Histone deacetylase inhibitor SAHA epigenetically regulates miR-17-92 cluster and MCM7 to upregulate MICA expression in hepatoma

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Background: Epigenetic therapy using histone deacetylase inhibitors (HDACi) has shown promise in clinical trials for the treatment of human malignancies. In addition to the immediate effects on the tumour cell growth, HDACi upregulates the expression of MHC class I-related chain molecules A and B (MICA and MICB), resulting in an enhanced susceptibility of tumour cells to natural killer cell-mediated lysis. The molecular mechanism underlying is still unclear.

Methods: The transcriptional regulation mechanism underlying suberoylanilide hydroxamic acid (SAHA)-mediated regulation of MICA and related miRNA expression was investigated using promoter acetylation assays, bioinformatics analysis and chromatin immunoprecipitation assay.

Results: SAHA upregulates the transcription of MICA/B by promoting MICA-associated histone acetylation while suppressing the MICA/B-targeting miRNAs miR-20a, miR-93 and miR-106b. The mechanism by which SAHA repressed miRNAs transcription involved repression of their host genes (miR-17-92 cluster and MCM7). SAHA downregulated the miR-17-92 cluster by abolishing tyrosine phosphorylation of STAT3 and decreased MCM7 transcription through localised histone deacetylation.

Conclusions: The HDACi SAHA epigenetically upregulates MICA expression through regulating the expression of miR-17-92 cluster and MCM7 in hepatoma, thus enhancing the sensitivity of HCC to natural killer cell-mediated lysis. This novel mechanism of action provides promise for HDACi in therapy of HCC.

Epigenetic therapy using histone deacetylase inhibitors (HDACi) has shown promise in clinical trials for the treatment of human malignancies (Dawson and Kouzarides, 2012). Suberoylanilide hydroxamic acid (SAHA) has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma, and many other HDACi are undergoing clinical trials (Duvic et al., 2007; Sato, 2013). HDACi specifically induce cancer cell to differentiate, undergo cell cycle arrest and initiate apoptosis by altering the expression of genes involved in apoptosis signal transduction pathways or cell cycle modulation (Bolden et al., 2013). It is hypothesised that the accumulation of acetylated proteins, particularly histones, results in the upregulation of genes that have become epigenetically silenced (Khan and Khan, 2010). In particular, the gene encoding the cell cycle kinase inhibitor p21, is commonly...
HDACi epigenetically regulates MICA expression

Cell culture. The human HCC cell lines HepG2 (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences) and H7402 (Institute of Basic Medical Sciences, Shandong Academy of Medical Science, Jinan, China) were cultured in RPMI-1640 medium (GIBCO/BRL, Grand Island, NY, USA) containing 10% foetal bovine serum. The human NK cell line NKL, generously provided by Dr Jin Boquan (Fourth Military Medical University, Xi’an, China), were cultured in RPMI-1640 medium containing 10% foetal bovine serum and 100 U ml⁻¹ rIL-2 (Changsheng, Changchun, China). All cells were incubated at 37°C in a 5% CO₂ atmosphere and passaged once every 2–3 days. Experiments were initiated when cells showed logarithmic growth.

Reagents. SAHA, which was synthesised by Department of Medicinal Chemistry, School of Pharmacy, Shandong University, Shandong, China, was stored at −20°C, dissolved in DMSO to a 5 mM stock, and diluted with culture medium as needed. IL-6, a kind gift of Professor Ru Sun (Department of Microbiology and Immunology, School of Life Sciences, University of Science and Technology of China, Hefei, China), was stored at 4°C and dissolved as a 1 × 10⁵ U ml⁻¹ stock in 1× phosphate buffered saline.

Cytotoxicity assay. After treatment with 2 μM SAHA for 48 h, HepG2 and H7402 cells were used as target cells and plated in 96-well plates at a density of 1 × 10⁴ cells per well. NK cells were co-cultured at an effector-to-target ratio between 4:1 and 1:1 for 6 h. Subsequently, 20 μl of 1 mM methylthiazolyldiphenyl-tetrazolium (5 mg ml⁻¹) was added to each well and incubated for an additional 4 h (Qu et al., 2014). Absorbance at 570 nm and 630 nm was measured using a microplate reader (Synergy 2, BioTek, Winoski, VT, USA). Cytotoxicity was calculated using the following formula: cytotoxicity (%) = 1 - (ODE + T - ODT)/ODE × 100% (ODE: OD value of the effector cell group; ODT: OD value of the effector cell and target cell group; ODE: OD value of the effector cell group; OD T: OD value of the target cell group).

Flow cytometry. For cell surface protein expression analysis, cells were harvested and washed with phosphate buffered saline, and incubated with antibodies for 1 h at room temperature. The following antibodies were used: anti-MICA/B mAb (eBioscience, San Diego, CA, USA); anti-MICA mAb (R&D Systems, Minneapolis, MN, USA); and anti-HLA-A/B/C mAb (BD Pharmingen, San Diego, CA, USA). Measurements were performed using a flow cytometer (FACScalibur; BD Biosciences, San Jose, CA, USA) and analysed with WinMDI 2.9 software (Scripps Institute, La Jolla, CA, USA).

RNA isolation and quantitative reverse transcription-PCR analysis of mRNA and miRNA. RNA was extracted using a TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA). MiR-20a, miR-93, miR-106b, miR-17, miR-18a, miR-19a were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using specific BulgeLoop miRNA qRT-PCR primers purchased from Guangzhou Ribobio (Guangzhou, China) and miR-106b in hepatoma cells were purchased from GenePharma.

miRNA mimics transfection. MiRNA mimics (double-stranded oligonucleotides) used for the overexpression of miR-20a, miR-93 and miR-106b in hepatoma cells were purchased from GenePharma.

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MATERIALS AND METHODS

upregulated in tumour cells treated with these agents (Richon et al., 2000; Yang et al., 2014). In addition to the immediate effects of HDACi on tumour cell growth, HDACi-induced recognition of tumour cells by immune cells also contributes to their anticancer effects (Setiadi et al., 2008). For example, we and others have reported that HDACi promote the expression of MICA or MICB, ligands of the natural killer (NK) cell activating receptor NK2G2D on tumour cells, thus increasing their susceptibility to NK cell-mediated lysis (Armeau et al., 2005; Zhang et al., 2009). The molecular mechanisms underlying the involvement of HDACi-regulated genes in immune recognition, however, are not fully understood. An increased understanding of the molecular mechanisms by which HDACi elicit immunostimulatory effects would undoubtedly contribute to their clinical development as anticancer agents.

MicroRNAs (miRNAs) are small, non-coding RNAs that can regulate various target genes. Computational and biological evidence suggests that miRNA-mediated gene regulation represents a fundamental mechanism of post-transcriptional regulation with diverse functional effects. Stern-Ginossar et al. (2008) identified six cellular miRNAs, miR-20a, miR-93, miR-106b, miR-372, miR-373 and miR-520d, that target the mRNA of the MHC class I-related chain molecules A and B (MICA and MICB). Interestingly, among these miRNAs, both miR-106b and miR-93 are in the miR-106b-93-25 cluster while miR-20a is in the miR-17-92 cluster. Both miRNA clusters have been shown to accumulate in different types of cancer and have been designated oncomiRs, with miR-17-92 being designated oncomir-1 (He et al., 2005). Maintenance complex component 7 (MCM7), the host gene of the miR-106b-93-25 cluster, is also observed to have a higher expression level in many cancers and is regarded as an indicator of poor prognosis (Poliseno et al., 2010). The identification of miRNA target genes has been extensively pursued, whereas little is known about the mechanism of regulation of miRNA by epigenetic alterations. MICA/B-targeting miRNAs are overexpressed in tumours, and were found to promote tumour progression and contribute to avoidance of immune recognition via their suppression of MICA and MICB expression.

Early models of regulation of transcription by acetylation focused on the physical interactions of basic histone proteins with negatively charged DNA. The addition of an acetyl group is believed to neutralise the positive charge of histones, decreasing their interactions with negatively charged DNA. This results in de-compaction of chromatin and greater access to DNA for transcription factors, leading to a transcriptionally active genomic locus. There is, however, considerable evidence that these models are oversimplified (Drummond et al., 2005). Not all genes are upregulated by HDACi treatment. In fact, the ratio of upregulated to downregulated genes is close to 1:1. This suggests that the regulation of gene expression by acetylation is highly selective and also likely involves chromatin-associated non-histone proteins. Additionally, HDACi can downregulate certain oncogenes (Kim et al., 2001; Mitsiades et al., 2004; Chou et al., 2011) via reduced transcription because of local histone hypoacetylation after HDAC and HAT dissociation from the respective promoter regions (Duan et al., 2005; Fiegler et al., 2013).

In this present study, we investigated the molecular mechanisms underlying SAHA-mediated regulation of MICA and miRNA expression in two hepatocellular carcinoma (HCC) cell lines. The results demonstrated that SAHA significantly increased MICA/B levels by promoting localised histone acetylation, and simultaneously suppressed the miRNAs targeting MICA/B by localised histone deacetylation or by abolishing tyrosine phosphorylation of STAT3. Both mechanisms likely contribute to SAHA—induced increases in the expression of MICA and MICB proteins. Expression of MICA/B enhances immune recognition of tumours by innate immune cells, and ultimately enhances their sensitivity to cytolysis by NK cells.
(Shanghai, China). H7402 or HepG2 cells were seeded at a density of 1.5 × 10^6 cells ml⁻¹, and transfected with miR-20a/miR-93/miR-106b mimics with Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. The miRNA mimics were used at final concentration of 100 nM. Significant controls of miRNA mimics (mNC) were transected as matched controls (Su et al., 2011).

**Chromatin immunoprecipitation (ChIP) assay.** H7402 cells and HepG2 cells were plated at a density of 1 × 10^6 cells in 10-cm dishes and cultured with 2 μM SAHA for 48 h. Chromatin immunoprecipitation (ChIP) was performed using the EZ ChIP kit (Millipore, Temecula, CA, USA) according to the manufacturer’s recommendations. Briefly, cells were lysed and crosslinked with 1% formaldehyde. Lysates were sonicated to shear DNA to lengths between 200 and 1000 base pairs. An aliquot of the chromatin preparation was set aside and designated as the input fraction. Immunoprecipitations were carried out in ChIP Dilution Buffer with the following antibodies: IgG, anti-acetyl histone H3 (AcH3), anti-acetyl histone H4 (AcH4; Millipore), and anti-p-Tyr705-STAT3 (Cell Signalling Technology, Danvers, MA, USA). Immunoprecipitates were collected using protein G-sepharose beads, washed and eluted according to the manufacturer’s protocol. Immunoprecipitated DNA was recovered by reversing crosslinking, and purified using the QIAGEN Purification Kit (QIAGEN, Hilden, Germany) and analysed by PCR. The sequences of the PCR primers used for MICA, miR-17-92 or MCM7 promoter analysis are listed in supplementary Table 3. Negative control primers (Millipore) amplified a non-related GAPDH promoter sequence.

**Western blot.** H7402 cells and HepG2 cells were treated with SAHA (1-2 μM) for 48 h. Western blotting assays were carried out as described previously (Zhang et al., 2014). The following antibodies were used: anti-p-Ser727-STAT3, anti-MCM7 mAb (BBI; Shanghai SangoniBiotech, CHN); anti-STAT3, anti-p-Tyr705-STAT3 (Cell Signalling Technology); AcH3 mAb, and AcH4 mAb.

**Statistical analysis.** Significant differences were determined by Student’s t-test and one way-ANOVA. P < 0.05 was considered significant. Data are presented as the mean ± s.d. of three independent experiments. All analyses were performed with GraphPad Prism 5 (La Jolla, CA, USA).

## RESULTS

SAHA treatment significantly increased the susceptibility of HCC cell lines to cytolyis by NK cells. In many cases, the functions of NK cells in cancer patients are impaired and tumours can escape NK cell-mediated cytotoxicity. The HCC cell lines HepG2 and H7402 were treated with 2 μM SAHA, a concentration that does not induce significant cell apoptosis (data not shown) for 48 h. Sensitivity of these cells to NK-mediated lysis was then evaluated. The results showed that SAHA treatment significantly increased the susceptibility of the two HCC cell lines to cytolyis by NKL cells (Figure 1A). Furthermore, we found that the expression of MICA/B was upregulated, while there were no significant changes in expression of HLA-ABC in the two HCC cell lines when exposed to SAHA (Figure 1B). These results suggest that SAHA treatment may increase expression of MICA/B in hepatoma cells, and further promote the interaction between NKG2D and its ligands, MICA and MICB, thus increasing the susceptibility of HCC cells to cytotoxic NK cells.

Expression of miRNAs targeting MICA/B is downregulated in SAHA-treated HCC cells in a dose-dependent manner. MiR-20a, miR-93, miR-106b, miR-372, miR-373 and miR-520d regulate the expression of MICA and MICB (Stern-Ginossar et al., 2008), whereas miR-20a, miR-93 and miR-106b have been implicated in tumourigenesis. To explore the molecular mechanisms of SAHA in the regulation of MICA/B expression, we sought to examine whether SAHA could regulate the expression of these miRNAs. Among the panel of miRNAs, only miR-20a, miR-93 and miR-106b were detectable in the cell lines tested. SAHA treatment resulted in a two- to four-fold dose-dependent decrease in the expression of miR-20a and miR-106b in both HCC cell lines (Figure 2). MiR-93 also showed a modest decrease in HepG2 cells. Downregulation of these miRNAs was consistent with upregulation of MICA/B, suggesting that SAHA increased MICA/B expression through downregulation of these MICA/B-targeting miRNAs, further enhancing susceptibility of HCC cells to NK cell-mediated lysis.

Expression of miR-17-92 cluster and MCM7, the host genes of miRNA targeting MICA/B, is downregulated in SAHA-treated HCC cells in a dose-dependent manner. Approximately 500–1000 miRNAs are expressed in human cells, and their expression signatures vary depending on the tissue and cell type examined (O’Connell et al., 2012). MiRNAs are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs) that undergo sequential processing to produce mature miRNAs (Lee et al., 2004). There are at least two types of miRNA promoters. Some miRNAs have their own promoters, while others are located within an miRNA or intron and are transcribed as part of the host gene (Takada and Asahara, 2012). MiR-17-92, which is processed from the transcript of Chromosome 13 open reading frame 25 (C13orf25), is a target gene for 13q31–q32 amplification (Ota et al., 2004) in some lymphomas and solid tumours (Li et al., 2011), is the host gene of miR-20a. In addition to miR-20a, miR-17, miR-18a and miR-19a, all members of miR-17-92 cluster and located at the upstream of miR-20a. Minichromosome MCM7 is the host gene of miR-93 and miR-106b. To address whether SAHA might repress miR-20a, miR-93 and miR-106b transcription by suppressing the expression of their host genes, we examined the expression of MCM7 and miR-17, miR-18a, miR-19a in the miR-17-92 cluster. Primers for qPCR to detect pri-miRNA transcripts of miR-17-92 were also designed (Nagel et al., 2009; Yan et al., 2009). When HCC cells were treated with SAHA (0.5, 1, 2, 4 μM) for 24 and 48 h, dose- and time-dependent decreases in the levels of miR-17, miR-18a and miR-19a were observed (Figure 3A). Interestingly, they were repressed to the same extent as miR-20a was repressed (Figure 2). Furthermore, the levels of pri-miR-17-92 and MCM7 also decreased significantly in both a dose- and time-dependent manner (Figure 3B and C). Together, these results demonstrate that treatment with SAHA results in reduced expression of the host genes of miRNA targeting MICA/B miR-17-92 cluster and MCM7 in the two HCC cell lines.

miR-20a, miR-93 and miR-106b specifically downregulate MICA expression in HCC cell lines. We next examined whether these miRNAs could affect MICA surface protein levels. MiRNA mimics or control miRNAs were transiently transfected into HepG2 or H7402 cells, which had high expression of both MICA and MICB. As shown in Figure 4, all three miRNAs significantly downregulated MICA surface expression. Our findings are consistent with previous studies from Stern-Ginossar, which showed that miR-20a, miR-93 and miR-106b target the 3’ untranslated region of MICA and MICB, and suppress MICA/B expression (Stern-Ginossar et al., 2008). These data suggest a novel molecular mechanism by which HDACi regulate MICA expression. HDACi may promote the expression of the NKG2D ligand MICA by downregulating the expression of miRNAs (miR-20a, miR-93 and miR-106b) targeting MICA/B.

SAHA induces the accumulation of acetylated histones in chromatin associated with the MICA gene, but decreases the binding of AcH4 to the MCM7 promoter. To verify the activity of the HDACi in our studies, HepG2 cells were treated with SAHA
for 48 h, and AcH3 and AcH4 protein levels were determined by western blot analysis. As shown in Figure 5A, neither AcH3 nor AcH4 were detectable in HepG2 cells treated with the vehicle (DMSO) control. Following treatment with SAHA, the levels of AcH3 and AcH4 were increased in a dose-dependent manner \( (P < 0.05) \).

As demonstrated above, SAHA induces MICA/B expression in HCC cells when compared with vehicle (DMSO) control \( (P < 0.05; \) Figure 1B). To determine whether these changes were associated with histone acetylation status in the promoter region of MICA, ChIP was performed using primers corresponding to -1105 (P1), -439 (P2) and -308 (P3) bp upstream of the translation initiation site (ATG) of the MICA gene (Figure 5B). Chromatin fragments from HCC cells cultured with 2 \( \mu M \) SAHA were immunoprecipitated with antibody to AcH3 or AcH4. DNA from the immunoprecipitation was isolated, and a 213-bp fragment of the MICA promoter region was amplified (Figure 5C). Following culturing with SAHA for 48 h, a 3.7-fold increase in MICA promoter DNA in H7402 cells and 3.2-fold increase in MICA promoter DNA in HepG2 cells was associated with highly AcH3, compared with the same region isolated from HCC control cells. PCR analysis with the P2 primer, amplifying a 423-bp fragment of

Figure 1. SAHA sensitises HCC cells to IL-2–activated NK cell-mediated cytotoxicity by upregulating MICA. (A) Human HCC cell lines H7402 and HepG2 were untreated (Ctrl) or treated with SAHA (2 \( \mu M \)). After 48 h, cells were coincubated with NKL cells for 6 h, and the cytotoxicity of NK cells was measured in the MTT assay. (B) HCCs cells were treated with SAHA. After 48 h, the expression of HLA-ABC, MICA/B and MICA was analysed by flow cytometry. Shown is one representative experiment. The data represent the mean \( \pm \) s.d. of three independent experiments. \( *P < 0.05, \) vs Ctrl group.

Figure 2. Expression of miRNAs targeting MICA is downregulated in a dose-dependent manner in SAHA-treated HCC cell lines. qRT-PCR analysis was performed of miR-20a, miR-93, and miR-106a in H7402 and HepG2 cells treated with varying concentrations of SAHA (0.5, 1, 2, 4 \( \mu M \)) for 48 h. Relative expression levels were normalised to U6 gene expression levels. Error bars represent the s.d. of mean values (expressed as percent of control) from three independent experiments. **\( P < 0.01; *P < 0.05, \) paired t-test.

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the MICA promoter region and the P3 primer, amplifying a 310-bp fragment of the MICA promoter region was also performed (Figure 5C). The accumulation of AcH4 in chromatin associated with the MICA gene was verified in HepG2 cells (Figure 5C). These results strongly suggest that the enhancement in acetylation of histones associated with the MICA promoter is involved in the upregulation of MICA induced by SAHA.

We also examined histone modification in the promoter region of miR-17-92. ChIP primers were designed corresponding to -4913 (A), -4181 (B) (Woods et al, 2007), -4003 (C) and -3927 (D)bp upstream of the translation initiation site of the miR-17-92 gene (Figure 5D). No increases in the levels of AcH3 and AcH4 were found at the promoter region of miR-17-92 in HCC cells treated with SAHA (Figure 5E). We further performed quantitative ChIP assays to examine the binding of acetylated histones to the MCM7 promoter regions. Primers were designed at -2273 (PM1) and -2194 (PM2) base pairs relative to the transcription initiation site of the MCM7 gene (Figure 5F) (Scian et al, 2008; Zhao et al, 2012). Consistent with SAHA-induced MCM7 transcriptional repression, ChIP analysis showed a significant reduction of AcH4 binding to both MCM7 promoters, although SAHA treatment did not alter binding of AcH3 to either promoter region (Figure 5G). This demonstrates the selectivity of gene regulation by acetylation. The observed histone H4 hypoacetylation could be the result of reduced cooperative HDAC/HAT binding and activity at the MCM7 5'-UTR, even though SAHA strongly enhances global histone acetylation levels. Taken together, these data suggest that HDACi may regulate the transcription of MCM7 by modifying histone acetylation, and regulate the transcription of MCM7 by a localised decrease of histone H4 acetylation at its promoters. Histone acetylation did not affect transcription of the miR-17-92 cluster directly during SAHA treatment.

SAHA downregulated the miR-17-92 cluster by abolishing tyrosine phosphorylation of STAT3. To investigate the mechanisms of downregulation of the miR-17-92 cluster by SAHA, we analysed the sequences of the miR-17-92 cluster to predict the exact location of its promoter with Promoter 2.0. The binding sites of transcription
MICA primers for the regions of the MCM7 promoter. HepG2 cells were treated with SAHA (1-2 μM) for 48 h. (A) Western blot analysis showed that SAHA induced an increase in AcH3 and AcH4 in HepG2 cells. (B) Schematic representation of the human MICA gene. Primer sets are indicated as P1, P2, and P3. (C) Schematic representation of the human miR-17-92 gene. Primer sets are indicated as A miR, B miR, C miR, D miR. (D) Schematic representation of the human MCM7 gene. Primer sets are indicated as PM1, PM2, and PM3.

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Figure 5. SAHA induces accumulation of acetylated histones in chromatin associated with the MICA gene, but decreases the binding of AcH4 to the MCM7 promoter. H7402 cells and HepG2 cells were treated with SAHA (1-2 μM) for 48 h. (A) Western blot analysis showed that SAHA induced an increase in AcH3 and AcH4 in HepG2 cells. (B) Schematic representation of the human MICA gene. Primer sets are indicated as P1, P2, and P3. (C) Schematic representation of the human miR-17-92 gene. Primer sets are indicated as A miR, B miR, C miR, D miR. (D) Schematic representation of the human MCM7 gene. Primer sets are indicated as PM1, PM2, and PM3.
MiR-20a The levels of p-STAT3 (Tyr705) and AcH3 protein were examined by Western blotting.

experiments. We and others have shown that treatment with HDACi immunotherapy. NKG2D is a pivotal activation receptor for NK cells. Therefore, has been regarded as an important approach to cancer manipulation of NK cell activation, as anticancer agents. The functions of NK cells are usually not fully understood. Improved understanding of the molecular mechanisms underlying the anticancer effects of these drugs are the efficacy of standard chemotherapy. However, the molecular mechanisms underlying the anticancer effects of these drugs are the efficacy of standard chemotherapy. However, the molecular mechanisms underlying the anticancer effects of these drugs are not fully understood. Improved understanding of the molecular mechanisms would undoubtedly assist their clinical development as anticancer agents. The functions of NK cells are usually impaired in cancer patients. Manipulation of NK cell activation, therefore, has been regarded as an important approach to cancer immunotherapy. NKG2D is a pivotal activation receptor for NK cells. We and others have shown that treatment with HDACi induces expression of the NKG2D ligands MICA/B on some tumour cells, rendering them more sensitive to NK cell-mediated killing (Armeanu et al, 2005; Zhang et al, 2009). The present study further explores the molecular mechanism of regulation of MICA/B expression by the histone deacetylase inhibitor SAHA from the perspective of epigenetics and miRNA regulation. Our results demonstrate that HDACi selectively regulate gene expression, with increased histone acetylation near the MICA promoter and inhibition of the transcription of MICA/B-targeting miRNAs.

Histone acetylation status is assumed to be an important factor that controls the accessibility of transcription factors to DNA and subsequent gene transcription. Both AcH3 and AcH4 are associated with an open chromatin structure and active gene expression. For example, the p21WAF1 protein, an inhibitor of cyclin-dependent kinases, stalls cell cycle progression in the G1 phase. The induction of p21WAF1 by HDACi is associated with the accumulation of acetylated histones at the promoter region of the gene (Yang et al, 2014). In the present study, we demonstrated that the significant upregulation of MICA induced by SAHA was mainly a result of enhanced acetylation of histones H3 and H4.

![Figure 6](image1.png) SAHA downregulates the miR-17-92 cluster by abolishing phosphorylation of STAT3. (A, B) H7402 cells and HepG2 cells were treated with SAHA (1-2 μM) for 48 h. Cell lysates were subjected to Western blot analysis with anti-STAT3, anti-p-STAT3 (Tyr705), anti-p-STAT3 (Ser727) and anti-MCM7 antibodies. (C) The levels of STAT3 and MCM7 in H7402 cells stimulated with IL-6 were examined by Western blotting. (D) The levels of p-STAT3 (Tyr705) and p-STAT3 (Ser727) in H7402 cells and HepG2 cells stimulated with IL-6 for indicated times were examined by Western blotting. (E) qRT-PCR analysis of miR-20a expression in H7402 and HepG2 cells treated with IL-6. (F) qRT-PCR analysis of pri-miR-17-92 expression in H7402 cells treated with IL-6. (G, H) H7402 cells and HepG2 cells were stimulated with IL-6 alone or with IL-6 combined with 2 μM SAHA for 24 h. The levels of p-STAT3 (Tyr705) and AcH3 protein were examined by Western blotting. miR-20a expression in H7402 and HepG2 cells was detected by qRT-PCR. *P < 0.05, vs vehicle control group. **P < 0.05, vs IL-6 group. Data shown are mean ± s.d. of at least three independent experiments.

![Figure 7](image2.png) SAHA suppresses the binding of activated STAT3 to the promoter region of the miR-17-92 cluster. (A) Schematic representation of the human miR-17-92 gene. Primer sets are indicated as PI, PII, PIII, occupying predicted STAT3 binding sites within the promoter and 3′-untranslated regions of the miR-17–92 cluster. (B) H7402 cells were treated with 2 μM SAHA for 48 h. A ChIP assay was performed using anti-p-Tyr705-STAT3 antibody or IgG antibody as a negative control.

**DISCUSSION**

HCC cells display a marked resistance to conventional cytostatic agents resulting in disappointing clinical outcomes when currently available chemotherapeutic treatment strategies are employed. There is growing evidence that HDAC inhibitors might improve the efficacy of standard chemotherapy. However, the molecular mechanisms underlying the anticancer effects of these drugs are not fully understood. Improved understanding of the molecular mechanisms would undoubtedly assist their clinical development as anticancer agents. The functions of NK cells are usually impaired in cancer patients. Manipulation of NK cell activation, therefore, has been regarded as an important approach to cancer immunotherapy. NKG2D is a pivotal activation receptor for NK cells. We and others have shown that treatment with HDACi induces expression of the NKG2D ligands MICA/B on some tumour cells, rendering them more sensitive to NK cell-mediated...
associated with the MICA promoter. Interestingly, the miR-17-92 cluster and its related paralogues (miR-106a/363 and miR-106b/25) showed decreased expression rather than activation. Although the mechanism of HDACi in the upregulation of gene expression in transformed cells has been extensively studied, the mechanism of downregulation of gene expression by HDACi is not fully understood. It has been reported that HDACi can suppress the expression of target genes, such as cyclin D1 (Alao et al, 2006), cyclinB1 (Mateen et al, 2012), bcr-abl (Nimmanapalli et al, 2003), ErbB2 (Drummond et al, 2005) and steroidogenic factor 1 (Chen et al, 2007), through proteasomal degradation or ubiquitin-dependent protein degradation at the protein level, whereas the repression of miRNAs by HDAC inhibitors occurs at the level of transcription. Understanding the regulatory mechanism of HDACi on miRNA expression is challenging.

We analysed the sequence of the miR-17-92 cluster to predict the exact location of its promoter using Promoter 2.0. The results revealed that the promoter of miR-17-92 cluster is most likely located approximately -4300 upstream of the transcription initiation site (ATG) of the miR-17-92 cluster. The sequences of predicted miR-17-92 promoters were then analysed for potential transcription factor binding sites using the JASPAR database and TFSEARCH. We found potential binding sites for GATA2 and STAT3 in the upstream promoter region of the miR-17-92 gene. SAHA suppressed the expression of GATA-2 in a dose-dependent manner, however, regarding its precise regulatory mechanism, is not fully understood. It has been reported that HDACi can suppress the expression of target genes, such as cyclin D1 (Alao et al, 2006), cyclinB1 (Mateen et al, 2012), bcr-abl (Nimmanapalli et al, 2003), ErbB2 (Drummond et al, 2005) and steroidogenic factor 1 (Chen et al, 2007), through proteasomal degradation or ubiquitin-dependent protein degradation at the protein level, whereas the repression of miRNAs by HDAC inhibitors occurs at the level of transcription. Understanding the regulatory mechanism of HDACi on miRNA expression is challenging.

Previous reports have suggested that STAT3 transcriptionally activates the miR-17-92 cluster (Brock et al, 2009). There is little direct evidence, however, regarding its precise regulatory mechanism. As an oncogene, STAT3 is persistently activated in many human cancers and transformed cell lines. After activation, STAT3 proteins dimerise and translocate to the nucleus, where they bind to specific DNA-response elements in the promoters of target genes and regulate gene expression. STAT3 was found in both the cytoplasm and nucleus, whereas activated p-STAT3 (Tyr705) was restricted to the nucleus. Our previous studies have demonstrated that STAT3 is constitutively activated in human HCC cell lines, and blockade of STAT3 activation suppresses growth of cancer cells, promotes cancer cell apoptosis and inhibits cell cycle progression in vitro (Sun et al, 2008). It is important to note that the activity of STAT3 can be post-translationally modulated by acetylation and phosphorylation, and both processes are affected by HDAC inhibitors. Gupta reported that treating cells with the HDACi LBH589 (panobinostat) increases STAT3 acetylation and results in decreased levels of STAT3 in the nucleus in a significant decrease in nuclear p-STAT3 (Tyr). These results suggest that HDAC inhibition may enhance STAT3 export from the nucleus to the cytoplasm or prevent entrance of STAT3 to the nucleus (Gupta et al, 2012). Here, we demonstrated that SAHA treatment resulted in the dephosphorylation of STAT3 in a dose-dependent manner in HCC cells. We further demonstrated that p-STAT3 could directly bind to the miR-17-92 cluster promoter, with a reduction of p-STAT3 at the miR-17-92 promoter after SAHA treatment. Although HDAC inhibitors increase histone acetylation in treated cells, no increases occur at the miR-17-92 promoter region. Combined with decreased binding of p-STAT3 at the promoter, this results in the transcriptional repression of miR-17-92 by HDAC inhibitors. Besides, c-Myc (O’Donnell et al, 2005) and E2F1 (Petrocca et al, 2008) have been shown to transcriptionally activate the miR-17-92 cluster and paralogous clusters, while p53 (Yan et al, 2009) represses them (Grillari et al, 2010). Consistent with these reports, we verified that SAHA decreased the levels of E2F1 and c-myc in two HCC cell lines (data not shown). C-Myc and E2F1 may also be involved in the transcriptional repression of miR-17-92 in SAHA treated HCC cells.

The miR-106b-93-25 cluster is composed of the highly conserved miR-106b, miR-93 and miR-25, which have been shown to accumulate in different types of cancer. MCM7, the host gene of the miR-106b-93-25 cluster, is a transcription factor. High expression levels of MCM7 are also observed in many cancers and are regarded as an indicator of poor prognosis. In this study, we found that SAHA treatment significantly suppressed expression of the miR-106b-93-25 cluster as well as its host gene MCM7 in HCC cells. We further demonstrated that the transcriptional repression of the miR-106b-93-25 cluster and MCM7 by SAHA was associated with deacetylation of histone H4 but not H3 localised at the MCM7 promoter. Consistent with these findings, similar mechanisms have also been proposed for HDACi-induced regulation of expression of other genes. Studies by Ferguson (Ferguson et al, 2003) suggested that HDAC inhibitors could induce localised promoter histone deacetylation. Studies by Duan (Duan et al, 2005) showed that, although HDACi increased the global accumulation of acetylated histones, decreased AcH3 binding to the bel-2 promoter regions was correlated with the transcriptional repression of bel-2, while AcH4 binding did not change significantly. Noh also reported TSA (trichostatin A) augmented acetylation of the p21WAF1 promoter but reduced acetylation of the cyclin B1 promoter (Noh and Lee, 2003), suggesting a relationship between TSA-induced modulation of histone acetylation and differential expression of these genes. Fiegler (Fiegler et al, 2013) reported that HDACi downregulated B7-H6 through decreased B7-H6 promoter activity and reduced histone acetylation at the B7-H6 promoter. Local histone hypoacetylation induced by HDACi affects transcription by preventing recruitment of RNA polymerase II (You et al, 2008), as well as by reducing the binding of transcription factors (Duan et al, 2005). These observations are surprising because, in striking contrast to the usual correlation, histone acetylation is associated with decreased transcriptional activity (Struhl, 1998). The contribution of individual HATs to modulation of MCM7 expression requires further investigation. Although Li (Li et al, 2013) reported that STAT3 may regulate the expression of MCM7 in mouse, no direct evidence was provided. We also attempted to determine whether STAT3 was involved in the regulation of MCM7 expression. We performed human MCM7 promoter analysis for transcription factor binding sites, and the result revealed several putative STAT3 sites scattered within the promoter region of MCM7 (data not shown). However, as shown in Figure 6C, although IL-6 stimulated the activation of STAT3 in H7402 cells, the expression of MCM7 was not significantly changed. These results suggested that STAT3 activation had no effect on the expression of MCM7 in HCC cells. In future studies, it will be important to perform a detailed characterisation of the MCM7 promoter to identify the transcription factors that are involved in the regulation of MCM7 expression by HDACi.

Based on our results, we propose a model to explain the role of SAHA in the regulation of gene expression in HCC cells (Supplementary Figure 2). On the one hand, SAHA induces MICA mRNA transcription through increases in MICA-associated histone acetylation; on the other hand, SAHA suppresses the miRNAs targeting MICA/B to reduce the threshold for MICA and MICB expression. Both mechanisms could contribute to the SAHA-induced increase in the expression of MICA and MICB protein, which promotes immune recognition of tumours by innate immune cells. We demonstrated that the influence of HDACi on transcriptional activity is not only dependent on primary histone targets but also on targeting non-histone protein. SAHA repressed the transcription of miR-20a, miR-93 and miR-106b by repressing their host genes (miR-17-92 cluster and MCM7). SAHA decreased
miR-17-92 transcription by decreasing phosphorylation of tyrosine residues in STAT3, and reduced MCM7 mRNA transcription through localised promoter histone deacetylation. miRNAs can, therefore, alter the epigenetic machinery and also be regulated by epigenetic alterations. This suggests that HDAC inhibitors may have therapeutic potential for patients with tumours that over-express pSTAT3, MCM7, as well as the miR-17-92 cluster.

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