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Epigenetic regulation of miR-122 by PPAR γ and hepatitis B virus X protein in hepatocellular carcinoma cells

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Abstract

MiR-122, a pivotal liver specific miRNA, has been implicated in several liver diseases including hepatocellular carcinoma (HCC) and hepatitis C and B viral infection. This study aimed to explore epigenetic regulation of miR-122 in human hepatocellular carcinoma (HCC) cells and to examine the effect of hepatitis C virus (HCV) and hepatitis B virus (HBV). We performed microRNA microarray analysis and identified miR-122 as the most up-regulated miRNA (6-fold) in human hepatocellular cancer cells treated with 5'-aza-2'-deoxycytidine (5-Aza-CdR, DNA methylation inhibitor) and 4-phenylbutyric acid (PBA, histone deacetylation inhibitor). Real-time PCR analysis verified significant upregulation of miR-122 by 5'-aza and PBA in HCC cells, and to a lesser extent in primary hepatocytes. Peroxisome proliferator activated receptor-gamma (PPAR γ) and retinoid X receptor alpha (RXR α) complex was found to be associated with the DR1 and DR2 consensus site in the miR-122 gene promoter which enhanced miR-122 gene transcription. 5-Aza-CdR and PBA treatment increased the association of PPAR γ /RXR α , but decreased the association of its co-repressors (N-CoR and SMRT), with the miR-122 DR1 and DR2 motifs. The aforementioned DNA-protein complex also contains SUV39H1, a H3K9 histone methyl transferase, which downregulates miR-122 expression. Our findings establish a novel role of the PPAR γ binding complex for epigenetic regulation of miR-122 in human HCC cells. Moreover, we show that hepatitis B virus X protein (HBX) binds PPAR γ and inhibits the transcription of miR-122, whereas hepatitis C viral particles exhibited no significant effect; these findings provide mechanistic insight into reduction of miR-122 in patients with HBV but not with HCV infection.

Keywords

miR-122; PPAR γ ; HCC; epigenetic regulation; Hepatitis B virus X protein; HCV; liver; hepatocytes

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No conflicts of interest exist

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and the third most common cause of cancer-related death worldwide(1). Major risk factors for HCC include infection of hepatitis C virus (HCV) and hepatitis B virus (HBV), alcoholic and nonalcoholic fatty liver diseases(2). The global burden of HCC is expected to increase in the next decades, with HCV infection responsible for the rising incidence of HCC in the United States and HBV infection as the leading cause of HCC globally. Most of the HCC risk factors are known to cause epigenetic changes such as DNA methylation and histone modification, although the precise gene targets and the underlying mechanisms remain incompletely defined.

MicroRNAs have emerged as important regulators of gene expression in both normal and disease states(3, 4). Recent evidence suggests deregulation of miRNAs in hepatocarcinogenesis and tumor progression(5, 6). In this context, it is noticeable that epigenetic modification is being recognized as a key mechanism for regulation of miRNA expression(7), although it remains largely unknown whether miRNAs in the liver are epigenetically regulated.

miR-122 is the most highly expressed miRNA in the liver(8) and is implicated in several important aspects of liver pathobiology, including hepatocarcinogenesis, HCV replication, lipid metabolism and iron homeostasis(9–13). miR-122 is known to bind 5'-UTR of the HCV genome and stimulate the translation of HCV RNA(13); accordingly, inhibition of miR-122 decreases HCV viral load in cultured cells and in chimpanzee model of HCV infection(14). The level of miR-122 is decreased in patients with HBV infection(15), although the mechanism for HBV-mediated reduction of miR-122 is not known. Deletion of miR-122 in mice has been shown to cause hepatosteatosis, liver inflammation and fibrosis, and ultimately hepatocellular carcinoma(16, 17). Several liver enriched transcription factors (LETEs) are known to regulate miR-122 expression(18, 19); however, little is known about epigenetic regulation of miR-122 expression in the liver. Additionally, while recent studies have documented the effect of miR-122 in the liver(20), it remains unknown how miR-122 expression is regulated in hepatocellular cancer cells and in liver diseases.

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily(21). PPAR γ forms a heterodimer with retinoid X receptor α (RXR α) and binds to the DNA response element consisting of a direct repeat of two hexanucleotides spaced by one or two nucleotide (DR1 or DR2 motif, respectively)(22, 23). In the absence of ligands, PPAR γ /RXR α associate with corepressors such as the nuclear receptor corepressor protein (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT); this nuclear receptor co-repressor complex modify the chromatin environment through recruitment of histone deacetylase (HDAC) or histone methyltransferase (HMT), thereby downregulating transcriptional activity(24, 25). In the presence of PPAR γ ligands, the corepressors become dissociated from PPAR γ /RXR α , thus enabling gene transcription.

In the present study, we performed miRNA microarray analysis and our data showed that miR-122 is one of the most up-regulated miRNA in HCC cells treated with the epigenetic drugs (5-Aza-CdR and PBA). Given that the promoter region of miR-122 contains DR1 and DR2 motifs(19), we postulated that PPAR γ /RXR α complex might be implicated in epigenetic regulation of miR-122 during hepatocarcinogenesis. Indeed, our experimental results demonstrate that PPAR γ /RXR α associate with DR1 and DR2 motifs of the miR-122 promoter to regulate miR-122 expression in HCC cells and the effect is dependent on two PPAR γ corepressors, N-CoR and SMRT, and a key HMT, SUV39H1. Moreover, our data

show that hepatitis B virus X protein (HBX) binds PPAR γ and inhibits the transcription of miR-122 gene, which provides mechanistic explanation for the intriguing differential regulation of miR-122 by hepatitis B and C viruses.

EXPERIMENTAL PROCEDURES

Cell culture and reagents

Cells were maintained at 37°C and 5% CO₂. Human hepatocellular cancer cell lines (HepG2, Huh7 and Hep3B cells) were obtained from the American Type Culture Collection (Rockville, MD). HepG2 and Hep3B cells were cultured in minimum essential medium (MEM) and Huh7 cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco) and antibiotic, respectively. Huh7.5 cells line was obtained from the laboratory of Charlie Rice (The Rockefeller University, New York) and were cultured in DMEM with 10% FBS and antibiotics. Primary human hepatocyte cultures were purchased from Lonza (Walkersville, MD) and cultured in collagen I coated plates (BD Bioscience, Bedford, MA) with hepatocyte basal medium supplemented with HCM SingleQuots growth factors (Lonza, Walkersville, MD). HepG2.2.15 cells were maintained in DMEM containing 10% FBS as previously described(26). The differentiated HepaRG cells(27) were purchased from Invitrogen (Carlsbad, CA) and maintained in William's medium E with GlutaMax-1, supplemented with General Purpose Working Medium (Invitrogen). The immortalized untransformed human neonatal liver NeHepLxHT cells were purchased from American Type Culture Collection and cultured as described(28). The 5-Aza-2'-deoxycytidine (5-Aza-CdR), 4-phenylbutyric acid (PBA), Chaetocin and 9-cis retinoic acid were obtained from Sigma-Aldrich (St. Louis, MO). Pharmacological PPAR γ ligands (rosiglitazone, troglitazone, ciglitazone, 15-keto prostaglandin E₂, 15-deoxy-12, 14-prostaglandin J₂) were purchased from Cayman chemical (Ann Arbor, MI). Antibodies against di-methyl and tri-methyl histone H3K9, PPAR γ (ChIP grade), SMRT and acetyl histone were obtained from Abcam (Cambridge, MA). Anti-N-CoR antibody was purchased from Millipore (Billerica, MA). Anti-C/EBP α , anti-Akt, anti-PTEN, anti-mTOR, anti-phospho-mTOR, anti-Smad3, anti-Smad 4 and anti-SAPK/JNK were obtained from Cell signaling (Beverly, CA). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Hepatitis C virus infection and detection

HCV virus infection was performed as described previously(29, 30). Huh7.5 cells were transfected with 20 μ g of in vitro transcribed full-length HCV JFH1-GFP RNA by electroporation method. After 72 hours, the lysates and supernatant were collected by scraping and freeze-thaw cycle in dry-ice and infectious virus was clarified by centrifugation at 3,400 rpm for 5 minutes. Huh7.5 cells were plated on 6 well plates and the infectious culture medium containing HCV viral particles or control spent medium were added. 96 hours post-infection, the HCV infected GFP-positive cells were detected by fluorescence microscopy and quantitative real-time RT-PCR as previously described(29, 30) [the forward primer sequence is 5'-TCTTCACGCAGAAAGCGTCTA-3'; the reverse primer sequence is 5'-CGGTTCCGCAGACCACTATG-3'; the probe sequence is 5-'TGAGTGTCTGTCAGCCTCCAGGA-3', labeled at the 5' end with FAM (6-carboxyfluorescein) fluorophore reporter molecule and at the 3' end with TAMRA (6-carboxytetramethylrhodamine) quencher molecule].

Hepatitis B virus infection and detection

The HepG2.2.15 cell line was used for production of Hepatitis B viral particles. HBV inoculum was prepared from freshly collected supernatants of HepG2.2.15 cells by ultracentrifugation in Beckman rotor at 40,000 rpm for 1 hr at 4°C. The pellet was

resuspended in Williams E medium. HepaRG cells were incubated with concentrated infectious source diluted 2 fold in culture medium supplemented with 4% PEG 8000 for 20 hr at 37°C. At the end of the incubation, the cells were washed three times with the culture medium and maintained for 7 days (the medium was exchanged every 2 days). For detection of HBV DNA, the cells and supernatants were collected and DNA was extracted and purified with Qiagen DNeasy kit (Qiagen). The purified total DNA was used as template for quantitative RT-PCR. RT-PCR was performed using SYBR green PCR kit (Qiagen). Primers for amplification of the HBV DNA were 5'-ATCTTCTTGTGGTTCTTCT-3' (forward) and 5'-CTGAAAGCCAAACAGTGG-3' (reverse). For detection of HBX, total RNA isolated using Trizol reagent (Invitrogen) was reversely transcribed with Superscript II RT reagent kit (Invitrogen, Carlsbad, CA), followed by quantitative RT-PCR. Primers for amplification of the HBX mRNA were 5'-TCTCAGCAATGTCAACGAC-3' (forward) and 5'-TTTATGCCTACAGCCTCCT-3' (reverse), and for the glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA were 5'-TTGCCATCAATGACCCCTTCA-3' (forward) and 5'-GCCCCACTTGATTTTGA-3' (reverse).

RESULTS

Epigenetically regulated miRNAs in human hepatocellular carcinoma cells

To identify epigenetically regulated miRNAs in HCC, we performed miRNA microarray in human hepatocellular cancer cells (HepG2) treated with the DNA methylation inhibitor (5-aza-2'-deoxycytidine, 5-Aza-CdR) and the histone deacetylase inhibitor (4-phenylbutyric acid, PBA). The microarray data were analyzed by using hierarchical clustering of the log₂ value and displayed in a heatmap (Figure 1A). Out of 837 human miRNAs that were analyzed, 43 miRNAs were differentially expressed in 5-Aza-CdR and PBA treated cells compared to control vehicle treated cells (at the level of $p < 0.01$). The up-regulated miRNAs include miR-122, miR-30e, miR-3922-5p, miR-125-5p, and miR-224; the down-regulated miRNAs include miR-654-3p, miR-4481, miR-133a, and miR-133b. Among these, miR-122 was identified as the most up-regulated miRNA (6.6 fold, Figure 1B).

Given that miR-122 is the dominant hepatocyte-specific miRNA (accounting for approximately 70% of the liver's total miRNAs)(8, 31) and its expression is decreased during hepatocarcinogenesis(16, 32), we elected to focus on epigenetic regulation of miR-122. As determined by qRT-PCR analysis, 5-Aza-CdR/PBA treatment increased the levels of mature miR-122 in two hepatocellular cancer cell lines, HepG2 and Huh7, by 11.6- and 4.2-folds, respectively (Figure 1C). 5-Aza-CdR and PBA treated HepG2 and Huh7 cells also showed significantly higher expression of pri-miR-122 level (6.3- and 5-fold increase, respectively) (Figure 1D), suggesting that epigenetic up-regulation of miR-122 occurs predominantly at the transcriptional level. We observed that primary human hepatocytes express much higher basal level of miR-122 compared to hepatocellular cancer cell lines (HepG2, Huh7 and Hep3B) (Figure 1E). However, in primary human hepatocytes, miR-122 expression was up-regulated to a lesser extent by 5-Aza-CdR/PBA (1.8-fold, Figure 1F). These results suggest that the expression of miR-122 in HCC cells is epigenetically suppressed to a greater extent than in primary hepatocytes.

5-Aza-CdR and PBA induce PPAR γ /RXR α binding to DR1 and DR2 motifs of miR-122 gene promoter

The promoter region of miR-122 gene contains specific binding sites for the liver-enriched transcription factors (LETFs), such as hepatocyte nuclear factors-4 α (HNF-4 α) and CCAAT/enhancer-binding proteins (C/EBPs), which regulate miR-122 gene transcription(18, 19). To determine whether 5-Aza-CdR/PBA induce miR-122 expression through induction of these LETFs, we examined the protein levels of HNF-4 α , C/EBP α and

related signaling molecules. As shown in Supplemental Figure S1, the levels of these molecules were not significantly altered by 5-Aza-CdR and PBA treatment. We observed that the level of E-cadherin expression is increased in HepG2 cells treated with 5-Aza-CdR and PBA; this finding is consistent with the notion that E-cadherin expression is frequently suppressed by epigenetic mechanisms such as promoter hypermethylation in HCC(33).

Given that the promoter region of miR-122 contains DR1 and DR2 motifs which are recognized by several members of the nuclear hormone receptor superfamily including PPAR γ (19), we performed DNA-pull down assay using biotinylated DR1 or DR2 oligonucleotides corresponding to the miR-122 promoter. As shown in Figure 2A, PPAR γ and RXR α bound to miR-122 DR1 and DR2 motifs and the association was significantly enhanced by 5-Aza-CdR and PBA. In contrast, PPAR α did not bind to either DR1 or DR2 consensus site. Transient transfection with a PPAR γ expression vector further enhanced 5-Aza-CdR/PBA-induced PPAR γ binding to miR-122 DR1 and DR2 motifs (Figure 2B). To further determine PPAR γ association with miR-122 promoter DNA, we performed chromatin immunoprecipitation (ChIP) assay using three specific primer sets corresponding to the three DR1 and DR2 regions in miR-122 gene promoter. As shown in Figure 2C, 5-Aza-CdR/PBA treatment significantly enhanced PPAR γ binding to the DR1 and DR2 regions of the miR-122 promoter. To analyze for miR-122 gene promoter transcription activity, we generated a luciferase reporter construct containing the DR1 and DR2 regions of the miR-122 promoter and our data showed that 5-Aza-CdR/PBA treatment significantly increased miR-122 gene promoter luciferase reporter activity (Figure 2D). Therefore, 5-Aza-CdR and PBA treatment induces PPAR γ /RXR α complex association with the DR1 and DR2 element and this mechanism is implicated in 5-Aza-CdR/PBA-induced miR-122 expression.

As the activity of PPAR γ /RXR α is influenced by specific ligands, we next examined the effect of PPAR γ and RXR α ligands on miR-122 expression. For these experiments, HepG2 cells were treated with the PPAR γ agonists, 15-deoxy-prostaglandin J₂ (15d-PGJ₂, 10 μ M) or 15-keto-prostaglandin E₂ (15-keto-PGE₂, 10 μ M), and the RXR α agonist, 9-cis-retinoic acid (9-cis RA, 10 μ M). As shown in Figure 2E, the expression of miR-122 was increased by these three agonists and the effects were further augmented when PPAR γ protein was overexpressed. Treatment with additional PPAR γ agonists (rosiglitazone, troglitazone, ciglitazone) also increased the expression of miR-122 in PPAR γ overexpressed HepG2 cells (Figure 2F). To evaluate the effects of PPAR γ on miR-122 expression in non-malignant hepatocytes, NeHepLxHT cells (immortalized untransformed neonatal hepatocytes) were transfected with PPAR γ siRNA or expression vector. As shown Figure 2G, knockdown of PPAR γ decreased miR-122 expression, whereas overexpression of PPAR γ increased it. These results demonstrate that miR-122 expression is positively regulated by PPAR γ and RXR α in cells of hepatocyte origin.

5-Aza-CdR and PBA induce N-CoR and SMRT dissociation from PPAR γ and DR1/DR2 complex

Given that N-CoR and SMRT are co-repressors of PPAR γ (34), we performed DNA-pull down assay to determine their association with the miR-122 DR1 and DR2 motifs. Our data showed that 5-Aza-CdR and PBA treatment decreased the binding of N-CoR and SMRT to DR1 and DR2 oligonucleotides (Figure 3A). Accordingly, co-immunoprecipitation assay showed that 5-Aza-CdR and PBA treatment led to dissociation of N-CoR and SMRT from PPAR γ (Figure 3B), although the protein levels of N-CoR and SMRT were not altered. These findings suggest that dissociation of N-CoR and SMRT from PPAR γ and DR1/DR2 complex contribute to 5-Aza-CdR/PBA-induced miR-122 expression.

The role of SUV39H1 and histone modification in miR-122 expression

Epigenetic regulation of gene expression is known to involve DNA methylation and histone modifications (acetylation and/or methylation). As miR-122 gene promoter contains no CpG island, we performed further experiments to determine whether histone modification might be involved in miR-122 regulation. As shown in Figure 3C, 5-Aza-CdR/PBA treatment decreased the level of SUV39H1, a H3K9 histone methyl transferase (HMT), in both HepG2 and Huh7 cells. Consistent with this, the association of SUV39H1 with miR-122 DR1 and DR2 motifs was also reduced after 5-Aza-CdR/PBA treatment (Figure 3D). Thus, SUV39H1 is a negative regulator for miR-122 gene expression; this assertion is consistent with the well-documented repression of gene transcription by SUV39H1 and its enzymatic products (H3K9 dimethyl and trimethyl)(35, 36). To further determine the role of SUV39H1 in miR-122 expression, we assessed miR-122 levels in cells transfected with SUV39H1 targeting siRNAs. As shown in Figure 3E, knockdown of SUV39H1 by two different siRNAs enhanced miR-122 expression by 5.3- and 4.3-folds, respectively. Similarly, inhibition of SUV39H1 by its pharmacological inhibitor, chaetocin, increased miR-122 expression in both HepG2 and Huh7 cells (Figure 3F). These findings are consistent with the observation that the levels of H3K9 dimethyl and trimethyl were decreased in human primary hepatocytes compared to hepatocellular cancer cells (Supplemental Figure S2). Furthermore, ChIP assay revealed that histone acetylation around DR1 and DR2 regions of the miR-122 promoter was increased in cells treated with 5-Aza-CdR and PBA (Figure 3G). Taken together, these results suggest the role of SUV39H1-mediated histone H3K9 methylation and histone acetylation in regulation of miR-122 expression.

Hepatitis C virus does not influence miR-122 expression

Hepatitis C and hepatitis B virus infection are major epigenetic factors associated with HCC. miR-122 has been shown to bind 5'-UTR of HCV RNA leading to HCV accumulation(13). To investigate whether HCV infection might influence miR-122 expression, we utilized Huh7.5 cells (which are optimal for HCV infection). In this system, more than 80% of the Huh7.5 cells become infected 96 hours after addition of the hepatitis C virus (clone JFH1-GFP), as visualized by GFP fluorescence; successful infection is also verified by qRT-PCR analysis for HCV RNA (Figure 4A). As shown in Figure 4B, the levels of miR-122 expression were not significantly different between HCV-infected and control cells. Likewise, HCV infection did not significantly alter miR-122 promoter luciferase reporter activity (Figure 4C). Thus, HCV infection does not significantly influence miR-122 expression.

Hepatitis B virus down-regulates miR-122 expression

The expression of miR-122 is known to be down-regulated in patient with HBV infection(15). To investigate the relationship between miR-122 expression and HBV, we utilized HepG2.2.15 cells, which are derived from HepG2 cells and characterized by having stable HBV expression and replication in culture systems(37). As shown in Figure 5A, successful HBV replication was confirmed by the presence of HBV DNA in HepG2.2.15 cells but not in HepG2 cells, whereas the expression of miR-122 was markedly down-regulated in HepG2.2.15 cells compared to HepG2 cells. Additionally, we performed HBV infection experiments by using supernatants of HepG2.2.15 cells in the presence of 4 % polyethylene glycol (PEG). As shown in Figure 5B and C, HBV infected HepaRG and primary human hepatocytes showed significantly decreased miR-122 compared to uninfected cells (optimal infection efficiency was confirmed by detecting HBV DNA and HBX mRNA in infected cells). These results suggest that HBV infection down-regulates the expression of miR-122.

The effect of hepatitis B virus X protein (HBX) on miR-122 expression

Given that hepatitis B virus X protein (HBX) plays a pivotal role in HBV-mediated hepatocarcinogenesis and that HBX is known to modulate transcription machinery via protein-protein interaction(38), we investigated the potential effect of HBX on miR-122 expression in our system. As shown in Figure 6A, transfection of HBX decreased miR-122 expression in HepG2 and Huh7 cells as well as in primary human hepatocytes. Accordingly, transfection of HBX also reduced miR-122 promoter luciferase reporter activity (Figure 6B). On the other hand, siRNA knockdown of HBX in HepG2.2.15 cells significantly increased the expression of miR-122 (Figure 6C). These results suggest that HBX protein is able to down-regulate miR-122 expression. Co-immunoprecipitation assay showed that HBX bound to PPAR γ (Figure 6D), which is consistent with the previous report that HBX binds to the DNA binding domain of PPAR γ and suppresses PPAR γ -mediated transactivation(39). These observations suggest that HBX protein negatively regulates miR-122 expression through binding and inhibiting PPAR γ . The role of PPAR γ for suppression of miR-122 gene transcription is further corroborated by the observation that overexpression of PPAR γ prevented HBX-induced reduction of miR-122 mature and pri-miRNA levels (Figure 6E and 6F). Taken together, these results provide mechanistic explanation for reduction of miR-122 in HBV-infected patients as recently reported by Wang and colleagues(15).

DISCUSSION

The present study discloses a novel epigenetic regulatory mechanism for miR-122 expression in HCC cells, which involves PPAR γ /RXR α binding to DR1 and DR2 motifs of the miR-122 promoter. Our findings suggest that this process is influenced by the PPAR γ co-repressors (N-CoR and SMRT) and by the histone methyl transferase (SUV39H1). We observe that PPAR γ and RXR α bind to DR1 and DR2 motifs of the miR-122 promoter and their association is significantly increased in HCC cells treated with 5-Aza-CdR and PBA. The association is specific for PPAR γ isoform, as PPAR α did not bind to DR1 and DR2 motifs. Consistent with these findings, we observed that treatment with the PPAR γ and RXR α agonists increased the expression of miR-122 in HCC cells. Additionally, overexpression and knockdown studies showed that PPAR γ also regulated the expression of miR-122 in non malignant hepatocytes. These findings suggest that PPAR γ and RXR α are positive regulators for miR-122 expression. On the other hand, we observed that 5-Aza-CdR and PBA treatment reduced the interaction of N-CoR/SMRT with PPAR γ /RXR α and with DR1 and DR2 elements in the miR-122 promoter, suggesting that the PPAR γ co-repressors, N-CoR and SMRT, are negative regulators for miR-122 expression. In addition, we found that 5-Aza-CdR and PBA treatment inhibited the expression of SUV39H1 (a H3K9 methyltransferase that catalyzes the formation of H3K9 dimethyl and trimethyl, leading to suppression of gene transcription) and decreased SUV39H1 binding to the DR1 and DR2 regions of the miR-122 promoter. The role of SUV39H1 for miR-122 suppression is further supported by the observation that knockdown or inhibition of SUV39H1 enhanced miR-122 expression in HCC cells. The latter finding is also corroborated by the observation that human primary hepatocytes contain lower levels of H3K9 dimethyl and trimethyl compared to HCC cells. Thus, SUV39H1 is another negative regulator for miR-122 expression in HCC cells. Collectively, our findings suggest that PPAR γ and RXR α -mediated miR-122 expression is suppressed by N-CoR/SMRT/SUV39H1 in HCC cells (illustrated in Figure 7). It is plausible that reduction of SUV39H1 by 5-Aza-CdR and PBA may lead to dissociation of N-CoR/SMRT/SUV39H1 from the PPAR γ /RXR α and DR1/DR2 binding complex, thus allowing transcription of the miR-122 gene. Furthermore, we observed that 5-Aza-CdR and PBA treatment also increased histone acetylation around miR-122 promoter regions. Therefore, epigenetic regulation of miR-122 in HCC cells is a complicated process which

involves the PPAR γ /RXR α /N-CoR/SMRT/SUV39H1/DR1/DR2 binding complex, histone acetylation, and histone H3K9 methylation.

Previous studies have shown that miR-122 expression is associated with the level of essential liver-enriched transcription factors (LETFs) including HNF4 α and C/EBP α (11, 18, 19). However, HNF4 α and C/EBP α do not appear to be implicated in epigenetic silencing of miR-122 in HCC cells, as their levels were not significantly altered by 5-Aza-CdR and PBA treatment in our system. It is worth mentioning that C/EBP α is epigenetically silenced in several extrahepatic cancers including lung cancer and acute myelogenous leukemia(40, 41) and HNF4 α is decreased due to epigenetic silencing of transcription factor 2 (TCF2) in ovarian cancer(42). Thus, susceptibility to epigenetic modifications may depend on specific tissue context and different tumor types.

Hepatitis C and B viruses are major epigenetic environmental factors that are closely associated with HCC development. In this study, we investigated the mechanism for differential regulation of miR-122 by HCV and HBV. It is well known that miR-122 is essential for HCV RNA accumulation by binding to the 5'-UTR of the viral genome. Previous reports have shown that miR-122 stimulates HCV viral protein translation and binds HCV RNA in association with Ago2 protein thereby slowing decay of the viral RNA in infected cells. Silencing of miR-122 with a locked nucleic acid (LNA)-modified oligonucleotide leads to suppression of HCV viremia and improvement of HCV-induced liver pathology in chronic HCV infected chimpanzees model(14). However, whether HCV is able to regulate miR-122 expression was not known prior to the current study. In our study, we did not observe significant changes in miR-122 expression and promoter activity in Huh7.5 cells infected with HCV particles. Thus, HCV infection does not appear to significantly influence the level of miR-122, despite that miR-122 positively regulates HCV replication and stability. Our findings are also consistent with the previous report showing no correlation between HCV RNA and miR-122 levels in liver biopsies from patients with chronic hepatitis C(43).

In contrast to HCV infection, miR-122 was significantly down-regulated in patients with HBV infection(15). miR-122 is known to down-regulate cyclin G1 and interrupt cyclin G1 interaction with p53, thus abrogating p53-mediated inhibition of HBV replication(15). However, the mechanism for HBV-mediated down-regulation of miR-122 was not known prior to the current study. In this paper, we employed three HBV infection systems (stable HBV expression in HepG2.2.15 cells, transient HBV infection in HepaRG cells, and transient HBV infection in primary human hepatocytes); all of these systems showed that HBV infection down-regulated miR-122 expression. Our data indicate that HBX overexpression significantly decreases miR-122 gene promoter activity and down-regulates miR-122 expression, whereas siRNA knockdown of HBX enhances miR-122 expression. A previous study reports that HBX binds PPAR γ and thus blocks nuclear localization and suppresses PPAR γ -mediated transactivation(39). In our system, we also observed that HBX directly interacted with PPAR γ , although we did not observe nuclear translocation of PPAR γ by HBX, as PPAR γ is localized in the nucleus and HBX is localized in both cytoplasm and nucleus in HepG2 cells (Supplemental Figure S3). Our findings provide novel evidence for HBX-mediated inhibition of miR-122 transcription by PPAR γ .

In summary, this study provides the first evidence for epigenetic regulation of miR-122, a pivotal liver specific miRNA, by PPAR γ /RXR α complex through interaction with the co-repressors and H3K9 histone methyltransferase (SUV39H1) in HCC cells. Our data also provide important mechanistic insight into differential regulation of miR-122 by HBV and HCV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-Aza-CdR	5'aza-2'deoxyctidine
C/EBP	CCAAT/enhancer-binding protein
ChIP	chromatin immunoprecipitation
HBV	hepatitis B virus
HBX	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAC	histone deacetylase
HMT	histone methyltransferase
HNF-4α	hepatocyte nuclear factors-4 α
LETFs	liver-enriched transcription factors
N-CoR	nuclear receptor corepressor protein
PBA	4-phenylbutyric acid
PPARγ	peroxisome proliferator activated receptor-gamma
RXRα	retinoid X receptor alpha
SMRT	silencing mediator of retinoid and thyroid hormone receptors

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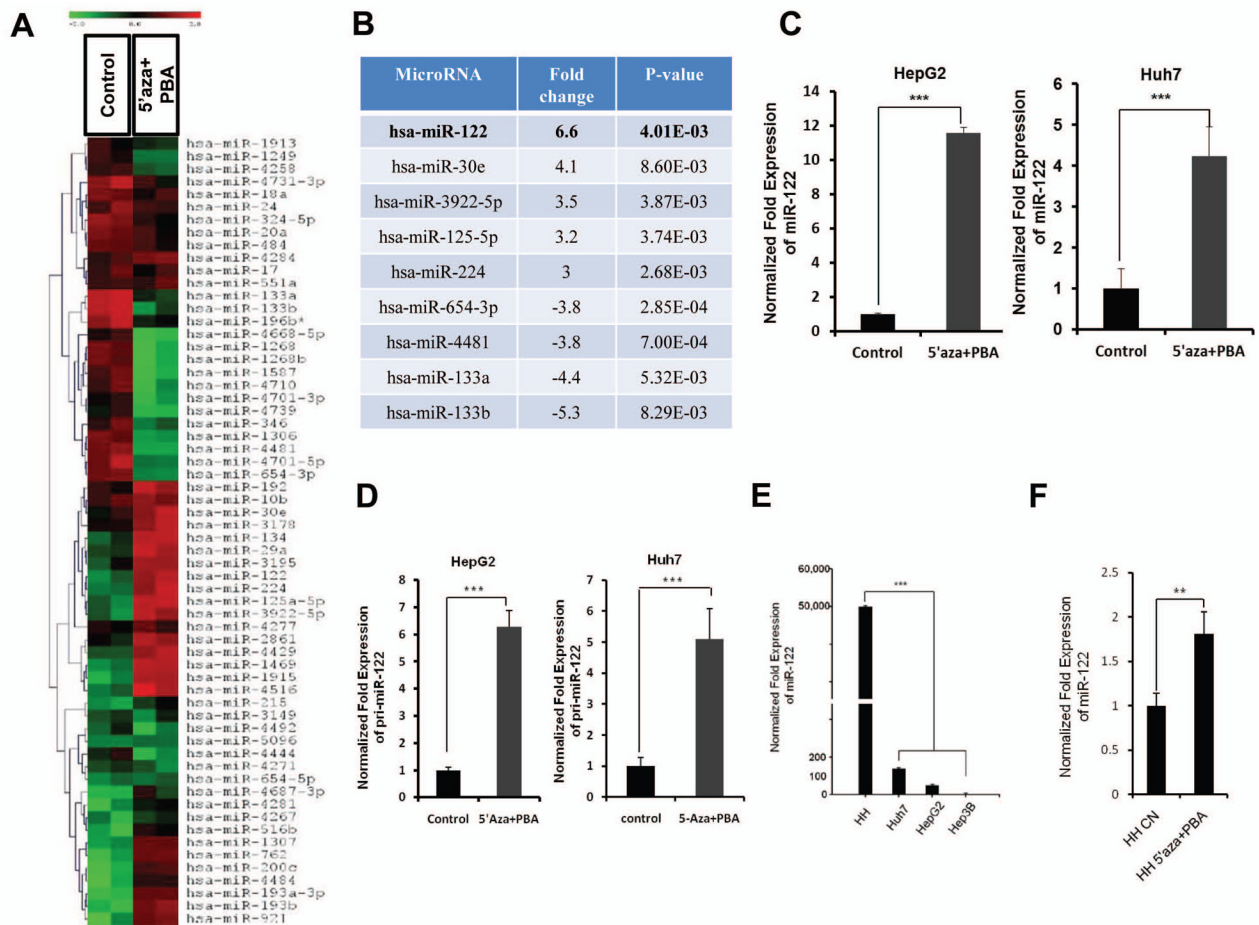


Figure 1. The expression of miR-122 is epigenetically suppressed in HCC cells

(A) MiRNA expression heat map depicting miRNAs differentially expressed ($p < 0.01$) in HepG2 cells treated with control vehicle or with 5-Aza-CdR ($3 \mu\text{M}$) and PBA (3mM) for 48 hours. (B) Summary of miRNA microarray data with at least 3 fold changes (compared to control) (p-value was calculated by ANOVA). (C) qRT-PCR analysis of mature miR-122 in HepG2 and Huh7 cells treated with control vehicle or with 5-Aza-CdR and PBA for 48 hours. The data were normalized to U6 RNA. (D) qRT-PCR analysis of pri-miR-122 in HepG2 and Huh7 cells treated with control vehicle or with 5-Aza-CdR and PBA for 48 hours. (E) qRT-PCR of miR-122 expression in HCC cell lines compared to human primary hepatocytes. (F) qRT-PCR of miR-122 expression in human primary hepatocytes treated with 5-Aza-CdR ($3 \mu\text{M}$) and PBA (3mM) for 48 hours. The data represent mean + SD (** $P < 0.001$, $n = 3$).

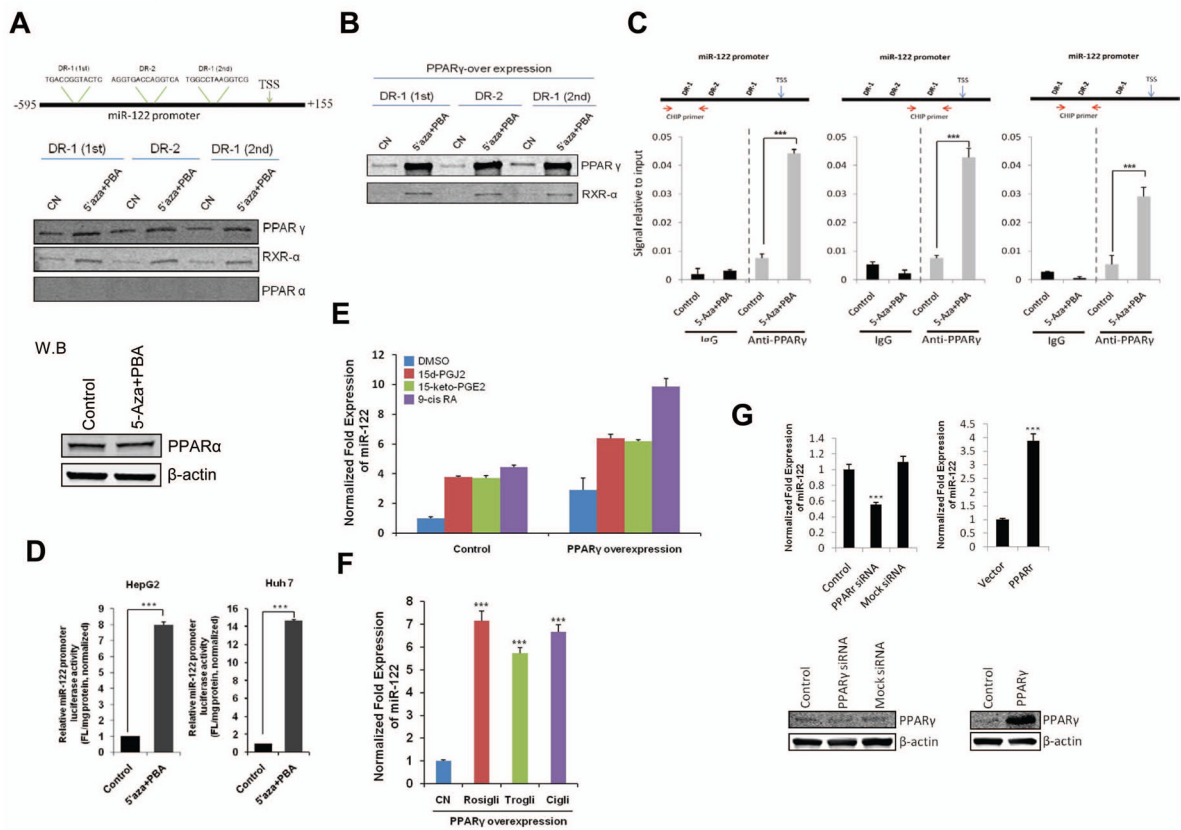


Figure 2. PPAR γ /RXR α complex in 5-Aza-CdR/PBA-induced miR-122 expression
(A) 5-Aza-CdR/PBA induce the binding of endogenous PPAR γ /RXR α to the DR1 and DR2 consensus site. (*Upper panel*) schematic representation of putative PPAR γ /RXR α binding sites in human miR-122 gene promoter. (*Mid panel*) Equal amount of cell lysates from HepG2 cells were incubated with biotinylated double-stranded oligonucleotides corresponding to the DR1 and DR2 motifs in miR-122 promoter and with streptavidin-agarose beads. The precipitated complexes were subjected to SDS-PAGE and Western blotting. (*Lower panel*) Western blot for PPAR α in HepG2 cells with or without 5-Aza-CdR/PBA treatment. **(B)** 5-Aza-CdR/PBA induce PPAR γ /RXR α binding to miR-122 DR1 and DR2 motifs in HepG2 cells with PPAR γ overexpression. After transient transfection of PPAR γ expression vector, the cells were treated with 5-Aza-CdR/PBA for 48 hours and the cell lysates were obtained for DNA pull down assay. **(C)** ChIP assay. The chromatin extracted from HepG2 cells treated with 5-Aza-CdR/PBA or control vehicle were subjected to immunoprecipitation with PPAR γ antibody and the precipitates were subjected to qRT-PCR analysis using primers to amplify DR1 and DR2 regions as indicated in the schematic diagram (the arrowheads show the primer regions in the miR-122 promoter). Normal rabbit IgG was used as the negative control. **(D)** Effect of 5-Aza-CdR and PBA on miR-122 promoter luciferase activity in HepG2 and Huh7 cells. After transient transfection of miR-122-Luc promoter vectors, the cells were treated 5-Aza-CdR and PBA for 48 hours and the cell lysates were obtained for luciferase activity. **(E)** qRT-PCR for mature miR-122 in HepG2 cells treated with the PPAR γ agonist (15-d-PGJ₂, 15-keto-PGE₂) or RXR α agonist (9-cis RA). The cells were transiently transfected with the PPAR γ expression vector or control vector and the cells were incubated for 24 hours with DMSO or 10 μ M agonist. qRT-PCR was performed to measure mature miR-122. **(F)** qRT-PCR for mature miR-122 in PPAR γ overexpressed HepG2 cells treated with 10 μ M of the PPAR γ agonists

(rosiglitazone, troglitazone and ciglitazone) or the vehicle control (DMSO). (G) qRT-PCR for mature miR-122 in NeHepLxHT cells transfected with the PPAR γ siRNA or the PPAR γ expression vector (*Upper panel*). Knockdown or overexpression of PPAR γ in NeHepLxHT cells were confirmed by western blotting (*Lower panel*). The data represent mean + SD from (***) $P < 0.001$, n = 3).

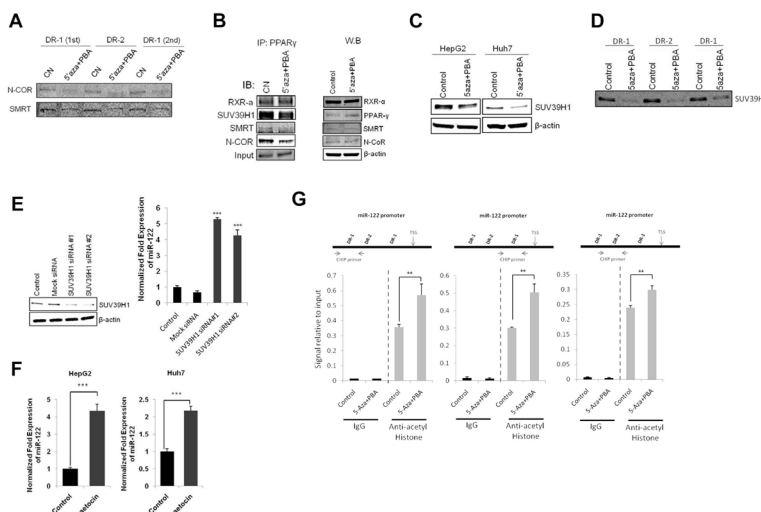


Figure 3. 5-Aza-CdR/PBA-induced miR-122 expression is associated with N-CoR/SMRT corepressor dissociation and inhibition of SUV39H1
(A) Binding of endogenous N-CoR and SMRT to miR-122 DR1 and DR2 motifs in HepG2 cells treated with control vehicle or with 5-Aza-CdR/PBA. **(B)** Effect of 5-Aza-CdR and PBA on PPAR γ association with N-CoR, SMRT and SUV39H1 in HepG2 cells. (*Left panel*) The cell lysates were immunoprecipitated with anti-PPAR γ antibody followed by immunoblotting with indicated antibodies. (*Right panel*) Conventional western blotting using indicated antibodies. **(C)** Effect of 5-Aza-CdR and PBA on SUV39H1 expression in HepG2 and Huh7 cells. The cells were treated with 5-Aza-CdR/PBA for 48 hours and the cell lysates were obtained for Western blotting with anti-SUV39H1 antibody. **(D)** Binding of SUV39H1 to miR-122 DR1 and DR2 motifs. HepG2 cells were treated with 5-Aza-CdR and PBA for 48 hours and the cell lysates were incubated with biotinylated DR1 and DR2 oligonucleotides. The samples were subjected to western blotting using anti-SUV39H1. **(E)** The effect of SUV39H1 knockdown on miR-122 expression. HepG2 cells were transfected with two different siRNAs targeting SUV39H1 (100 nM) or mock siRNA as control. The efficiency of SUV39H1 knock-down was analyzed by western blotting 72 hours after transfection (*left panel*). qRT-PCR for mature miR-122 was performed (*right panel*). **(F)** The SUV39H1 inhibitor, chaetocin, increased miR-122 expression in HepG2 and Huh7 cells. The cells were treated with 200 nM chaetocin for 48 hours and qRT-PCR was performed to determine the level of miR-122. **(G)** Effect of 5-Aza-CdR/PBA on histone acetylation (chromatin immunoprecipitation assay). Chromatins extracted from HepG2 cells treated with 5-Aza-CdR/PBA or control vehicle were subjected to immunoprecipitation with anti-acetyl histone antibody. The precipitates were subjected to qRT-PCR analysis using primers to amplify DR1 and DR2 regions of the miR-122 gene promoter as indicated in the schematic diagram. The data represent mean + SD (**P<0.01, ***P<0.001; n = 3).

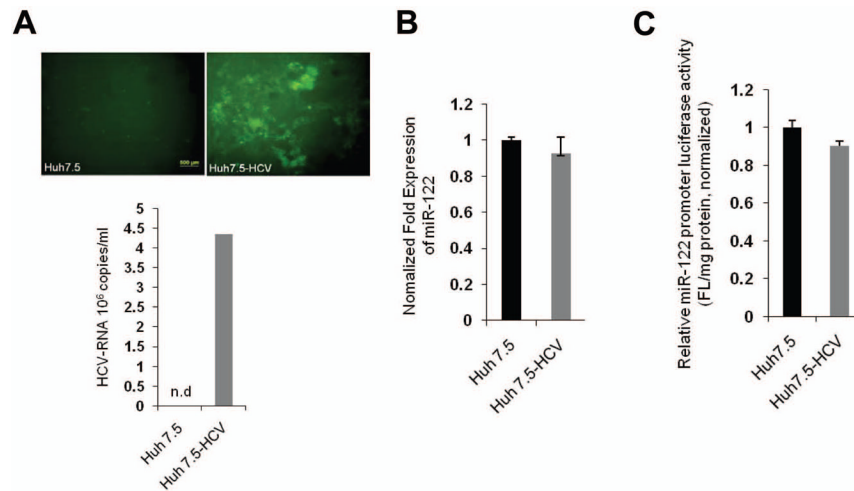


Figure 4. Effect of HCV on miR-122 expression

(A) Huh7.5 cells were infected with JFH1-GFP-HCV virus for 96 hours. (*Upper panel*) Fluorescence microscopy showing intracellular expression of GFP in cells infected with JFH1-GFP-HCV. (*Lower panel*) qRT-PCR analysis for HCV RNA in cells with or without JFH1-GFP-HCV infection. (B) qRT-PCR analysis for miR-122 in Huh7.5 cells with or without JFH1-GFP-HCV infection for 96 hours. The results were normalized to the level of U6 RNA. (C) miR-122 promoter luciferase reporter activity in Huh7.5 cells with or without JFH1-GFP-HCV infection for 96 hours.

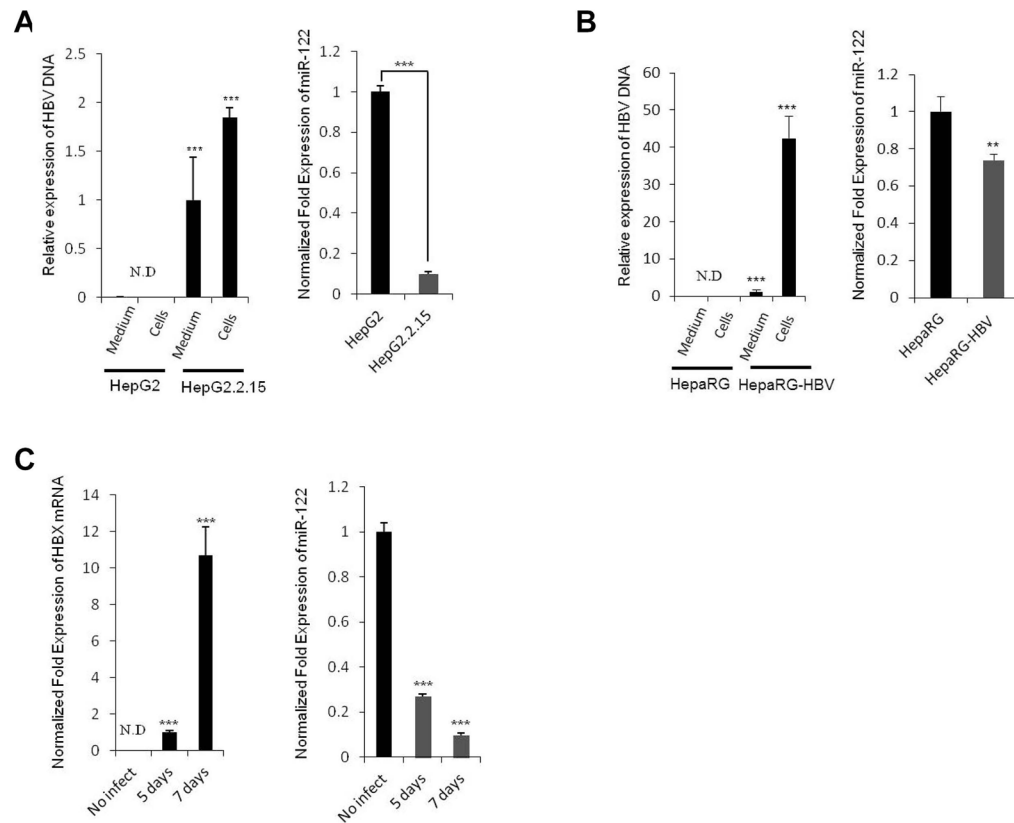


Figure 5. Effect of HBV on miR-122 expression

(A) HepG2.2.15 cells were cultured for 72 hours; DNA was extracted from HepG2.2.15 cells or supernatants (400 μ l). HBV DNA was detected by qRT-PCR using HBV-specific primers (*Left panel*). HepG2 cells and their supernatants were used as negative controls. qRT-PCR analysis of mature miR-122 was performed in HepG2 and HepG2.2.15 cells (*Right panel*). (B) HepaRG cells were infected with HBV inoculums for 20 hours and incubated for 7 days. The medium was changed every 2 days. At the end of incubation, HBV DNAs were detected in the culture medium and HBV-infected cells (*Left panel*). Total RNAs extracted from uninfected or HBV-infected HepaRG cells were subjected to qRT-PCR analysis for miR-122 (*Right panel*). (C) Human primary hepatocytes were infected with HBV inoculums for 5 or 7 days. The HBX mRNA levels (*Left panel*) and miR-122 expression levels (*Right panel*) were determined by qRT-PCR. Levels of GAPDH mRNA and U6 were used as an internal control. The data represent mean + SD (***) $P < 0.001$, **) $P < 0.01$; $n = 3$).

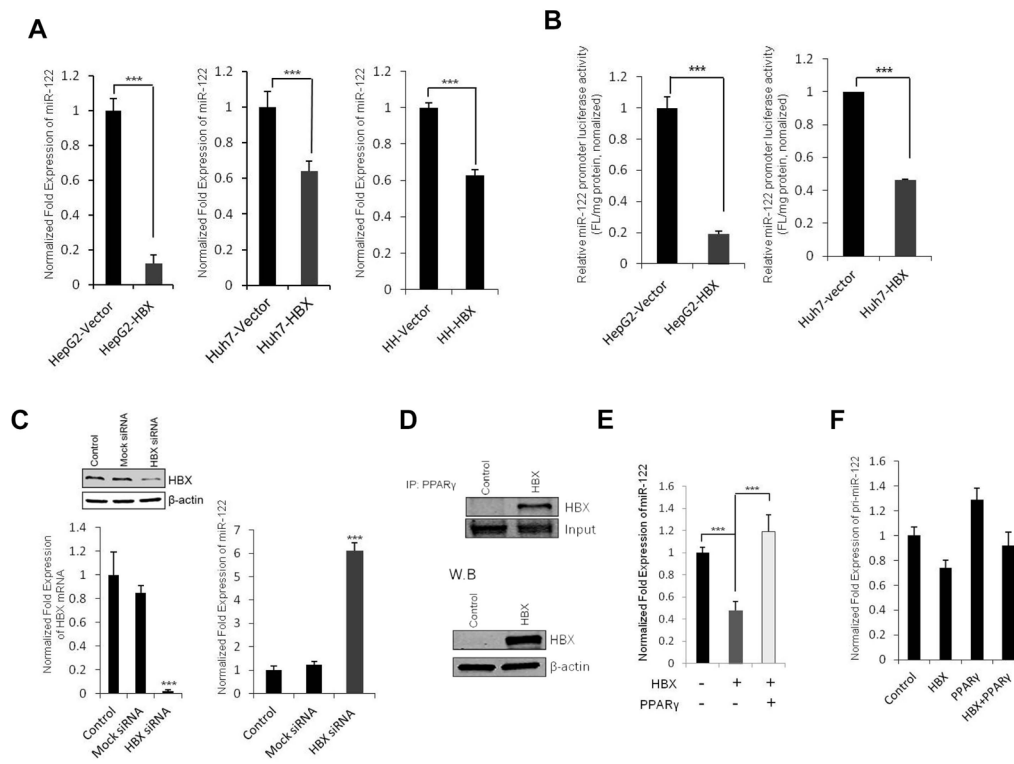


Figure 6. Effect of HBX on miR-122 expression

(A) qRT-PCR analysis of miR-122 in HepG2 and Huh7 cells 48 hours after transfection with HBX expression vector or control vector (*left and mid panel*). Human primary hepatocytes were transfected with HBX for 72 hours and miR-122 expression levels were measured by qRT-PCR (*right panel*). (B) miR-122 promoter luciferase reporter activity in HepG2 and Huh7 cells 48 hours after transfection with HBX expression vector or control vector (***) ($p < 0.001$). (C) Mock-siRNA and HBX-siRNA (100 nM) were transfected into HepG2.2.15 cells using lipofectamine. The levels of HBX protein (*Left-upper panel*) or mRNA (*Left-lower panel*) were detected by western blot and qRT-PCR (72 hours after transfection). The levels of miR-122 expression were measured by qRT-PCR in control and HBX-siRNA transfected HepG2.2.15 cells (*Right panel*). (D) HBX associates with PPAR γ . HepG2 cells were transfected with the HBX expression vector or control vector and the cell lysates were immunoprecipitated with anti-PPAR γ antibody followed by immunoblotting with anti-HBX antibody (*upper panel*). Conventional western blotting for HBX and β -actin in HBX transfected or vector control cells are shown at the *lower panel*. (E) qRT-PCR analysis for mature miR-122 in HepG2 cells transfected with HBX and/or PPAR γ . The data represent mean + SD (***) ($P < 0.001$, n = 3). (F) qRT-PCR analysis for pri-miR-122 in HepG2 cells transfected with HBX and/or PPAR γ .

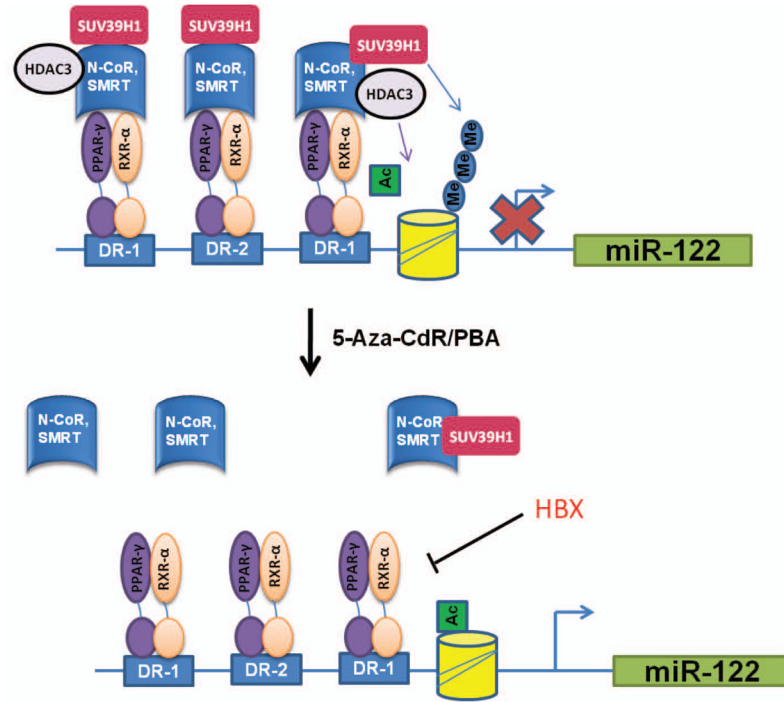


Figure 7. Schematic illustration of mechanisms for epigenetic regulation of miR-122
 Epigenetic regulation of miR-122 in HCC cells and hepatocytes involves the PPAR γ /RXR α /N-CoR/SMRT/SUV39H1/DR1/DR2 binding complex, histone acetylation, and histone H3K9 methylation. 5-Aza-CdR and PBA treatment inhibit SUV39H1 expression and cause dissociation of the corepressor complex (N-CoR/SMRT/SUV39H1) from PPAR γ /RXR α /DR1/DR2, thus allowing PPAR γ /RXR α -induced miR-122 gene transcription. Hepatitis B viral X protein inhibits PPAR γ through direct binding and thus down-regulate miR-122 expression.