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Reciprocal regulation of farnesoid X receptor α activity and hepatitis B virus replication in differentiated HepaRG cells and primary human hepatocytes

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ABSTRACT: Hepatitis B virus (HBV) and bile salt metabolism seem tightly connected. HBV enters hepatocytes by binding to sodium taurocholate cotransporting polypeptide (NTCP), the genome of which contains 2 active farnesoid X receptor (FXR) α response elements that participate in HBV transcriptional activity. We investigated in differentiated HepaRG cells and in primary human hepatocytes (PHHs) effects of FXR activation on HBV replication and of infection on the FXR pathway. In HepaRG, FXR agonists (6-ethyl chenodeoxycholic acid and GW4064), but no antagonist, and an FXR-unrelated bile salt inhibited viral mRNA, DNA, and protein production (IC_{50}, 0.1–0.5 μM) and reduced covalently closed circular DNA pool size. These effects were independent of the NTCP inhibitor cyclosporine-A, which suggests inhibition occurred at a postentry step. Similar results were obtained in PHH with GW4064. Infection of these cells increased expression of FXR and modified expression of FXR-regulated genes *SHP*, *APOA1*, *NTCP*, *CYP7A1*, and *CYP8B1* with a more pronounced effect in PHH than in HepaRG. FXR agonists reversed all but one of the HBV-induced FXR gene profile modifications. HBV replication and FXR regulation seem to be interdependent, and altered bile salt metabolism homeostasis might contribute to the persistence of HBV infection.—Radreau, P., Porcherot, M., Ramière, C., Mouzannar, K., Lotteau, V., André, P. Reciprocal regulation of farnesoid X receptor α activity and hepatitis B virus replication in differentiated HepaRG cells and primary human hepatocytes. FASEB J. 30, 000–000 (2016). www.fasebj.org

KEY WORDS: cccDNA · bile salt · nuclear receptor · FXR agonist

Hepatitis B remains a public health problem despite an efficient and safe vaccine, with one third of the world population already infected by hepatitis B virus (HBV) and 240 million chronically infected individuals with the inherent risk of progression to cirrhosis and hepatocellular carcinoma (1). The natural history of HBV infection varies widely depending on age of infection. In most cases, HBV infection becomes chronic in children and newborns, whereas it remains self-limited in adults, evolving to a state of functional cure characterized by virus clearance from plasma and HBe and HBs seroconversions; however, for a minority of adults, primary infection persists with continuous replication of the virus in the liver and sustained viremia. For these patients, current treatments with pegylated IFNs or with nucleotide analogs generally fail to induce a sustained virological response, defined by off-treatment suppression of viral replication, loss of plasma HBe and HBs proteins, and undetectable HBV DNA (2). A better understanding of the cellular status that favors persistence of viral replication in chronically infected patients, either preexisting or induced by the virus, should lead to original therapeutic strategies to restore the physiologic conditions that predominate in the majority of patients with self-limited disease.

Hepatitis B virions enter hepatocytes by following HBs that bind to heparan sulfate proteoglycans and to the sodium taurocholate cotransporting polypeptide (NTCP), a

ABBREVIATIONS: 6ECDCA, 6-ethyl chenodeoxycholic acid; cccDNA, covalently closed circular DNA; CyA, cyclosporine A; dHepaRG, differentiated HepaRG cells; EnhII/Cp, enhancer II/core promoter region; FXR, farnesoid X receptor; GFP, green fluorescent protein; HBV, hepatitis B virus; HEK 293T, human embryonic kidney 293T; LAM, lamivudine; NTCP, sodium taurocholate cotransporting polypeptide; PHH, primary human hepatocyte; Tak, FXR inhibitor CAS936123-05-6; UDCA, ursodeoxycholic acid

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bile salt transporter expressed at the basolateral membrane of hepatocytes (3, 4). After entry, viral capsids migrate to nuclear pores where the genome is released into the nucleus (5). The circular, partially double-stranded DNA viral genome is then completed and closed to form the covalently closed circular DNA (cccDNA) that associates with histones and other cellular and viral proteins—predominantly the core protein HBc and the multifunctional HBx protein (6, 7). The resulting cccDNA complex is organized into minichromosomes that persist in the nuclei and are transmitted to daughter cells when hepatocytes divide (8, 9). Thus, cccDNA is the molecular complex responsible for the persistence, latency, and reactivation of the virus. cccDNA contains 4 promoters and 2 enhancers that regulate its transcription and synthesis of viral mRNA, including the pregenomic RNA (10). Several binding sites for ubiquitous and liver-specific nuclear receptors and transcription factors are located in these *cis*-regulating regions (11). Regarding liver-restricted nuclear receptors, we previously identified 2 response elements for the farnesoid X receptor (FXR) α (nuclear bile acid receptor) located within the enhancer II/core promoter region (EnhII/Cp) (12). Expression by transfection of FXR in cells of nonhepatic origin is sufficient to allow transcription and expression of viral RNAs upon activation by bile acids or synthetic FXR agonists. Moreover, peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and sirtuin 1 (SIRT1), 2 key metabolic sensors, form with FXR a network of proteins that regulate transcriptional activity of the HBV EnhII/Cp region in an FXR-dependent manner (13). Of interest, PGC-1α modulates HBV replication *via* nutritional stimuli, which suggests that the cell metabolic environment controls HBV infection (14, 15).

HBsAg and hepatitis B virions compete with bile salts to bind NTCP, and this competition likely limits NTCP functions leading to modifications of bile salts internalization and intracellular concentrations (16). Indeed, quantification of FXR expression and other genes under its control in HBV-infected liver biopsies in chronically infected patients or in liver of humanized mouse model showed that infection is associated with down-regulation of FXR activity, with ensuing modifications of expression of FXR-regulated genes, including its own overexpression that results from inhibition of the negative feedback exerted by activated FXR, the overexpression of APOA1, and decreased expression of SHP, 2 paradigm genes that are negatively and positively, respectively, controlled by FXR (17). HBV infection thus seems to be characterized by important modifications of bile acid metabolism and of FXR expression and activity. This intimate link between bile acid metabolism and HBV replication suggests that a particular bile acid metabolic status may generate a favorable cellular environment for HBV replication.

The aim of this study was to further investigate the interplay between the bile acid metabolism pathway and HBV replication. We therefore tested the effect of various FXR ligands on HBV replication in differentiated HepaRG cells (dHepaRG) as well as in primary human hepatocytes (PHHs), and conversely, the effect of HBV infection on FXR expression and activity. We found that FXR agonists, but not antagonists or FXR-irrelevant bile acids, have a strong inhibitory effect on HBV mRNA, DNA, and protein synthesis. We also showed that HBV infection modifies the expression profile of FXR and genes under its regulation, and that FXR agonists, at least in part, reverse virus-induced modifications of FXR-dependent gene expression profile.

MATERIALS AND METHODS

Chemicals

FXR agonist GW4064 was purchased from Sigma-Aldrich (Lyon, France), and 6-ethyl chenodeoxycholic acid (6ECDCA) from Metabrain Research (Chilly Mazarin, France). Bile acid ursodeoxycholic acid (UDCA) was obtained from Sigma-Aldrich, cyclosporine-A (CyA) from CliniScience (Nanterre, France), and Poly(I:C)-LMW and Poly(I:C)-HMW (tlrl-picw and vac-pic, respectively) from InvivoGen (San Diego, CA, USA). FXR inhibitor CAS936123-05-6, herein referred to as Tak (described in patent WO 2007052843 A1 20070510; Takeda Pharmaceuticals, Osaka, Japan), was synthesized by Edelris, Lyon, France.

Cell culture

HepaRG cells were cultured in a 5% CO_2 atmosphere at 37°C, in William's E medium (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) that was supplemented with 10% v/v HyClone FetalClone II serum (Thermo Fisher Scientific Life Sciences), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1% v/v insulin-transferrin-selenium, 20 μg/ml gentamicin (Thermo Fisher Scientific Life Sciences), and 50 μM hydrocortisone hemisuccinate (Sigma-Aldrich). Cell differentiation was carried out as previously described (18, 19). Cells were seeded in 6-well plates and maintained for 2 wk in growth medium (proliferation phase). Cells were then cultured for an additional 2 wk in medium that was supplemented with 1.8% v/v DMSO (Sigma-Aldrich) and 5 ng/ml epidermal growth factor (Roche, Meylan, France). All experiments were carried out with cells grown on collagen I–coated plates (BD BioCoat; Becton Dickinson, Le Pont de Claix, France).

Freshly plated PHHs were obtained from Human HepCell (Paris, France). Upon arrival, cells were placed in a 5% CO_2 atmosphere at 37°C, in William's medium that was supplemented with 2% v/v HyClone FetalClone II serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1% v/v insulin-transferrin-selenium, 20 μg/ml gentamicin (Thermo Fisher Scientific Life Sciences), 50 μM hydrocortisone hemisuccinate, and 1.8% v/v DMSO (Sigma-Aldrich).

Human embryonic kidney 293T (HEK 293T) cells were cultured in a 5% CO_2 atmosphere at 37°C, in DMEM (Thermo Fisher Scientific Life Sciences) that was supplemented with 10% v/v FCS (Dutscher, Brumath, France), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific Life Sciences).

HBV infection

HBV inoculum was prepared from stably transfected HepG2.2.15 cell line (20) as previously described (18). Cell inoculation was performed with 100 genome-equivalent per cell in culture medium that contained 4% v/v PEG 8000 (Sigma-Aldrich) for 24 h at 37°C. After HBV infection, cells were washed and maintained in adequate culture medium during all treatments.

For PHHs and innate immunity-related experiments, infection was carried out in a lower volume (700 μl/well of a 6-well plate) of medium that contained only 2% v/v HyClone FetalClone II for 4 h. Extra PEG-containing medium was then added for 20 h.

HBs and HBe antigens

HBs and HBe antigens secreted into cell supernatants were quantified by using HBsAgII and HBeAg kits, after dilutions when needed, on the Architect apparatus (Abbott Diagnostics, Rungis, France).

Analysis of viral DNA

Secreted DNA was extracted from cell supernatant by using the easyMAG sample extraction platform following manufacturer recommendations (BioMérieux, Marcy l'Etoile, France). Eluates were directly used for quantification of secreted viral DNA by quantitative PCR experiments using primers for rcHBV DNA: forward 5′-GGGGAGGAGATTAGGTTAAAGGTC-3′, reverse 5′-CACAGCTTGGAGGCTTGAACAGTGG-3′, and SYBR green master mix on a LightCycler 480 II (Roche).

Quantification of total intracellular HBV DNA, cccDNA, and rcHBV DNA was performed as previously described (21). In brief, after treatment with FXRα agonists, total DNA from HepaRG cells was extracted by using the MasterPure DNA purification kit (Epicentre, Madison, WI, USA). A 5-μg DNA aliquot from each sample was treated with Plasmid-Safe ATP-dependent DNase before quantitative PCR experiments were carried out. LightCycler β-globin control kit DNA (Roche) was used for normalization.

Analysis of viral and cellular RNA

Total RNA was isolated from HepaRG cells by using RNeasy mini kit (Qiagen, Courtaboeuf, France) according to manufacturer instructions. After DNA digestion with Ambion Turbo DNase (Thermo Fisher Scientific Life Sciences), 500 ng RNA was reverse transcribed by using the High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific Life Sciences). cDNAs were then subjected to 45 PCR cycles with FastStart Universal SYBR green PCR master mix (Roche) using a LightCycler 480 II. The following primers were used for Preg/Precore: forward 5′-CTTTTTCACC TCTGCCTAATCATC-3′, reverse 5′-CACAGCTTGGAGGCTT GAACAGTGG-3′; FXRα forward 5′-AGAGATGGGAATGTT GGCTGA-3′, reverse 5′-GCATGCTGCTTCACATTTTTT-3′; SHP forward 5′-GGCTGGCAGTGCTGATTCAG-3′, reverse 5′-T GGGGTGTGGCTGAGTGAAG-3′ and APOA1 forward 5′-CCCAGTTGTCAAGGAGCTTT-3′, reverse 5′-TGGATGTG CTCAAAGACAGC-3′; NTCP forward 5′-GGCTTTCTGCT GGGTTATGT-3′, reverse 5′-CATGCTGACAGTGCGTCTG-3′; CYP7A1 forward 5′-GCTTATTCTTGGAATTAGGAGAAGG-3′, reverse 5′-TTGGCACCAAATTGCAGAG-3′; and CYP8B1 forward 5′-GCCTGTCCTTTGTAATGCTGA-3′, reverse 5′-GAAGC GAAAGAGGCTGTCC-3′. Normalization was carried out by using the following 3 housekeeping genes: OAZ1 forward 5′-GGAT AAACCCAGCGCCAC-3′, reverse 5′- TACAGCAGTGGAGGG AGACC-3′; S9 forward 5′-CCGCGTGAAGAGGAAGAATG-3′, reverse 5′-TTGGCAGGAAAACGAGACAAT-3′; and HPRT1 forward 5′- CATCACTAATCAGGACGCCAGGG-3′, reverse 5′- GCGAACCTCTCGGCTTTCCCG-3′. Northern blot experiments were carried out by using 3–4 μg Turbo DNAse-treated RNA separated on a 1% agarose-formaldehyde gel and transferred onto a positively charged nylon membrane. Probe was synthesized by PCR using the following primers: forward 5′-CTGAATCCTGCGGACGACCCTTCTC-3′ and reverse 5-GCCCAAAGCCACCCAAGGCAC-3′. [^{32}P]-Probe labeling was performed by using klenow polymerase and α-[^{32}P]-dCTP nucleotides. Quantification of HBV mRNAs was performed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

HepaRG cells were grown on collagen I–coated coverslips (BD biocoat) and cultured as in other experiments. After various treatments, cells were fixed with 4% v/v paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% v/v Triton X-100 for 10 min. All washes were made in PBS. Saturation was performed by using 4% w/v bovine serum albumin in PBS for 30 min at 37°C. Cells were stained with a rabbit polyclonal anti-HBc antibody (B0586; Dako, Trappes, France). They were visualized with Alexa Fluor 488–conjugated anti-rabbit secondary antibody (A-11070; Thermo Fisher Scientific Life Sciences). Chromatin was stained with Hoescht to reveal nuclei. Coverslips were mounted with fluorescence mounting medium (Dako) and images were obtained by using an axio-imager Zeiss fluorescent microscope (Zeiss, Jena, Germany).

shFXR lentiviral particle production and HepaRG transduction

HEK 293T cells (6×10^6) were transfected with jet-PEI (Polyplus Transfection, Illkirch, France) with 8 μg p8.91 plasmid, 2.5 μg pVSVg, and 8 μg pPLKO.1-puro-shFXR (5′-CCGGGCCTGACT-GAATTACGGACATCTCGAGATGTCCGTAATTCAGTCAGG CTTTTT-3′; NM_005123.1-1660s1c1; Sigma-Aldrich). Green fluorescent protein (GFP) control lentiviral particles were generated by transfecting p8.91, pVSVg, and PLKO.1 puro-CMV-turboGFP (Sigma-Aldrich). For both productions, media was changed 24 h post-transfection and lentivirus particles were harvested and filtered at 96 and 120 h post-transfection.

Confluent (80%) undifferentiated HepaRG cells were transduced by using HEK 293T transfected supernatant that contained lentiviral particles for 24 h post-transduction. Medium was then replaced with complete William's E medium for another 24 h. Cells were then selected and maintained with 3 μg/ml puromycin until end of the experiment.

RESULTS

FXR agonists inhibit production of HBV proteins in an FXR-dependent manner

Induction of differentiation of HepaRG cells increased FXR expression at RNA and protein levels as shown in Supplemental Fig. S1. FXR was detected only in cells with hepatocyte-like morphology (18) and in both nuclei and cytoplasm with some heterogeneity; some cells expressed FXR more in the cytoplasm than in the nucleus and vice versa, which probably reflects various degrees of differentiation. dHepaRG cells were infected with HBV produced in HepG2.2.15 cells for 24 h. As expected, significant viral protein production in culture supernatant was observed after 12 d of culture (**Fig. 1**). At d 2 postinfection, cultures were treated or not for 10 d with 2 FXR agonists (GW4064 and the bile salt derivative 6ECDCA) with the FXR antagonist Tak or with UDCA, a bile salt that does not activate

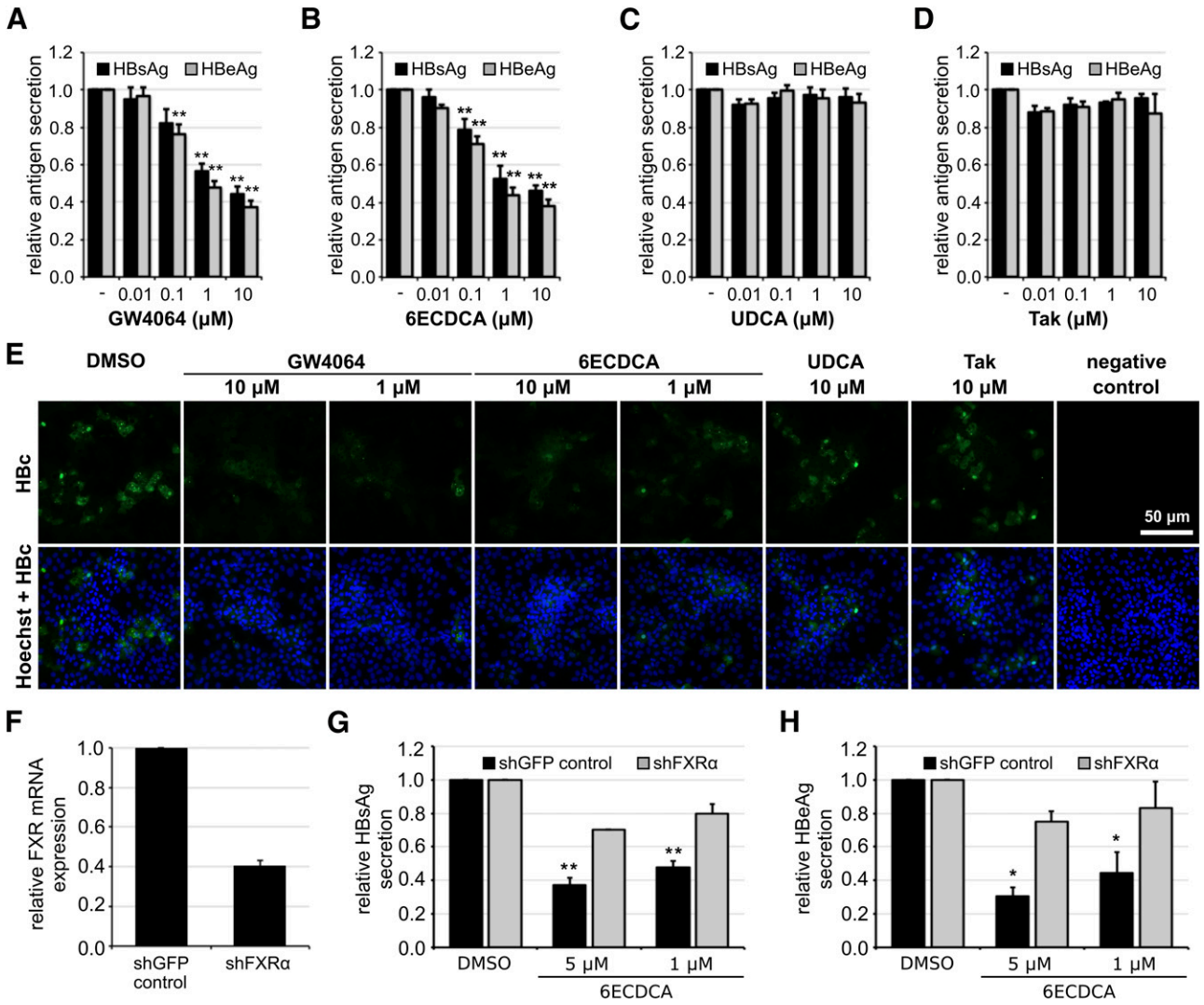

Figure 1. FXR agonists inhibit viral proteins production. Relative secretion of HBsAg and HBeAg in dHepaRG cells treated postinfection with a range (10 nM–10 μM) of GW4064 (*A*), 6ECDCA (*B*), UDCA (*C*), and Tak (*D*). *E*) Immunofluorescence showing nuclear (blue) and HBcAg (green) staining of dHepaRG cells treated or not with FXR modulators. Data are means ± SEM of at least 3 independent experiments; Student's *t* test. ** $P < 0.001$ and * $P < 0.01$. *F*) FXR extinction in dHepaRG cells after shFXR transduction and HBV infection. Relative secretion of HBsAg (*G*) and HBeAg (*H*) in shFXR-transduced dHepaRG cells treated postinfection with 2 doses (1 and 5 μM) of 6ECDCA. Data are means ± SEM of at least 3 independent experiments; Student's *t* test. ** $P < 0.01$ and * $P < 0.05$, for effect of FXR agonist treatments compared with DMSO, except for FXR mRNA expression in shGFP control *vs.* shFXR, where $n = 2$ (*F*).

FXR at these concentrations. In this model of HBV infection, the 2 FXR agonists significantly repressed expression of viral proteins HBsAg, HBeAg, and HBc (Fig. 1*A*, *B*, *E*), whereas antagonist Tak (Fig. 1*C*, *E*) as well as UDCA (Fig. 1*D*, *E*) had no activity. Both FXR agonists repressed HBsAg and HBeAg secretion in a dose-dependent manner with IC_{50} values around 1 μM without detectable toxicity (Supplemental Fig. S2). To confirm that effects of the 2 FXR agonists were indeed FXR dependent, we silenced its expression in HepaRG cells with a shFXR lentiviral vector. Partial knockdown of FXR expression in differentiated cells significantly decreased the repression of viral protein secretion by FXR agonist 6-ECDCA (Fig. 1*F*, *G*, *H*). When treatment was introduced during the infection period and continued thereafter (**Fig. 2*A*, *B***), IC_{50} values were close to 0.1 μM for 6ECDCA and 0.3 μM for GW4064.

FXR agonists repress HBV replication at a postentry step

Inhibition of HBV protein expression was thus more potent when agonists were present during the infection period, which suggests that the effect could also occur during the virus entry process. Indeed, bile salt derivative 6ECDCA, for instance, could compete with the virus for binding to the NTCP receptor and act as an entry inhibitor. CyA binds to NTCP at a site that overlaps that of bile salts and HBV and is a potent inhibitor of viral entry. As expected, addition of increasing concentrations of CyA during the 24 h of infection reduced, in a dose-dependent manner, HBV protein secretion in supernatant measured after 10 d of culture (Fig. 2*C*). Addition of FXR agonist for 10 d further reduced HBsAg and HBeAg secretion. Conversely, CyA did not affect inhibition of

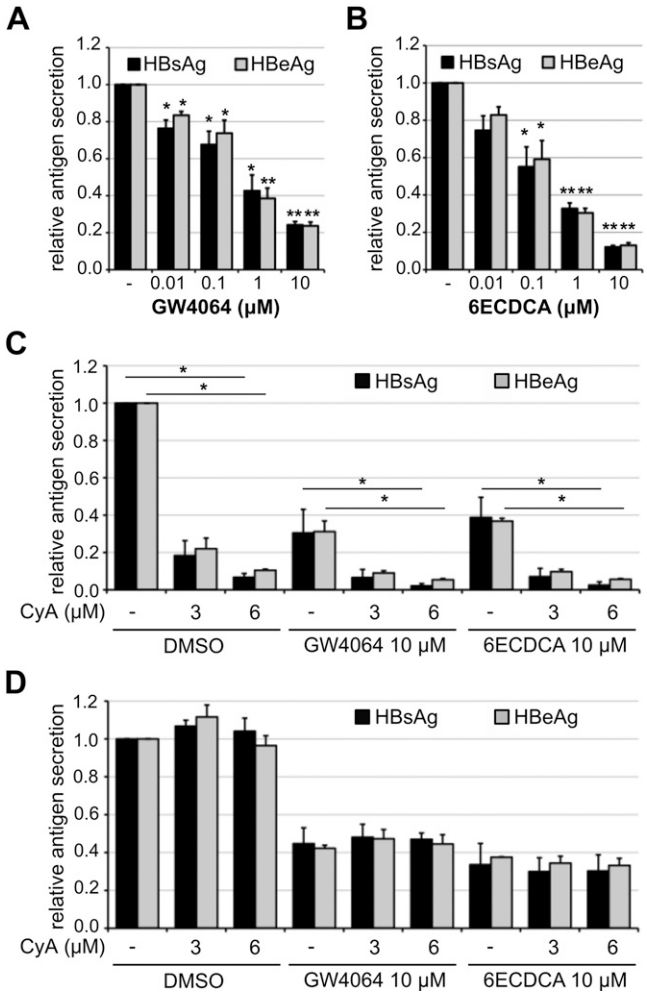

Figure 2. FXR agonists repress HBV replication at a postentry step. Relative secretion of HBsAg and HBeAg by dHepaRG cells treated with a range (10 nM–10 μM) of GW4064 (*A*) and 6ECDCA (*B*). Treatments were applied during infection (24 h) and continued until d 12 postinfection. *C*, *D*) Relative antigen secretion by dHepaRG cells treated with CyA (3 and 6 μM) during 24 h of viral infection (*C*) or during the first 3 d of culture with FXR agonists (*D*). Data are means ± SEM of at least 3 independent experiments; Student's *t* test. $^{**}P < 0.001$ and $^{*}P < 0.05$.

HBsAg and HBeAg secretion by the 2 agonists when added after inoculation medium change during the first 3 d of culture with FXR agonists (Fig. 2*D*). These data confirm that there is little or no propagation of the virus after initial infection in the dHepaRG model of infection (22), and indicates that effects of the modulation of FXR on HBV expression do not result from competition for the entry receptor but, more likely, proceed at postentry steps.

FXR agonists repress expression of viral RNA as well as synthesis and secretion of HBV DNA

We next tested the effect of FXR agonists on expression and replication of the HBV genome. The 2 FXR agonists similarly repressed secretion of HBV DNA in supernatant during the last 3 d of the 10-d treatment in a dose-dependent manner (**Fig. 3***A*). The 2 agonists were similarly active, with IC_{50} values around 0.2 μM. This finding was corroborated by concomitant inhibition of the expression of pregenomic mRNA and precoreRNA (Fig. 3*B*) and of the intracellular relaxed circular DNA level (Fig. 3*D*). All HBV mRNAs were similarly affected by agonists (Fig. 3*C*). We then investigated whether FXR agonists had an effect on the HBV minichromosome and found a reduction of 50–60% of the quantitative signal measuring the cccDNA pool at d 12 postinfection (Fig. 3*E*). Again, as for HBs and HBe secretion, FXR silencing significantly suppressed the effect of FXR agonists on viral RNA expression (data not shown).

FXR activation reduces HBV replication in PHH

Effects of FXR agonists on HBV replication were also investigated in PHH. All PHH expressed FXR in both cytosol and nuclei, with the highest level of expression always in nuclei (Supplemental Fig. S1). Cells were infected at d 2 postseeding and treated or not for the next 2 wk. Treatment of noninfected cells with GW4064 had no effect on cell morphology as judged by phase contrast examination (**Fig. 4***A*). HBV infection was associated with mild cytopathic effect characterized by some detaching and rounded cells compared with noninfected culture. Of interest, this was not observed when infected cells were treated with FXR agonists (Fig. 4*A*), and no toxicity was noted by protein/total RNA quantification or lactate dehydrogenase release (Supplemental Fig. S2). Moreover, as observed in the dHepaRG cell culture system, FXR agonist GW4064 had a strong inhibitory effect on HBsAg and HBeAg secretion (Fig. 4*B*). Similarly, expression of viral pg/precore RNAs and viral intracellular and secreted DNA was significantly reduced (Fig. 4*C*, *D*). Finally, the cccDNA pool also seemed to be reduced by treatment (Fig. 4*E*). Similar effects of FXR agonists were observed with PHH from another donor (Supplemental Fig. S1).

Effects of HBV infection and FXR agonists on FXR-regulated genes

Consequences of HBV infection and of FXR agonists on expression of genes regulated by FXR were investigated in dHepaRG and PHH by monitoring mRNA expression level of FXR, SHP, APOA1, NTCP, CYP7A1, and CYP8B1, with normalization against 3 housekeeping genes (**Fig. 5**) (21). In dHepaRG (Fig. 5*A*), despite the low proportion of infected cells (<20%), HBV infection induced a small but significant detectable increase of FXR and APOA1 mRNA expression compared with noninfected dHepaRG cells ($P = 0.05$ and $P = 0.03$, respectively). HBV infection did not significantly modify expression of other mRNA in these conditions. Conversely, in PHH for which the proportions of infected cells were high (Fig. 5*B* and Supplemental Fig. S3), expression of all genes was increased by HBV infection by a factor of 1.5–4. Changes were similar for the 2 PHH

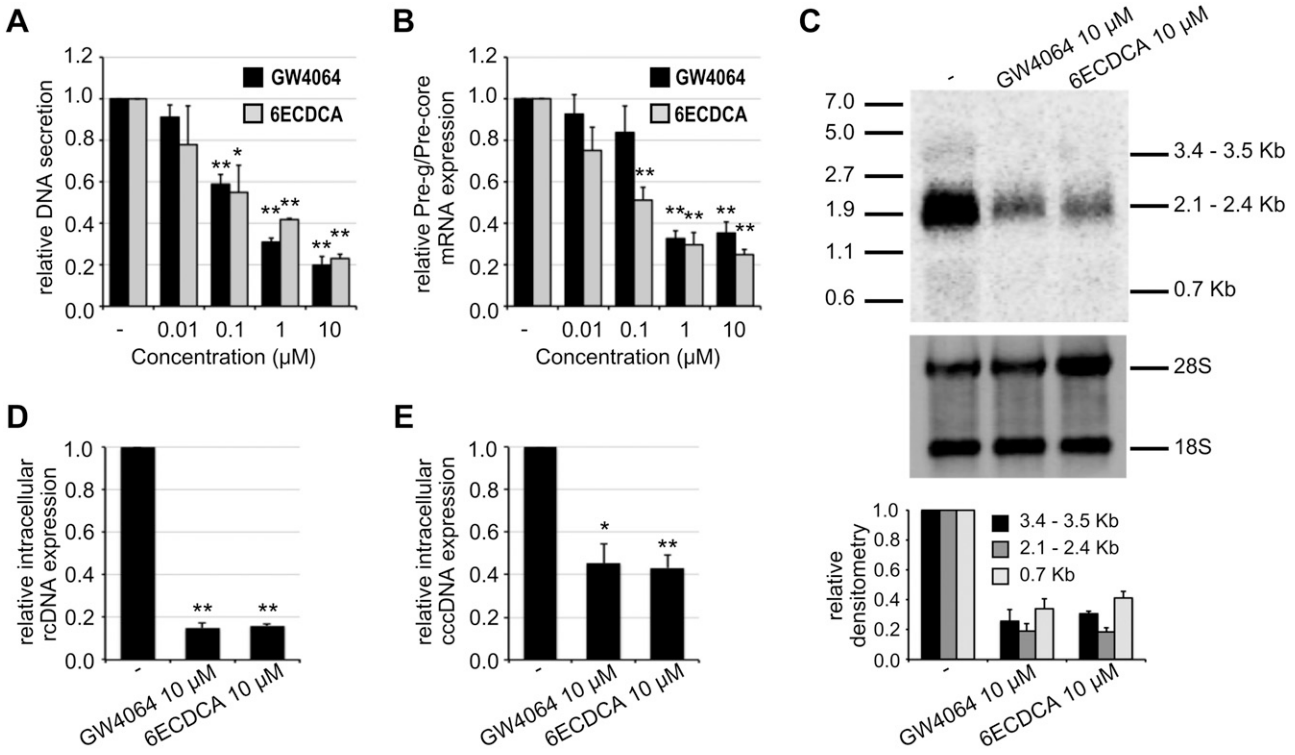

Figure 3. Treatment with FXR agonists represses expression of viral RNA and expression and secretion of HBV DNA. *A*) Secretion of viral DNA in cell supernatant during the last 3 d of treatment quantified by quantitative PCR. *B*) Preg/precore mRNA expression at d 12 postinfection by quantitative RT-PCR. *C*) Northern blot showing all HBV transcripts at d 12 postinfection; quantification of the densitometry of 3 independent experiments was carried out by using ImageJ software. Relative quantification of intracellular relaxed circular DNA (*D*) and cccDNA (*E*) by quantitative PCR. Data are means ± SEM of at least 3 independent experiments; Student's *t* test: $^{**}P < 0.001$ and $^{*}P < 0.01$.

donors. As expected, treatment with FXR agonists repressed mRNA expression of genes under the negative control of FXR, that is, expression of FXR itself as well as that of APOA1, NTCP, CYP7A1, and CYP8B1, and increased the expression of SHP mRNA, a gene positively regulated by FXR. FXR agonists and HBV infection, thus, had opposite effects on the profile of genes under the negative control of FXR.

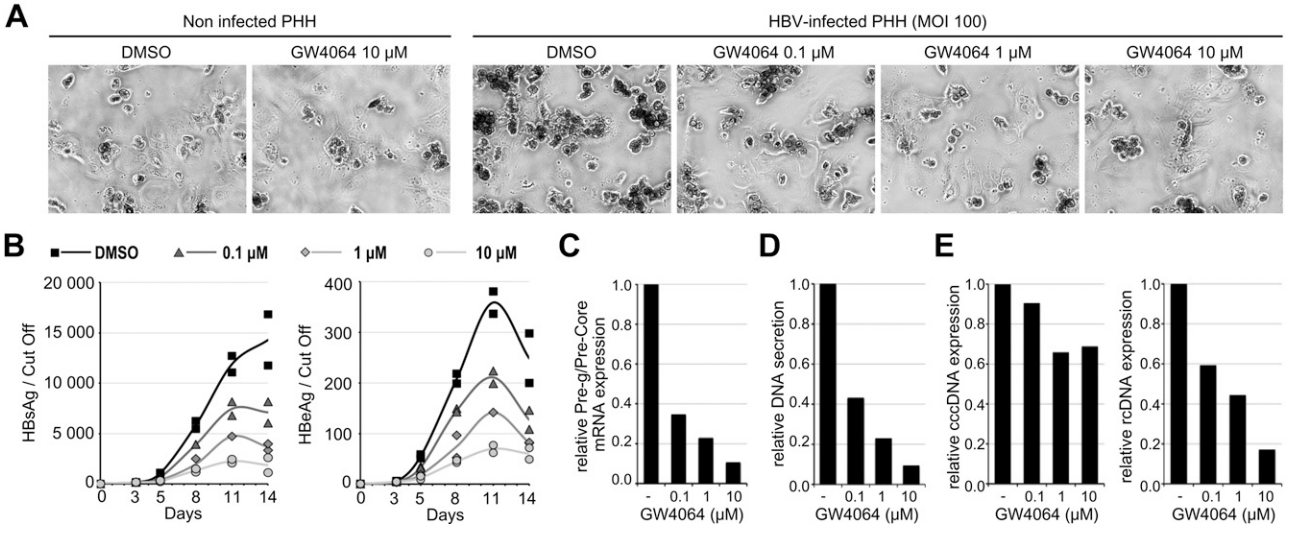

Figure 4. FXR activation by GW4064 down-regulates HBV replication in PHH. The following data were obtained in PHH from a 57-yr-old male donor. *A*) Phase-contrast micrographs of PHH at d 14 postinfection. *B*) Kinetics of HBsAg and HBeAg secretion after GW4064 treatments, from infection to d 14. Each symbol represents secretion from a single well and each curve represents the mean of 2 wells from the same condition. *C*) Preg/precore mRNA expression at d 14 postinfection by quantitative RT-PCR. *D*) Secretion of viral DNA in cell supernatant during the last 3 d of treatment quantified by quantitative PCR. *E*) Relative quantification of intracellular relaxed circular DNA and cccDNA at d 14 postinfection by quantitative PCR. Original magnification, ×20.

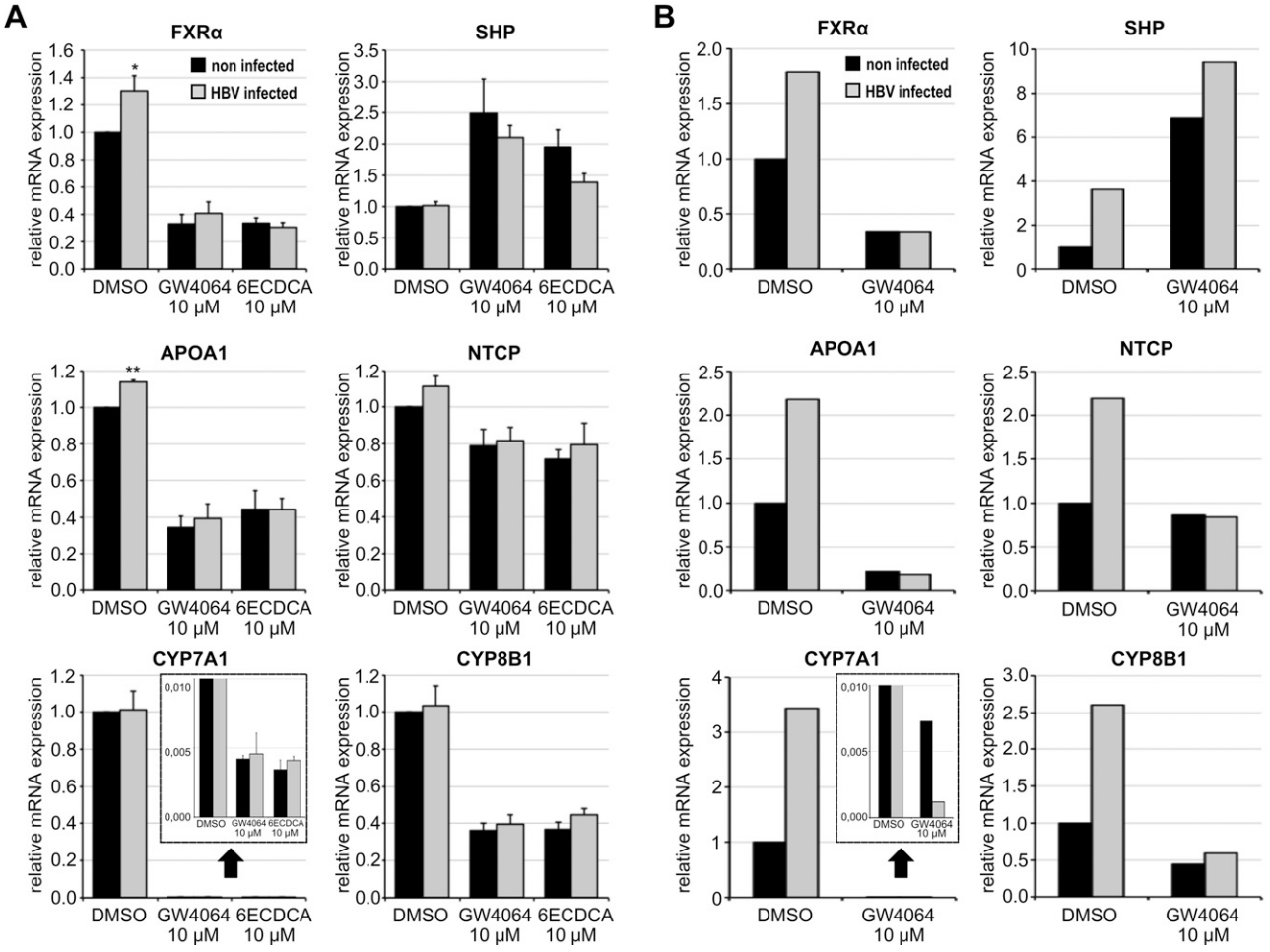

Figure 5. Effects of HBV infection and FXR agonists on bile acids that regulate genes. FXR, SHP, APOA1, NTCP, CYP7A1, and CYP8B1 mRNA expression at d 12 and 14 postinfection by quantitative RT-PCR in dHepaRG cells (*A*) and PHH (*B*), respectively. Data from dHepaRG cells are means ± SEM of at least 3 independent experiments; Student's *t* test. $^{**}P < 0.01$ and $^{*}P < 0.05$ for the effect of HBV infection. All FXR agonist treatments were $P < 0.05$ (not shown on graphs). Data from PHH are mean of 2 independent culture wells with cells from the same donor as in Fig. 4. Data were normalized against 3 housekeeping genes mRNA quantification. Inserts show enlargement of the indicated graph areas.

FXR agonist treatment rescued partial inhibition of HBV DNA synthesis by a polymerase inhibitor at suboptimal concentration

Because we observed that FXR agonists decreased expression of pg/precore RNAs, we investigated whether partial repression of viral DNA replication by suboptimal concentrations of the reverse transcriptase inhibitor, lamivudine (LAM), could be enhanced by treatment with FXR agonists. In this system and as expected, LAM treatment had no effect on HBsAg and HBeAg secretion regardless of whether cells were treated with FXR agonists (**Fig. 6*A***). Low LAM concentrations already reduced viral DNA synthesis and release at 70% of control, and addition of GW4064 further and significantly limited viral DNA secretion, reducing it 15% (Fig. 6*B*).

DISCUSSION

This study further reveals the complex interdependency of HBV infection and the FXR pathway in 2 cell culture systems permissive to HBV infection. HBV infection modifies expression of FXR and its regulated genes in both cell culture systems, even if effects were more impressive in PHH than in dHepaRG. This likely reflects the lower proportion of infected cells in dHepaRG culture than in PHH, which results in signal dilution. This also further strengthens the significance of variations of FXR and APOA1 mRNA expression induced by HBV infection that are observed in both systems. Overall, increases of FXR, CYP7A1, and APOA1 mRNA expression are consistent with those observed *ex vivo* in liver biopsies of infected patients and/or in liver of humanized mouse model of HBV infection (17). In addition, we found that expression of NTCP and CYP8B1 was also increased by HBV infection in PHH. Altogether, data suggest that HBV infection maintains FXR at a low activity level, which allows the transcription of genes that, directly or indirectly, are negatively controlled by FXR—CYP7A1, CYP8B1, NTCP, APOA1, and FXR itself—which thus appeared significantly overexpressed in both cell models (23–25). Repression of FXR activity by HBV infection, as previously proposed (17), is likely a result of the decrease of intracellular concentrations of

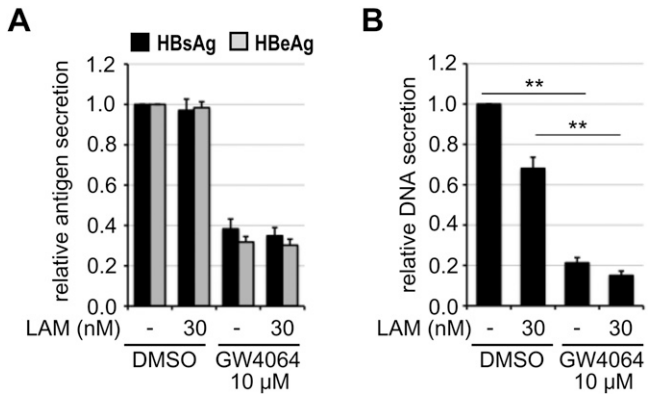

Figure 6. FXR agonist treatment rescued partial inhibition of HBV DNA synthesis by polymerase inhibitor at suboptimal concentrations. Relative secretion of antigens (*A*) and viral DNA (*B*) in dHepaRG cell supernatant during the last 3 d of treatment with GW4064 and/or LAM. Data are means ± SEM of 3 independent experiments; Student's *t* test. $^{**}P < 0.001$.

natural FXR ligands that results from competition between circulating virions or secreted HBs and bile salts for NTCP. One intriguing observation is that HBV increases SHP expression in PHH, whereas its expression is not modified in dHepaRG or is even decreased in liver of infected patients (17). These differences might be explained by variations in cellular models and experimental conditions that do not totally mimic the liver environment; however, as SHP expression is positively controlled by FXR, this suggests that other mechanisms might be involved. Additional studies are clearly warranted to fully understand how HBV changes FXR expression and activity.

As many other cellular functions—beyond bile acid metabolism—are modulated by FXR (24), the HBV-induced change of its activity may likely contribute to creation of a specific cellular metabolic environment favorable to HBV replication. For example, it was recently reported that FXR activation represses transcriptional regulation of autophagy by cAMP response element binding protein and peroxisome proliferator-activated receptor-α (26, 27). Because the autophagic pathway is required for HBV replication and envelopment, and HBV modulates several components of this pathway (28–31), modulation of FXR activity by HBV may contribute to maintenance of sustained autophagic flux. Of interest, FXR agonists reverse all observed modifications of gene expression but that of SHP. Indeed, the most striking consequence of the 10–12-d-long treatment with FXR agonists was repression of FXR expression, which was likely a result of the retro-control of FXR on its own expression mediated by SHP (23). FXR expression repression was noticed at both mRNA and protein levels. FXR became barely detectable by immunofluorescence, thus dramatically contrasting and counterbalancing overexpression induced by HBV.

We show here that 2 well-characterized FXR agonists, 6-ECDCA and GW4064, with 2 different structures have strong inhibitory effects on HBV replication in dHepaRG cells and in PHH. In contrast, the FXR-independent bile acid, UDCA, or FXR antagonist had no effect. In principle, FXR ligands could compete with HBV virions for binding to NTCP; however, this possibility was ruled out as the HBV entry inhibitor, CyA, did not modify the course of infection when added after the infection step, whereas agonists inhibited production of all viral elements in the same conditions (22). In addition, knockdown of FXR in dHepaRG almost completely suppressed the effects of FXR agonists. Altogether, these data suggest that FXR agonists interfere in viral replication at later steps of the viral cycle by directly acting on FXR and controlling the transcriptional activity of cccDNA. Inhibition of viral DNA and protein synthesis could mainly be the consequences of reduced viral mRNA expression.

These findings may seem surprising as previous reports have shown that the effect of FXR expression and activation favor expression of the viral genome (12, 13); however, in previous studies, effects of FXR agonists on EnII/Cp were exemplified with luciferase gene reporter constructs under control of the EnII/Cp region and with overexpression of FXR and RXR. Without concomitant overexpression of these factors, effects of FXR agonists on transcriptional activity varied with the basal expression of FXR and other cofactors (PGC-1α and SIRT1) in the cell lines tested—Huh-7 *vs.* HepG2 or HEK293T, a cell line of nonhepatic origin. When full-length or 1.3 full-length HBV genomes were transfected in the same test cell lines, effect of FXR agonists were again more pronounced when FXR and RXR were also overexpressed. These studies clearly demonstrate that FXR binds to the 2 putative FXR response elements we identified in the EnhII/Cp region, somehow in a dose-dependent manner, and that it is functional, regulating transcriptional activity of the promoter. It is worthwhile to mention that, in these experiments, effects of FXR agonists on reporter or viral genes were only monitored during the first 24 or 48 h after transfection and treatment with FXR ligands. Under these conditions, any regulatory feedback loops that could possibly interfere in the transcriptional activity of the EnhII/Cp region could not be detected.

Conversely, effects of FXR ligands were tested over a 10-d, complete HBV replication cycle in the present study, which allowed deployment of long-term effects of FXR stimulation, such as sustained FXR expression down-regulation. FXR agonist–induced repression of HBV replication might thus result from shutdown of the FXR-dependent activation of the transcriptional activity of EnhII/Cp, which has proved sufficient to induce HBV expression in cells of nonhepatic origin (12, 13). Differential binding affinity of FXR to viral and cellular promoters as a result of FXRE sequence differences, or to FXR post-translational modifications, depending on recruitment of cofactors on these DNA regions, could explain why low FXR expression levels might not be sufficient to activate the viral promoter while remaining high enough for cellular gene regulatory regions. Of note, treatment with FXR antagonist, Tak, did not further increase expression of FXR in these experimental conditions (Supplemental Fig. S1), and no modification of HBV replication was noticed.

It is therefore tempting to envision FXR activation as a new therapeutic option to control HBV replication. FXR activation could directly repress HBV transcription and correct virus-induced metabolic modifications, indirectly rendering the cellular environment less favorable to viral

replication. Of interest, FXR agonists have already been tested, with interesting results in long-term clinical trials for primary biliary cirrhosis or nonalcoholic steatohepatitis, with few adverse effects (32, 33). Of note, suboptimal concentrations of the reverse transcriptase inhibitor LAM only partially inhibit viral DNA synthesis, and we found that FXR agonist add-on further restrains viral DNA synthesis, which somehow rescues incomplete reverse transcriptase inhibition. Altogether, these findings suggest that FXR agonists could be used in combination with nucleotide analogues to repress the replication of HBV by additional mechanisms that act upstream of reverse transcription. FJ

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