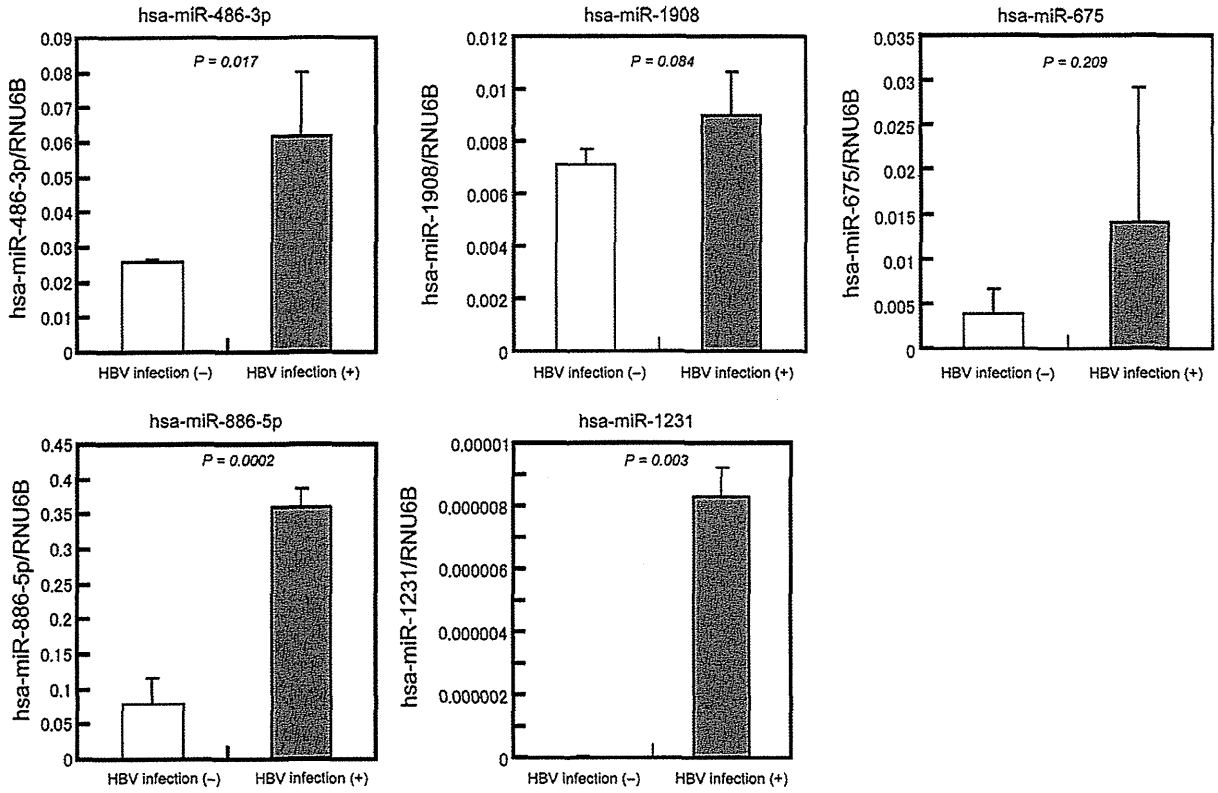

Fig. 1 Upregulation of microRNA by HBV infection. Signal intensities of five upregulated miRNAs were compared between HBV-infected and noninfected mouse livers. All 5 miRNAs were significantly upregulated by HBV infection. *P* values were calculated by the Mann–Whitney *U*-test.

Associations between signalling pathways and the upregulated miRNAs

To analyse the influence of miRNA upregulation on signalling pathways, pathway analysis was performed. However, there are several obstacles in analysing the association between miRNAs and pathways, such as the lack of reliable miRNA target prediction algorithms, differences in the results among target prediction systems, and the small number of validated target genes. To improve the reliability of the targets, we performed the pathway analysis in combination with four prediction tools (miRWalk, TargetScan, miRanda and miRDB). After this operation, 482 targets were predicted (hsa-miR-1231: 203 targets, hsa-miR-1908: 3 targets, hsa-miR-486-3p: 251 targets, hsa-miR-675: 25 targets), and these 482 targets were submitted to the PANTHER classification system for pathway analysis. As shown in Table 1, several immunological pathways such as inflammation mediated by chemokine and cytokine signalling pathway, and the interleukin signalling pathway were identified, but it was difficult to identify characteristic pathways.

Suppression of HBV replication with miR-1231 overexpression

Because hsa-miR-1231 was most the highly upregulated among these four miRNAs and had a high homology with the HBV genome, we focused on hsa-miR-1231. Using GENETYX ver. 8.2.1 (GENETYX, Tokyo, Japan), the hsa-miR-1231 sequence was predicted to hybridize at the HB core and X regions of the HBV genome (Fig. 3). To analyse the influence of hsa-miR-1231 on HBV replication, changes in HBV replication intermediates were evaluated using an *in vitro* HBV replication model. As shown in Fig. 4a, HBV replication intermediates were significantly reduced by hsa-miR-1231 overexpression, and the suppression of HBV RNA and HBc proteins were also observed by hsa-miR-1231 overexpression (Figs 4b,c). Thus, HBV replication was concluded to be inhibited by hsa-miR-1231 at the post-transcriptional level.

Specific regulation of HBV-related protein levels with hsa-miR-1231 overexpression

As the preceding results indicated an association between the production of HBV-related protein or HBV particles and

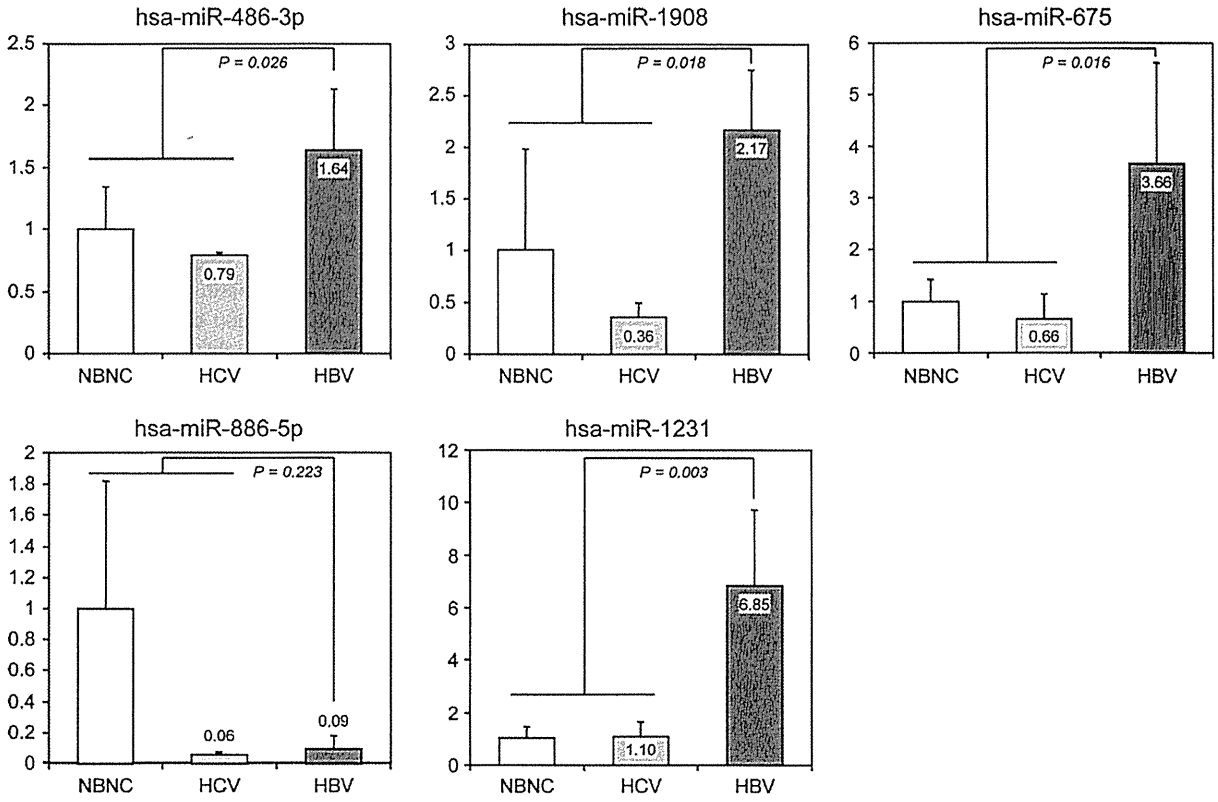

Fig. 2 Comparison of microRNA expression in clinical liver tissues. Quantification of miRNAs was performed by real-time PCR using nine human liver tissues obtained from the patients who had chronic hepatitis B ($N = 3$), C ($N = 2$) or alcoholic liver dysfunction ($N = 4$). Expression levels of four miRNA were significantly higher in the chronic hepatitis B patients than in those of other liver diseases. The results of miR-886-5p levels were not statistically significant. *P* values were assessed by Mann–Whitney *U*-test.

hsa-miR-1231 expression, further analysis was performed to identify the region hybridized by hsa-miR-1231. As shown in Fig. 5, HBc protein expression was remarkably reduced by hsa-miR-1231 expression, but no reduction in HBx protein was observed. These results indicate that hsa-miR-1231 might interact with HBV core mRNA and suppress HBV replication by inhibiting HBV core protein production.

The effects of hsa-miR-1231 on the expression of interferon-stimulated genes

Alternatively, hsa-miR-1231 might suppress HBV replication through activation of the interferon signalling pathway. We thus evaluated mRNA expression of interferon-stimulated genes (ISGs) with or without hsa-miR-1231 overexpression. None of the examined ISGs (MxA, PKR, OAS-1 and SOCS1) were regulated by hsa-miR-1231 expression (Fig. S3). These results suggest that hsa-miR-1231 suppresses HBV replication at the post-transcriptional level but not through the activation of interferon signalling.

DISCUSSION

Previously, we have demonstrated that human hepatocyte chimeric mice can be chronically infected with hepatitis B and C viruses [25,30,31]. This mouse model facilitates analysis of the effect of viral infection under immunodeficient conditions. In the present study, we performed miRNA array analysis using this mouse model and obtained miRNA expression profiles reflecting the direct influence of HBV infection on human hepatocytes. Furthermore, we found a novel mechanism for HBV replication mediated by hsa-miR-1231.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice were used in which liver tissue was largely (>90%) replaced by human hepatocytes. Although it is feasible to use microarray analysis in this chimeric mouse model [32], signals from miRNA array analysis may be influenced by cross-hybridization with mouse miRNA from a small amount of contaminated mouse-derived cells because of the high homology between the human and mouse genomes. To compensate

Table 1 Pathways associated with the 4 miRNAs upregulated by HBV infection

Pathway	Number of gene hits	Ratio of genes %
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	11	2.60
Angiogenesis (P00005)	10	2.30
Integrin signalling pathway (P00034)	9	2.10
Gonadotropin releasing hormone receptor pathway (P06664)	7	1.60
Wnt signalling pathway (P00057)	7	1.60
Parkinson disease (P00049)	7	1.60
EGF receptor signalling pathway (P00018)	7	1.60
Alzheimer's disease–presenilin pathway (P00004)	6	1.40
PDGF signalling pathway (P00047)	6	1.40
B-cell activation (P00010)	6	1.40
Interleukin signalling pathway (P00036)	5	1.20
Huntington disease (P00029)	5	1.20
FGF signalling pathway (P00021)	5	1.20
Cadherin signalling pathway (P00012)	5	1.20
VEGF signalling pathway (P00056)	4	0.90
Toll receptor signalling pathway (P00054)	4	0.90
T-cell activation (P00053)	4	0.90
Ras pathway (P04393)	4	0.90
Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha-mediated pathway (P00026)	4	0.90
Endothelin signalling pathway (P00019)	4	0.90

for contamination, mice that were negative for HBV infection were set up as negative controls.

Only 5 miRNAs showed more than 2.0-fold upregulation with HBV infection under miRNA array analysis using chimeric mouse livers (Fig. S1). Comparing these results with our previous study using patient sera, only hsa-miR-486-3p showed a similar change in sera from chronic hepatitis B patients, but no upregulation of the other 4 miRNAs was observed [15]. These results suggest that miRNA expression in sera from chronic hepatitis B patients might be regulated not only by HBV infection but also by human immune responses. In addition, it might be difficult to analyse changes in expression of miRNAs that are expressed at low levels in human hepatocytes, including hsa-miR-1231, using human serum.

To identify targets of miR-1231, we searched using four prediction systems. Although 632 target genes were identified (data not shown), and involvement of a number of pathways was indicated (Table S1), critical targets associated with human immunity or HBV replication could not be identified. Interferon signalling was also a potential mechanism of HBV suppression, but several ISG mRNAs were not induced by hsa-miR-1231 overexpression *in vitro* (Fig. S2). Therefore, we concluded that hsa-miR-1231 does not suppress HBV replication via interferon signalling.

To examine the possibility that miR-1231 directly regulates HBV replication by interacting with HBV-related mRNAs, we searched for hsa-miR-1231-binding motifs and found two candidate sequences in the HBV core and X genes (Fig. 3). As shown in Fig. 5, one target in the HBV core region could hybridize with hsa-miR-1231, and HBc expression was found to be suppressed by hsa-miR-1231 overexpression. The hsa-miR-1231-binding motif in the HBV core region was conserved in more than 90% of the HBV sequences in GenBank, regardless of HBV genotype (data not shown). Thus, we speculate that hsa-miR-1231 binds to the HBc target region and suppresses HBc production to inhibit HBV replication.

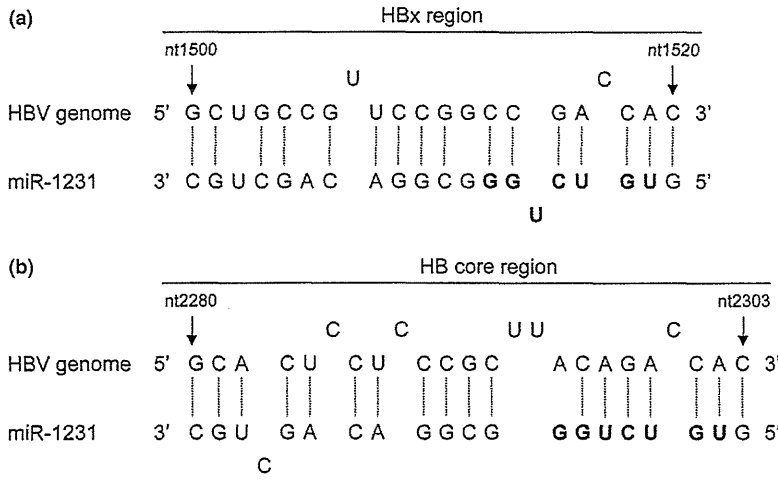

Fig. 3 Alignment of hsa-miR-1231 to HBV genome. Alignment of hsa-miR-1231 to the HBV genome was performed. MiR-1231 sequence was predicted to hybridize at the HBV core (a) and HBV X region (b).

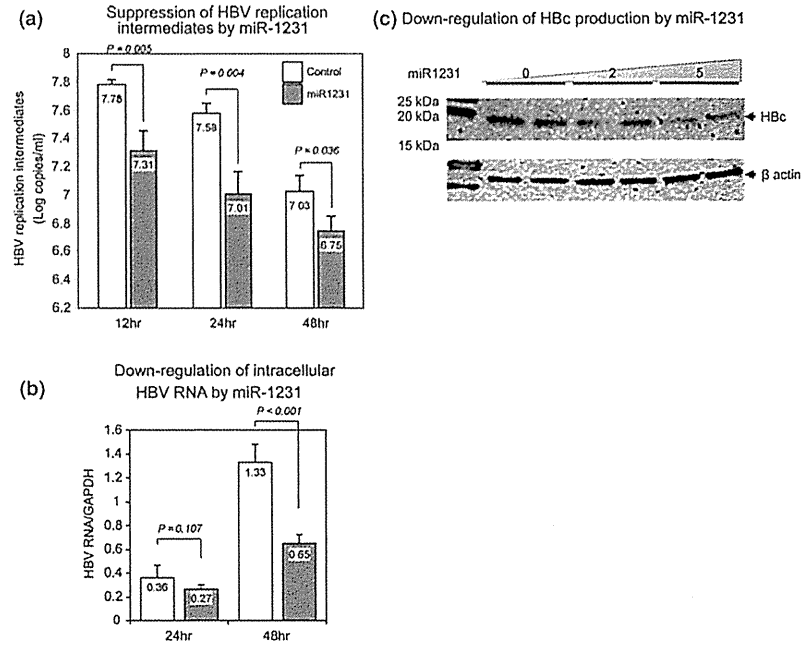

Fig. 4 Suppression of HBV replication by miR-1231. HBV replication intermediates were measured using an *in vitro* HBV replication model. (a) Production of HBV replication intermediates was significantly suppressed in cells transfected with both HBV and miR-1231 expression plasmids. (b, c) The levels of HBV RNA and HBc protein were also reduced by miR-1231 expression at 24 and 48 h after transfection.

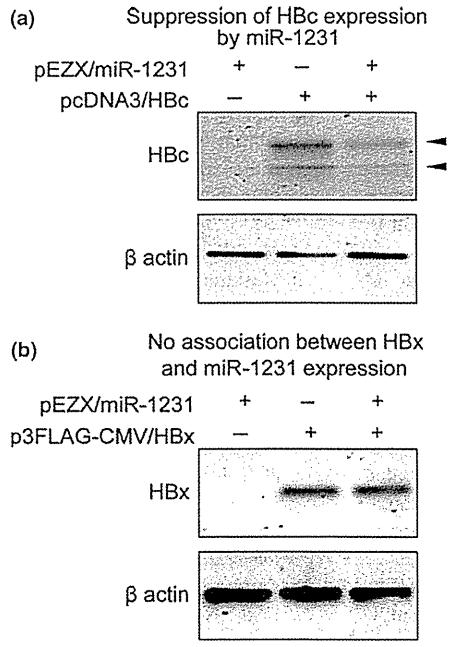

Fig. 5 Identification of miR-1231 target region in HBV genome. To determine the target for miR-1231, HBc or HBx expression plasmid was transfected into HepG2 cells with miR-1231 expression plasmid, and changes in protein levels were analysed by Western blot. HBc protein levels were reduced by miR-1231 expression (a), but HBx protein levels were not reduced (b).

To confirm the association between hsa-miR-1231 and HBV replication, we also tried to suppress hsa-miR-1231 expression using a miRNA inhibitor *in vitro*. However, no significant effects of miR-1231 inhibition on HBV replication were observed *in vitro*. As mentioned previously, expression levels of hsa-miR-1231 are quite low in HepG2 cells and human hepatocytes, and therefore, significant effects of hsa-miR-1231 inhibition could not be observed. The level of hsa-miR-1231 activity was also a factor. As shown in Fig. 4, HBV replication intermediates and HBc expression were significantly suppressed by hsa-miR-1231 overexpression, but the reduction rate was quite small even when 5-fold volume of hsa-miR-1231 plasmid and a volume of HBV expression plasmid were transfected into HepG2 cells. Therefore, it was difficult to observe changes in HBV replication by miRNA inhibition when HBV was replicating vigorously.

In conclusion, we performed miRNA array analysis using human hepatocyte chimeric mice and were able to analyse the direct effects of HBV infection without the confounding effects of the lymphocyte immunological response. We obtained evidence that hsa-miR-1231 was upregulated in response to HBV infection in human hepatocytes, whereupon hsa-miR-1231 suppressed replication of HBV.

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FINANCIAL DISCLOSURE

Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Takahashi S, Ochi H, Hayes CN, Chayama K: None to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: HBV infection regulated expression of several microRNAs. Complete linkage hierarchical clustering analysis was performed using Euclidean distance. Among the 900 miRNAs, 10 miRNAs showed more than 2.0-fold change between groups. Five of the 10 miRNAs were upregulated by HBV, and the other five were downregulated.

Figure S2: No effect of miR-1231 expression on IFN signalling. To analyse the influence of miR-1231 expression on interferon signalling, four interferon-stimulated genes (ISGs) were quantified by real-time PCR. None of the four ISGs (MxA, PKR, OAS-1 and *SOCS1*) were suppressed by miR-1231 expression.

Table S1: Pathway analysis of miR-1231 target genes.