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Negative regulation of hepatitis B virus replication by forkhead box protein A in human hepatoma cells

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ABSTRACT

Hepatitis B virus (HBV) replication is controlled by liver-enriched transcriptional factors, including forkhead box protein A (FOXA) members. Here, we found that FOXA members are directly and indirectly involved in HBV replication in human hepatic cells. HBV replication was elevated in HuH-7 treated with individual FOXA members-specific siRNA. Reciprocally, the downregulation of HBV replication was observed in FOXA-induced HuH-7. However, the mechanism of downregulation is different among FOXA members at the level of HBV RNA transcription, such as precore/pg RNA and 2.1 kb RNA. In addition, FOXA1 and FOXA2 suppressed nuclear hormone receptors, such as HNF4α, that are related to HBV replication.

1. Introduction

Hepatitis B virus (HBV) is one of the major causes of acute and chronic hepatitis leading to liver cirrhosis and to hepatocellular carcinoma (HCC). HBV has a partially double-stranded circular 3.2 kb genome which carries four viral genes, C (for core and e antigen), P (for DNA polymerase), S (for surface antigens), and X (for X protein). The expression of viral transcripts is regulated by four promoters (Cp, S1p, S2p, and Xp) and two enhancers (Enhancer I and II) [1]. The binding of liver-specific transcriptional factors such as hepatocyte nuclear factors (HNFs) and CCAAT/enhancer-binding protein family (C/EBP) members to those promoters and enhancers is thought to determine the liver tropism of HBV [2].

There are no cell culture systems that reflect the HBV life cycle because differentiated phenotypes of the liver are partially diminished or changed in the culture. For example, the lack of Na^+/taurocholate cotransporting polypeptide (NTCP), which was characterized as a functional HBV receptor, was reported in HuH-7 and HepG2 cells [3]. It has also been reported that C/EBPα is involved in the terminal differentiation of the liver and its upregulation in some HCC cell lines contributes to cell growth [4]. These results suggested that the intracellular environment of HCC-derived cell lines, including the expression of liver-specific transcriptional factors, was not suitable for HBV replication.

Forkhead box protein A (FOXA), also known as hepatic nuclear factor 3 (HNF3), consists of three members, FOXA1 (HNF3α), FOXA2 (HNF3β) and FOXA3 (HNF3γ). FOXA is one of the liver-enriched transcriptional factors and plays important roles in both liver development and liver metabolism [5,6]. FOXA is also thought to be a key regulator of HBV replication, because all HBV promoters and enhancers contain a FOXA-binding motif. In fact, FOXA has been shown to activate the transcriptional activity of HBV promoters and enhancers in a reporter assay [7–11]. However, pregenomic

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RNA (pgRNA) expression was repressed by FOXA2 in NIH3T3 cells that stimulate HBV replication by transfecting both HBV- and HNF4α-encoded plasmids [12]. Pervious studies were performed using non-hepatic cells. Therefore, further studies using genome-length HBV and human hepatic-derived cells will be needed to understand the roles of FOXA members in HBV replication. There are several reports indicating that HBV is regulated by FOXA2 in vivo. For instance, HBV replication was decreased in HBV transgenic mice transfected with rat FOXA2 [13]. Moreover, the distribution of HBV replication was negatively correlated with FOXA2 expression in the liver of patients with chronic hepatitis B [14]. These results suggested that, at the very least, FOXA2 negatively regulated HBV replication. To further elucidate the role of FOXA in HBV replication, studies describing other FOXA members are required. In this report, we investigated the role of all FOXA members in HBV replication using human hepatic-derived cell culture systems.

2. Materials and methods

2.1. HBV plasmid, antibodies, and siRNAs

HBV plasmid (pUC19/C_JPNAT) was kindly provided by Dr. Tanaka (Nagoya City University). Anti-FOXA1 antibody (Ab) (Anti-FOXA1 (ab2)) was obtained from Sigma (St. Louis, MO). Anti-FOXA2 Ab (D56D6) was obtained from Cell Signaling Technology (Beverly, MA). Anti-FOXA3 Ab (ab108454) and anti-HNF4α Ab were obtained from Abcam (Cambridge, MA). Anti-HBsAg (bs-1557G) Ab was obtained from Bioss (Boston, MA). siRNAs were obtained as siGENOME SMARTpool siRNA (human FOXA1: M-010319-01; human FOXA2: M-010089-01; human FOXA3: M-010319001; and Non-Targeting siRNA Control pool: D-001206-13) from Thermo Fisher Scientific (Waltham, MA).

2.2. Silencing of FOXA gene expression by RNA interference

HuH-7 cells were plated on a collagen-coated plate at a density of 2×10^4 cells/cm^2 and precultured in 10% FBS/DMEM for 24 h. The precultured HuH-7 cells were transfected with control, FOXA1-, FOXA2-, or FOXA3-specific siRNA by using a transfection reagent, Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). Twenty-four hours after the treatment, the medium was replaced and then the cells were transfected with pUC19/C_JPNAT using FuGENE HD (Promega, Madison, WI). Finally, the medium was replaced at 24 h after transfection and the samples were collected 2 days later.

2.3. Establishment of Tet-inducible FOXA-expressing cells

Tet-inducible FOXA-expressing HuH-7 cells were established using a Retro-X™ Tet-On Advanced Inducible Expression System (Takara-Bio Inc., Shiga, Japan). Briefly, we infected HuH-7 cells with a retrovirus vector, pRetroX-Tet-On Advanced, and used G418 to select the cells with stable RetroX-Tet-On Advanced HuH-7 clones. We next infected the clone with a retrovirus vector, either pRetroX-Tight-Pur-FOXA1, FOXA2 or FOXA3 and selected the cells with puromycin to generate Tet-inducible FOXA-expressing HuH-7 cells (HuH-7/Tet/FOXA). HuH-7/Tet/FOXA cells were plated on a collagen-coated plate at a density of 6×10^4 cells/cm^2 and precultured in 10% tetracycline-free FBS (Takara) containing DMEM for 24 h, and then the medium was replaced with ±1 μg/ml doxycycline (dox)-containing medium to induce FOXA expression. At the same time point, cells were transfected with pUC19/C_JPNAT using FuGENE HD. The medium was replaced at 24 h after transfection and samples were collected 3 days later.

2.4. Western blot analysis

Total cellular protein was extracted with RIPA buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was determined using a BCA protein assay kit (Thermo). Five micrograms of total protein extract was subjected to SDS–PAGE. After the electrophoresis, proteins that had migrated on the gel were transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with a skim milk solution. The membrane was first incubated with the primary Ab and then with the horseradish peroxidase-conjugated secondary Ab. The protein bands were visualized by using a Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA). The intensity of each band was quantified with image analyzer (Image J, NIH, Bethesda, MD, USA).

2.5. Detection of HBV RNA

Total RNA was extracted from cells by using Isogen reagent (Nippon Gene, Tokyo, Japan). Total RNA was treated with RNase-free DNase I (Promega) to remove contaminated plasmid DNA. Northern blot was performed to detect HBV transcripts. Five micrograms of DNase-treated total RNA was subjected to agarose/formaldehyde gel electrophoresis, then transferred onto Hybound P$^+$ membrane (GE). HBV RNA was hybridized with DIG-labeled 0.4 kb HBV DNA probe designed at X ORF, then detected by DIG detection kit (Roche). Real-time RT-PCR was performed to analyze precore and pregenomic RNA (pgRNA) levels by the fluorescent dye SYBR Green I method using the SYBR Premix Ex Taq, Perfect Real Time (Takara) with a LightCycler Nano System (Roche Diagnostics, Basel, Switzerland). The primer pairs for precore RNA or precore/core RNA were designed according to previous report [15]. The level of pgRNA was calculated by subtracting the value of precore RNA from that of precore/core RNA.

2.6. Detection of capsid associated HBV DNA

Intracellular capsid HBV DNA was detected by Southern blot as described previously with minor modifications [16,17]. Briefly, cells were lyzed with 1% NP-40, 1 mM EDTA, 50 mM Tris–HCl (pH7.5) and protease inhibitor cocktails (Roche), then centrifuged to remove nuclei. The supernatant was treated with DNase I, and then proteins were digested with SDS and proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Nucleic acid was purified with 2 times phenol/chloroform extractions and ethanol precipitation. Southern blot was performed by using DIG High Prime DNA Labeling and Detection Kit (Roche). DIG-labeled 3.2 kb whole HBV genome (C_JPNAT) was used to detect HBV replicative intermediates.

2.7. Detection of HBV DNA in the culture supernatant

The supernatant of HuH-7 cells after transfection of HBV plasmid was centrifuged at 15 000 rpm for 5 min to remove cell debris. The supernatant was treated with DNase I in the presence of 100 mM $MgCl_2$ and 10 mM $CaCl_2$ at 37 °C, then the reaction was stopped by the addition of EDTA. Viral DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). Real-time PCR was performed to detect HBV DNA with the specific primers described previously [18].

2.8. Analysis of host gene expression

Real-time RT-PCR was performed to detect host gene expression as described elsewhere. The primer pairs used in this experiment were showed in supplementary Table S1.