MicroRNA-581 promotes hepatitis B virus surface antigen expression by targeting Dicer and EDEM1

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Hepatitis B virus surface antigen (HBsAg) is an important risk factor for hepatocellular carcinoma (HCC) and is downregulated during hepatocarcinogenesis. MicroRNAs (miRNAs) are frequently deregulated in HCC tissues. However, whether the deregulation of certain miRNAs in HCC has an impact on HBsAg expression remains unclear. We found here that microRNA-581 (miR-581), which is deregulated during hepatocarcinogenesis, promoted HBsAg expression. Additionally, miR-581 targeted Dicer and endoplasmic reticulum degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) and repressed their expression. Although Dicer cannot process HBV transcripts, Dicer knockdown led to increased HBsAg secretion, most likely due to a reduction in the levels of Dicer-processed 7SL RNA fragments. Moreover, Dicer-processed 7SL RNA fragments partially inhibited the ability of miR-581 to stimulate HBsAg expression. Furthermore, we found that forced DEmE1 expression inhibited miR-581-mediated induction of HBsAg. Finally, transfection of miR-581 into HepG2.2.15 cells promoted cell proliferation and led to upregulation of genes involved in development, cell proliferation and protein secretion. Altogether, we conclude that miR-581 promotes HBsAg expression by targeting Dicer and EDEM1. Our findings suggest that downregulation of miR-581 during hepatocarcinogenesis may lead to a reduction in HBsAg expression and impede HCC development.

Introduction

MicroRNAs (miRNAs) are a group of small non-coding regulatory RNAs that have critical functions in various physiological and pathological processes. They are transcribed as primary miRNAs that are processed by Dicer, resulting in ~21-nt RNA duplexes. One strand of the duplex, the mature miRNA, preferentially enters the RNA-induced silencing complex to mediate cleavage or translational repression of mRNAs by binding to their 3’-untranslated regions (UTRs) (1).

Materials and methods

Cell culture, miRNAs, small interfering RNAs, 7SL RNA fragments, plasmids and transfection

HepG2.2.15 and HepG2 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Huh-7 cells were grown in Dulbecco’s modified Eagle’s medium (Hyclone, Logan, UT) supplemented with 10% FBS. All cultures were maintained at 37°C in a moist atmosphere containing 5% CO2. HepG2 cells were purchased from ATCC. Huh-7 cells were purchased from GeneChem (Shanghai, China). HepG2.2.15 cells were described previously (19). Cell line authentication was performed by STR profiling before initiation of this project.

Small interfering RNAs (siRNAs) were obtained from Invitrogen (Shanghai, China). The siRNA sequences are listed in Supplementary Table S1, available at Carcinogenesis Online. miRNA mimics, the miR-581 inhibitor (anti-miR-581) and the respective negative controls (miR-Con and anti-miR-Con) were purchased from Ribobio (Guangzhou, China). 7SL RNA fragments were prepared as described previously (20).

The HBV expression plasmid pCH-9/3091 was described previously (21). The EDEM1 expression plasmid (pEDEM1) was purchased from OriGene Technologies (Beijing, China).

RNA transfection was performed using siPORT NeoFX (Ambion, Austin, TX) as described previously (22). Plasmid transfection or co-transfection with

Abbreviations: EDEM1, endoplasmic reticulum degradation-enhancing alpha-mannosidase-like protein 1; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miRNAs, microRNA; siRNA, small interfering RNA; UTR, untranslated region.

These authors contributed equally to this work.
RNA was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions.

RNA sequencing and bioinformatic analysis
Small RNA sequencing was performed in our previous study (23). HBV-derived small RNAs were identified in the sequencing data sets using BLAST and SOAP2 software (24). Transcriptome sequencing was performed using an Illumina HiSeq 2000 system, and gene expression was analyzed with TopHat and Cufflinks software (25).

Northern blot
Total RNA was prepared using TRIzol reagent (Invitrogen). To detect HBV transcripts, 10 μg of total RNA was subjected to 1% agarose formaldehyde gel electrophoresis and transferred to a nylon membrane (Sigma, St Louis, MO). To detect the 7SL RNA fragments, 5 μg of total RNA was separated on an 8% polyacrylamide gel and transferred to a nylon membrane. RNA probe labeling, hybridization and signal detection were performed using the DIG Northern Starter Kit (Roche, Mannheim, Germany). The DNA templates for in vitro RNA probe synthesis were prepared from total RNA by RT–PCR. The primer sequences are listed in Supplementary Table S2, available at Carcinogenesis Online.

Quantitative real-time RT–PCR
Total RNA was treated with RNase-free DNase I (Promega, Madison, WI) for 30 min and reverse transcribed using the M-MLV reverse transcription kit (Promega). SYBR Green real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Grand Island, NY). The data for all genes were normalized to GAPDH and then to their own controls, the expression levels of which were set to 1. Primer information is shown in Supplementary Table S2 and Supplementary Figure S1, available at Carcinogenesis Online. The primers for precore and pregenome RNAs have been described previously (26).

Western blot analysis
Cells were lysed in RIPA buffer, and equal amounts of denatured cell lysate were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The blots were then incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detection with ECL plus reagents (Millipore). The secondary antibodies used included anti-Dicer (Abcam, Cambridge, MA), anti-HBsAg (Santa Cruz, Dallas, TX), anti-EDEM1 (Sigma) and anti-γ-tubulin (Boster, Wuhan, China).

Quantification of viral proteins
The HBV proteins in the culture medium, including HBsAg and HBV e antigen (HBeAg), were measured using ELISA kits (Kehua, Shanghai, China). The concentration of viral proteins in the culture medium was normalized to the cell number, and then to its own controls, the concentrations of which were set to 1.

Luciferase reporter assay
To prepare the firefly luciferase reporter constructs, the 3′-UTRs of the target genes were cloned into the pGL3-control vector (Promega) at the XbaI site immediately downstream of the stop codon of firefly luciferase. The primers used for cloning are shown in Supplementary Table S2 and Supplementary Figure S2, available at Carcinogenesis Online. The cells were co-transfected with 0.4 μg of the firefly luciferase reporter vector, 0.02 μg of the Renilla luciferase control vector (pRL-CMV) and 25 pmol small RNAs, as indicated, using Lipofectamine 2000 in a 24-well plate. Luciferase assays were performed 24 h after transfection, using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to the Renilla luciferase activity and then to its own control, the activity of which was set to 1.

Statistical analysis
Statistical analyses were performed using Student’s t-test. All data represent the mean ± SD of at least three independent experiments.

Results
MicroRNA-581 promotes HBV surface antigen expression
miR-18b*, miR-296-5p, miR-557 and miR-581 are downregulated in liver tumor and paratumor tissues compared with normal liver tissues (16). To determine whether these miRNAs regulate HBV expression, mimics of these miRNAs were transfected into HepG2.2.15 cells that constitutively express HBV (19), and the viral antigens in the culture medium were quantified. Compared with miR-Con transfection, miR-581 transfection led to a 2.5-fold increase in HBsAg in the medium, whereas the level of HBeAg increased only slightly (Figure 1A). However, the other miRNAs tested did not affect viral antigen secretion (Figure 1A). Northern blot and real-time RT–PCR analyses indicated that the levels of HBV transcripts were not significantly affected by miR-581 transfection (Supplementary Figure S3A and B, available at Carcinogenesis Online). Moreover, intracellular HBsAg protein increased upon miR-581 transfection (Figure 1B). To ensure that the observed effect of miR-581 on HBsAg expression was not cell line-specific, the HBV expression plasmid, pCH-9/3091, was co-transfected with miR-581 or miR-Con into Huh-7 cells. The results revealed that miR-581 also induced HBsAg expression in Huh-7 cells (Figure 1C and D), without affecting the levels of HBV transcripts (Supplementary Figure S3C, available at Carcinogenesis Online).

To further validate the effect of miR-581 on HBsAg expression, the miR-581 inhibitor (anti-miR-581) was co-transfected with the
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pCH-9/3091 plasmid into Huh-7 cells. Compared with anti-miR-Con, anti-miR-581 repressed HBsAg expression (Figure 1E and F), but did not affect the levels of HBV transcripts (Supplementary Figure S4A, available at Carcinogenesis Online). Surprisingly, transfection of anti-miR-581 did not affect HBsAg expression in HepG2.2.15 cells (Supplementary Figure S4B–D, available at Carcinogenesis Online), possibly because miR-581 is not expressed in HepG2.2.15 cells, according to our small RNA sequencing data (data not shown).

MicroRNA-581 inhibits Dicer and EDEM1 expression by targeting the 3′-UTR

Using the Sanger miRNA database and the TargetScan software, we found that miR-581 might target SON, EDEM1, MTOR, TR2 and Dicer. To validate these targets, we constructed a series of luciferase reporters bearing the 3′-UTRs of these genes (Supplementary Figure S2, available at Carcinogenesis Online). The luciferase assay revealed that miR-581 repressed the expression of the Luc-EDEM1 3′-UTR, Luc-Dicer 3′-UTR1 and Luc-Dicer 3′-UTR3 constructs, but had no obvious effect on the Luc-Dicer 3′-UTR2, Luc-TR2 3′-UTR, Luc-SON 3′-UTR and Luc-MTOR 3′-UTR constructs in HepG2.2.15 cells (Figure 2A). Furthermore, anti-miR-581 increased the luciferase activities of the Luc-EDEM1 3′-UTR, Luc-Dicer 3′-UTR1 and Luc-Dicer 3′-UTR3 constructs in Huh-7 cells, indicating that Dicer and EDEM1 are potential targets of miR-581 (Figure 2B).

We then examined the effect of miR-581 on the expression of endogenous Dicer and EDEM1. Western blot analysis showed that transfection of miR-581 resulted in a significant reduction in the levels of both Dicer and EDEM1 proteins (Figure 2C). Real-time RT-PCR analysis indicated that the EDEM1 mRNA level also reduced, although to a lesser extent than the protein level, whereas the Dicer mRNA level was nearly unchanged (Figure 2D). Interestingly, anti-miR-581 transfection led to an increase in the expression of Dicer and EDEM1 in Huh-7 cells but not in HepG2.2.15 cells, which do not express miR-581 (Figure 2E). Thus, we concluded that miR-581 targets Dicer and EDEM1 and represses their expression.

Dicer does not process HBV RNAs in HepG2.2.15 cells

Dicer regulates viral gene expression by processing viral dsRNAs into siRNAs (27). To determine whether Dicer processes HBV RNAs, we analyzed our previous small RNA sequencing data from Dicer knockdown and control HepG2.2.15 cells (23). Small RNAs mapping to the HBV genome represented only 0.003% of cellular small RNAs, which is approximately 10–1000 × lower than that of virus-derived small RNAs from RNA virus-infected mammalian cells (28). HBV-derived small RNAs were heterogeneously distributed and spanned the entire HBV genome, with some localized hotspots (Figure 3A), and were all from the positive strand (Supplementary Table S3, available at Carcinogenesis Online). Dicer-processed products are 21–23 nt in length and preferentially begin with a uridine residue (29). The length distribution and the first nucleotide bias indicated that the HBV-derived small RNAs were not Dicer-processed products (Figure 3B and C). In addition, Dicer knockdown did not affect the abundance of HBV-derived small RNAs (Supplementary Table S3, available at Carcinogenesis Online). Moreover, northern blot analysis and real-time RT–PCR data showed that the HBV transcripts were not significantly increased following Dicer depletion (Figure 3D and E). Therefore, we concluded that Dicer does not process HBV transcripts in HepG2.2.15 cells.

Dicer-processed 7SL RNA fragments mediate the effect of microRNA-581 on HBV surface antigen expression

We have demonstrated that Dicer processes 7SL RNA into different fragments that function as dominant-negative regulators of the
full-length 7SL RNA and inhibit ER-mediated protein secretion. Dicer knockdown enhances ER-mediated protein secretion due to a decrease in the levels of Dicer-processed 7SL RNA fragments (20,23). Consistently, we observed that Dicer knockdown led to a significant increase in HBsAg and a slight increase in HBeAg in the culture medium (Figure 4A and Supplementary Figure S5A, available at Carcinogenesis Online). However, the level of intracellular HBsAg protein remained constant upon Dicer knockdown (Figure 4B and Supplementary Figure S5B, available at Carcinogenesis Online). The effect of Dicer knockdown on HBsAg secretion was not due to a non-specific effect of Dicer siRNA, because knockdown of Dicer using two additional siRNAs also significantly increased the levels of secreted HBsAg (Supplementary Figure S6, available at Carcinogenesis Online). Transfection of Dicer-processed 7SL RNA fragments into HepG2.2.15 cells or pCH-9/3091-transfected Huh-7 cells led to a decrease in the level of HBsAg in the medium (Figure 4C and Supplementary Figure S5C, available at Carcinogenesis Online). However, the levels of HBV transcripts and intracellular HBsAg protein were not significantly affected (Supplementary Figure S7, available at Carcinogenesis Online). To determine whether the effect of Dicer knockdown on HBsAg secretion was due to a decrease in the levels of Dicer-processed 7SL RNA fragments, a mixture of these fragments (termed 7SL mix) was transfected into Dicer knockdown HepG2.2.15 cells or Huh-7 cells that had been co-transfected with Dicer siRNA and pCH-9/3091 plasmid. As expected, transfection of the 7SL mix partially restored HBsAg secretion in Dicer knockdown cells. As a negative control, transfection of LacZ RNA did not affect HBsAg secretion (Figure 4D and Supplementary Figure S5D, available at Carcinogenesis Online).

The levels of Dicer-processed 7SL RNA fragments were reduced in Dicer-knockdown cells (23), and miR-581 inhibited Dicer expression. Consistently, we observed that the levels of Dicer-processed 7SL RNA fragments were decreased in miR-581-transfected cells (Figure 4E and Supplementary Figure S5E, available at Carcinogenesis Online). To determine whether elevated HBsAg expression in miR-581-transfected cells was due to a decrease in the levels of Dicer-processed 7SL RNA fragments, the 7SL mix was co-transfected with miR-581 into HepG2.2.15 cells or pCH-9/3091-transfected Huh-7 cells. As shown in Figure 4F and Supplementary Figure S5F, available at Carcinogenesis Online, the 7SL mix partially restored the levels of HBsAg secreted from miR-581-transfected cells, whereas the control RNA did not affect HBsAg secretion.

EDEM1 mediates the effect of microRNA-581 on HBV surface antigen expression

We demonstrated above that miR-581 targets EDEM1 and inhibits its expression. Additionally, EDEM1 has been reported to mediate HBsAg degradation, and EDEM1 knockdown leads to increased HBsAg expression (9). To determine whether the effect of miR-581 on HBsAg expression is mediated by EDEM1, HepG2.2.15 cells or pCH-9/3091-transfected Huh-7 cells were co-transfected with miR-581 and an EDEM1-encoding plasmid that lacks the 3’-UTR of EDEM1, thereby preventing inhibition by miR-581. As shown in Figure 5 and Supplementary Figure S8, available at Carcinogenesis Online, forced EDEM1 expression partially inhibited the ability of miR-581 to stimulate HBsAg secretion, indicating that increased HBsAg expression upon miR-581 transfection was partially mediated by EDEM1.

Discussion

Dicer can cleave viral dsRNAs into siRNAs to specifically target viral genomes and mrRNAs for degradation in plants and invertebrates (27). However, whether mammalian cells mount a similar antiviral RNAi response remains controversial. The evidence from small RNA cloning data is not sufficiently compelling to conclude that siRNAs of viral origin are present in virus-infected mammalian cells (30). However, deep sequencing data have indicated that virus-derived siRNAs do exist in mammalian cells infected with virus RNAs (28). HBV is an enveloped DNA virus, and only one strand of the genomic DNA is transcribed (2). In addition, our strand-specific transcripome sequencing data revealed that the HBV transcripts in HepG2.2.15 cells were all from the positive strand (data not shown). Therefore, HBV transcripts are unable to form double-stranded RNAs and are thus not natural substrates of the Dicer enzyme. Consistently, our deep sequencing data of small RNAs indicates that the HBV-derived siRNAs are most likely to be random degradation products rather than Dicer-processed RNAs. We have demonstrated previously that Dicer regulates 7SL RNA-mediated protein secretion (20,23). In this study, we found that Dicer knockdown increased HBsAg secretion, which was partially inhibited by transfection of Dicer-processed 7SL RNA fragments. Our data indicate that although Dicer cannot cleave HBV transcripts, it does regulate HBsAg secretion.

Earlier, we demonstrated that Dicer processes a proportion of 7SL RNA into small fragments and that these fragments are decreased in Dicer knockdown cells (23). Here we found that miR-581 targeted...
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Dicer and repressed its expression, and consistently, the 7SL RNA-derived fragments were reduced in miR-581-transfected cells. We also demonstrated that miR-581 functioned as a positive regulator of HBsAg expression and that the effect of miR-581 on HBsAg expression was partially repressed by Dicer-processed 7SL RNA fragments. Moreover, we observed that EDEM1 is another target of miR-581. A recent study indicated that EDEM1 is involved in the degradation of HBV envelope proteins and that knockdown of EDEM1 leads to an increase in HBsAg expression (9). Here, we demonstrated that the miR-581-mediated increase in HBsAg was partially inhibited by overexpression of EDEM1. Moreover, we showed that simultaneous knockdown of Dicer and EDEM1 mimics the effect of miR-581 on HBsAg expression (Supplementary Figure S9, available at Carcinogenesis Online). Taken together, we conclude that miR-581 promotes HBsAg expression by targeting Dicer and EDEM1. The miRNA regulates the expression of its target genes through translation inhibition and/or mRNA degradation. We found that miR-581 repressed the expression of Dicer at the protein level but not at the mRNA level and that it inhibited EDEM1 expression at both protein and mRNA levels, although the reduction is less pronounced at the mRNA level than at the protein level. These results suggest that miR-581 represses Dicer expression via translational inhibition, whereas it inhibits EDEM1 expression via mRNA degradation and translational inhibition.

HBV expression is downregulated during the process of hepatocarcinogenesis (17,18,31). Katayama et al. (16) have demonstrated that miR-581 is downregulated in liver tumor and paratumor tissues compared with normal liver tissues. Here, we found that miR-581 promotes HBsAg expression. Therefore, future studies should investigate whether downregulation of miR-581 is the cause of HBsAg reduction during in vivo hepatocarcinogenesis. HBsAg is an important risk factor for HCC (32), and HBsAg seroclearance is associated with a low risk of HCC development (33). In addition, HBsAg transgenic

Fig. 4. Dicer-processed 7SL RNA fragments repress the effect of miR-581 on HBsAg secretion in HepG2.2.15 cells. (A) HepG2.2.15 cells were transfected with siDicer or the control siRNA, and the secreted viral antigens were quantified 72 h after transfection; *P < 0.01. (B) Representative western blot of intracellular HBsAg protein in cells described in A. (C) Quantification of secreted HBsAg from HepG2.2.15 cells transfected with different 7SL RNA fragments; *P < 0.01. (D) The 7SL RNA fragment mixture was co-transfected with siDicer into HepG2.2.15 cells that were pretreated with siDicer for 48 h. The level of HBsAg in the medium was quantified 48 h after the second transfection; *P < 0.05. (E) Northern blot of 7SL RNA fragments in HepG2.2.15 cells transfected with miR-581 or miR-Con. (F) The 7SL RNA fragment mixture was co-transfected with miR-581 into HepG2.2.15 cells, and the level of HBsAg in the medium was determined 48 h after transfection; *P < 0.05, **P < 0.01.

Fig. 5. Forced EDEM1 expression inhibits miR-581-mediated induction of HBsAg in HepG2.2.15 cells. HepG2.2.15 cells were co-transfected with pEDEM1 or the empty vector and miR-581 or miR-Con as indicated. (A) Representative western blot of intracellular HBsAg and EDEM1 proteins. (B) Quantification of HBsAg in the medium; *P < 0.05, **P < 0.01.
mice spontaneously develop HCC (34). Here, we observed that fetal stage–specific genes were upregulated and that the proliferation of HepG2.2.15 cells slightly increased upon miR-581 transfection. Altogether, these findings suggest that miR-581 plays an important role in HBV-associated hepatocarcinogenesis.

It has been reported that disruption of Dicer in mouse hepatocytes leads to upregulation of fetal stage-specific genes, whereas the expression pattern of genes normally found in mature hepatocytes is preserved (35), and here we found that miR-581 inhibits Dicer expression. Consistently, our transcriptional profiling data revealed that several fetal stage-specific genes were upregulated in miR-581-transfected HCC cells, whereas the expression levels of liver-enriched transcription factors and other genes present in mature hepatocytes, including Alb, Sdh and Tdo, were not significantly changed upon miR-581 transfection (Supplementary Figures S10 and S11 and Supplementary Table S4, available at Carcinogenesis Online). Dicer disruption also resulted in induction of cell cycle-promoting genes and repression of steroid biosynthesis-related genes (35). However, we observed that these genes were not regulated by miR-581 in HepG2.2.15 cells (Supplementary Table S4, available at Carcinogenesis Online). Interestingly, we found that genes involved in cell proliferation were upregulated in miR-581-transfected cells (Supplementary Figures S10 and S11 and Supplementary Table S4, available at Carcinogenesis Online) and that miR-581 transfection promoted cell proliferation (Supplementary Figure S12, available at Carcinogenesis Online). Moreover, we observed that several genes involved in protein secretion were upregulated upon miR-581 transfection (Supplementary Figures S10 and S11 and Supplementary Table S4, available at Carcinogenesis Online).

Although miR-581 regulates a common set of genes in HepG2 and HepG2.2.15 cells, the effects of miR-581 on the expression of the majority of genes are cell type-specific (Supplementary Figure S13, available at Carcinogenesis Online). Specifically, although miR-581 induces the expression of AQP, H19, IG2, ACHE, CD79A, EMP1, PYY, RBP4, TGFBI, TNSFSF4, RAB37, GOLM1, CHST4, ARHGIB and SLC15A in HepG2.2.15 cells, it only promotes the expression of IGF2, PYY, RAPGEF3, RBP4, CHST4 and ARHGIB in HepG2 cells (Supplementary Figures S10 and S11 and Supplementary Table S4, available at Carcinogenesis Online). By analyzing our previous small RNA deep-sequencing data (23,36), we found that miR-581 is not expressed in both HepG2 and HepG2.2.15 cells (data not shown). Therefore, the cell type-specific effect of miR-581 on gene expression is not a consequence of the different expression levels of endogenous miR-581 in these cells. As HepG2.2.15 cells were developed by transfecting HepG2 cells with an HBV-expressing plasmid (19), we therefore speculate that HBV may itself modulate the effect of miR-581 on gene expression in HCC cells. Interestingly, although miR-581 was able to stimulate the proliferation of both HepG2 and HepG2.2.15 cells, its ability to induce proliferation-associated genes was weaker in the former cell line (Supplementary Figures S10–S12 and Supplementary Table S4, available at Carcinogenesis Online). These results suggest that in addition to upregulation of proliferation-promoting genes, other mechanism(s) may contribute to miR-581’s ability to stimulate cell proliferation. As miR-581 is downregulated in both HBV-positive and HBV-negative HCC tissues, we proposed that it also plays pivotal roles in HBV-independent hepatocarcinogenesis.

HBV replication is closely associated with cell proliferation status and that loss of the differentiation status in hepatocytes during hepatocarcinogenesis reduces the ability of these cells to support HBV expression (10,17,37). However, our results indicated that although miR-581 transfection resulted in a slight increase in cell proliferation and upregulation of fetal stage-specific genes, HBV transcripts were not significantly reduced in miR-581 transfected cells. This finding may be explained by the observation that ectopic expression of miR-581 did not significantly alter the expression of liver-enriched transcription factors, which are essential for HBV expression (3). Further work is required to elucidate the precise molecular mechanisms underlying this phenomenon.

In summary, we demonstrated that miR-581 induced the expression of HBsAg and genes involved in development and cell proliferation and promoted cell proliferation in HCC cells. Our findings suggest that downregulation of miR-581 during hepatocarcinogenesis may impede HCC development. Future studies should investigate whether miR-581 can be used as a diagnostic and prognostic molecular marker and whether it is a therapeutic target for HCC treatment.

Supplementary material
Supplementary Figures S1–S13 and Supplementary Tables S1–S4 can be found at http://carcin.oxfordjournals.org/

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