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Suppressive Effect of the Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid (SAHA) on Hepatitis C Virus Replication

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ABSTRACT

The histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) has a clinical promise for treatment of cancer including hepatocellular carcinoma (HCC). To investigate effect of SAHA on hepatitis C virus (HCV) replication, we treated the HCV replicon cell OR6 with SAHA. HCV replication was significantly inhibited by SAHA at concentrations below 1 μM with no cellular toxicity. Another HDAC inhibitor, tricostatin A, also showed reduction of HCV replication. The microarray analysis and quantitative RT-PCR demonstrated up-regulation of *osteopontin* (*OPN*) and down-regulation of *apolipoprotein-A1* (*Apo-A1*) after SAHA treatment. Direct gene induction of *OPN* and knockdown of *Apo-A1* also showed reduction of HCV replication. The liver specific *microRNA-122*, which is involved in HCV replication, was not affected by SAHA treatment. These results suggest that SAHA has suppressive effect on HCV replication through alterations of gene expression such as *OPN* and *Apo-A1* in host cells. Epigenetic treatment with HDAC inhibitors may be a novel therapeutic approach for diseases associated with HCV infection such as chronic hepatitis, liver cirrhosis, and HCC. J. Cell. Biochem. 114: 1987–1996, 2013.

KEY WORDS: SUBEROYLANILIDE HYDROXAMIC ACID; HEPATITIS C VIRUS; OR6; *MIR-122*; *OSTEOPONTIN*; *APOLIPOPROTEIN-A1*

Hepatitis C virus (HCV) infection is a major worldwide health problem and approximately 170 million people are infected with the virus. Roughly 60–80% of who carry HCV progress to chronic infection, in which liver cirrhosis and hepatocellular carcinoma (HCC) are frequent complications [Lauer and Walker, 2001]. Treatment of HCV infection has been progressed with introduction of protease inhibitors. Although the combination therapy with pegylated interferon (PEG-IFN), ribavirin, and protease inhibitors has shown a higher sustained virological response (SVR) rate, the SVR rate of patients with genotype 1b and a high viral load has not been satisfactory [Ikeda et al., 2006; Ebinuma et al., 2012; Takayama et al., 2011]. Effective drugs without severe side effects are needed for treatment of chronic infection with HCV genotype 1b.

HCV itself cannot replicate or proliferate with its own protein and nucleic acids, and instead relies on various host factors [Tu et al., 1999; Gao et al., 2004; Hamamoto et al., 2005; Okamoto et al., 2006]. It is possible to attenuate viral replication by stimulating some of these host factors [Kaul et al., 2009; Chen et al., 2010; Vaillancourt et al., 2012]; one such possibility involves epigenetic alterations. Epigenetics is an acquired modification of methylation and/or acetylation of chromatin DNA or histone proteins, which regulates downstream gene expression. Epigenetic alterations can be induced by aging, chronic inflammation, or viral infection.

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Aberrations in DNA methylation and histone modification may induce inactivation of tumor suppressor genes and play critical roles in the initiation and progression of human cancer [Gal-Yam et al., 2008]. Moreover, a recent study has demonstrated that the liver-specific microRNA (miRNA) *miR-122* binds to the 5′ noncoding region of the HCV genome and facilitates HCV replication [Jopling et al., 2005; Sarasin-Filipowicz et al., 2009]. MiRNAs are small non-coding RNAs that function as endogenous silencers of various target genes and are expressed in a tissue-specific manner. MiRNAs play important roles in cell proliferation, apoptosis, and differentiation [Schickel et al., 2008]. We have recently reported that some miRNAs are regulated by epigenetic alterations such as DNA methylation and histone modification at their CpG island promoters [Saito et al., 2006].

Unlike genetic alterations, which are almost impossible to reverse, epigenetic aberrations are potentially reversible, allowing the malignant cell population to revert to a more normal state [Kurita et al., 2010]. Chromatin-modifying drugs such as DNA methylation inhibitors and histone deacetylase (HDAC) inhibitors have clinical promise for cancer therapy [Yoo and Jones, 2006]. The HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) is emerging as a promising agent for epigenetic therapy of human malignancies including HCC [Butler et al., 2002]. SAHA was recently approved in Japan for the treatment of cutaneous T-cell lymphoma [Watanabe et al., 2010].

In the present study, we investigated the effect of SAHA on HCV RNA replication in the replicon cell OR6, in which genotype 1b full-length HCV RNA replicates and HCV RNA replication can be monitored by luciferase reporter assay [Ikeda et al., 2005]. Here we show that SAHA has a suppressive effect on HCV replication through changes of gene expression in OR6 cells, suggesting that HDAC inhibitors could be promising drugs for diseases associated with chronic HCV infection.

MATERIALS AND METHODS

CELL CULTURE

OR6 cells were cultured in Dulbecco′s modified Eagle′s medium (DMEM; Gibco) with 4.5 g/L glucose (Gibco) with 10% heat inactivated fetal bovine serum (FBS; BioWest), 1% penicillin–streptomycin solution (Sigma–Aldrich Japan, Tokyo), and 50 mg/ml G-418 (Roche Diagnostics Co.) in 5% CO_2 in humidified air at 37°C as described previously [Nakamura et al., 2008].

SAHA AND TRICHOSTATIN A (TSA) TREATMENT

SAHA (Sigma–Aldrich) and TSA (Sigma–Aldrich) were dissolved in dimethylsulphoxide (DMSO, Sigma–Aldrich) and ethanol, respectively, and added to culture medium. As a control, DMSO or ethanol diluted with culture medium was used.

MTT ASSAY

Cell proliferation was also measured using the MTT Cell Proliferation Assay Kit (Cayman Chemical). OR6 cells were seeded at 1.0×10^4 cells/well in a 96-well plate and cultured for 24 h. SAHA (0.1, 0.5, 1.0, 1.5, 3.0, and 5 μM) was added and the cells were further cultured for 72 h. MTT reagent (10 μM) was added to each well and the plate was the shaken for 1 min and subsequently incubated at 37°C for 3 h. A sample from each well was mixed with 100 μl Crystal Dissolving solution and shaken for 30 min, and the absorption was measured at 570 nm.

CELL NUMBER COUNTING

OR6 cells were seeded at 1.0×10^5 cells/well in a 6-well plate, and 1.0 μM of SAHA was added after 24 h and the cells were then cultured for 72 h. Cells were collected after treating with trypsin (Sigma–Aldrich) and number of cells number was counted using a hemocytometer and the Scepter 2.0 Handheld Cell Counter (Millipore).

CELL VIABILITY ASSAY

The viability of OR6 cells treated with SAHA was analyzed using Vi-CELL XR cell viability counter (Beckman Coulter). Five hundred microliters of cell suspension were mixed with trypan blue, and then images were taken to determine cell concentration and viability.

LUCIFERASE ASSAY

OR6 cells were seeded at 4.0×10^4 cells/well in a 24-well plate for 24 h. SAHA (0.1, 0.5, 0.8, or 1.0 μM) was added and the cells were then cultured for 72 h. Luciferase activity was measured with Renilla Luciferase Assay System (Promega) using a luminometer (Lumat CB 9507, Berthold Technologies). One hundred microliters of Renilla Luciferase Assay Reagent and 20 μl of cell lysate were mixed in tube, and then luminescence was measured. Luciferase activity was normalized with cell number.

RNA EXTRACTION

Total RNA including small RNA was extracted from OR6 cells using the mirVana miRNA isolation kit (Ambion). The sample is first lysed in a denaturing lysis solution which stabilizes RNA and inactivates RNases. The lysate is then extracted once with Acid-Phenol: Chloroform which removes most of the other cellular components, leaving a semi-pure RNA sample. This is further purified over a glass-fiber filter to yield total RNA enriched in miRNAs.

QUANTITATIVE RT-PCR OF *miR-122*

Levels of miRNA expression were analyzed by quantitative RT-PCR using the TaqMan microRNA assay for *miR-122* (Applied Biosystems) according to the manufacturer′s instructions. Expression levels were normalized to that of U6 RNA.

MICROARRAY ANALYSIS

Microarray analysis was carried out using the Human Oligo chip 25K (ID QH0ZG35) by Toray (http://www.3d-gene.com/). In brief, total RNA was amplified using an Amino Allyl aRNA kit (Ambion). RNA from SAHA treated cells was labeled with Cy3 Mono-Reactive Dye (GE Healthcare), and control RNA was labeled with Cy5 Mono-Reactive Dye (GE Healthcare). After purification, each 1 μg sample was mixed and hybridized at 37°C for 16 h. After washing, the hybridized chip was scanned using a 3D-Gene Scanner 3000 (Toray). Background was subtracted from the raw data and the values were normalized according to a median Cy3/Cy5 ratio of 1.

QUANTITATIVE RT-PCR OF *OPN* AND *Apo-A1*

After incubation with DNase I (Promega) to eliminate DNA contamination, 1 mg of total RNA was applied for RT reaction with random primers using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Then, quantitative RT-PCR was performed with

SYBR Green PCR Master Mix (Applied Biosystems) using CFX96 real-time PCR system (Bio-Rad). The primer sequences for quantitative RT-PCR of *OPN* and *Apo-A1* were obtained from the previous reports [Hahnel et al., 2010; Haas et al., 2011]. *GAPDH* was used as an internal control.

OPN forward: 5′-TGGCCGAGGTGATAGTGTG-3′, *OPN* reverse: 5′-CGGGGATGGCCTTGTATG-3′, *Apo-A1* forward: 5′-AGCTTGCTGAAGGTGGAGGT-3′, *Apo-A1* reverse: 5′-ATCGAGTGAAGGACCTGGC-3′, *GAPDH* forward: 5′-CTCCTCCTGTTCGACAGTCAGC-3′, *GAPDH* reverse: 5′-CCCAATACGACCAAATCCGTTG-3′.

The PCR conditions were as follows; *OPN*: 95°C for 15 min, 40-cycles following reaction; 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. *Apo-A1*: 95°C 3 min, 40 cycles; 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min.

WESTERN BLOTTING OF OPN AND Apo-A1

Protein extracts were separated by SDS/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were hybridized with the mouse anti-OPN monoclonal antibody (sc-21742, Santa Cruz Biotechnology) and the goat anti-Apo-A1 polyclonal antibody (ab7613, Abcam). β-actin was used as the internal control.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP assay for acetylation of histone H3 around the promoter region of *OPN* was performed using acetyl-histone H3 ChIP assay kit (Millipore) according to the manufacturer's instructions. In brief, 1×10^7 of OR6 cells were cross-linked with 1% formaldehyde at 37°C for 10 min. Crude cell lysates were sonicated to generate DNA fragments of 200–1,000 bp. ChIP was performed with anti-acetyl-histone H3 antibody as well as control IgG. Quantitative PCR analysis was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a CFX96 real-time PCR system (Bio-Rad). The primer sequence for ChIP assay of *OPN* was designed using the UCSC Genome Browser (http://genome.ucsc.edu/) and the Primer3 (http://frodo.wi.mit.edu/).

OPN (ChIP) forward: 5′-TCATACAGGCAAGAGTGGTTGCAGA-3′, *OPN* (ChIP) reverse: 5′-GCTCCCACACTTCCCCCTCTGGTTT-3′.

The primer sequence for ChIP assay of *Apo-A1* was obtained from the previous report [Mishiro et al., 2009].

Apo-A1 (ChIP) forward: 5′-CAAGGCCTGAACCTTGAGC-3′, *Apo-A1* (ChIP) reverse: 5′-TTAGAGACTGCGAGAAGGAGGT-3′.

The PCR conditions were as follows; 95°C for 3 min, 40-cycles following reaction; 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The fraction of immunoprecipitated DNA was calculated as follows: [immunoprecipitated DNA (IP) with anti-acetyl-histone H3 antibody−nonspecific antibody control (NAC)]/(input DNA−NAC). Fold changes relative to control that was set to 1.0 were compared.

TRANSFECTION OF THE *OPN* EXPRESSION VECTOR AND THE *Apo-A1* siRNA

The expression vector containing the *OPN* open reading flame and the pre-designed siRNA for the *Apo-A1* gene (#s1467) were purchased from InvivoGen and Ambion, respectively. They were transfected into OR6 cells using lipofectamine (Invitrogen) in accordance with the manufacturer's instructions. Forty-eight hours after transfection, the cells were collected and luciferase activity was analyzed as described above. OR6 cells treated with lipofectamine only were used as controls.

STATISTICS

The data were expressed as mean and standard deviation (SD) for at least three independent experiments. The data were analyzed using the SPSS statistics software and differences with P values of less than 0.05 were considered significant.

RESULTS

TREATMENT OF OR6 CELLS WITH 1.0 MM OF SAHA DID NOT AFFECT CELL VIABILITY

To investigate the cellular toxicity of SAHA in OR6 cells, MTT assay was performed, and the results showed no significant difference in absorbance at concentrations less than 1.5 μM of SAHA, whereas the absorbance at 3 and 5 μM was significantly reduced ($P < 0.01$, Fig. 1A).

We next examined cell number and cell viability after treatment with SAHA using Scepter Handheld Cell Counter and Vi-CELL autoanalyzer, respectively. Cell viability did not differ after treatment with 1.0 μM of SAHA for 72 h as compared to control (Fig. 1B). We also confirmed that there was no significant difference in cell number among control cells and cells treated with 1.0 μM of SAHA and 0.1 μM of another HDAC inhibitor TSA for 24, 48, and 72 h (Fig. 1C). These findings indicate that treatment with SAHA at concentration of 1.0 μM for 72 h did not affect the viability of OR6 cells.

TREATMENT OF OR6 CELLS WITH SAHA AND TSA REDUCED HCV REPLICATION

We next evaluated HCV replication by measuring luciferase activity in OR6 cells. Luciferase activity of OR6 cells was measured after treatment with SAHA at concentrations of 0.1, 0.5, 0.8, or 1.0 μM for 72 h. As shown in Figure 2A, treatment of OR6 cells with SAHA (<1.0 μM) reduced luciferase activity in a dose-dependent manner. Treatment with SAHA at concentrations of 1.0 and 0.8 μM significantly reduced luciferase activity compared to control (Fig. 2A, 1.0 μM: $P < 0.01$, 0.8 μM: $P < 0.05$).

To investigate whether the effect of SAHA was agent-specific or a general consequence of HDAC inhibitors, we treated OR6 cells with another HDAC inhibitor TSA at concentrations of 0.1 and 0.5 μM and confirmed that TSA also suppresses HCV replication (Fig. 2B).

LACK OF INVOLVEMENT OF *miR-122* IN THE SUPPRESSIVE EFFECT OF SAHA ON HCV REPLICATION

The liver-specific miRNA *miR-122* has been reported to bind to the 5′ noncoding region of the HCV genome to facilitate replication of the viral RNA [Jopling et al., 2005]. To determine whether the suppressive effect of HDAC inhibitors on HCV replication is mediated through alterations in *miR-122* expression, we performed quantitative RT-PCR to measure *miR-122* expression in OR6 cells cultured with 1 μM SAHA or 0.1 μM TSA for 72 h. As shown in Figure 3, *miR-122* expression did not differ between control cells and cells treated with

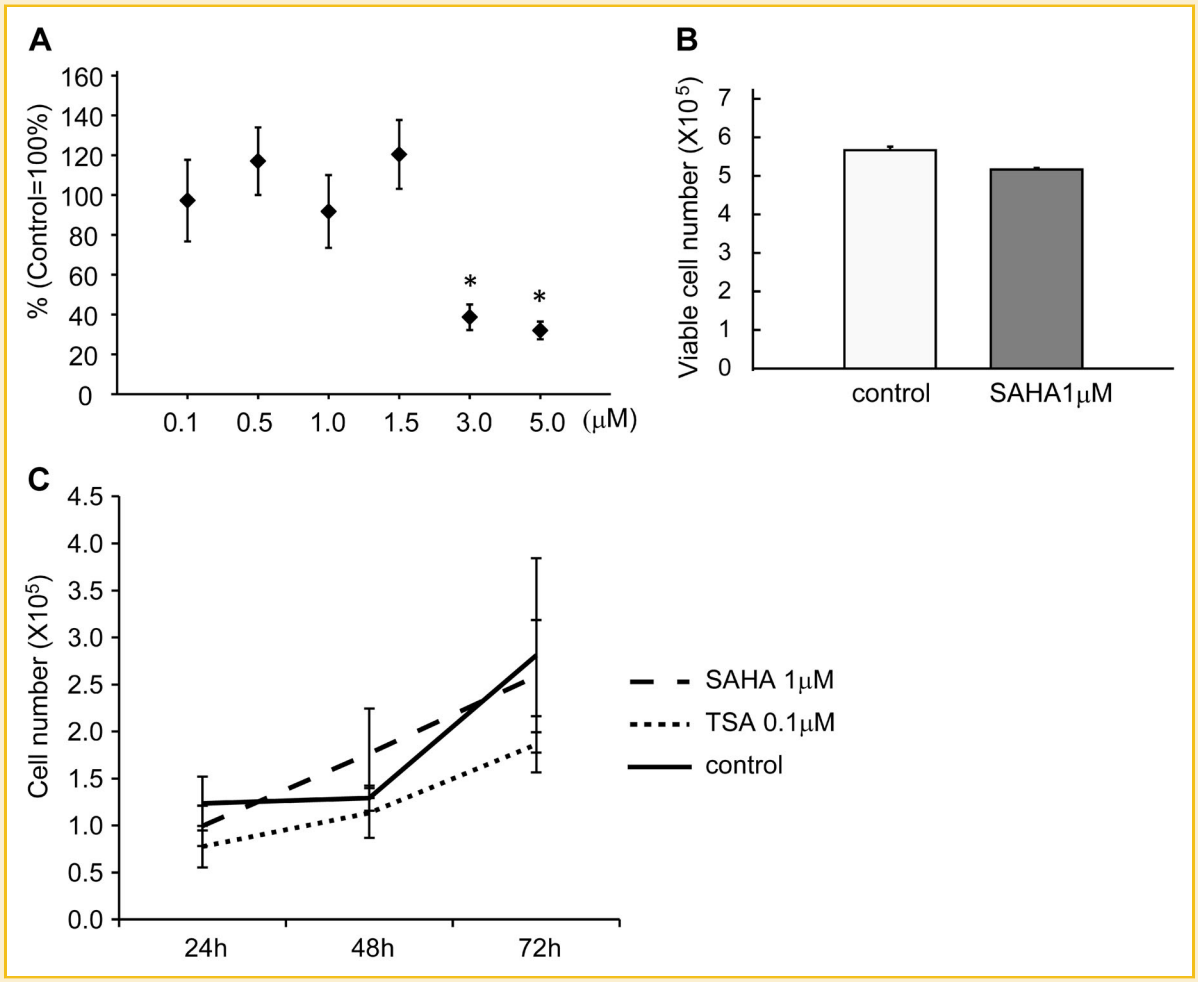

Fig. 1. A: MTT assay for cell viability of OR6 cells after SAHA treatment The viability of OR6 cells was measured by MTT assay. OR6 cells were treated with SAHA at concentrations of 0.1, 0.5, 1.0, 1.5, 3.0, or 5.0 μM for 72 h and MTT assay was then performed to determine the number of viable cells. Absorbance of cells treated with 3.0 and 5.0 μM of SAHA was significantly decreased compared with control. No toxic effect was observed at SAHA concentrations at or below 1 μM. Dots and bar indicate means and SDs for four experiments. $^{*}P < 0.01$. B: Cell viability of OR6 cells after SAHA treatment OR6 cells were treated with 1 μM SAHA for 72 h. Cell viability was tested using a Vi-CELL autoanalyzer. There was no significant difference in the number of viable cells between the control and 1.0 μM SAHA conditions. Means and SDs of viable cell numbers for three experiments are shown. C: Cell number of OR6 cells treated with 1.0 μM SAHA and 0.1 μM TSA The cell number in OR6 cells treated with 1.0 μM SAHA and 0.1 μM TSA for 24, 48 and 72 h did not differ significantly versus control cells. Cell number was measured by the Scepter Handheld Cell Counter and means and SDs for five experiments are shown.

SAHA or TSA, indicating that *miR-122* is not involved in the suppressive effect of HDAC inhibitors on HCV replication.

ALTERATION OF *OSTEOPONTIN* (*OPN*) AND *APOLIPOPROTEIN-A1* (*Apo-A1*) EXPRESSION LEVELS AFTER TREATMENT OF OR6 CELLS WITH SAHA

To determine comprehensive gene expression changes induced by SAHA treatment, microarray analysis was applied in SAHA-treated OR6 cells (Fig. 4A). We selected genes with expression levels of more than 100 and more than a fourfold difference relative to control. The genes that were up-regulated and down-regulated in SAHA-treated OR6 cells are summarized in Tables I and II. Among genes whose expression was largely changed, we selected genes intimately related to HCV replication from Tables I and II by means of document retrieval. From the literature search, *OPN* was picked-up from the up-regulated genes since *OPN* is an important cytokine for host resistance to viral infection through the initiation of the Th1 immune response [Patarca et al., 1993; Ashkar et al., 2000]. *Apo-A1* was selected from among the down-regulated genes since *Apo-A1* is closely involved in the HCV life cycle [Mancone et al., 2011].

Quantitative RT-PCR shown in Figure 4B and C demonstrated that expression of *OPN* was significantly up-regulated (8.6-fold, $P < 0.001$), and expression of *Apo-A1* was significantly down-regulated (0.39-fold, $P < 0.001$) by treatment with 1 μM SAHA for 72 h. We confirmed the increased protein expression of OPN and the decreased protein expression of Apo-A1 after treatment with 1 μM SAHA by Western blotting (Fig. 4B,C). To investigate whether the changes in expression of these two genes affect HCV RNA replication in OR6 cells, we overexpressed the *OPN* gene using the expression vector containing the *OPN* open reading frame and knocked down the *Apo-A1* gene using the specific siRNA in OR6 cells and examined

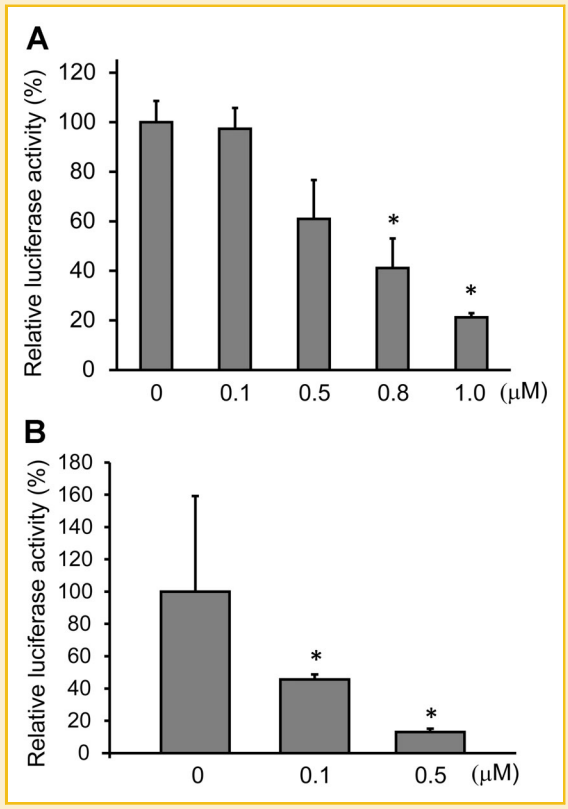

Fig. 2. A: HCV replication in OR6 cells after SAHA treatment HCV replication was evaluated by luciferase activity in OR6 cells treated with SAHA. OR6 cells were treated with 0.1, 0.5, 0.8, or 1.0 μM SAHA for 72 h and luciferase activity was measured. Means and SDs of luciferase activities for three experiments are shown. Luciferase activity in SAHA-treated OR6 cells was dose-dependently reduced, and the reduction at concentrations of 0.8 and 1.0 μM were statistically significant. $^*P < 0.05$. B: HCV replication in OR6 cells after TSA treatment HCV replication was evaluated by luciferase activity in OR6 cells treated with TSA. Means and SDs of luciferase activities for three experiments are shown. OR6 cells were treated with 0.1 and 0.5 μM TSA, and the luciferase activity in TSA-treated OR6 cells was significantly reduced. $^*P < 0.05$.

changes in luciferase activity. As shown in Figure 5A,B, we confirmed overexpression of OPN and knockdown of Apo-A1 after transfection of *OPN*-expressing vector and siRNA for *Apo-A1*, respectively. After transfection of *OPN*-expressing vector, luciferase activity was significantly reduced to around 40% of control ($P < 0.01$), and knockdown of *Apo-A1* by siRNA resulted in decrease of luciferase activity to around 60% of control ($P < 0.05$).

INCREASED HISTONE H3 ACETYLATION AT THE PROMOTER REGIONS OF *OPN* AND *Apo-A1* AFTER TREATMENT OF OR6 CELLS WITH SAHA

Since histone acetylation generally activates gene expression, we further examined histone acetylation levels around the promoter regions of *OPN* and *Apo-A1* by ChIP assay in OR6 cells treated with 1 μM SAHA.

We designed the ChIP primers according to the report of Liang et al. [2004]. They have demonstrated that histone H3 acetylation is localized within 500 bp of transcriptional start sites (TSSs) of transcriptionally active genes. Therefore we designed the ChIP primers within 500 bp of TSSs of the *OPN* gene and the *Apo-A1* gene, and confirmed the location of the ChIP primers using the UCSC Genome Browser (http://genome.ucsc.edu/). The ChIP-PCR products for the *OPN* gene and the *Apo-A1* gene are 279 and 78 bp, respectively (Fig. 6A,B).

The results of the ChIP assay showed that histone H3 acetylation levels of the promoter regions of *OPN* and *Apo-A1* were significantly higher in SAHA-treated OR6 cells compared to control ($P < 0.01$, Fig. 6A,B). These results suggest that up-regulation of the *OPN* gene is induced by histone H3 acetylation around its promoter region through SAHA treatment of OR6 cells. Although expression level of *Apo-A1* was significantly decreased after SAHA treatment of OR6 cells, histone H3 acetylation level of the promoter region of *Apo-A1* was increased after SAHA treatment.

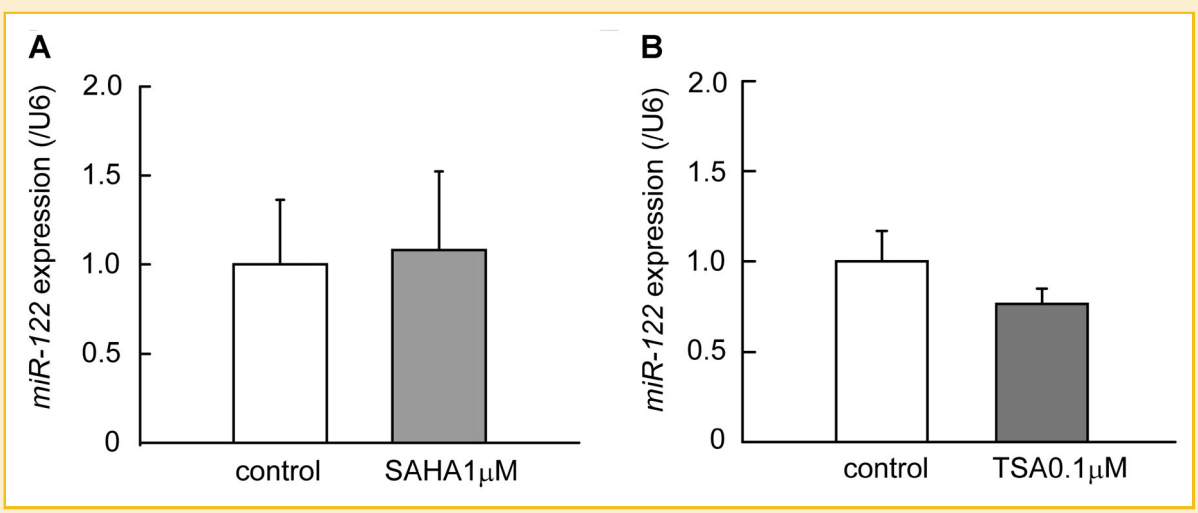

Fig. 3. Expression of *miR-122* in OR6 cells after treatment with SAHA and TSA OR6 cells were treated with 1 μM SAHA (A) or 0.1 μM TSA (B) for 72 h. Expression levels of *miR-122* were examined by TaqMan® quantitative RT-PCR. Means and SDs of *miR-122* expression normalized with U6 for three experiments are shown. There was no significant difference in *miR-122* expression between control and treatment with SAHA or TSA.

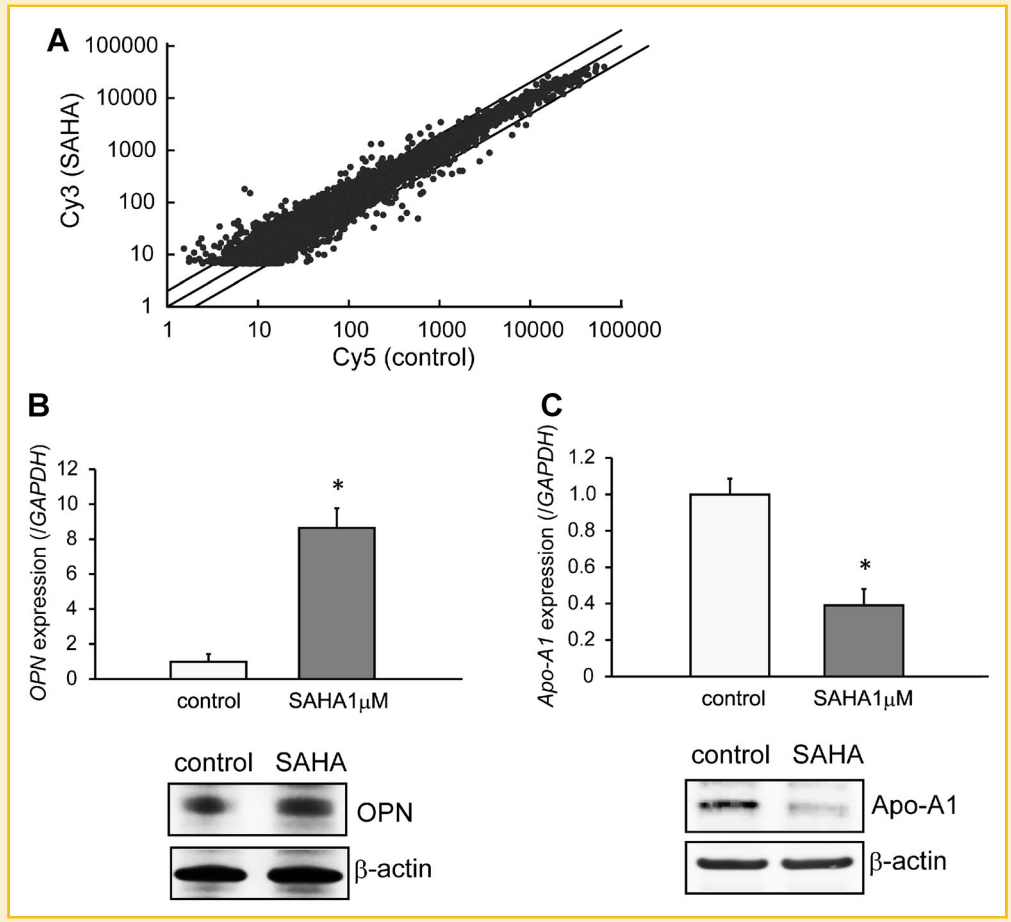

Fig. 4. A: Microarray analysis of OR6 cells after SAHA treatment Standardized data of Cy3 (SAHA)- and Cy5 (control)-stained RNAs are plotted. Global normalization was performed by adjusting to a median Cy3/Cy5 to 1. Lines outside the median line represent twofold difference. Values were normalized according to a median Cy3/Cy5 ratio of 1. B,C: Expression levels of *OPN* and *Apo-A1* in OR6 cells after SAHA treatment Expression levels of *OPN* and *Apo-A1* were examined by quantitative RT-PCR and Western blotting. Means and SDs of gene expression normalized with GAPDH for three experiments are shown. *OPN* expression was significantly up-regulated after treatment with 1 μM SAHA for 72 h compared to control (B: $^{*}P < 0.001$). *Apo-A1* expression was significantly down-regulated after treatment with 1 μM SAHA for 72 h compared to control (C: $^{*}P < 0.001$).

DISCUSSION

In the present study, we investigated whether the HDAC inhibitor SAHA affects HCV replication via epigenetic alterations in host cells. To this objective, we used OR6 cells, in which full-length HCV RNA replicates, were used as the replicon system.

The replicon cell culture system was first established by Lohmann et al. [1999] and this system finally developed to the infectious HCV particle producing cell system [Wakita et al., 2005]. In the replicon system, modified HCV genomes can replicate to high levels in human hepatocellular carcinoma cells (HuH-7) and many stably transfected replicon cell lines are established after continuous drug selection with

TABLE I. Up-Regulated Genes in SAHA-Treated OR6 Cells

Name	Description	SAHA/control	$\log_2$ (SAHA/control)	Up
ANXA1	Annexin A1	25.23	4.66	[a]
SPP1	Osteopontin	7.43	2.89	[b]
UBE2L6	Ubiquitin/ISG15-conjugating enzyme E2L6	5.80	2.54	[b]
IFIT1	Interferon-induced rotein with tetratricopeptide repeats 1	5.30	2.40	[b]
TUBA1A	Tubulin alpha-1A chain	4.86	2.28	[b]
KCNJ8	ATP-sensitive inward rectifier potassium channel 8	4.55	2.19	[b]
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	4.43	2.12	[b]
KRT23	Keratin, type I cytoskeletal 23	4.07	2.03	[b]

OR6 cells were treated with 1 μM SAHA for 72 h and total RNA was analyzed by microarray (Human Oligo chip 25K). Genes with expression levels of more than 100 and more than a fourfold difference relative to control were selected.

[a] >eightfold.

[b] >fourfold.

TABLE II. Down-Regulated Genes in SAHA-Treated OR6 Cells

Name	Description	SAHA/control	$\log_2$ (SAHA/control)	Down
SERPINC1	Antithrombin-III	0.08	3.58	[a]
GJB1	Gap junction beta-1 protein	0.14	2.80	[b]
F12	Coagulation factor XII	0.17	2.54	[b]
SLCO183	Solute carrier organic anion transporter family member 183	0.22	2.19	[b]
C3	Complement C3	0.22	2.18	[b]
AKR1810	Aldo-keto reductase family 1 member 810	0.23	2.11	[b]
GAL	Galanin	0.24	2.07	[b]
FABP1	Fatty acid-binding protein, liver	0.24	2.06	[b]
APOA1	Apolipoprotein A1	0.25	2.00	[b]

OR6 cells were treated with 1 μM SAHA for 72 h and total RNA was analyzed by microarray (Human Oligo chip 25K). Genes with expression levels of more than 100 and less than 1/4 of control were selected.

[a] $<1/16$.

[b] $<1/4$.

neomycin (G418). This cell culture system has been widely used as a tool in the study of HCV virology and drug development [Horscroft et al., 2005]. Development of OR6 cell was originally reported by Ikeda et al. [2005]. The *Renilla* luciferase reporter gene and neomycin resistant gene were introduced in the 5′ untranslated region of genome-length HCV RNA (genotype 1b). This construct containing full-length HCV RNA robustly replicated in the HuH-7 cells after the electroporation and one of the colonies designated OR6 was selected by G418.

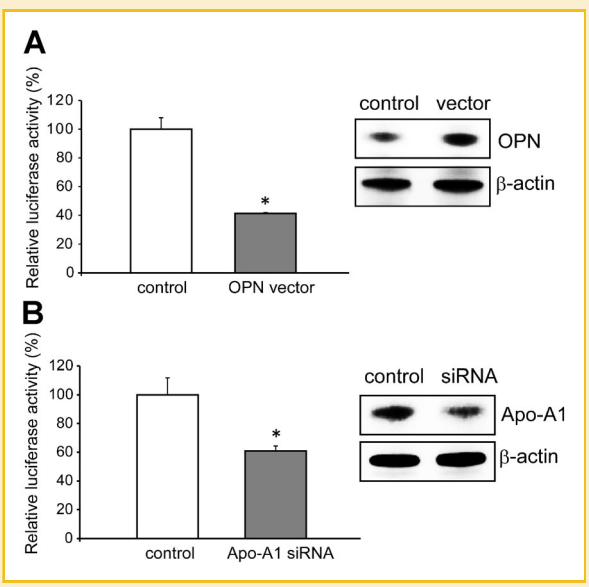

Fig. 5. Reduced HCV replication in OR6 cells after gene transfer. A: HCV replication in OR6 cells after overexpression of the *OPN* gene HCV replication was evaluated by luciferase activity in OR6 cells after transfection with the *OPN* expression vector. Means and SDs of luciferase activities for three experiments are shown. The luciferase activity in OR6 cells after overexpression of the *OPN* gene was significantly reduced. $^*P < 0.01$. B: HCV replication in OR6 cells after knockdown of the *Apo-A1* gene HCV replication was evaluated by luciferase activity in OR6 cells after transfection with siRNA for the *Apo-A1* gene. Means and SDs of luciferase activities for three experiments are shown. The luciferase activity in OR6 cells after knockdown of the *Apo-A1* gene was significantly reduced. $^*P < 0.05$.

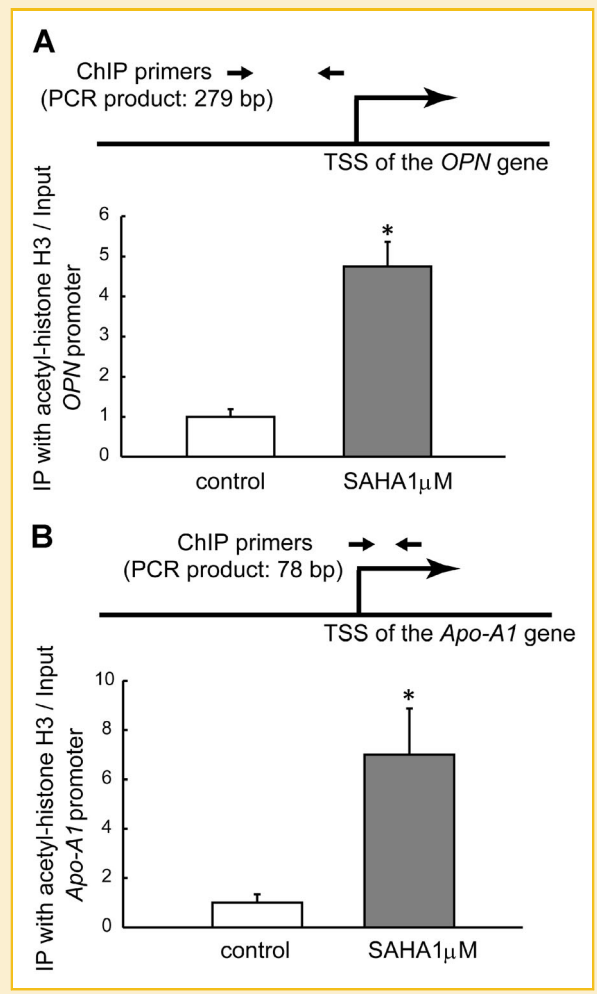

Fig. 6. ChIP assay at the promoter regions of *OPN* and *Apo-A1* in OR6 cells after SAHA treatment levels of acetylated histone H3 around the promoter regions of *OPN* (A) and *Apo-A1* (B) after SAHA treatment were analyzed by ChIP assay. The ChIP primers are located near the TSSs of these genes and should be suitable for histone acetylation assay. The ChIP-PCR products for the *OPN* gene and the *Apo-A1* gene are 279 and 78 bp, respectively. The fraction of immunoprecipitated DNA was calculated as: immunoprecipitated DNA (IP) with anti-acetyl-histone H3 antibody/input DNA. Means and SDs of acetylated histone H3 levels for three experiments are shown. The acetylated histone H3 levels around the promoter regions of *OPN* and *Apo-A1* were significantly increased after SAHA treatment. $^*P < 0.001$.

The OR6 cell system facilitates monitoring of HCV RNA replication. However, if cell viability is decreased as a result of drug toxicity, luciferase activity cannot be evaluated accurately. Therefore, we first examined the cellular toxicity of SAHA in OR6 cells and confirmed that SAHA treatment was non-toxic at concentrations below 1 μM as determined by cell counting, viability assay, and MTT assay. Lu et al. have reported the inhibitory effect of HDAC inhibitors including SAHA on hepatocellular carcinoma (HCC). They treated several HCC cell lines with SAHA at various concentrations and show similar results to our results [Lu et al., 2007]. In contrast, the HCV replication rate evaluated by luciferase activity was significantly reduced by treatment with SAHA at concentration below 1 μM in a dose-dependent manner. We also confirmed that another HDAC inhibitor TSA suppresses HCV replication (Fig. 2B). These results indicate that the HDAC inhibitors SAHA and TSA reduced HCV replication without cellular toxicity in OR6 cells.

We next investigated the molecular mechanisms underlying the suppressive effect of SAHA on the HCV replication rate. *miR-122* is a liver-specific miRNA and plays an important role in fatty-acid metabolism in the liver [Esau et al., 2006]. Recent studies have reported that *miR-122* is down-regulated in HCC and has multiple functions as a tumor suppressor during hepatocarcinogenesis [Coulouarn et al., 2009; Fornari et al., 2009]. Moreover, *miR-122* directly binds to the 5′ noncoding region of the HCV genome and modulates HCV RNA replication [Jopling et al., 2005]. It is noteworthy that *miR-122* facilitates HCV replication via binding to the 5′ noncoding region of the viral genome, whereas miRNAs generally function to repress expression of their target genes by binding to 3′ noncoding regions. Epigenetic alterations such as DNA methylation and histone modification are important mechanisms for the regulation of miRNA expression [Saito et al., 2006, 2011]. In this study, we investigated the effect of treatment with HDAC inhibitors on *miR-122* expression in OR6 cells and found that there was no significant difference in *miR-122* expression after treatment with 1 μM SAHA or 0.1 μM TSA, suggesting that *miR-122* is not involved in the suppressive effect of HDAC inhibitors on HCV replication. These findings led us to hypothesize that histone acetylation induced by SAHA treatment does not modulate *miR-122* expression and that HCV replication was inhibited through other pathways.

Microarray analysis showed differential expression of various genes in OR6 cells induced by SAHA treatment for 72 h. Notably, several cytoskeletal genes such as *tubulin alpha-1A chain*, *extracellular matrix protein 1*, and *keratin* were significantly up-regulated by SAHA treatment. We have reported that a close correlation might exist between malignant transformation of HCC and cytoskeletal changes [Kanamori et al., 2011]. The results of this study are similar to those of our previous studies showing that sodium butyrate, another HDAC inhibitor, changed the mobility, fluidity, and adhesive properties of cancer cells into less a malignant phenotype [Saito et al., 1998; Masuda et al., 2000; Wakabayashi et al., 2000; Nakamura et al., 2001; Kaneko et al., 2004]. Another interesting result of the microarray was the up-regulation of interferon-induced protein by SAHA treatment. This finding is also consistent with our previous study, in which sodium butyrate induced interferon-related gene expression [Wakabayashi et al., 2005], suggesting that there may be crosstalk between the interferon-inducing pathway and epigenetic changes by HDAC inhibitors. Up-regulation of interferon-induced protein by HDAC inhibitors may result in suppression of HCV replication.

Besides cytoskeletal genes and interferon-induced proteins, we focused our present study on *OPN* and *Apo-A1*. The *OPN* gene is a key regulator of the Th1-type immune system and Th1-type immunity plays an important role in viral elimination [Patarca et al., 1993; Ashkar et al., 2000]. Recent studies showed that the promoter activity of *OPN* was correlated with the efficacy of interferon-based therapy [Mochida et al., 2004; Naito et al., 2005]. These findings suggest that increased expression of *OPN* can eliminate HCV by activating the Th1-type immune system. Among the genes that were down-regulated by SAHA treatment, we focused on the *Apo-A1* gene because this protein is essential for HCV replication [Mancone et al., 2011]. The *Apo-A1* gene is a protein component of high-density lipoprotein particles and is thought to be necessary for HCV particle formation and maintenance of infectivity. To confirm the results of the microarray analysis, we examined expression of *OPN* and *Apo-A1* in OR6 cells after SAHA treatment by quantitative RT-PCR. We found a significant increase of *OPN* expression and significant reduction of *Apo-A1* expression, which may lead to suppression of HCV replication. Moreover, we overexpressed *OPN* and knocked down *Apo-A1* in OR6 cells using an expression vector and siRNA procedure, respectively. As shown in Figure 5A,B, the luciferase activities were significantly reduced after overexpression of *OPN* and knockdown of *Apo-A1* in OR6 cells. These results indicate that overexpression of *OPN* and reduced expression of *Apo-A1* play critical roles in SAHA-induced suppression of HCV replication.

Since histone acetylation generally activates gene expression, we presumed that up-regulation of *OPN* is mediated by histone H3 acetylation around its promoter region. The ChIP assay results demonstrated that histone H3 acetylation levels of the promoter regions of *OPN* and *Apo-A1* were remarkably increased in SAHA-treated OR6 cells compared to control. Acetylation of lysine residues on histones H3 is correlated with active or open chromatin, which allows various transcription factors access to the promoters of target genes [Yoo and Jones, 2006]. This suggests that up-regulation of *OPN* is induced by histone H3 acetylation through SAHA-mediated epigenetic mechanisms. On the other hand, *Apo-A1* transcription was significantly down-regulated by SAHA treatment in OR6 cells despite acetylation of its promoter region. Although up-regulation of *OPN* expression by SAHA treatment is reasonable, we could not prove the molecular mechanism underlying down-regulation of *Apo-A1* by SAHA treatment at this time. Studies have demonstrated that HDAC inhibitors suppress expression of specific genes such as *adiponectin* and *bcl-2* [Duan et al., 2005; Qiao et al., 2006]. Further studies regarding *Apo-A1* inactivation induced by HDAC inhibitors are necessary.

To our knowledge, this study is the first report showing that the HDAC inhibitor SAHA significantly inhibits HCV replication. We presume that this inhibitory effect on HCV replication may be mediated by up-regulation of *OPN* via SAHA-mediated histone modification in host cells, which is expected to inhibit HCV replication. Another significant benefit of SAHA treatment on HCV replication may be down-regulation of *Apo-A1*, which is essential for the HCV life cycle. These findings suggest that epigenetic therapy

with HDAC inhibitors represents a novel potential treatment strategy for diseases associated with HCV infection such as chronic hepatitis, liver cirrhosis, and HCC.

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