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Original Article

The susceptibility of human hepatoma-derived oval-like cells to hepatitis B virus infection

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Abstract: Human hepatocytes are a primary site of infection and replication of the hepatitis B virus (HBV). It is tempting to conclude that tissue specificity is controlled via virus-hepatocyte specific interactions at various steps during the viral lifecycle. However, the molecular mechanisms underlying hepatotropism of HBV are not fully clear. To address this issue, this study aims to identify hepatic factors that contribute to the regulation of the lifecycles of hepatitis viruses- especially HBV- and to clarify their regulatory mechanisms. We established oval-like cell lines (Hdo cells) by introducing a set of reprogramming factors (OCT3/4, SOX2, KLF4, LIN28, and NANOG) into human hepatoma HuH7 cells that are susceptible to HBV. Hdo cells exhibit a bi-directional differentiation potential. We found that Hdo cells maintained support for the replication of HBV but not of HCV. The level of particle-associated HBV DNA secreted into the culture medium was higher in the Hdo cells. Still, the HBs antigen level was lower than in parental HuH7 cells, suggesting that the regulation of HBV gene expression was affected by the reprogramming of HuH7 cells. A microarray analysis determined that the expression of host factors was largely comparable among of HuH7 and Hdo cells. In contrast, Hdo cells lost their susceptibility to HCV infection and to replication of the viral subgenome replicon RNA. Our results suggest that epigenetic reprogramming of human hepatoma cells potentially changes their permissivity to HBV. Furthermore, Hdo cells can be used as powerful tools to identify cellular determinants that change their expression during reprogramming or hepatic differentiation.

Keywords: Hepatitis B virus, hepatic factors, oval-like cell lines, susceptibility

Introduction

About 400 million individuals worldwide are infected with hepatitis B virus (HBV), causing 1.2 million deaths annually from cirrhosis, chronic hepatitis B, hepatocellular carcinoma, and liver damage [1-5]. Patients with chronic HBV have an elevated risk of chronic active hepatitis, cirrhosis, or primary hepatocellular carcinoma [6, 7].

Generally, the progression of HBV replication is promoted byviral covalently closed circular DNA, which transcribes four unspliced RNA transcripts: 3.5, 2.4, 2.1, and 0.7 kb from the core, preS1, preS2/S and X promoters, and a variety of spliced viral RNAs. The transcriptional regulation of HBV genes is a prerequisite for the control of viral replication [8]. A variety of liver-enriched and ubiquitous transcription factors target the promoter and enhancer regions to regulate viral transcription and replication [9, 10]. In order to support the complete propagation of HBV [11], HuH7, which is a well-differentiated hepatocellular carcinoma cell line, has been commonly used for HBV research. We know of human induced pluripotent (iPS) and embryonic stem (ES) cells, but they are rarely used for HBV studies. Previous studies have demonstrated that hepatic progenitor cells, which have the characteristic features of oval cells, can be isolated in rodent livers [12-16].

Although it has long been thought that rodent oval cell counterparts can exist in the human liver, human progenitor cells well-characterized as oval cells have not been established to date. Also, virus-hepatocyte specific interactions at various steps during the viral lifecycle, such as entry into cells [17-20], genome replication [21-

24], translation, virion assembly and egress [25] have been reported on in a number of studies, but the molecular mechanisms underlying the hepatotropism of HBV are not fully understood yet.

To address this issue, this study aims to identify the hepatic factors that contribute to the regulation of the lifecycles of hepatitis viruses, especially HBV. To investigate the viruses' regulatory mechanisms, we introduced a set of reprogramming factors, OCT3/4, SOX2, KLF4, LIN28, and NANOG, into human hepatoma HuH7 [26] cells that are susceptible to HBV. New cells termed Hdo (HuH7-Derived Oval-like) cells exhibited a bi-directional differentiation potential and are established cell lines possessing oval-like cell features. We found that Hdo cells lost their susceptibility to HCV infection and replication but maintained support to propagate JEV, another Flaviviridae member and HBV, another hepatotropic virus.

Materials and methods

Plasmids

Plasmids containing the 1.3-fold HBV genomes derived from HBV genotypes Ae, Bj, and D60, pUC-HB-Ae, pUC-HB-Bj and pUC-HB-D60 [27, 28] respectively, were gifts from Dr. Mizokami (National Center for Global Health and Medicine, Japan). pUC-HB-Ce, contains the 1.3-fold HBV genome derived from a consensus sequence of HBV genotype Ce as previously described. pcDNA3.1/CT-GFP-TOPO was purchased from Invitrogen. pSV40-HBs was obtained from Dr. Tetsuro Suzuki (Department of Virology and Parasitology, Hamamatsu University School of Medicine).

Cell culture

Human hepatoma HuH7 cells (a gift from Francis V. Chisari, the Scripps Research Institute) were maintained as described [29]. Hdo cells were cultured in mTeSR1 (STEMCELL Technologies, Vancouver, Canada) on a CELLstart CTS (Thermo Fisher Scientific, Rockford, IL) pre-coated dish.

RNA extraction and qRT-PCR

Total RNA from the cells was isolated by the TRI reagent (Cosmo Bio, Tokyo, Japan) and transcribed by the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) as described [30]. Aliquots of cDNAs were subjected to 45 cycles of PCR amplification. The primer sequences were 5'-TCCCTCGCCTCGCAGACG-3' and 5'-GTTTCCCACCTTATGAGTC-3'. qRT-PCR was performed in the CFX Connect Real-Time System (Bio Rad) using THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan). Briefly, reversed transcribed cDNAs together with 6 pmol of forward and reverse primers were used for PCR. The thermal cycling conditions comprised 1 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. The RNA expression data were normalized to levels of GAPD using the comparative threshold cycle method.

Quantification of HBV DNA

HBV DNA was determined as previously described [31]. To quantify the particle-associated HBV DNA, culture supernatants collected from transfected cells were treated with PNE solution (8.45% PEG, 0.445 M NaCl, 13 mM EDTA) for 1 h on ice. To remove the free nucleic acids, the pellets were incubated at 37°C for 1 h with DNase I and RNase. After treatment with proteinase K at 56°C overnight, HBV DNAs were isolated with phenol/chloroform extraction and ethanol precipitation. HBV DNA copies were determined by quantitative PCR (qPCR) with primers 5'-TCCCTCGCCTCGCAGACG-3' and 5'-GTTTCCCACCTTATGAGTC-3'.

Electroporation

Trypsinized cells were washed with phosphate buffered saline (PBS) and resuspended at 1×10^5 cells/10 uL with a BTXpress buffer (BTX, Holliston, MA). 1 ug of DNA was mixed with 10 uL of cell suspension and electroporated by Neon (Thermo Fisher Scientific). The condition of electroporation was at 1400 V, 20 msec, and 2 pulse. Transfected cells were immediately seeded into 24-well plates.

Immunoblotting

Immunoblotting was performed as previously described with a slight modification [32]. Briefly, cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with an antibody against HBc, which was generated by immunizing rabbits with bacterially-expressed HBc protein, HBs (Institute of Immunology, Tokyo, Japan) or GAPDH (Santa

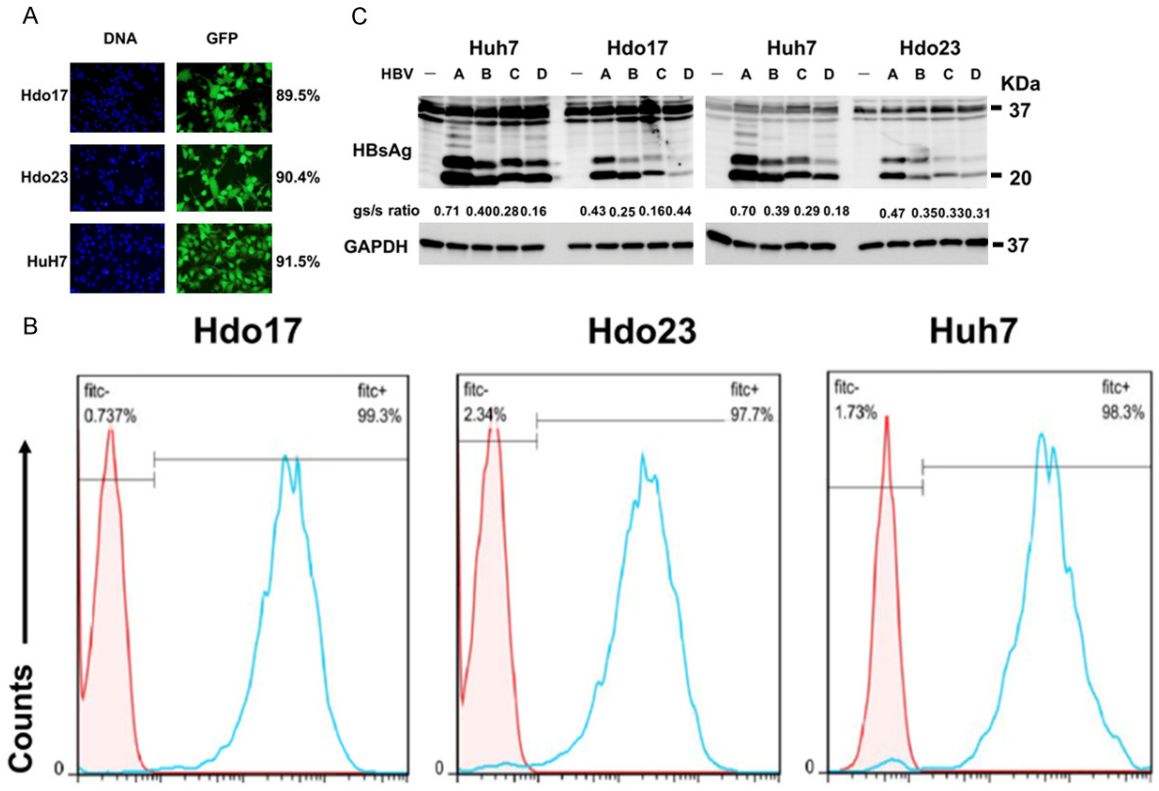

Figure 1. Determination of transfection efficiency between Hdo and HuH7 cells. A. Hdo and HuH7 cells were transfected with pCMV-emGFP by electroporation. After 2 days' transfection, GFP expression and the cells that were stained with Hoechst 33342 to label nuclei were checked by fluorescence microscope. B. GFP positive cells were screened by flow cytometry. C. HBV genomes of genotypes Ae, Bj, Ce and D60 were introduced into Hdo cells as well as HuH7 cells by electroporation, respectively. 3 days post-transfection later, HBs expression was detected from the HuH7 and Hdo cells.

Cruz Biotechnology, Dallas, TX) for 1 h. After washing, the membranes were incubated with an HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) for 0.5-1 h. Antigen-antibody complexes were detected using the ChemiDoc™ Imaging System (BIO-RAD Laboratories, Tokyo, Japan).

Results

Determination of transfection efficiency between Hdo and HuH7 cells

To generate undifferentiated cells derived from HuH7, cell reprogramming was induced via transduction with retroviral vectors expressing the Oct3/4, Sox2, Klf4, Nanog, and Lin28 genes, which are essential for the establishment and maintenance of the pluripotent state.

First, to test the transfection efficiency of the two kinds of Hdo and HuH7 cells, we performed electroporation with pCMV-emGFP. The GFP expression in the cells that were stained with Hoechst 33342 to label the nuclei was determined on day 2 post-transfection using a fluorescence microscope (**Figure 1A**). Demonstrate transfection efficiency was similar among Hdo17, Hdo23, HuH7cells. Also, flow cytometry data (**Figure 1B**) showed similar results. Further, to examine the susceptibility of Hdo cells to HBV replication, the viral genomes of genotypes Ae, Bj, Ce and D60 were introduced into Hdo cells as well as HuH7 cells by electroporation, followed by harvesting the cells at day 3 post-transfection. The HBs expression from the HuH7 cells was higher than the expression from the Hdo cells (**Figure 1C**). These results suggest that Hdo cells maintain support of the replication of HBV.

Genotype preference of HBV replication in Hdo and HuH7 cells

Next, we wanted to investigate whether the genotype preference of HBV replication was also the same in Hdo cells. First, HuH7 and Hdo

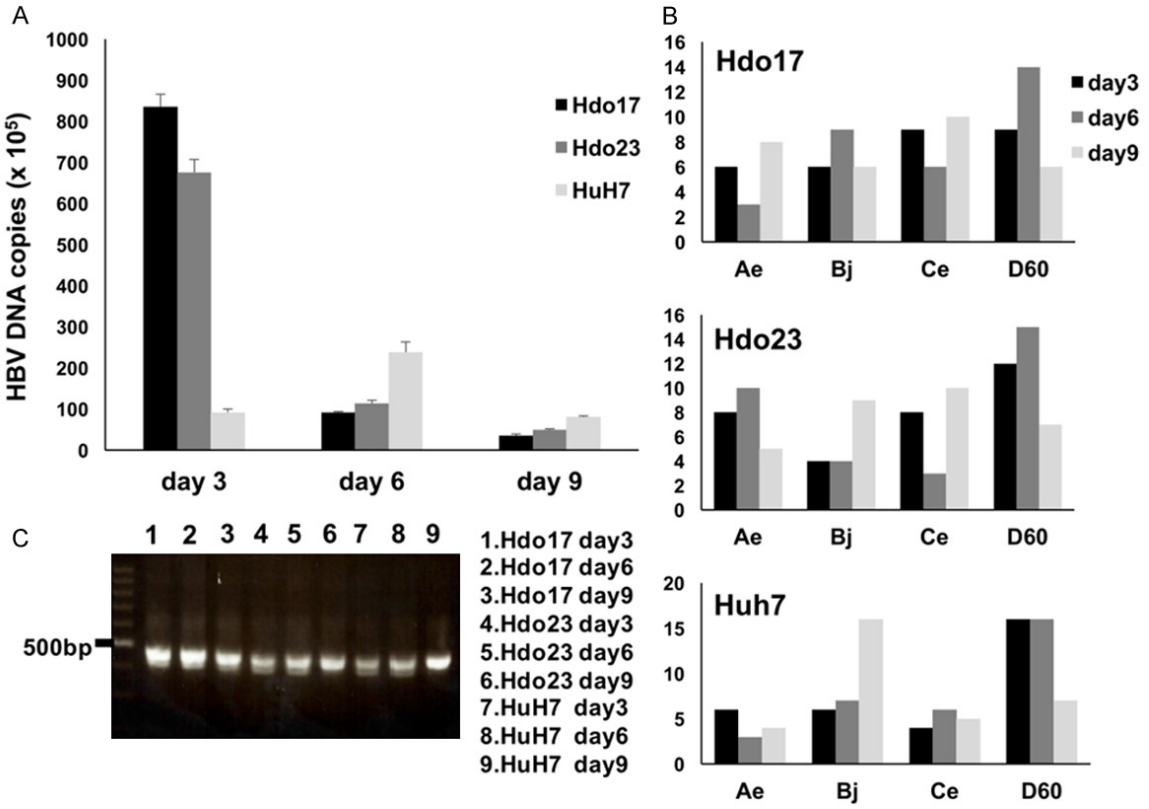

Figure 2. The genotype preference of HBV replication in Hdo and HuH7 cells. A. HBV DNA copies associated with the particles were detected from culture supernatants of Hdo17, Hdo23, Huh7 at days 3, 6, and 9 after transfection with HBV plasmids (genotypes A, B, C and D mixture). B. An HBV DNA fragment for the PreS region was amplified by checking it through electrophoresis and cloning it into a pCR 2.1-TOPO vector. C. Thirty clones from each time-point/cell sample were sequenced and the numbers of clones detected were indicated.

cells were transfected with 1 μg/well plasmid mixture with equimolar pUC-HB-Ae, pUC-HB-Bj, pUC-HB-Ce and pUC-HB-D60, which harbors a 1.3-fold size of the HBV genome. After 3, 6, and 9 days of transfection, the particle-associated HBV DNA copy numbers in the supernatants were examined by real-time PCR. We found the HBV genome replicated more efficiently in Hdo cells than in HuH7 cells, at least at an early time point such as day 3 post-transfection. Following this, the cells were transfected with the mixed plasmids as indicated in **Figure 2A**. The culture supernatants were collected at days 3, 6, and 9 post-transfection, followed by DNA extraction. An HBV DNA fragment for PreS region (**Figure 2B**) was amplified and cloned into a pCR 2.1-TOPO vector. Thirty clones from each time-point/cell sample were sequenced and the number of clones detected are indicated in **Figure 2C**. It seems that the genotype preference of HBV replication is different between HuH7 and Hdo cells.

HBV genome replication is more efficient but has a lower expression of HBs and HBc in the Hdo cells

To investigate whether replication of HBV occurs in Hdo cells as well as in HuH7 cells, the cells were transfected with plasmids containing 1.3-fold HBV genome derived from genotypes Bj and Ce at 3 and 6 days post-transfection, followed by determining the particle-associated HBV DNA (**Figure 3A**) in the culture supernatants by qPCR and the viral proteins in the cells by western blot **Figure 3B**. Although the HBV genome replicates 3- to 5-fold higher in the Hdo transfected cells than in the HuH7 transfected cells, the expression levels of HBs and HBc antigen in the transfected Hdo cells seemed moderately lower than those in the transfected HuH7 cells. Unlike the case of HCV, immunostaining indicated that the efficiency of JEV infection and replication was comparable in Hdo and HuH7 cells. Thus, the impaired sus-

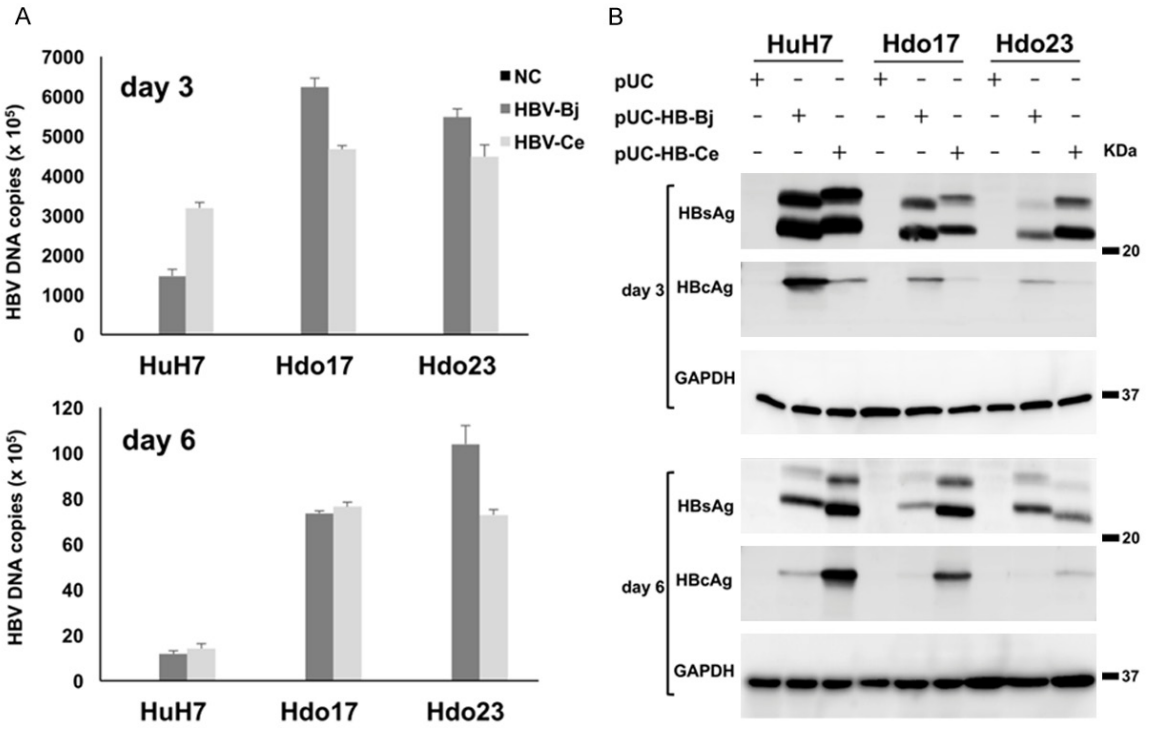

Figure 3. HBV replication in Hdo and Huh7 after transfection with HBV genomes with genotype Bj or Ce. HuH7 and Hdo were transfected with pUC-HB-Bj and pUC-HB-Ce. After 3 and 6 days of transfection, the particle-associated HBV DNA copies in the culture supernatants (A) and protein level in cells (B) were determined by real-time PCR and immunoblotting.

ceptibility to infection and replication during the directed reprogramming of HuH7 cells appears not to be a trait that applies to this variety of viruses but is a characteristic of this particular virus.

Pregenomic promoter activities and RNA level in Hdo cells compared to HuH7 cells

To demonstrate whether the HBV genome replicated more efficiently in Hdo cells than in HuH7 cells from pregenomic promoter activities and RNA expression, we further examined pregenomic promoter activities and the RNA levels in the HuH7 and Hdo cells. HuH7 and Hdo cells were transfected with the promoter reporter pGL4.74-HBpg-Bj, pGL4.74-HBpg-Ce. At day 3, GFP positive cells (an equal number of GFP positive cells of three types cell were screened by flow cytometry) were lysed by a passive lysis buffer and the luciferase activity was measured by illuminometer (**Figure 4A**). Also, HuH7 and Hdo cells were transfected with the 1.3 fold HBV full genome plasmids pUC-HB-Bj and pUC-HB-Ce by electroporation. Three days after transfection, the HBV pregenome RNA level was examined by real-time PCR (**Figure 4B**). We found the promoter activities for the HBV pregenome as well as the pregenomic RNA level were higher in Hdo compared to the levels in HuH7 cells. These results demonstrate that HBV genome replication is more efficient in Hdo cells because of more efficient pregenomic promoter activity in Hdo cells.

Quantification of HBV particles secreted in the culture supernatants of HuH7 and Hdo cells

To further assess the level of HBV particles secreted, we conducted an OptiPrep density gradient fractionation of the culture supernatants, followed by western blotting and qPCR. The supernatants collected from both the HuH7 and Hdo cells transfected with pUC-HB-Ce or pSV40-HBs were subjected to density fractionation followed by a quantification of the HBV DNA (**Figure 5A**) and a determination of the western blot of HBs protein (**Figure 5B**). The distribution of the viral DNA in fractions of the culture supernatant showed a similar pattern,

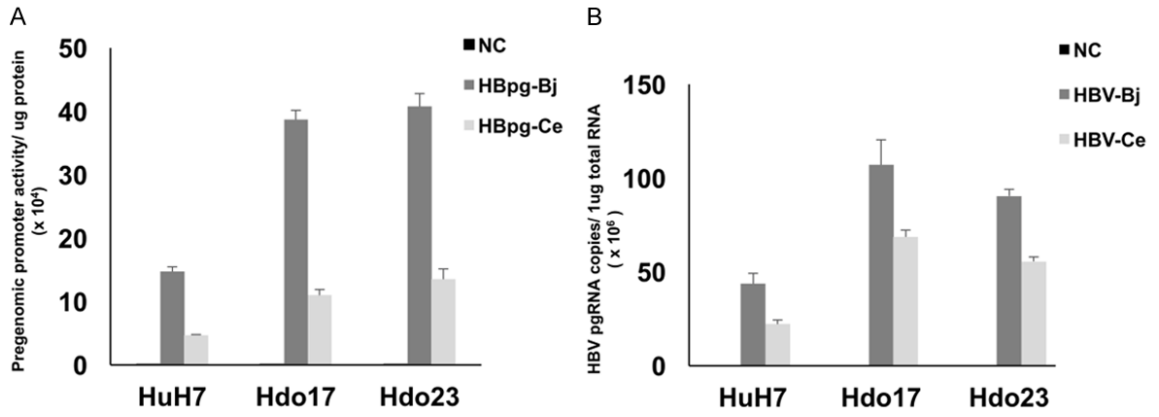

Figure 4. HBV pregenome promoter activities and RAN level of GT-Bj or GT-Ce in HuH7 and Hdo cells. A. HuH7 and Hdo cells were transfected with pGL4.74-HBpg-Bj, pGL4.74-HBpg-Ce. After 3 days of transfection, the GFP positive cells (equal cell numbers of three kinds of cells) were lysed by a passive lysis buffer and the luciferase activity was measured by a luminometer. B. The HBV pregenome RNA copy number of GT-Bj or GT-Ce in transfected HuH7 and Hdo cells was examined by real-time PCR, 3 days after transfection.

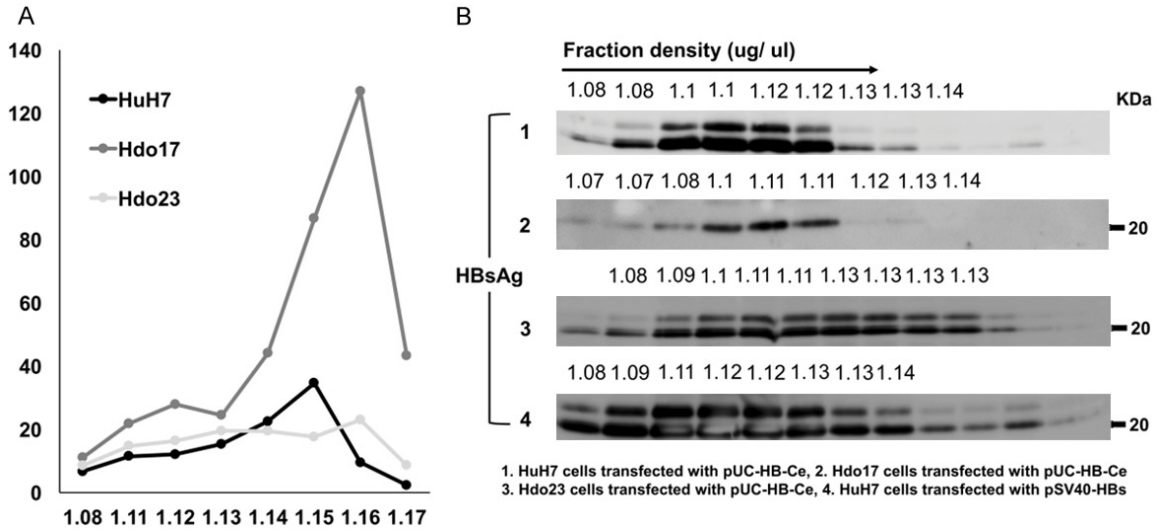

Figure 5. Quantification of HBV particles secreted in the culture supernatants of HuH7 and Hdo cells transfected with HBV Ce. A and B. HuH7 and Hdo cells were transfected with pUC-HB-Ce or pSV40-HBs by electroporation. After 3 days post-transfection, the supernatants collected from both transfected HuH7 and Hdo cells were subjected to density fractionation followed by quantification of HBV DNA and determination of the western blot of HBs protein.

with the highest DNA levels observed in the fraction with the Hdo17 cells. As for the Hdo cell-derived fractions, the distribution pattern of HBs antigen differed considerably from that of the HuH7 cells.

Discussion

Infection by hepatitis B virus (HBV) causes many hepatocyte changes due to the direct action of a protein coded for by the virus, HBx, and due to indirect changes caused by a large increase in intracellular reactive oxygen species (ROS) after infection [32]. HBx appears to dysregulate a number of cellular pathways. HBx causes dysregulation in part by binding to genomic DNA, changing the expression patterns of miRNAs, affecting histone methyltransferases, binding to the SIRT1 protein to activate transcription, and cooperating with histone methylases and demethylases to change cell expression patterns [33]. HBx is partly respon-

sible for the approximate 10,000-fold increase in intracellular ROS upon chronic HBV infection [34]. Increased ROS can be caused, in part, by the localization of HBx to the mitochondria where HBx decreases the mitochondrial membrane potential [35]. In addition, another HBV protein, HBsAg, also increases ROS through interactions with the endoplasmic reticulum. Increased reactive oxygen species (ROS) after HBV infection cause inflammation, in turn further increasing ROS [36]. ROS causes more than 20 types of DNA damage.

Hepatotropism is a primary research orientation of infection and replication of the hepatitis B virus (HBV) and the hepatitis C virus (HCV), and it is tempting to conclude that tissue specificity is controlled via virus-hepatocyte specific interactions at various steps during the viral lifecycle such as entry into cells, genome replication, translation, virion assembly, and secretion [37, 38]. To address this issue, we established oval-like cell lines (Hdo cells) by introducing a set of reprogramming factors; OCT3/4, SOX2, KLF4, LIN28, and NANOG into human hepatoma HuH7 cells that are susceptible to HBV and HCV. The Hdo cells exhibited a bi-directional differentiation potential. Under the culture for induction of hepatocyte differentiation, the expression of the mature hepatocyte marker albumin and of the hepatoblast marker alpha-fetoprotein increased and decreased, respectively.

Comparing the transfect Hdo and HuH7 cells with the HBV genome, we find the HBV genome replicated more efficiently in the Hdo cells than in HuH7 cells, at least at an early time point such as day 3 post-transfection (**Figures 2A** and **3A**). It seems that the genotype preference of HBV replication is different between the HuH7 and Hdo cells (**Figure 2C**). The expression levels of the HBs and HBc antigens were lower in the Hdo cells compared to those in the HuH7 cells (**Figure 3B**). The promoter activities for HBV pregenome (**Figure 4A**) as well as the pregenome RNA level (**Figure 4B**) were higher in Hdo compared to those in the HuH7 cells. The level of particle-associated HBV DNA secreted into the culture medium was rather higher in the Hdo cells but the HBs antigen level was lower than parental HuH7 cells, suggesting that the regulation of HBV gene expression was possibly affected by the reprogramming of HuH7 cells. From the microarray analysis between the HuH7 and Hdo cells, the expression of the host factors known to be involved in HBV pregenome RNA expression was largely comparable between the cells.

Based on these findings, we suggest that the epigenetic reprogramming of human hepatoma cells changes their permissivity to HBV, and that established Hdo cells can be powerful tools to identify cellular determinants which change their expression during reprogramming or hepatic differentiation and are important for viral infection and replication.

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Disclosure of conflict of interest

None.

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