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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

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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 NKG2D on tumour cells, thus increasing their susceptibility to NK cell-mediated lysis (Armeanu *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.

MATERIALS AND METHODS

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} rhIL-2 (Changsheng, Changchun, China). All cells were incubated at 37 °C in a 5% CO_2 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 Rui 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 1×10^7 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 1.0×10^4 cells per well. NK cells were coincubated at an effector-to-target ratio between 4 : 1 and 1 : 1 for 6 h. Subsequently, 20 μl methylthiazolyldiphenyl-tetrazolium bromide (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, Winooski, VT, USA). Cytotoxicity was calculated using the following formula: cytotoxicity (%) = $1 - (OD_{E+T} - OD_E)/OD_T \times 100\%$ (OD_{E+T}: OD value of the effector cell and target cell group; OD_E: 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) with U6 small nuclear RNA as an internal reference (Hou *et al*, 2014). The mRNA expression levels of *pri-miR-17-92* and *MCM7* were determined by qRT-PCR. Expression levels of the target genes were normalised to GAPDH. The sequences of the PCR primers are listed in Supplementary Tables 1 and 2. Real-time PCR was carried out using SYBR green mix (FastStart Universal SYBR Green Master, Roche, Indianapolis, IN, USA).

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

(Shanghai, China). H7402 or HepG2 cells were seeded at a density of 1.5×10^5 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. Negative controls of miRNA mimics (mNC) were transfected 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 Signaling 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 SangonBiotech, CHN); anti-STAT3, anti-p-Tyr705-STAT3 (Cell Signaling 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 cytolysis 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 cytolysis 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 mRNA 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*), a target gene for 13q31–q32 amplification (Ota *et al*, 2004) in some lymphomas and solid tumours (Ji *et al*, 2011), is the host gene of *miR-20a*. In addition to *miR-20a*, *miR-17*, *miR-18a* and *miR-19a* are also memebers 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

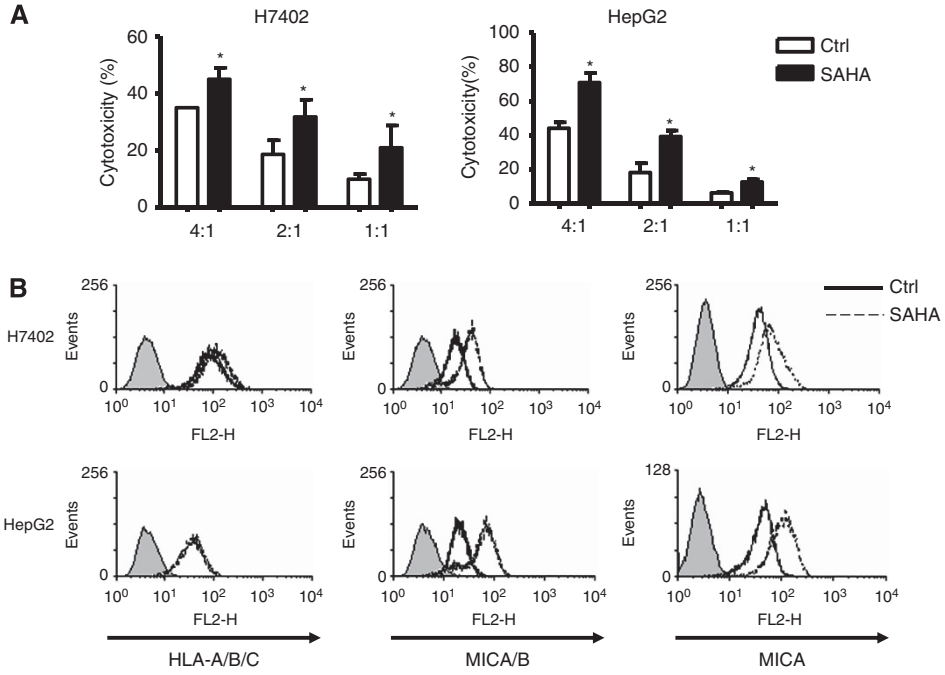

Figure 1. SAHA sensitises HCC cells to IL-2–activated NK cell-mediated cytotoxicity by upregulating MICA. (**A**) Human HCC cell lines H7402 and HepG2 were untreated (Ctrl) or treated with SAHA (2 μM). After 48 h, cells were coincubated with NKL cells for 6 h, and the cytotoxicity of NK cells was measured 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 ± s.d. of three independent experiments. *$P<$ 0.05, *vs* Ctrl group.

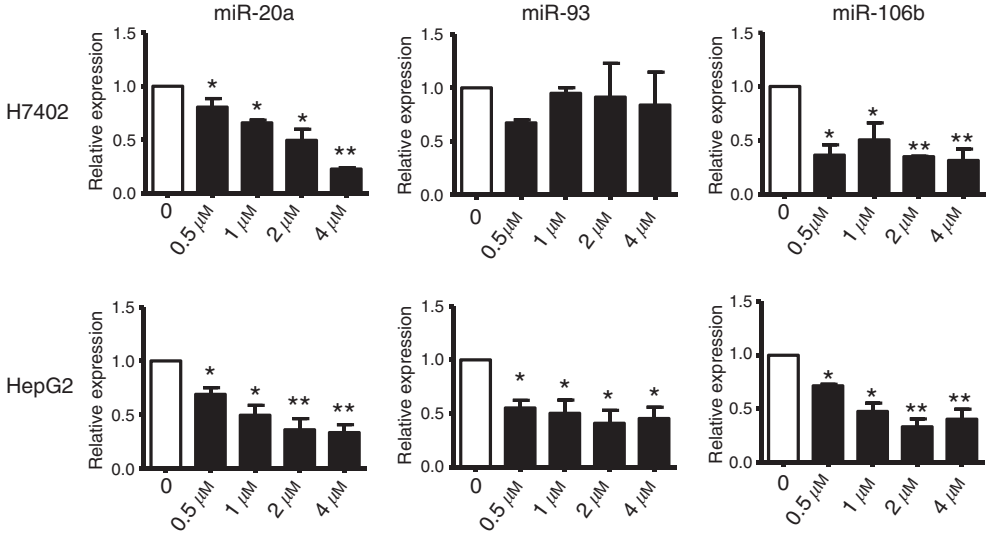

Figure 2. Expression of miRNAs targeting MICA is downregulated in a dose-dependent manner in SAHA-treated HCC cell lines. qRT-PCR analysis was performed of *miR-20a*, *miR-93*, and *miR-106a* in H7402 and HepG2 cells treated with varying concentrations of SAHA (0.5, 1, 2, 4 μM) for 48 h. Relative expression 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.

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 μM 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

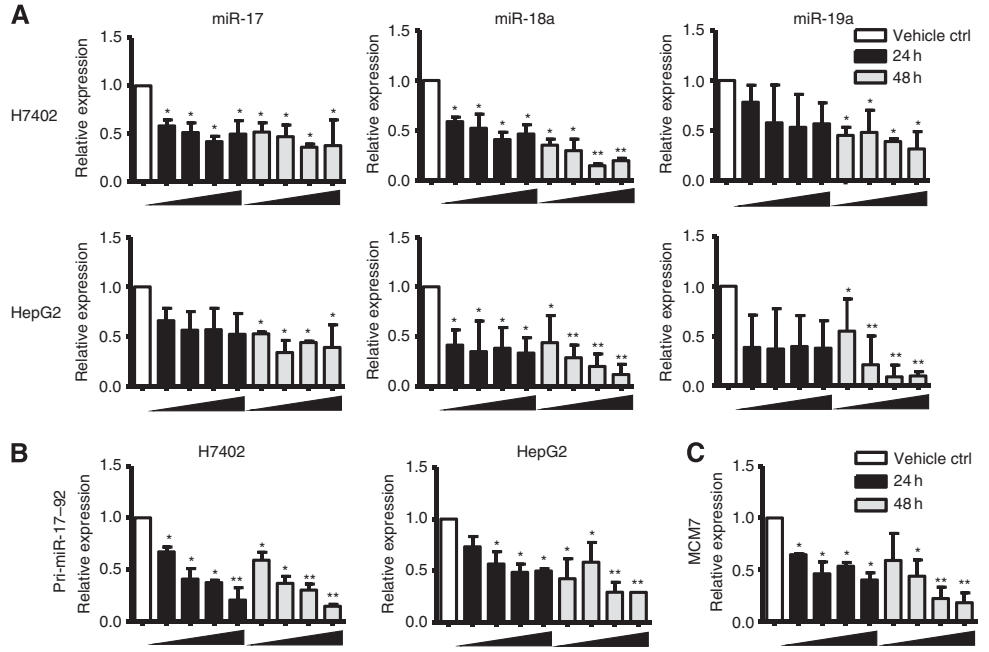

Figure 3. Expression of *miR-17*, *miR-18a* and *miR-19a* in *C13ORF25* BOX and *MCM7* is downregulated in a dose-dependent manner in SAHA-treated HCC cell lines. (**A**) qRT-PCR analysis of *miR-17*, *miR-18a*, *miR-19a* and (**B**) the primary miRNA transcript (*pri-miR-17-92*) of their host gene *miR-17-92* expression in H7402 and HepG2 cells treated with SAHA at different concentrations (0.5, 1, 2, 4 μM) for 24 h and 48 h.(**C**) qRT-PCR analysis of *MCM7* (the host gene of *miR-93*, *miR-106b*) expression in H7402 cells.

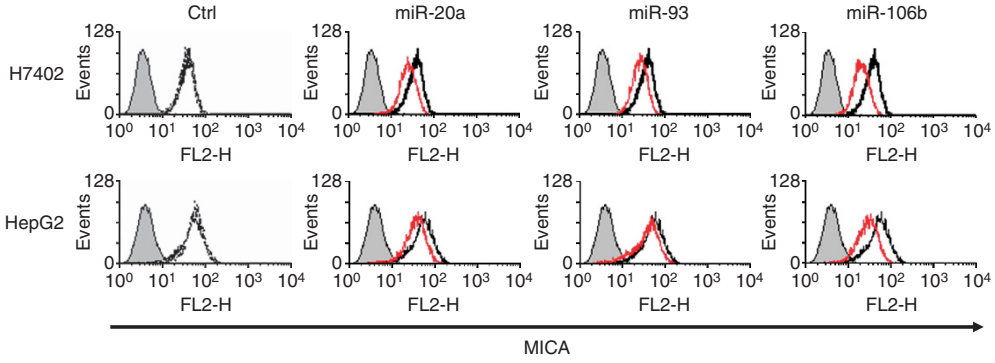

Figure 4. *MiR-20a*, *miR-93* and *miR-106b* specifically downregulate MICA expression in HCC cell lines. H7402 cells and HepG2 cells were transfected with 100 nM of the indicated human miRNA (red lines) or control miRNA (black lines) or untransfected (dotted lines). After 24 h, MICA expression was analysed by flow cytometry. Filled histograms represent staining with a control antibody. Shown is a representative trace selected from at least three independent experiments.

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 *MICA* 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

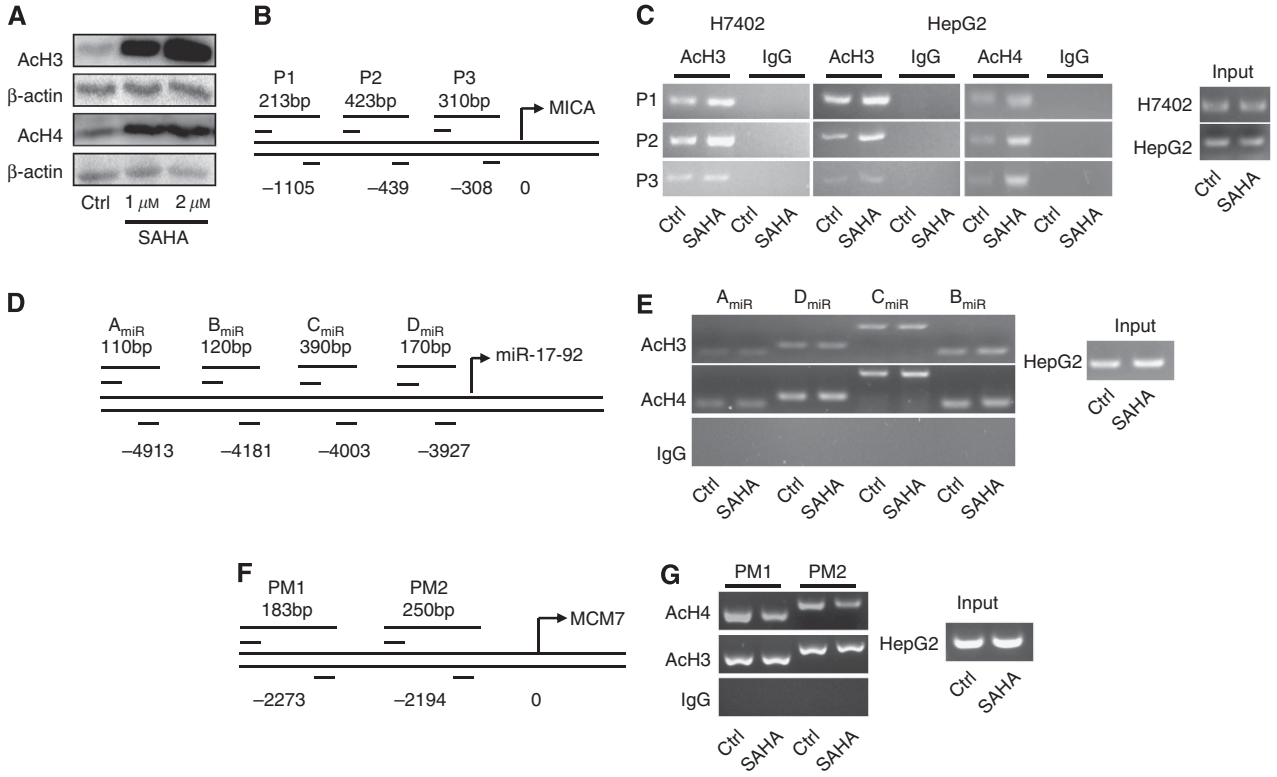

Figure 5. SAHA induces accumulation of acetylated histones in chromatin associated with the *MICA* gene, but decreases the binding of AcH4 to the *MCM7* promoter. H7402 cells and HepG2 cells were treated with SAHA (1-2 μM) for 48 h. (**A**) Western blot analysis showed that SAHA induced an increase in AcH3 and AcH4 in HepG2 cells. (**B**) Schematic representation of the human *MICA* gene. Primer sets are indicated as P1, P2, and P3. (**D**) Schematic representation of the human miR-17-92 gene. Primer sets are indicated as A_{miR}, B_{miR}, C_{miR}, D_{miR}. (**F**) Schematic representation of the human *MCM7* gene. Primer sets are indicated as PM1, PM2.(**C**, **E**, **G**) Soluble chromatin was immunoprecipitated with indicated antibodies. PCR primers for the regions of the *MICA* gene, *miR*-17-92 gene, *MCM7* gene as indicated above were used to amplify the DNA isolated from the immunoprecipitated chromatin. Experiments were carried out at least three times.

factors in the promoter regions of *miR-17-92* were predicted using the JASPAR database and TFSEARCH. The promoter regions of *miR-17-92* were determined to contain putative transcription factor binding sites, such as, GATA-1, GATA2, GATA-3, SP1, E2F, c-myc and STAT3. Previous studies have demonstrated that both E2F and c-myc can transcriptionally activate the *miR-17-92* cluster (O'Donnell *et al*, 2005; Petrocca *et al*, 2008). We found that expression of SP1 was unchanged following SAHA treatment (data not shown). GATA-3, an essential regulator of T-lymphocyte differentiation (Ting *et al*, 1996), does not appear to play a role in the development of hepatic carcinoma. We, therefore, focused on the tumour-associated transcription factors GATA-2 and STAT3 (Luesink *et al*, 2012). Although the protein levels of GATA-2 were decreased after SAHA treatment (Supplementary Figure 1A), overexpression of GATA-2 (Supplementary Figure 1B, 1C) had no effect on the expression of *miR-17-92* (Supplementary Figure 1D). STAT3 has been reported to transcriptionally activate the *miR-17-92* cluster and paralogous clusters (Brock *et al*, 2009). Increasing evidence suggests that the regulation of gene expression by STATs requires class I HDAC activity (Gupta *et al*, 2012). Therefore, we next attempted to determine whether SAHA treatment had any effect on the expression or activation of STAT3 in HCC cells. As shown in Figure 6A, both H7402 and HepG2 cells showed high levels of phosphorylation at tyrosine 705 of STAT3. SAHA treatment resulted in the dephosphorylation of STAT3 in both HCC cell lines within 48 h with little effect on total STAT3 levels (Figure 6A). Similar results were obtained with regards to phosphorylation of STAT3 at serine 727 (Figure 6B). We also observed decreases in the protein levels of MCM7, the host gene of *miR-93* and *miR-106b*, in response to SAHA treatment in whole cell lysates (Figure 6B).

IL-6 is a known inducer of the STAT3 pathway. We stimulated the two HCC cell lines with IL-6 to induce the phosphorylation of STAT3. As shown in Figure 6C and D, higher levels of total STAT3 and phosphorylation of STAT3 was observed at 30 min, and sustained for at least 1 h. After stimulation with IL-6 for 24 h, the expression of *miR-20a* and *pri-miR-17-92* increased significantly in both cell lines (Figure 6E and F). A relationship between activation of STAT3 and induction of *miR-17-92* expression is possible. Importantly, SAHA treatment alleviated IL-6-induced STAT3 activation and the corresponding upregulation of miR-20a expression (Figure 6G and H).

Transcription Element Search System-mediated (JASPAR database) sequence analysis was performed and revealed three putative STAT3 sites scattered within the promoter region of the *miR-17-92* cluster (Figure 7A). To determine whether activated STAT3 could directly bind to the promoter region of the *miR-17-92* cluster and regulate transcription, ChIP analysis was performed in H7402 cells to validate promoter binding using an antibody to p-STAT3(Tyr705) followed by PCR using the primers covering the putative STAT3 binding sites. The results showed that the -4234 and -3709 sites had STAT3 occupancy in H7402 cells, while no binding of STAT3 was found at the -3317 site. As shown in Figure 7B, we verified that p-STAT3 could bind to the *miR-17-92* promoter, with a reduction in STAT3 levels at the *miR-17-92* promoter after SAHA treatment. We concluded that STAT3 promotes the expression of the *miR-17-92* cluster, while SAHA treatment downregulates the *miR-17-92* cluster via suppression of STAT3 phosphorylation.

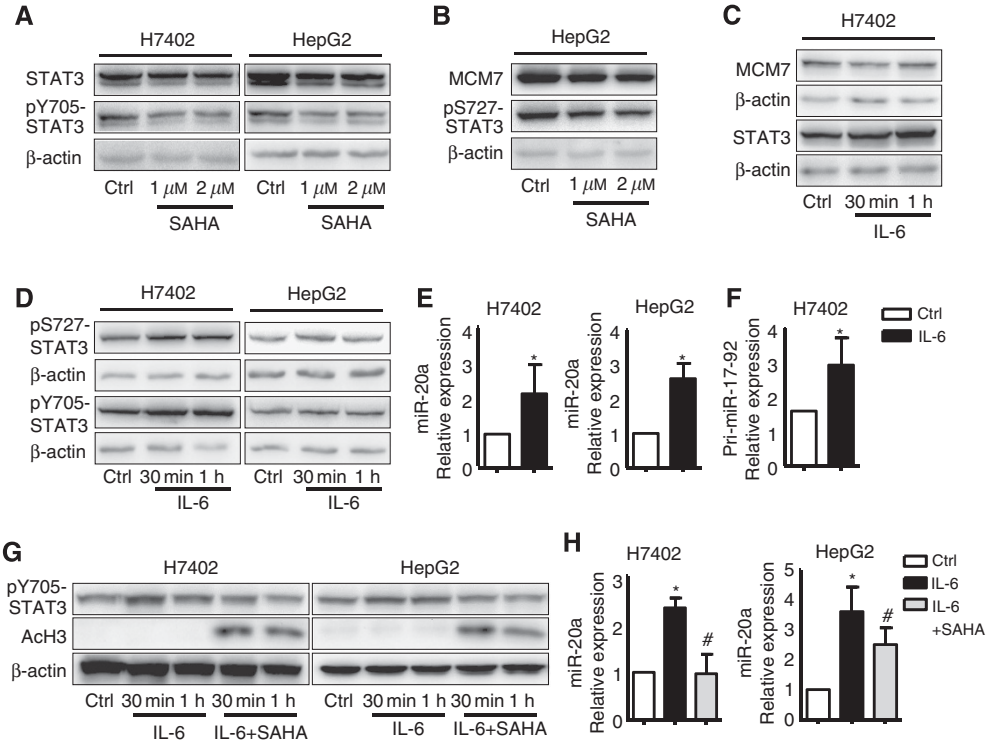

Figure 6. SAHA downregulates the *miR-17-92* cluster by abolishing phosphorylation of STAT3. (**A**, **B**) H7402 cells and HepG2 cells were treated with SAHA (1-2 μM) for 48 h. Cell lysates were subjected to Western blot analysis with anti-STAT3, anti-p-STAT3 (Tyr705), anti-p-STAT3 (Ser727) and anti-MCM7 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.

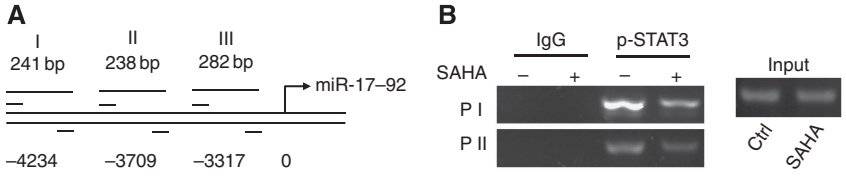

Figure 7. SAHA supresses the binding of activated STAT3 to the promoter region of the *miR-17-92* cluster. (**A**) Schematic representation of the human *miR-17-92* gene. Primer sets are indicated as PI, PII, PIII, occupying predicted STAT3 binding sites within *miR-17-92* promoter. (**B**) H7402 cells 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 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 $p21^{WAF1}$ protein, an inhibitor of cyclin-dependent kinases, stalls cell cycle progression in the G1 phase. The induction of $p21^{WAF1}$ 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

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, especially in K562 cells (Supplementary Figure 1). Over-expression of GATA-2 led to no changes in the expression of the mature transcripts of *miR-20a*, suggesting that GATA-2 may not be involved in the repression of *miR-17-92* expression induced by SAHA.

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 blockage 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 along with 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 *bcl-2* promoter regions was correlated with the transcriptional repression of *bcl-2*, while AcH4 binding did not change significantly. Noh also reported TSA (trichostatin A) augmented acetylation of the $p21^{WAF1}$ 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 deceased transcriptional activity (Struhl, 1998). The contribution of individual HATs to modulation of MCM7 expression requires further investigation. Although Li (Li *et al*, 2013) reported 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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