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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.
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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.

2.9. Statistical analysis

Data represent the mean ± standard error of at least triplicate experiments. *P*-value were determined by Student's *t*-Test. $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. FOXA gene silencing increased HBV replication

To clarify the role of FOXA2 in HBV replication, we performed silencing of the FOXA2 gene using a FOXA2-specific siRNA in human-hepatoma derived HuH-7 cells. We confirmed all FOXA members were expressed at the protein level in HuH-7 cells. With FOXA2-specific siRNA treatment, FOXA2 protein expression in HuH-7 cells was obviously suppressed (Fig. S1a). Cell growth was not changed in FOXA2-specific siRNA-treated cells (Fig. S1d). Under these conditions, we observed the expression of 3.5 kb, 2.1 kb and 0.7 kb HBV RNA were increased in FOXA2-specific siRNA-treated cells by Northern blot analysis (Fig. 1a). We could not compare the expression of 2.4 kb RNA because the expression level was low in our experimental system. We further investigated the expression of precore/pg RNA by real time-RT-PCR using their specific primers. Although precore RNA was not changed by FOXA2 gene silencing, the expression of pgRNA was elevated in FOXA2-specific siRNA-treated cells (Fig. 1b). The expression ratio of precore/pg RNA was decreased by FOXA2 gene silencing (Fig. 1b). The HBV replicative intermediates were increased in cells treated with FOXA2-specific siRNA (Fig. 1c). The synthesis of small S proteins (gp27 and p24) was elevated in FOXA2-specific siRNA-treated cells (Fig. 1d). Secreted HBV DNA in the culture medium from FOXA2-specific siRNA-treated cells was significantly elevated in comparison with that from control siRNA-treated cells (Fig. 1e). These results indicated that HBV replication was elevated in FOXA2 siRNA-treated cells.

We next investigated the role of other FOXA members, FOXA1 and FOXA3, in HBV replication (Fig. 2). FOXA1 and FOXA3 protein expression was suppressed with FOXA1- and FOXA3-specific siRNA, respectively (Fig. S1b). Cell growth was decreased in FOXA1-specific siRNA-treated cells and slightly decreased in FOXA3-specific siRNA-treated cells (Fig. S1e). HBV replication was increased in both FOXA1- and FOXA3-specific siRNA-treated cells, as indicated by HBV RNA expression (Fig. 2a), pgRNA expression (Fig. 2b), HBV replicative intermediates (Fig. 2c), small S protein level (Fig. 2d) and the supernatant HBV DNA level (Fig. 2e). Since the redundant function was observed in the individual FOXA-specific siRNA treatment, we investigated the effect of combination treatment of each FOXA-specific siRNA on the HBV replication (Fig. 2f). The supernatant HBV DNA level was 4-fold increased in all FOXA-specific siRNA mixture treated cells. The results of a series of FOXA gene-silencing experiments showed that HBV replication was elevated in HuH-7 cells treated with FOXA siRNA, but the phenotype was slightly different among FOXA members.

3.2. Induction of FOXA reduced HBV replication

To further study the role of FOXA in HBV replication, we established dox-inducible FOXA expressing HuH-7. We investigated whether the expression of each type of FOXA was induced by dox treatment (Fig. S1c). Cell growth was not changed by the induction of each FOXA gene (Fig. S1f). HBV transcription and replication were strongly suppressed by the induction of either FOXA1 or FOXA2 gene, and slightly suppressed by the induction of FOXA3 (Fig. 3a and c). FOXA members inhibited pgRNA expression rather than precore RNA expression (Fig. 3b). Interestingly, the ratio of

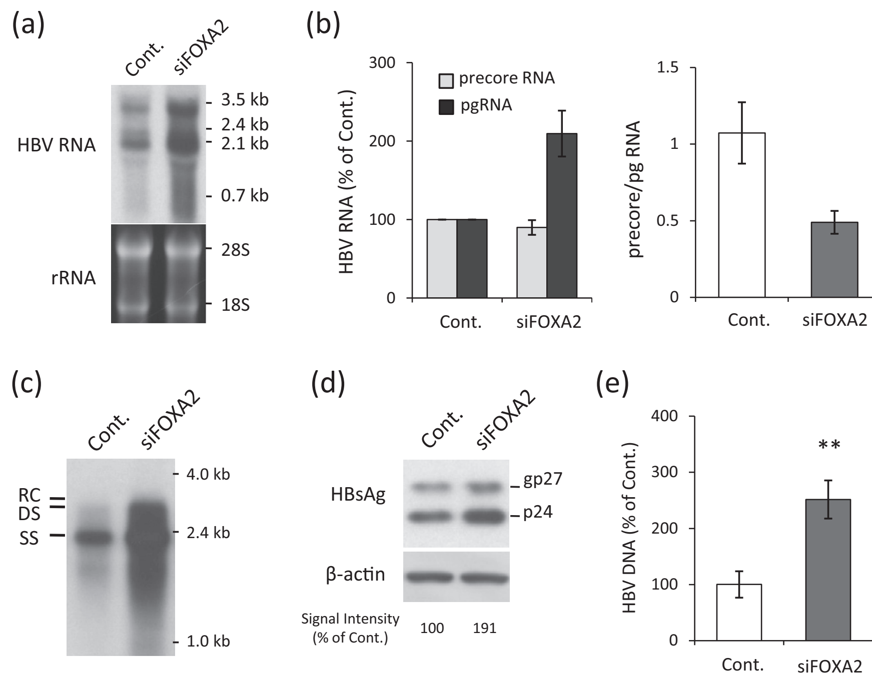

Fig. 1. FOXA2 gene silencing promoted HBV replication in HuH-7 cells. (a) HBV transcripts were analyzed by Northern blot. Ribosomal RNA (rRNA) was used as internal control. The data shows representative results of at least three independent experiments. (b) The expression of precore RNA and pgRNA were measured by real-time RT-PCR. Left graph shows the amount of precore RNA and pgRNA mRNA expression, which were expressed as the percent of that in cells treated with control siRNA (Cont.). The ratio of precore RNA to pgRNA was calculated and shows on the right graph. (c) HBV replicative intermediates were analyzed by Southern blot. RC (relaxed circular), DS (double-stranded) and SS (single-stranded) DNA were indicated (d) The intracellular HBsAg level was assayed by Western blot analysis. The bands gp27/p24 were indicated as small S protein respectively. The band intensity was quantified by densitometric analysis and indicated below each lane. The value of the amount of gp27/p24 was normalized to that of β-actin, and expressed as percent of control siRNA (Cont.). (e) HBV DNA in the culture medium at 3 days after HBV plasmid transfection (= 4 d after siRNA transfection) was quantified by real-time PCR after DNase I treatment. Data were expressed as the percent of that in cells treated with control siRNA (Cont.).

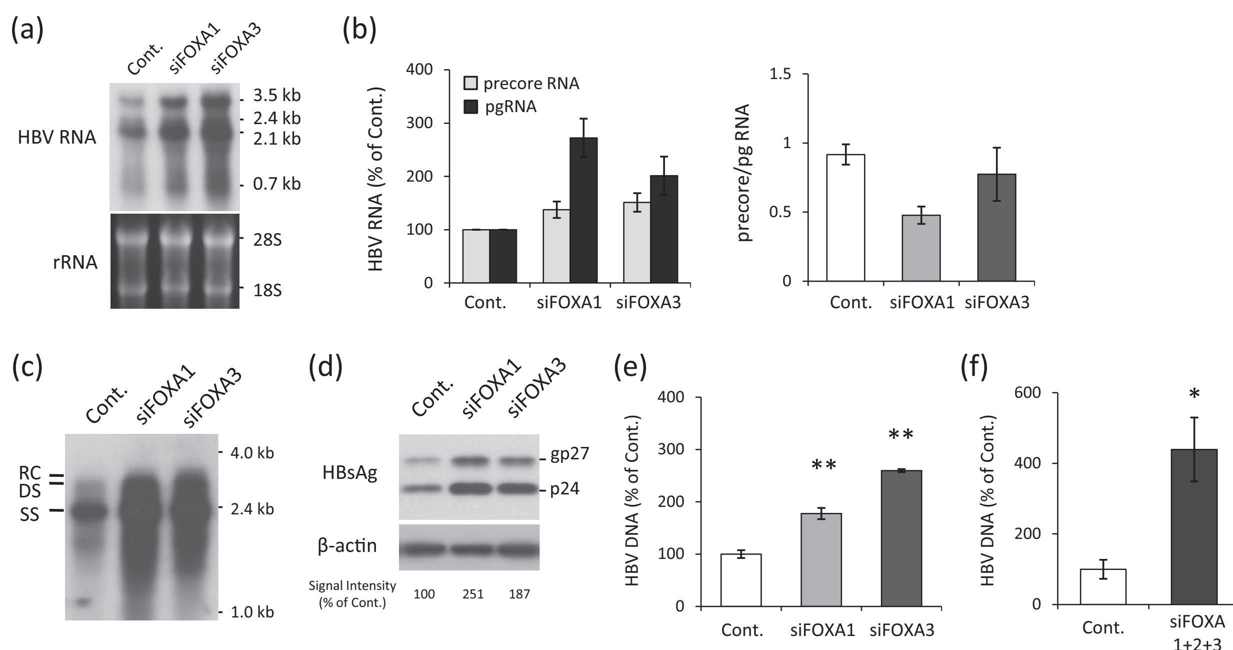

Fig. 2. FOXA1 and FOXA3 gene silencing promoted HBV replication in HuH-7 cells. (a) HBV transcripts were analyzed by Northern blot. Ribosomal RNA (rRNA) was used as internal control. The data shows representative results of at least three independent experiments. (b) The expression of precore RNA and pgRNA were measured by real-time RT-PCR. Left graph shows the amount of precore RNA and pgRNA mRNA expression, which were expressed as the percent of that in cells treated with control siRNA (Cont.). The ratio of precore RNA to pgRNA was calculated and shows on the right graph. (c) HBV replicative intermediates were analyzed by Southern blot. RC (relaxed circular), DS (double-stranded) and SS (single-stranded) DNA were indicated (d) The intracellular HBsAg level was assayed by Western blot. The bands gp27/p24 were indicated as small S protein. The band intensity was quantified by densitometric analysis and indicated below each lane. The value of the amount of gp27/p24 was normalized to that of β-actin, and expressed as percent of control siRNA (Cont.). (e) HBV DNA in the culture medium at 3 days after HBV plasmid transfection (= 4 d after siRNA transfection) was quantified by real-time PCR. Data were expressed as the percent of that in cells treated with control siRNA (Cont.). (f) HuH-7 cells were transfected with 50 nM of control (Cont.) or combination of FOXA1 (10 nM), FOXA2 (30 nM) and FOXA3 (10 nM)-specific siRNA (siFOXA1 + 2 + 3) by using a transfection reagent. HBV DNA in the culture medium at 3 days after HBV plasmid transfection was quantified by real-time PCR. Data were expressed as the percent of that in cells treated with control siRNA (Cont.).

precore/pg RNA was increased only in FOXA2-induced cell (Fig. 3b). Small S proteins were decreased in FOXA1- and FOXA2-induced cells, but not in FOXA3-induced cells (Fig. 3d). Secreted HBV DNA in the culture supernatant was significantly decreased in cells overexpressing any of the FOXA members (Fig. 3e). These results indicated that FOXA induction suppressed HBV replication, but the mechanism was different among FOXA members.

3.3. Regulation of hepatic differentiation by FOXA members

Liver-enriched transcriptional factors control hepatic differentiated states in the liver and is thought to engage in crosstalk [19,20]. HNF4α is a central factor which involves in hepatic maturation and regulates many liver-specific genes, including albumin [21]. HNF4α has also been reported to be a positive regulator of HBV replication [22,23]. Therefore, we investigated the possibility that FOXA members regulated HBV replication via HNF4α and other nuclear hormone receptors by using a Tet-inducible FOXA-expressing system. HNF4α mRNA expression was significantly suppressed by approximately 50% in FOXA1- and FOXA2-induced cells (Fig. 4a). However, the induction of FOXA3 did not significantly affect the HNF4α level. We also obtained similar expression pattern regulated by FOXA members in RXRα and PPARα expression but not in HNF1α and HNF1β (Fig. 4b). These results suggested that HBV replication was negatively regulated by FOXA members, partly mediated via the downregulation of HNF4α and other nuclear hormone receptors expression.

4. Discussion

Previous studies demonstrated that all HBV promoters and enhancers contain at least one FOXA binding site [2]. In this study, we showed that the transcription of 3.5 kb, 2.1 kb and 0.7 kb RNA were regulated by FOXA members (Figs. 1a, 2a, and 3a). 3.5 kb RNA contains precore RNA and pgRNA. The former codes HBeAg, which is reported as a negative regulator for HBV [24]. The latter codes core and polymerase and also acts as a template for HBV DNA, so that pgRNA directly serves for HBV replication [25]. Actually, the mutations, A1762T and G1764A, which was frequently observed in chronic hepatitis B patients, suppressed precore RNA expression and shows high HBV replication [26]. Therefore, the change of the expression ratio of precore/pg RNA was important for HBV replication. Here we showed that FOXA members negatively regulate pgRNA expression rather than precore RNA expression (Figs. 1b, 2b, and 3b). However, the effect of FOXA on the precore/pg RNA ratio was somewhat different among members. Our results demonstrated that FOXA2 caused the greatest effect for precore/pg RNA ratio in both FOXA2 gene silencing and induction studies. On the contrary, FOXA3 showed less effect for precore/pg RNA ratio than other members (Figs. 2b and 3b). The studies using non-hepatic cell lines, which supported HBV replication by introducing nuclear hormone receptors, showed that FOXA1 and FOXA2 antagonize HBV replication [22]. It is also reported that FOXA1 and FOXA2 directly interfered with the elongation rate of pgRNA [12]. These results suggested that FOXA members negatively regulate HBV transcription at various transcriptional steps, but their contributions were different among members.

The HBV surface antigen is composed of large, middle, and small S proteins. The large S protein is transcribed from 2.4 kb preS1 RNA, whereas middle and small S proteins are transcribed from 2.1 kb preS2/S RNA. Different promoters, S1p and S2p, independently regulate these RNAs, respectively [1]. We had expected that HBV surface antigens would be activated by FOXA members, because FOXA activated both S1p and S2p in reporter

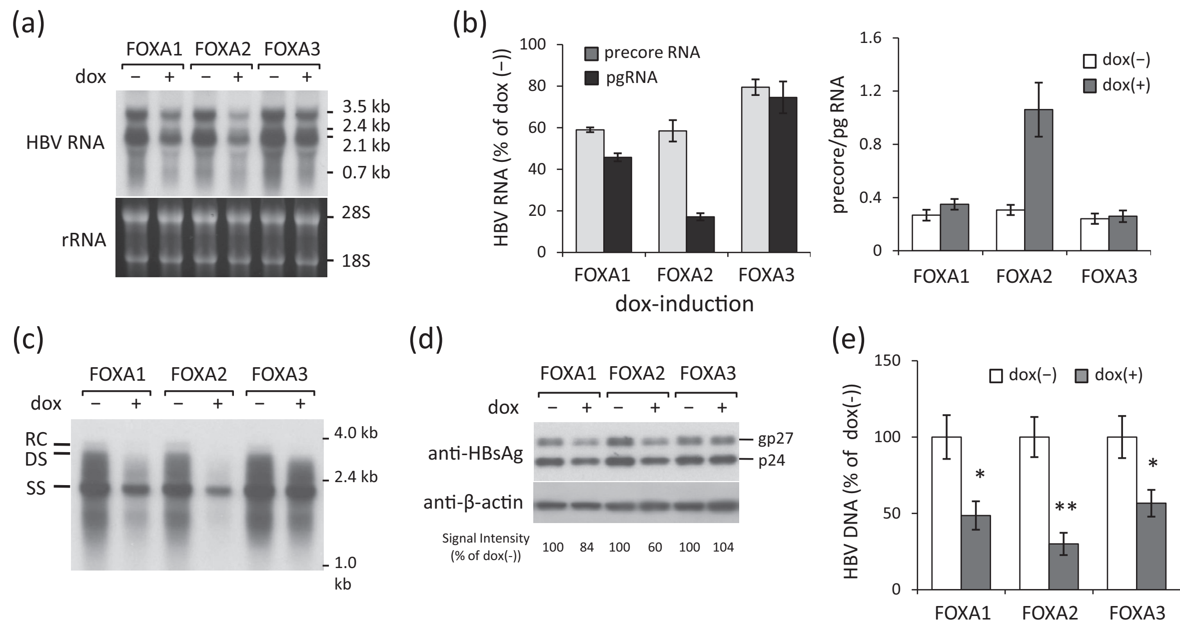

Fig. 3. Dox-induction of FOXA expression reduced the HBV replication in HuH-7 cells. (a) HBV transcripts were analyzed by Northern blot. Ribosomal RNA (rRNA) was used as internal control. The data shows representative results of at least three independent experiments. (b) The expression of precore RNA and pgRNA were measured by real-time RT-PCR. Left graph shows the amount of precore RNA and pgRNA mRNA expression, which were expressed as the percent of a percentage of that in dox-untreated. The ratio of precore RNA to pgRNA was calculated and shows on the right graph. (c) HBV replicative intermediates were analyzed by Southern blot. RC (relaxed circular), DS (double-stranded) and SS (single-stranded) DNA were indicated (d) The intracellular HBsAg level was assayed by Western blot. The bands gp27/p24 were indicated as small S protein. The band intensity was quantified by densitometric analysis and indicated below each lane. The value of the amount of gp27/p24 was normalized to that of β-actin, and expressed as percent of dox-untreated (dox(−)). (e) HBV DNA in the culture media at 4 day after HBV plasmid transfection was quantified by real-time PCR. Data were expressed as a percentage of that in the medium from dox-untreated (dox(−)) cells.

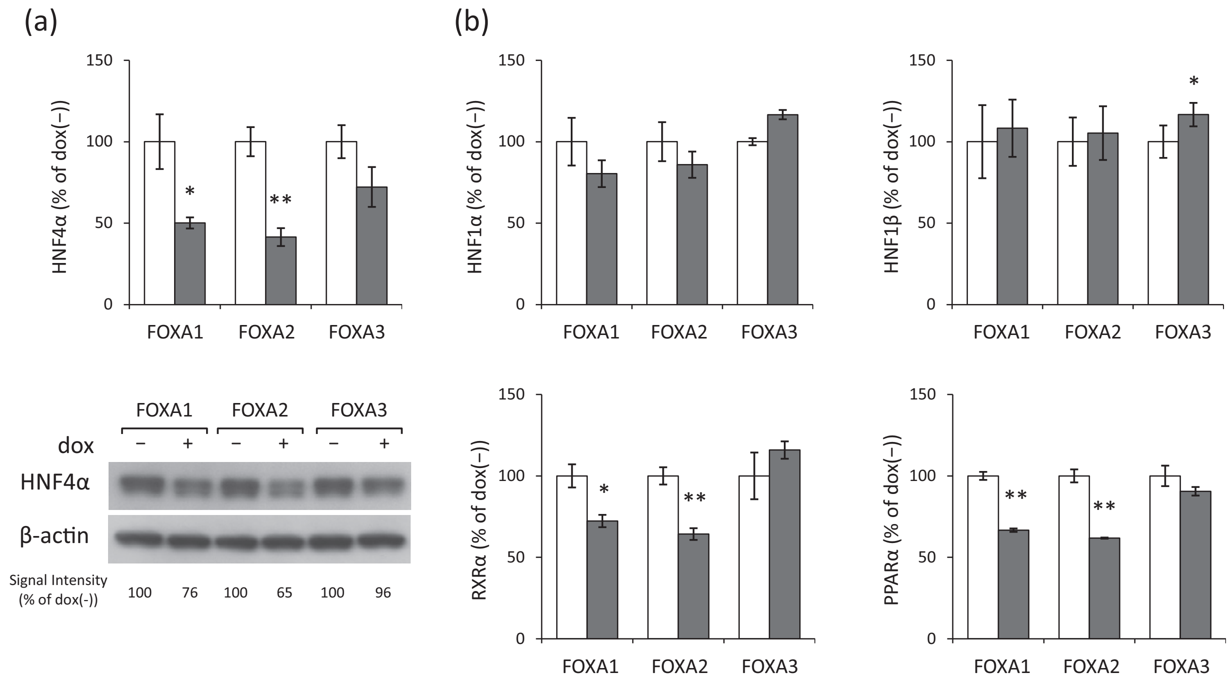

Fig. 4. Effect of FOXA induction by dox on the expression of liver enriched transcriptional factors in HuH-7 cells. (a) The expression of HNF4α was analyzed by real-time RT-PCR (graph) and Western blot (lower panel). The expression of HNF4α mRNA was expressed as a percentage of dox-untreated (dox(−)) cells. The protein band intensity was quantified by densitometric analysis and indicated below each lane. The value of HNF4α was normalized to that of β-actin, and expressed as percent of dox-untreated (dox(−)) cells. (b) The expression of HNF1α, HNF1β, RXRα, and PPARα mRNA were measured by real-time RT-PCR. Data were expressed as a percentage of dox-untreated (dox(−)) cells.

assays [10,11]. However, our results using 1.24-fold genome-length HBV indicated that only the small S protein was downregulated by FOXA members, especially FOXA1 and FOXA2. This was due to the methodological differences between the reporter assay and HBV replication system using a 1.24-fold HBV genome. As a report regarding HBV enhancer [27,28], HBV transcription thought

to be regulated by its multiple enhancers. In this respect, the studies using over genome-length HBV were thought to be more suitable for understanding the mechanism of HBV replication.

Liver function is controlled by the set of liver-enriched transcriptional factors [29]. FOXA members are also key regulators for liver development and liver-specific functions [5,30]. Based on the studies using knockout mice for various FOXA members, the function of FOXA in those events is thought to differ among members [31]. In this study, we found that the suppression of HNF4α, PPARα and RXRα expression was observed only in FOXA1- or FOXA2-expressing cells (Fig. 4). These nuclear hormone receptors are important for HBV replication [22]. As for HNF4α, the reduction of HNF4α expression by TGF-β1 resulted in the suppression of HBV replication [32]. The replication of HBV was inhibited by HNF4α-specific siRNA in HepG2 cell transfected plasmid containing 1.3-fold HBV genome [33]. These results suggested that FOXA1 and FOXA2 had indirect pathways leading to the suppression of HBV replication via nuclear hormone receptors. Moreover, we observed that the regulation of small S expression was different between FOXA1/2 and FOXA3 (Fig. 3). Because there were no HNF4-binding sequences in Sp2 [1], the regulation of small S by FOXA was thought to be independent of HNF4α. FOXA members bind similar DNA sequences via highly conserved Forkhead box motifs, but their gene regulation differs among various cell types [31,34]. These results suggested that the different regulatory roles between FOXA1/2 and FOXA3 in small S expression consisted of not only direct binding to the HBV genome but also indirectly regulation through FOXA target genes. Further studies will be needed to address these questions.

It has been reported that the infection of HBV in vitro was restricted only in differentiated-hepatocytes, such as human primary hepatocytes [35]. The development of HBV-susceptible cells has been attempted using HepaRG cells [36], HuS-E/2 cells [37], and umbilical cord matrix stem cells [38]. These results indicated that the differentiated state of these cells was important for viral infection. However, a method of persistent HBV infection using the established cell lines has not been developed yet. One of the reasons is that the HCC cell lines alter hepatic differentiated states, including by changing the expression of hepatic transcriptional factors, to maintain tumor phenotypes [4,39]. Here, we showed that the changes of FOXA expression levels altered the replication of HBV in HuH-7. These results suggested that the control of liver-enriched transcriptional factors in HCC cell lines is important for the development of effective HBV replication in cell culture systems.

In conclusion, we demonstrated that all FOXA members negatively regulated in HBV replication via downregulation of the level of HBV transcripts. Small S proteins were decreased in FOXA1- and FOXA2-, but not in FOXA3-induced cells. We also reported that the downregulation mechanism was different among FOXA members. It is hoped that these results will contribute to the establishment of a persistent HBV replication system, which could lead to the development of effective antiviral therapies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.03.022.

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