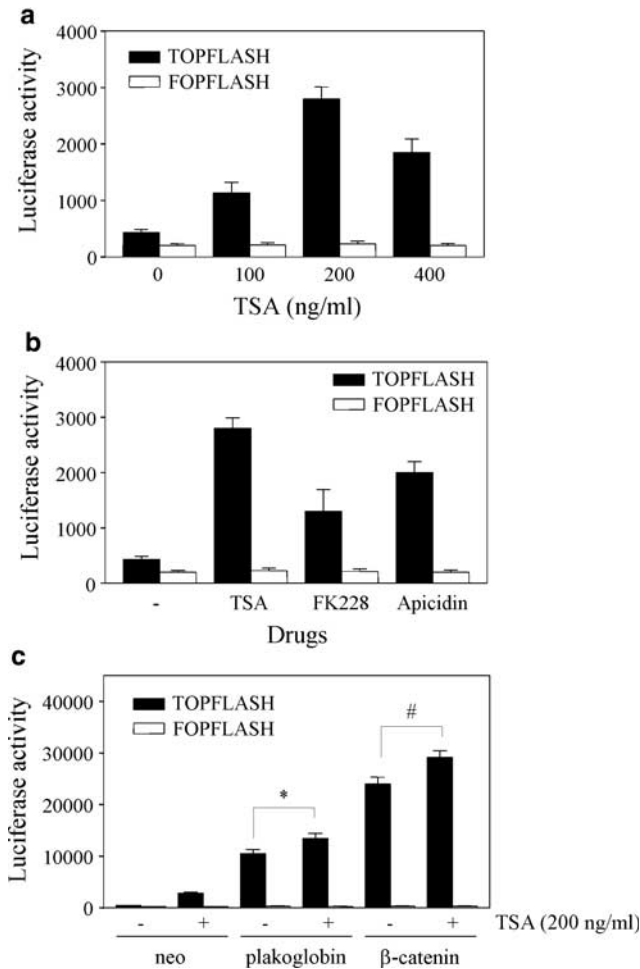


Figure 6 Tcf/Lef reporter assay in HT1080 cells. (a) HT1080 cells transfected either with pFOPFLASH or pTOPFLASH were treated with TSA at the indicated concentrations, and the luciferase assay was performed, as described in 'Materials and methods'. (b) Effects of various HDAC inhibitors on TOP/FOPFLASH reporter activities. (c) Effects of control vector (neo), plakoglobin, and β -catenin on TOP/FOPFLASH reporter activities. TSA was incubated with HT1080 cells 24h after the transfection. Statistical significances of * $P < 0.003$ and # $P < 0.0046$ were determined using Student's *t*-test. All data represent mean \pm s.e. from three independent experiments

HDAC-specific inhibitors, including FK228 and apicidin, also upregulated the plakoglobin expression level (data not shown). The inhibition of MeCP2 increased plakoglobin levels synergistically with an HDAC inhibitor, suggesting that an epigenetic alteration may be present in the promoter of plakoglobin in HT1080 cells (Potter *et al.*, 2001). However, the expression of β -catenin, a structural and functional homolog of plakoglobin, was not increased by an HDAC inhibitor, implying that there are distinct transcriptional regulations between two closely related proteins. Interestingly, the immunofluorescence analysis showed that TSA not only increases the protein level of plakoglobin but also induces nuclear localization of the protein. The nuclear localization of plakoglobin suggests its role in transcriptional regulation similar to β -catenin in HT1080 cells.

We also demonstrated that upregulation of plakoglobin by an HDAC inhibitor is through direct modification of the local chromatin structure of the plakoglobin promoter region. ChIP analysis suggests that the distal region (-945 ~ -646) of the plakoglobin promoter may have the target site for HDAC-mediated transcriptional repression. Consistent with this observation, Potter *et al.* (2001) have predicted the presence of a silencing element in the first 360 nucleotides (-965 ~ -594) in the plakoglobin promoter. However, the exact target element of HDAC binding and the recruiting factor for the HDAC-corepressor complex remain to be identified. Examination of the sequence of the plakoglobin distal promoter on the web (<http://www.gene-regulation.com>) revealed that several potential binding factors capable of interacting with HDAC are present. They include retinoic acid receptors (Lin *et al.*, 1998), Sp1 (Sowa *et al.*, 1997), and HNF-3 (Ogden *et al.*, 2001). Identification of the specific factors regulating plakoglobin repression will provide clues for elucidating the molecular mechanisms controlling its expression in certain tumors.

We finally investigated the functional role of plakoglobin in HT1080 cells. As described earlier, plakoglobin has two opposing functions, that is, oncogenic and tumor suppressive. The oncogenic function of plakoglobin is dependent on its capability to activate the Tcf/Lef transcription factor (Kolligs *et al.*, 2000). In contrast, the tumor-suppressive function of plakoglobin is dependent on its ability to inhibit β -catenin function followed by Tcf/Lef transcription factor activation (Winn *et al.*, 2002). Thus, plakoglobin can differently function according to the cell expressing the protein and expression level. Our present data suggest that, like β -catenin, plakoglobin may act as an oncogene in HT1080 cells, because it can activate the Tcf/Lef transcription factor. Moreover, TSA and other HDAC inhibitors highly increased the Tcf/Lef reporter activity, suggesting that the inhibition of HDAC activity results in activation of the Tcf/Lef target promoter, at least in part, via upregulation of plakoglobin expression. Although, overexpression of plakoglobin activates the Tcf/Lef transcription factor in HT1080 cells, inhibition of HDAC activity eventually suppresses the proliferation and the cell cycle progression of the cells (data not shown). Further studies on the role of plakoglobin overexpression and Tcf/Lef activation in HDAC inhibitor-induced cellular differentiation will be necessary to elucidate the biological significance of plakoglobin expression in HDAC-targeted cancer therapy.

Materials and methods

RNA and probe preparation

HT1080 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator adjusted with 5% CO₂. The cells were treated with or without TSA (200 ng/ml) for 12 h, and total cellular RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA, USA). Cy3 (control)- or Cy5 (drug-treated)-dUTP (Perkin-

Elmer Life Sciences, Inc., Boston, MA, USA) was incorporated into cDNA during reverse transcription by Superscript II (Life Technologies, Grand Island, NY, USA), and the cDNA was purified using PCR purification kit (Qiagen).

cDNA microarray and data analysis

Human cancer-focused 1024 genes-arrayed DNA chips (GenomicTree, Inc., Taejon, Korea) were used for cDNA microarray analysis. Microarray hybridization was performed as described previously (Kim *et al.*, 2002). A fluorescent image of the microarray was obtained using a GenePix 4000B scanner (Axon Instruments, Foster, CA, USA). Data obtained from the scanner were further analysed using GeneSight data analysis software, version 3.1 (BioDiscovery, Inc., Los Angeles, CA, USA).

RT-PCR and Western blot analysis

HT1080 cells were treated with either TSA (200 ng/ml) or 5Aza-dC (10 μ M), and total RNA was isolated by the RNeasy mini kit (Qiagen). The synthesis of cDNA and a standard PCR were performed as described previously (Kim *et al.*, 2002). Primer pairs used for RT-PCR are as the following: 5'-ATGGAGGTGATGAACCTG-3' and 5'-GATGGCATA-GAACAGGAC-3' for plakoglobin; 5'-ATGGCTACT-CAAGCTGATTT-3' and 5'-ACGAGCTGTTTCTACATC-AT-3' for β -catenin. For Western blot analysis, cells were seeded in 100 mm dishes, and TSA (200 ng/ml) was treated for the indicated time points. The cell lysates were separated by 10% SDS-PAGE, followed by transfer to PVDF membranes (Milipore, Bedford, MA, USA) using standard electroblotting procedures. Blots were then blocked and immunolabeled overnight at 4°C with the indicated antibodies (antiacetyl-H4 (Upstate Biotechnology, Lake Placid, NY, USA), anti-p21^{WAF1} (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-plakoglobin (Transduction Laboratories, Lexington, KY, USA), anti- β -catenin (Transduction Laboratories), and anti-tubulin (Upstate Biotechnology) antibodies). Immunolabeling was detected by enhanced chemiluminescence (ECL) kit (Amersham Life Science, Inc., Buckinghamshire, UK), according to the manufacturer's instructions.

Immunofluorescence microscopy

Cells were grown on glass coverslips in 24-well plates and treated with TSA (200 ng/ml) for 12 h. After washing with PBS, cells were fixed and permeabilized with a fixative solution containing 3.7% formaldehyde and 0.2% Triton X-100 in PBS at 37°C for 30 min. The cells were rinsed with PBS and incubated with blocking buffer containing 1% BSA in PBS at 37°C for 30 min. Either antiplakoglobin or anti- β -catenin antibody diluted in blocking buffer was applied to the coverslips, and the incubation was continued for 1 h at room temperature. After washing with PBS three times, the cells were incubated with FITC-conjugated anti-mouse IgG for 1 h at room temperature. The coverslips were washed and mounted, and the immunolabeled cells were observed under an IX70 fluorescence microscope (Olympus America, Inc., Melville, NY, USA) at an $\times 400$ magnification.

ChIP analysis

Cells grown in 100 mm dishes were treated with or without TSA (200 ng/ml) for the indicated time points. Before harvesting, the cells were treated with 1% formaldehyde for 10 min at 37°C to crosslink histones to DNA. The cells were scraped with ice-cold PBS containing protease inhibitors (1 mM

phenylmethylsulfonyl fluoride and a tablet of protease inhibitor cocktail) and centrifuged for 4 min at 700 g at 4°C. ChIP assay was performed using ChIP Assay Kit (Upstate Biotechnology), according to the manufacturer's instructions. Briefly, the chromatin solutions were sonicated to reduce the DNA length to between 200 and 1000 basepairs, and precleared with a salmon sperm DNA/protein A agarose slurry. The chromatin solutions were immunocaptured using antiacetyl-H4 antibody overnight at 4°C with gentle rotation. Aliquots (1 ml) of each chromatin solution were saved for no-antibody controls. Immune complexes were then collected with a salmon sperm DNA/protein A agarose slurry, and washed with wash buffer supplied from the kit. The immune complexes were eluted with elution buffer containing 1% SDS and 0.1 M NaHCO₃, and the elution was repeated twice. Combined eluates were treated with 5 M NaCl, and the crosslinks between DNA and histones were reversed at 65°C for 4 h. DNA from each eluate was recovered by phenol/chloroform extraction and ethanol precipitation. Specific promoter sequences from no antibody control and immunoprecipitated samples were detected by PCR analysis using the following primer sets: 5'-AAATGAGAGGAAGGAGGT-3' and 5'-GGTTTGTGAA-AGCACTCA-3' for P1, 5'-AAATTTCTAACCTTTAGCAG-3' and 5'-TGATTTTAGGCAAGTTACTT-3' for P2, and 5'-CATCCCTTCCATTGGTTT-3' and 5'-AAGGCAGCAACT-CAGTAA-3' for P3.

Plasmids and reporter assays

Plasmid constructs for plakoglobin and β -catenin, each subcloned into the eukaryotic expression vector pcDNA3, and reporter constructs, including pTOPFLASH and pFOPFLASH, were kindly provided by Dr Eric R Fearon (The University of Michigan Health System, Ann Arbor, MI, USA). HT1080 cells were seeded in six-well plates 24 h prior to transfection. Transfection was performed using Superfect Transfection Reagent (Qiagen), according to the manufacturer's instructions. To assess Tcf/Lef reporter activity in HT1080 cells, 0.5 μ g of either pTOPFLASH or pFOPFLASH was cotransfected with 0.3 μ g of pCMV- β Gal into the cells. To determine the ability of plakoglobin and β -catenin to activate Tcf transcription, HT1080 cells were transfected with 1 μ g of the respective pcDNA3 expression construct and 0.5 μ g of either pTOPFLASH or pFOPFLASH reporter construct. After incubation for 24 h, the cells were treated with or without HDAC inhibitors (200 ng/ml of TSA, 20 ng/ml of FK228, and 1 μ M of apicidin) and the incubation was continued for 12 h. The cells were then collected and resuspended in Luciferase Reporter Lysis Buffer (Promega, Madison, WI, USA). The cell lysates were centrifuged and aliquots (70 μ l) of the supernates were assayed for luciferase activity with a FL600 Microplate Fluorescence Reader (Bio-Tek Instrument, Inc., Winooski, VT, USA). Aliquots (5 μ l) of the supernates were assayed for β -galactosidase activity to control the transfection efficiency. Experiments were performed three times independently.

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References

- Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R and Birchmeier W. (1996). *Nature*, **382**, 638–642.
- Brunner E, Peter O, Schweizer L and Basler K. (1997). *Nature*, **385**, 829–833.
- Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, Clevers H, Peifer M and Bejsovec A. (1998). *Nature*, **395**, 604–608.
- Choi JH, Kwon HJ, Yoon BI, Kim JH, Han SU, Joo HJ and Kim DY. (2001). *Jpn. J. Cancer Res.*, **92**, 1300–1304.
- Deroanne CF, Bonjean K, Servotte S, Devy L, Colige A, Clausse N, Blacher S, Verdin E, Foidart JM, Nusgens BV and Castronovo V. (2002). *Oncogene*, **21**, 427–436.
- Hassig CA, Fleischner TC, Billin AN, Schreiber SL and Ayer DE. (1997). *Cell*, **89**, 341–347.
- He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW. (1998). *Science*, **281**, 1509–1512.
- Hoshikawa Y, Kwon HJ, Yoshida M, Horinouchi S and Beppu T. (1994). *Exp. Cell Res.*, **214**, 189–197.
- Karnovsky A and Klymkowsky MW. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 4522–4526.
- Kim DH, Kim M and Kwon HJ. (2003). *J. Biochem. Mol. Biol.*, **36**, 110–119.
- Kim JH, Shim JS, Lee SK, Kim KW, Rha SY, Chung HC and Kwon HJ. (2002). *Jpn. J. Cancer Res.*, **93**, 1378–1385.
- Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW and Kim KW. (2001). *Nat. Med.*, **7**, 437–443.
- Kolligs FT, Kolligs B, Hajra KM, Hu G, Tani M, Cho KR and Fearon ER. (2000). *Genes Dev.*, **14**, 1319–1331.
- Lin RJ, Nagy L, Inoue S, Shao W, Miller Jr WH and Evans RM. (1998). *Nature*, **391**, 811–814.
- Ogden SK, Lee KC, Wernke-Dollries K, Stratton SA, Aronow B and Barton MC. (2001). *J. Biol. Chem.*, **276**, 42057–42062.
- Pantel K, Passlick B, Vogt J, Stosiek P, Angstwurm M, Seen-Hibler R, Haussinger K, Thetter O, Izbicki JR and Riethmuller G. (1998). *J. Clin. Oncol.*, **16**, 1407–1413.
- Park JS, Kim EJ, Kwon HJ, Hwang ES, Namkoong SE and Um SJ. (2000). *J. Biol. Chem.*, **275**, 6764–6769.
- Peifer M and Wieschaus E. (1990). *Cell*, **63**, 1167–1176.
- Peifer M, Berg S and Reynolds AB. (1994). *Cell*, **76**, 789–791.
- Potter E, Braun S, Lehmann U and Brabant G. (2001). *Eur. J. Endocrinol.*, **145**, 625–633.
- Richon VM and O'Brien JP. (2002). *Clin. Cancer Res.*, **8**, 662–664.
- Richon VM, Sandhoff TW, Rifkind RA and Marks PA. (2000). *Proc. Natl. Acad. Sci. USA*, **97**, 10014–10019.
- Sambucetti LC, Fischer DD, Zabrudoff S, Kwon PO, Chamberlin H, Trogani N, Xu H and Cohen D. (1999). *J. Biol. Chem.*, **274**, 34940–34947.
- Simcha I, Geiger B, Yehuda-Levenberg S, Salomon D and Ben-Ze'ev A. (1996). *J. Cell Biol.*, **133**, 199–209.
- Simcha I, Shtutman M, Salomon D, Zhurinsky J, Sadot E, Geiger B and Ben-Ze'ev A. (1998). *J. Cell Biol.*, **141**, 1433–1448.
- Sowa Y, Orita T, Minamikawa S, Nakano K, Mizuno T, Nomura H and Sakai T. (1997). *Biochem. Biophys. Res. Commun.*, **241**, 142–150.
- Taunton J, Hassig CA and Schreiber SL. (1996). *Science*, **272**, 408–411.
- Tetsu O and McCormick F. (1999). *Nature*, **398**, 422–426.
- Van Lint C, Emiliani S and Verdin E. (1996). *Gene Expr.*, **5**, 245–254.
- Winn RA, Bremnes RM, Bemis L, Franklin WA, Miller YE, Cool C and Heasley LE. (2002). *Oncogene*, **21**, 7497–7506.