Adenosine deaminases acting on RNA-1 (ADAR1) involves adenosine to inosine RNA editing and microRNA processing. ADAR1 is known to be involved in the replication of various viruses, including hepatitis C and D. However, the role of ADAR1 in hepatitis B virus (HBV) infection has not yet been elucidated. Here, for the first time, we demonstrated ADAR1 antiviral activity against HBV. ADAR1 has two splicing isoforms in human hepatocytes: constitutive p110 protein and interferon-α (IFN-α)-responsive p150 protein. We found that overexpression of ADAR1 decreased HBV RNA in an HBV culture model. A catalytic-site mutant ADAR1 also decreased HBV RNA levels, whereas another adenosine deaminases that act on the RNA (ADAR) family protein, ADAR2, did not. Moreover, the reduction of HBV RNA by ADAR1 expression was positively correlated with ADAR1 expression, and exogenous miRNA-122 decreased both HBV RNA and DNA, whereas, conversely, transfection with a miRNA-122 inhibitor increased them. The reduction of HBV RNA by ADAR1 expression was abrogated by p53 knock-down, suggesting the involvement of p53 in the ADAR1-mediated reduction of HBV RNA. This study demonstrated, for the first time, that ADAR1 plays an antiviral role against HBV infection by increasing the level of miRNA-122 in hepatocytes.

Hepatitis B virus (HBV) is a major cause of liver disease, including hepatic fibrosis, cirrhosis, and hepatocellular carcinoma. More than 250 million people worldwide are chronically infected with HBV (1). Understanding the antiviral pathway against HBV is vital for the development of effective therapeutic approaches to prevent severe liver diseases (1–4). The HBV genome is 3.2 kb long and comprises four genes: core protein (HBc), X protein (HBx), surface protein (HBs), and P protein (P). After HBV enters a hepatocyte, viral DNA is transferred to the cell nucleus, where covalently closed circular DNA (cccDNA) is formed. cccDNA generates viral RNAs, including a replicative intermediate known as pregenomic (pg) RNA and viral mRNAs for the HBs and HBx proteins. HBc, P protein, and pgRNA are assembled into a nucleocapsid, and the P protein converts pgRNA into relaxed circular (RC)-DNA using its reverse transcriptase activity. The mature nucleocapsid finally associates with HBs prior to secretion as an infectious virion (3, 5, 6). However, until now, the mechanisms of antiviral immunity and the pathogenesis of HBV infection have not been fully clarified.

The abbreviations used are: HBV, hepatitis B virus; ADAR1, adenosine deaminases acting on RNA-1; AID, activation-induced cytokine deaminase; APOBEC3G, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G; cccDNA, covalently closed circular DNA; GFP, green fluorescent protein; IFN, interferon; NAGE, native agarose gel electrophoresis; NC, nucleocapsid-associated; pg, progenomic; pri-miRNA, primary miRNA; miRNA, microRNA; RC, relaxed circular; RISC, RNA-induced silencing complex; ZAP, zinc-finger antiviral protein; qPCR, quantitative PCR; nt, nucleotide; HPRT, hypoxanthine-guanine phosphoribosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sgRNA, single guide RNA.
ADAR1 inhibits HBV replication through miR-122

Several members of the ADAR (adenosine deaminases that act on RNA) family have been newly identified as types of antiviral factors. In humans, the ADAR family has three members: ADAR1, ADAR2, and ADAR3. Most of these enzymes can convert adenosine to inosine (Ala to Ile) via their adenosine deaminase activity (7–10). ADAR1 is the best-studied member of this family, because of its role in neural receptor RNA editing (9). It has also been suggested that ADAR1 possesses antiviral activities against various viruses, including HIV type 1 (11) and hepatitis C virus (HCV) (12). In the case of the hepatitis D virus, ADAR1 edits the viral antigenome RNA, to switch from replication to packaging (13). The role of ADAR1 in HBV infection, however, has not been previously investigated.

ADAR1 has two major alternative splicing isoforms: p110 is transcribed from constitutive promoters, whereas p150, from an IFN responsive promoter (9, 10, 14, 15). ADAR1 is also involved in miRNA processing (9, 16). miRNA is generated from its precursor, primary-miRNA (pri-miRNA), which is a several-hundred nucleotides (nt) long miRNA precursor with an ~80–nt RNA stem-loop. The Drosha and Dicer enzymes belong to the RNase III gene family and are key components of the miRNA processing machinery. In the nucleus, Drosha cleaves pri-miRNAs to produce intermediate precursors, pre-miRNAs, with the help of the dsRNA-binding protein DGCR8. Pre-miRNAs are cleaved by Dicer in the cytoplasm to release 21–24–nt mature miRNAs (17). Dicer forms a complex with other proteins and acts as the platform for RNA-induced silencing complex (RISC) assembly by recruiting Ago2 (18). ADAR1 directly interacts with Dicer and forms a protein complex, including Ago2, to promote miRNA processing (16).

In the present study, we investigated whether ADAR1 could restrict HBV replication using an HBV culture model. The results obtained in this study led us to propose ADAR1 as an anti-HBV factor. Its molecular mechanism is discussed.

Results
ADAR1 decreases the HBV transcript level

Because ADAR1 is an interferon-responsive gene (9–11, 14), we examined the expression of endogenous ADAR1 in which HBV was replicating. An HBV replicon plasmid (pPB) containing 1.04 copies of HBV genome (19, 20) was transfected into a human hepatocyte cell line (Huh7) to reproduce viral replication in vitro. The transfected cells were further treated with IFN-α at doses of 100, 500, and 1000 units/ml, selected to match the range reported as antiviral doses in human hepatocytes. Immunoblotting of ADAR1 revealed that the ADAR1 p150 isoform was increased by 3.45-, 5.32-, and 5.98-fold by 100, 500, and 1000 units/ml of IFN-α, respectively (Fig. 1A, top). In contrast, p110 was up-regulated by no more than 1.93-fold by 500 units/ml of IFN-α. RT-quantitative PCR (qPCR) analysis was performed in parallel, and copy numbers of p110 and p150 isoforms were individually quantified. We found that both were increased by IFN-α (Fig. 1A, bottom), consistent with the Western blot analysis. Although p110 was reported to constitutively express (11) 1000 units/ml of IFN-α significantly up-regulated p110 in Huh7 cells, albeit not robustly compared with p150.

Viral replication was also examined by measurement of total HBV RNA and viral DNA levels secreted into culture supernatants. As shown in Fig. 1B, the levels of both HBV RNA and secreted HBV DNA were decreased in the IFN-α–treated cells, in a dose-dependent manner. Furthermore, to verify whether ADAR1 expression affects HBV replication, ADAR1-p150 was expressed in HBV-replicating hepatocytes. RT-quantification PCR revealed decreased levels of HBV RNA in the ADAR1 transfectant, whereas IFN-α mRNA was unaffected (Fig. 1C and Fig. S1). A native agarose gel electrophoresis (NAGE) assay can differentially estimate the amounts of cytoplasmic nucleocapsid protein and its associated DNA, allowing for accurate assessment of viral replication. Using this method, we found that p150 expression also led to a decrease in both nucleocapsid production and secreted viral DNA (Fig. 1C). Collectively, these results suggest that ADAR1 is induced by IFN-α and can decrease HBV RNA levels.

Activation-induced cytidine deaminase (AID) and zinc finger antiviral protein (ZAP) have been reported to decrease HBV RNA and viral replication (20, 21). Indeed, we observed a reduction in both HBV RNA and secreted HBV DNA levels following the overexpression of AID, ZAP, and ADAR1 (Fig. S2, top). In contrast, another DNA deaminase, APOBEC3G, reduced the level of secreted HBV DNA, but not of viral RNA (Fig. S2, middle and bottom). This is consistent with previous studies, where APOBEC3G targeted HBV DNA, but did not directly affect the HBV RNA levels (20, 22).

Endogenous ADAR1 negatively regulates HBV RNA levels

IFN-α, a human type I interferon (23), is used clinically as an anti-HBV drug (24, 25), although the mechanism behind its antiviral effect is not yet fully understood. To investigate the possibility of whether the induction of ADAR1 suppresses HBV replication in IFN-α–treated hepatocytes, loss-of-function of ADAR1 approaches were adopted. First, siRNA against ADAR1 (or control siRNA; siNC) was transfected together with pPB. Then, one group was further treated with IFN-α and another group was left untreated. As shown in Fig. 2A, among the three kinds of siRNA targeting ADAR1, we found that two (1-1 and 1-2) ablated its expression to almost undetectable levels. Significant suppression of ADAR1 by siADAR1 was also confirmed by RT-quantification PCR analysis (Fig. 2B, top). As shown in Fig. 2B (lower panel), HBV transcript levels were reduced by IFN-α treatment (compare the first lane with fourth), whose effect was attenuated by transfection with siADAR1. Notably, ADAR1 knock-down cells saw increased HBV RNA levels even in the absence of IFN-α treatment, suggesting the involvement of constitutively expressed ADAR1 proteins (mainly p110) in the regulation of HBV transcript levels in an IFN-α-independent manner.

Second, we reduced the ADAR1 expression level by transfection with a CRISPR/Cas9-guide RNA vector targeting ADAR1. Of three sgADAR1 vectors we tested, we confirmed a reduction in ADAR1 in two transfectants (exons 3 and 4) to undetectable levels, as assessed by Western blot analysis (Fig. 2C, top). The HBV RNA levels of two transfectants (exons 3 and 4) were compared with that of the control transfectant, and
HBV RNA levels were found to be significantly higher in exons 3 and 4 transfectants compared with the control cells (Fig. 2, bottom). These results suggest that endogenous ADAR1 (both p110 and p150) negatively regulates HBV transcript levels.

**ADAR1 p110 and p150 increase microRNA-122**

ADAR1 catalyzes the conversion of adenosine to inosine in dsRNA by hydrolytic deamination (7, 9). To explore the mechanism by which ADAR1 suppresses HBV transcript levels, we first investigated the possibility of Ala to Ile RNA editing of HBV transcripts. Because NC-DNA is generated from pgRNA through reverse transcription, any Ala to Ile conversion in pgRNA should be transduced to be a Thr to Cys mutation in NC-DNA. Therefore, we sequenced NC-DNA purified from cells overexpressing ADAR1. The results showed no Thr to Cys mutations in five, independent clones (Fig. S3, 835 bases sequenced in total). Meanwhile, AID and APOBEC3G, known to be mutators of HBV DNA (22, 26), accumulated Gly to Ala mutations in nucleocapsid associated (NC)-DNA (Fig. S3). These results suggest that Ala to Ile RNA editing is unlikely to account for a reduction in HBV RNA levels by ADAR1.

The modulation of microRNA processing is another function of ADAR1 (9, 10, 16). Therefore, we next examined whether changes in ADAR1 expression had any effect on miRNA expression. To this end, the level of the miRNAs miR-122, -205, -196b, and -433 (27), which are abundantly expressed in hepatocytes, were compared between ADAR1 and mock-expressing Huh7 cells. As shown in Fig. 3A and Fig. S4, miR-122, a predominant miRNA in hepatocytes (28), was found to
be up-regulated by ADAR1 expression with concomitant decreases in HBV RNA levels. In contrast, the expression of another ADAR family member, ADAR2, did not affect either miR-122 or HBV RNA (Fig. 3B). Moreover, AID and ZAP did not alter the level of miR-122 either, suggesting that their anti-HBV activity is not dependent on miR-122 (Fig. 3C). Meanwhile, two ADAR1 isoforms, p110 and p150, both increased miR-122 levels and suppressed HBV transcript levels when individually overexpressed (Fig. 3D). Collectively, these results suggest that ADAR1 plays a role in the positive regulation of miR-122 in hepatocytes.

**The level of miR-122, but not that of pri-miR-122, is up-regulated by ADAR1 expression**

It has previously been reported that ADAR1 facilitates the conversion of pri-miRNA to miRNA, and that this process does not require deaminase activity (16); therefore, we examined the levels of both miR-122 and pri-miR-122 in ADAR1 and its mutant expressing Huh7 cells. ADAR1 has one deaminase catalytic domain that is composed of a single zinc finger. Mutation of histidine 910 and glutamine 912, which are located within the catalytic core CHAE motif, abolish ADAR1 deaminase activity (29, 30). As shown in Fig. 4, ADAR1 or its catalytic mutant (E912Q) was overexpressed, together with pPB. Six h post-transfection, 1000 units/ml of IFN-α was added and the cells were cultured for 3 days. Top, the ADAR1 protein levels were determined using Western blotting. Bottom, RT-qPCR analyses of ADAR1 and HBV transcripts. The level of ADAR1 and HBV transcripts in the control sgRNA sample is defined as value 1. **, p < 0.01.
and that deaminase activity is not necessarily required for ADAR1-mediated miR-122 up-regulation.

**ADAR1 down-regulates HBV RNA levels via miR-122**

To demonstrate the role of ADAR1 in the positive regulation of miR-122, a loss-of-function approach was employed, utilizing siRNA or CRISPR/Cas9-based genetic ablation of ADAR1 (Fig. 5A). In both experiments, decreased ADAR1 levels were associated with decreased miR-122 levels, consistent with the results presented in Figs. 3 and 4. Next, we examined whether miR-122 is responsible for the ADAR1-mediated down-regulation of HBV transcripts. In accordance with previous reports (31), transfection of a miR-122 mimic resulted in a dose-dependent reduction in HBV RNA levels (Fig. 5B, upper panel). Conversely, transfection of a miR-122 inhibitor increased HBV transcript levels in a dose-dependent manner (Fig. 5B, lower panel). These results suggest that the constitutively expressed miR-122 contributes to HBV RNA reduction.

To assess the contribution of miR-122 downstream of ADAR1 expression, Huh7 cells were co-transfected with pPB and the ADAR1/mock expression vector, in combination with the miR-122 inhibitor (Fig. 5C). RT-qPCR analysis was performed to quantify viral RNA levels and revealed that miR-122 inhibitor treatment of the ADAR1 transfectant increased viral RNA (lanes 3 and 4). In the presence of the miR-122 inhibitor, viral RNA levels were comparable between the mock and ADAR1 transfectants (lanes 1 and 3). Collectively, these results suggest that miR-122 negatively regulates HBV RNA, and that ADAR1 reduces HBV transcripts through the up-regulation of miR-122.

**Up-regulation of the p53 protein contributes to ADAR1-mediated down-regulation of HBV transcripts**

It has been previously reported that miR-122 and its target, cyclin G1, are involved in immunity to HBV (31, 32). It is known that cyclin G1 induces the ubiquitination of the p53 protein, and the degradation of p53 with the cooperation of MDM2 (33, 34). Therefore, we further examined whether ADAR1 uses cyclin G1 and p53 for the restriction of HBV replication. Expression vectors for green fluorescent protein (GFP)-tagged ADAR1 or mock (GFP-only), and the miR-122 mimic or miR-122 inhibitor were co-transfected with pPB in Huh7 cells. As expected, the ADAR1- and miR-122 mimic transfectants showed decreased cyclin G1 expression with concomitant up-regulation of the p53 protein (Fig. 6A). Conversely, the miR-122 inhibitor increased cyclin G1 expression with a concomitant
Down-regulation of p53 protein levels. Similarly, when we knocked down ADAR1 expression using siRNA against ADAR1, the cyclin G1 levels were up-regulated, whereas the p53 protein levels were concomitantly down-regulated. Additionally, NAGE analysis revealed an inverse correlation between p53 protein levels and nucleocapsid formation (Fig. 6B).

Next, we evaluated the relationship between cyclin G1 and p53 with ADAR1-mediated HBV RNA reduction. First, the overexpression of cyclin G1 resulted in higher HBV transcript levels compared with those of the GFP control (Fig. 6C). Conversely, the knock-down of cyclin G1 by siRNA (scyclin G1-1 and -2, but not -3) increased p53 protein levels (Fig. 6D), with a concomitant reduction in HBV transcripts, regardless of ADAR1 overexpression (Fig. 6E).

On the other hand, p53 reportedly inhibits HBV replication by interfering with HBV enhancer activity or by inducing apoptosis in infected cells (31, 35, 36). Consistent with these reports, we found that the overexpression and the knock-down of p53 resulted in decreased and increased HBV RNA levels, respectively (Fig. 6, F–H). Interestingly, the silencing of p53 expression by siRNA (sip53-2 and -3, but not -1) increased HBV RNA levels in Huh7 cells that both overexpressed and did not express ADAR1 (Fig. 6I), indicating that p53 expression regulated the level of HBV transcripts more tightly than ADAR1. Collectively, these data suggest that cyclin G1 levels negatively regulate p53, and that p53 negatively regulates HBV RNA levels. In addition, our findings suggest that ADAR1 adopts the cyclin G1 and p53 proteins as downstream molecules of miR122 to reduce the HBV RNA levels.

Dicer and Ago2 are important for ADAR1-mediated HBV RNA reduction

To elucidate how ADAR1 increases miR-122, we investigated the role of RISCs in the promotion of miRNA formation. One component, Ago2, was overexpressed in Huh7 cells, and an immunoprecipitation assay was performed. As suggested by Ota et al. (16), ADAR1 forms a complex with Ago2 in Huh7 cells (Fig. S5). Moreover, when Ago2 was silenced by siRNA transfection, miR-122 levels were reduced, and HBV transcript levels were up-regulated (Fig. 7A). Dicer is an endoribonuclease that produces miRNA by cleaving pre-miRNA, and facilitates the activation of the RISC complex (17). When Dicer was knocked-down, miR-122 levels were reduced and as a consequence the HBV RNA levels were increased (Fig. 7B). A reduction in HBV RNA by exogenous ADAR1 expression was no longer observed in Ago2 and Dicer knock-down cells (Fig. 7, A and B, comparing the first and fifth samples). Overall, these data suggest the involvement of RISC in ADAR1-mediated HBV RNA reduction.

Discussion

In this study, we demonstrated that IFN-α reduced HBV RNA levels and concomitantly up-regulated the expression of ADAR1 (mostly p150) in HBV-replicating hepatocytes (Fig. 1). Huh7 cells expressed ADAR1 p110 protein even in the absence of IFN-α stimulation (Fig. 1A). The knock-down of ADAR1 increased HBV RNA levels in both IFN-α–treated and -untreated hepatocytes (Fig. 2). Both ADAR1 p110 and p150, but not ADAR2, increased the anti-HBV miRNA, miR-122 (Fig. 3).

This up-regulation of miR-122 seemed to occur after the transcription of pri-miRNA in a deaminase-independent manner (Fig. 4). miR-122 levels were down-regulated by ADAR1 knockdown (Fig. 5). The knock-down of Ago2 and Dicer attenuated both the up-regulation of miR-122 and the down-regulation of...
HBV RNA. Therefore, we propose that ADAR1 increases miR-122 levels by facilitating miR-122 processing. miR-122 has been reported to reduce HBV RNA levels by reducing cyclin G1, a negative regulator of p53 (31). In this study, we also observed similar decreases in cyclin G1 levels and increases in p53 protein levels with miR-122 (Fig. 6).

In Fig. 7C, we propose a model for the reduction of HBV RNA levels by ADAR1. ADAR1 (mostly p110) is constitutively expressed in hepatocytes, and the level of expression, especially that of p150, is further up-regulated following IFN-α/H9251 stimulation. The level of total ADAR1 protein determines the efficiency of miR-122 processing by binding with Dicer and Ago2. The up-regulation of miR-122 suppresses the translation of its target, cyclin G1 (31), which increases the p53 protein level. p53 may induce apoptosis or growth arrest in HBV-replicating hepatocytes (36) and may inhibit HBV transcription by binding with HBV enhancers I and II (31, 35). It has also been reported that miR-122 may bind directly to HBV RNA, thus reducing HBV RNA stability (37). However, this pathway may not have contributed to ADAR1-mediated HBV RNA reduction in our experimental conditions, because the p53 knock-down completely abolished ADAR1-mediated HBV RNA reduction (Fig. 6H).

As shown in Fig. 2B, silencing of ADAR1 by siRNA resulted in the increased level of viral transcript, even in the absence of IFN-α, suggesting the involvement of not only inducible p150, but also constitutive p110. On the other hand, siADAR1 did not completely restore the HBV-RNA levels to those seen for the siNC control without IFN-α treatment (Fig. 2B, first, fifth, and sixth). This result implies the involvement of other effectors in IFN-α-mediated anti-HBV activity. Indeed, ZAP-s and ISG20 have been reported to reduce HBV RNA in IFN-α–treated hepatocytes (21, 38). Their possible association with ADAR1 remains to be verified.

Pujantell et al. (39) reported that ADAR1 negatively regulates IFN-β, DDX58 (RIG-I), and HCV replication in Huh7 cells. However, in the present study, whereas HBVRNA was reduced by ADAR1 overexpression (Fig. 1C), IFN-α expression was unaffected (Fig. S1). On the other hand, exogenous IFN-α increased ADAR1 expression, whereas decreasing HBV RNA (Fig. 1, A and B). Thus, the role of IFNs and ADAR1 in HBV replication appears to be different from that found in HCV.

Huh7 cells have a mutation (Y220C) in TP53. Previously, the Y220C mutant was believed to have lost its DNA-binding ability and to be transcriptionally inactive (40–42). However, later studies demonstrated that Y220C TP53 does possess DNA-binding ability, albeit of lower or comparable efficiency to that of the WT, and thus retains its transcriptional activity (43–47). The observations made in this study are in agreement with those of the later studies, because p53 knock-down was found to clearly up-regulate HBV RNA levels in this study (Fig. 6H), indicating the functional involvement of p53 in HBV replication, regardless of the presence of the Y220C mutation.

This study suggests that human ADAR1 enhances human miR-122 processing. Indeed, it was previously reported that mature miR-122 expression levels in a murine model were dramatically reduced in ADAR1 knock-out mice at E11.5 (16).
Although there is a small difference between human and murine pri-miR-122 in the loop sequence, mature miR-122s are highly conserved in these species. These findings and our data provide consistent support for the idea that ADAR1 is an important factor in facilitating the production of mature miR-122 from pri-miR-122 in hepatocytes (Fig. 7C). Meanwhile, pri-miR-122 was not affected by ADAR1 expression (Fig. 4).

From our results, we speculate that ADAR1 regulates the processing of pre-miR-122 without affecting the level of pri-miR-122. Alternatively, the amount of pri-miR122 may be too large to be affected by the overexpression of ADAR1, even if ADAR1 promotes the conversion of pri-miR-122 into pre-miR-122. Further studies are necessary to verify whether ADAR1 is involved in this process.

Wu et al. (48, 49) reported that rs4845384, an SNP in the 5′-UTR of the ADAR1 gene, is associated with HBV clearance and ADAR1 promoter activity in vitro. The allele rs4845384A is reportedly associated with a lower expression of ADAR1, and a

Figure 6. ADAR1 suppressed HBV replication in a cyclin G1-p53–dependent manner. A, pPB together with mock (GFP), GFP-tagged ADAR1, the miR-122 mimic, or the miR-122 inhibitor were transfected into Huh7 cells. Three days post-transfection, the indicated protein levels were determined using Western blotting. B, Huh7 cells were co-transfected with siADAR1 (or siNC) and pPB; 3 days post-transfection, HBV replication was evaluated. The NC-DNA and core protein levels in the crude cytoplasmic extracts were assessed using a NAGE assay. Protein levels in the same crude extracts were estimated using Western blotting. C, Huh7 cells were co-transfected with pPB and either GFP-tagged cyclin G1 or a mock expression vector. Three days post-transfection, the protein and HBV RNA levels were estimated using Western blotting (top) and RT-qPCR (bottom), respectively. The mock (GFP) sample is defined as value 1. D, Huh7 cells were co-transfected with pPB and either cyclin G1 or siNC. Three days post-transfection, protein levels were estimated using Western blotting. E, Huh7 cells were co-transfected with pPB and either cyclin G1 or siNC together, with or without an ADAR1 expression vector. Three days post-transfection, HBV RNA was estimated by RT-qPCR. The siNC sample is defined as value 1. F, Huh7 cells were co-transfected with pPB and either GFP-tagged p53 or mock expression vector. Three days post-transfection, protein and HBV RNA levels were examined using Western blotting (top panel) and RT-qPCR (bottom panel), respectively. The mock (GFP) sample is defined as value 1. G, Huh7 cells were co-transfected with pPB and either siNC or sip53. Three days post-transfection, the protein levels were determined using Western blotting. H, Huh7 cells were co-transfected with pPB and either siNC or sip53 together, with or without an ADAR1 expression vector. Three days post-transfection, the HBV RNA level was determined using RT-qPCR. The siNC sample is defined as value 1. **, p < 0.01.
poorer response to IFN-α therapy, compared with non-AA alleles. Interestingly, they also reported that the ADAR1 mRNA levels were lower in patients with chronic hepatitis B compared with healthy individuals. Thus, these studies suggest a potential antiviral role for ADAR1 in treating patients who have HBV.

In summary, to the best of our knowledge, this study demonstrates for the first time that ADAR1 can down-regulate HBV viral RNA by increasing miR-122 levels. However, the mechanism by which ADAR1 selectively increases miR-122 remains unclear. Further studies are required to clarify the detailed mechanism of ADAR1-mediated HBV RNA reduction as well as to verify its anti-HBV activity in vivo.

**Experimental procedures**

**Cell culture and transfection**

Plasmids were transfected into Huh7 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s instructions. The total amount of transfected plasmid per sample was equalized by supplementation with empty or mock expression plasmid. The co-transfection with plasmid and siRNA was also performed using Lipofectamine 2000. Stealth-grade siRNA, human ADAR1, cyclin G1, p53, and siNC were purchased from RiboBio Co., Ltd. (Guangzhou, China). siADAR1-1 and 1-2 target both p110 and p150 alternative splicing forms.

**NAGE assay**

The NAGE assay was performed as previously described (20, 22, 26). Briefly, intact nucleocapsid particles were separated from crude extracts of HBV-replicating cells using agarose gel electrophoresis. Nucleocapsid particles within the gel were denatured with alkaline and transferred onto nitrocellulose membrane (Roche Applied Science, Basel, Switzerland). Nucleocapsid DNA and core protein were detected by South-
ern and Western blotting, using a double-stranded HBV DNA probe spanning the entire viral genome and an anti-core antibody (Dako, Carpinteria, CA), respectively.

**Expression vectors and reagents**

Human IFN-α was purchased from HumanKine systems (EMD Millipore, Burlington, MA). The HBV replicon plasmid (pPB) contains 1.04 copies of HBV genomic DNA and expresses all viral transcripts, including pgRNA, in transfected cells (19). FLAG-Ago2 was a gift from Edward Chan (Addgene plasmid number 21538; RRID: Addgene_21538) (50). GFP-tagged ZAP-s and ADAR1 were generated by inserting their open reading frames into pEGFP vectors (Takara Bio, Shiga, Japan). Probe labeling and Northern and Southern blotting were developed using the AlkPhos Direct Labeling and Detection System (GE Healthcare). Signals for Northern, Southern, and Western blotting were analyzed using the LAS1000 Imager System (Fuji-film, Tokyo, Japan). The AID, A3C, and A3G expression vectors were used as previously described (20, 22, 26).

**Immunoprecipitation and Western blotting**

Cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.1), 20 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 2% glycerol, and protease inhibitor mixture (Roche). After centrifugation at 12,000 rpm for 10 min, supernatants were incubated with anti-FLAG M2-agarose beads 4 °C overnight (Sigma). Crude lysates were incubated with anti-FLAG M2 beads overnight. Western blotting was performed using the standard methodology. Rabbit-anti-GAPDH (Proteintech Group, Rosemont, IL), mouse anti-FLAG (Medical and Biological Laboratories Co., Ltd., Aichi, Japan), anti-core (Dako; B0586), rabbit anti-GFT (Santa Cruz Biotechnology, Dallas, TX), anti-rabbit IgG (LI-COR, Lincoln, NE), rabbit anti-cyclin G1 (Abclonal, MA), anti-mouse IgG (LI-COR, Lincoln, NE), rabbit anti-p53 (Abclonal), and mouse anti-ADAR1 (Santa Cruz Biotechnology) antibodies were used and the density of ADAR1 was measured using ImageJ software.

**qPCR and RT-PCR**

Total RNA was extracted using TRIzol (Thermo Fisher Scientific), treated with DNase I (TakaRa Bio) to eliminate the transfected plasmids, and reverse-transcribed with an oligo(dT) or specific primer using Superscript II (Thermo Fisher Scientific). qPCR was performed using SYBR Green ROX (TakaRa Bio) and qTower2.2 (Analytik Jena, Jena, Germany). The following PCR protocol was used: 95 °C for 1 min; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s; and one cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s (20). mRNA copy numbers of ADAR1, HBV, miR-122 RNA, HPRT, GAPDH, and β-actin were calculated using the ΔCt method. The RC-DNA from secreted virions or cytoplasmic lysate was purified by treatment with DNase I to eliminate transfected plasmids, then with proteinase K and SDS digestion to remove capsid protein, followed by phenol-chloroform extraction and isopropyl alcohol precipitation. The RC-DNA copy number was determined using an HBV plasmid standard curve. HPRT, β-actin, and GAPDH were used as internal controls to normalize expression levels in the qRT-PCR analyses. The primers used are listed in the Table S1. Unless noted, samples were tested in triplicate for each assay, and each dot in the figures indicates a technical replicate, which represents two biologically independent experiments.

**miRNA analysis**

miRNA was isolated from the total RNA using an miRNA Kit (Omega Bio-Tek Inc., Norcross, GA, China). The miRNA was polyadenylated using a poly(A) polymerase, and reverse-transcribed using a specific primer, RTQ, to synthesize the cDNA. The forward miR-122 primer and the universal reverse primer RTQ-UNIr (listed in the Table S1) were used for qRT-PCR analysis, as described in Ref. 51.

**ADAR1 knock-out using CRISPR/Cas9**

The construction of CRISPR vectors is described in Table S2. The lentiCRISPR version 2 was a gift from Feng Zhang (Addgene plasmid number 52961; RRID: Addgene_52961) (52). The vectors were transiently transfected into Huh7 cells. Two days post-transfection, cells were selected by culturing in 2 μg/ml of puromycin for 3 days, and knock-out efficiency was determined using Western blotting.

**Statistical analysis**

Statistical significance was calculated using a two-tailed unpaired Student’s t test. p < 0.05 was considered to be statistically significant.

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