Doctoral Dissertation

博士論文

Modulation of Hepatitis B Virus Infection by Epidermal Growth Factor

(B 型肝炎ウイルス感染に対する Epidermal growth factor の作用)

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Table of Contents

List of Abbreviations

APC: allophycocyanin

BMP: bone morphogenetic protein

cccDNA: covalently closed circular DNA

CIE: clathrin independent endocytosis

CME: clathrin mediated endocytosis

CQ: chloroquine

DMSO: dimethyl sulfoxide

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

FITC: fluorescein isothiocyanate

FLP: filipin

FGF: fibroblast growth factor

HBV: hepatitis B virus

HBsAg: hepatitis B surface antigen

HCC: hepatocellular carcinoma

HGF: hepatocyte growth factor

hiPSC: human induced pluripotent stem cell

HSC: hepatic stellate cell

HSPG: heparin sulfate proteoglycan

IKA: ikarugamycin

LSEC: liver sinusoidal endothelial cell

NPC: non-parenchymal cell

NTCP: sodium-taurocholate co-transporting polypeptide

OSM: oncostatin M

PEG: polyethylene glycol

STM: septum transversum mesenchyme

VEGF: vascular endothelial growth factor

Abstract

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family of viruses and enters hepatocytes via sodium-taurocholate co-transporting polypeptide (NTCP). After virus entry, it establishes a nuclear pool of episomal covalently closed circular DNA (cccDNA), which is then copied into RNA. The viral RNA is transported to the cytoplasm where it is reverse-transcribed into DNA and packed in a virus particle. This complicated life cycle is a major problem to develop effective antivirus therapeutics. For the development of antiviral therapeutics, it is necessary to develop an in vitro infection system that faithfully recapitulates the complicated viral life cycle. Primary human hepatocytes have been used for studies on HBV infection, but these cells are phenotypically unstable and the availability is limited. Because there are many genotypes of HBV and genetic backgrounds of individuals affect infection of each HBV genotypes, it would be beneficial to establish hepatocytes derived from human induced pluripotent stem cells (hiPSCs) with different genetic backgrounds. However, hiPSC-derived hepatocytes show immature phenotypes and also exhibit limited capacity for the infection and replication of HBV.

Previously it was shown that hiPSC-derived non-parenchymal cells, liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs), promoted hepatic-maturation of hiPSC-derived hepatocytes. I therefore developed a co-culture system of hiPSC-derived hepatocytes with

LSEC and HSC, and found that LSEC enhanced HBV infection to hepatocytes by secreting

epidermal growth factor (EGF). While EGF receptor (EGFR) is known as a co-receptor for HBV

entry, I found that EGF enhanced HBV infection by the activation of EGFR. Furthermore, I found

that the effect of EGF was dose dependent, i.e. EGF at a low dose enhanced HBV infection, whereas

it at a high dose suppressed HBV infection. EGFR is internalized by clathrin-mediated endocytosis

(CME) and clathrin-independent endocytosis (CIE) pathways depending on the dose of EGF. At a

high dose of EGF, the endocytosed EGFR is degraded in the lysosome *via* CIE. I found that HBV

is endocytosed *via* CME and CIE pathways at a low and high dose of EGF, respectively. In

conclusion, I have developed an *in vitro* system of HBV infection using iPSC-derived liver cells,

and revealed that EGF secreted from LSEC modulates HBV infection in a dose dependent manner

via different endocytosis pathways.

Introduction

Hepatitis B virus (HBV) infection is a significant global public health issue. More than 400 million people in the world are infected with HBV, leading to 1 million annual deaths due to hepatocellular carcinoma, HCC (Revill et al., 2016). Despite its severe medical and social problems, progress in HBV research has been limited by the lack of an appropriate *in vitro* system, in which the virus infects human liver cells.

Hepatitis B virus (HBV) is a member of the Hepadnaviridae family, comprised of a group of hepatotropic DNA viruses and divided into two genera. The Orthohepadnavirus genus includes members that infect mammals (woodchucks, ground squirrels, bats and primates) and the Avihepadnavirus genus infects birds such as ducks, herons, storks, geese and parrots (Locarnini, 2014). Although they share a common genomic organization, their species-specificity between each type still hinders the establishment of animal model for virus study. Besides, human HBV isolates worldwide were classified into ten genotypes (A to J) and further divided into subgenotypes based on nucleotide sequence divergence. The genotypes have a distinct geographical distribution and are used in tracing the evolution and transmission of the virus. Differences between genotypes cause a variety of the disease severity, complications, and response to treatment and possibly vaccination (Locarnini, 2014).

The virus particle, called Dane particle, is one of the smallest enveloped animal viruses with a virion diameter of 42 nm, consists of an outer envelope made of HBsAg in a lipid bilayer (Dane et al.,1970), wrapped around the nucleocapsid core of the virus, which in turn encloses the viral genome and a copy of its polymerase. The partially double-stranded DNA genome of the virus is a relaxed circle of 3.2 kb in length (rcDNA) (Robinson et al., 1974), contains 4 open reading frames: surface (PreS/S), core (C), polymerase (P) and X genes. The Pre-S/S ORF of the genome encodes for small (S), middle (M), and Large (L) envelope proteins on the outer envelope (Seeger et al., 2000).

During virus infection, HBV initiates the cell attachment by the interaction with heparin sulfate proteoglycan (HSPG) on the cell surface (Christianson and Belting, 2014). Sodium-taurocholate co-transporting polypeptide (NTCP) was identified as a specific host receptor and plays an essential role for HBV entry (Yan et al., 2012). Very recently, the epidermal grow factor receptor (EGFR) was reported as a cofactor of the NTCP-mediated HBV internalization (Iwamoto et al., 2019). Because of the dual pathways of EGFR internalization, the precise role of EGFR for HBV infection remains elusive as described below. After virus entry, it establishes a nuclear pool of episomal DNA that is covalently closed circular DNA (cccDNA). This cccDNA is copied into RNA that is transported to the cytoplasm, where it is reverse-transcribed into DNA, which is then packed

in a virus particle (Revill et al., 2016) (Fig.1). While drugs that interfere entry, replication or reverse-transcription have been developed, none can remove cccDNA, resulting in persistent infection and chronic disease. Therefore, considerable efforts have been made to develop agents that protect healthy hepatocytes from infection at each step of HBV life cycle.

In the past decades, the studies on infection and replication of HBV and the development of antiviral therapeutics have been hampered by the lack of an appropriate *in vitro* infection systems that recapitulate the complicated viral life cycle. Primary human hepatocytes have been used as the host cells for HBV infection, however, the availability is limited and they are phenotypically unstable and cannot maintain their permissiveness for HBV infection. As alternatives, chimeric humanized mice or cultured hepatocytes transfected with NTCP have been used (Thomas and Liang, 2016). As the preS1-domain of L protein in HBV capsid has been shown to interact with NTCP, a myristylated peptide containing the 2-48th amino acid region of preS1, which is known as Myrcludex-B, has been used to inhibit HBV infection in cell culture and in vivo mouse model (Meier et al., 2013). However, HBV dose not infect every cells overexpressing hNTCP, suggesting that the binding of HBV to NTCP is still not sufficient to trigger HBV entry. Moreover, there are 10 HBV genotypes that exhibit distinct geographical distributions, disease severity and response to

antiviral therapeutics, indicating the importance of specific host-virus interaction. None of the currently available experimental systems is sufficient for addressing such host-virus interaction.

Recently, EGFR was reported as an important co-factor for HBV internalization (Iwamato et al., 2019). EGFR is one of the best-characterized receptor tyrosine kinases (RTKs), and it can be activated by several ligands, of which EGF is most extensively studied (Bakker et al., 2017; Sigismund et al., 2008). In general, EGFR is the monomeric form that is inactive in the absence of ligand. Upon ligand binding, EGFR becomes the active dimeric form, which is then internalized by the clathrin-mediated endocytosis pathway (CME), i.e. the receptor is removed from the surface *via* clathrin-coated pits that are routed to the early endosome and then recycled back to the cell surface. Interestingly, the EGFR recycling *via* CME occurs by EGF stimulation at a low dose, however. activation of EGFR with a high dose of EGF results in hyperphosphorylation at the C-terminal domain, leading to the clathrin-independent endocytosis pathway (CIE). The highly phosphorylated EGFR is ubiquitinylated and then degraded in the lysosome (Sigismund et al., 2008) . Although EGFR is a co-factor for HBV entry, it remains unknown how EGFR internalization pathways affect HBV infection.

As induced pluripotent stem cells (iPSC) can be derived from any individuals and are capable of proliferate and differentiate to hepatocytes, iPSC can be an ideal cell source for HBV

infection. However, hepatocytes derived from hiPSCs, in most cases, exhibit immature hepatic

phenotypes. As liver non-parenchymal cells (NPCs), such as LSECs, HSCs, mesothelial cells (MCs)

and Kupffer cells, constitute 40% of total liver cells and intimately interact with hepatocytes, it is

highly likely that communication between NPCs and hepatocytes is essential not only for normal

liver functions but also for hepatogenesis. The development of the mouse liver begins with early

endoderm development (Tremblay and Zaret, 2005) . The cells of the ventral foregut endoderm are

induced to the hepatoblast stage by FGF and BMP signaling from the heart and septum transversum

mesenchyme (STM). Following induction, hepatoblasts proliferate and migrate into the STM to

form the liver bud with non-parenchymal cells. Finally, they differentiate into mature hepatocytes

and cholangiocytes through interactions with LSECs and HSCs. Therefore, I considered the

possibility that co-culture of iPS-derived hepatoblasts with NPCs would induce fully functional

hepatocytes. In addition, while NTCP is limitedly expressed on the basolateral membrane, which is

closely located with LSEC, the regulation of HBV intake from LSEC might be functional in the co-

culture system. In fact, our laboratory had previously established the protocols to induce LSEC and

HSC from hiPSCs, and demonstrated that NPC induced maturation of iPS-derived immature

hepatocytes (Koui et al., 2017) (Fig. 2).

In this study, I have developed a co-culture system using hepatocytes derived from human iPSC and NPC and found that LSECs enhance HBV infection by secreting EGF. I then investigated in detail the role of EGF and EGFR in HBV infection and found that EGF secreted from LSEC modulates HBV infection in a dose dependent manner *via* different endocytosis pathways.

Materials and Methods

Mice

C57BL/6J mice were purchased from CLEA Japan, Inc (Tokyo, Japan). All mouse experiments in

this study were performed according to the guidelines of the institutional Animal Care and Use

Committee of the University of Tokyo.

Cell lines and Culture

The hiPSC line TkDN4-M was obtained from the Institute of Medical Science, the University of

Tokyo (Takayama et al., 2010). hiPSCs were maintained in feeder-free culture using Essential 8

Medium (Thermo Fisher Scientific, Waltham, USA). hiPSC-derived LPCs, LSECs, and HSCs

were prepared according to previous protocols (Si-Tayeb et al., 2010; Koui et al., 2017) . HepG2-

NTCP cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad,

CA, USA) supplemented with 10% FBS, MEM non-essential amino acids solution, 100 U∕mL

penicillin, and 100 μg∕mL streptomycin. Primary human hepatocytes (PXB-cells) derived from

humanized mice were purchased from PhoenixBio (Hiroshima, Japan), maintained in its

commercial medium; during HBV infection, FBS and EGF were removed and 4% PEG8000 and

2% DMSO were added to the medium.

Isolation and culture of fetal mouse liver cells

LSEC and HSC progenitors were isolated from livers of E14.5 fetal mice. The fetal livers (each

sample contained 25-35 embryos) were minced and dissociated in Liver Digest Medium (Life

Technologies, California, US). Cells were treated with FcR blocking reagent and incubated with

FITC-conjugated anti-stab2 antibody and APC-conjugated anti-Ngfr antibody (Miltenyi biotec).

Stab2$^+$ cells and Ngfr$^+$ cells were isolated by a MoFlo XDP cell sorter (Beckman Coulter, Inc,

California, US). After sorting, cells were seeded onto a collagen I-coated dish at the density of

40,000 cells/cm^2 in Endothelial Cell Growth Basal Medium plus (LONZA, Switzerland)

supplemented with VEGF (50 ng/ml) and Y27632 (10 μM) under hypoxic condition. Quantitative

RT-PCR was performed using SYBR Premix EX TaqII (Takera bio, Shiga, Japan). Primers and

antibodies are listed in Table 1 and 3.

Endocytosis of EGFR

Cells were incubated with FBS-starved medium for 24 hours and then stimulated with EGF for 30 minutes at 37˚C, followed by HBV infection. To inhibit endocytosis, cells were incubated with 2 µM of ikarugamycin (Sigma-Aldrich Corporation, St. Louis, US) or 1 µg/mL of filipin (SIGMA) for 30 minutes before EGF stimulation. For lysosome inhibition, cells were incubated with 25 µM of chloroquine (Sigma-Aldrich Corporation, St. Louis, US) for 2.5 hours before EGF stimulation. To block EGF stimulation, cells were incubated with 10 µM gefitinib (Sigma-Aldrich Corporation, St. Louis, US) when EGF was added.

Co-culture system

Hepatocytes were derived from iPSCs in the lower chamber of trans-well as previously reported (Corning, New York, US). To induce hepatic maturation, cells were incubated in Hepatocyte Basal Medium (LONZA, Switzerland) supplemented with HCM SingleQuots (excluding EGF) and Oncostatin M (20 ng/ml) (PeproTech, New Jersey, US). NPCs were suspended in Endothelial Cell Growth Basal Medium plus (LONZA, Switzerland) supplemented with VEGF (50 ng/ml) and Y27632 (10 µM) and seeded into the upper chamber of trans-well (Corning, cat. 3495) at 120,000 cells/cm^2. Co-culture started at HGF stage day0. After 10 days of co-culture, cells were infected with HBV for 16 hours.

HepG2-NTCP cells were suspended in HepG2-NTCP maintenance medium and seeded on a

collagen I-coated plate at the density of 25,000 cells/cm^2. iPSC-derived NPCs were suspended in

NPC maintenance medium and seeded into the upper chamber of trans-well. Co-culture was

started at day 1, and after 6 days of co-culture, cells were infected with HBV for 16 hours.

Recombinant HBV infection and assay of NanoLuc activity

HBV/NL virus was prepared as previously described (Nishitsuji et al., 2015). Cells were infected

with HBV/NL at 100 Genome equivalent (GEq) /cell in the presence of 4% PEG8000 and 2%

DMSO for 16 hours. After washing to remove the virus, cells were maintained for 5 days. Activity

of NL was then measured using the NL Luciferase Assay Kit (Promega) according to the

manufacturers' protocols.

HBV infection assay

The culture supernatant of HepAD38 cells was used as an HBV source as previously described

(Ladner et al., 1997). Medium was harvested and the virus fraction prepared by precipitation with

13% PEG8000 (SIGMA) containing 0.75 M NaCl. Virus was then purified by precipitation at

1,750 xg for 30 minutes, suspended in Opti-MEM (Life Technologies, California, US) and stored

at −80°C until use. Cells were infected with the virus at 10,000 GEq/cell in the presence of 4%

PEG8000 and 2% DMSO for 16 hours. After washing to remove virus, cells were cultured for 14

days in the medium containing 4% PEG8000 and 2% DMSO. HBsAg was detected by ELISA

assay; HBV DNA and cccDNA were detected by qPCR and southern blot. Primers and probes are

listed in Table 2.

HBV attachment assay

HBV derived from the culture supernatant of HepAD38 cells was used for this assay. Cells were

incubated with the virus at 10,000 GEq/cell in the presence of 4% PEG8000 and 2% DMSO at

4°C for 3 hours. After washing out the free virus, HBV DNA was detected by qPCR. Primers and

probes are listed in Table 2.

HBV internalization assay

Following HBV attachment assay, the cells were washed to remove free virus and incubated at

37°C for 24 hours. The attached virus was removed by 0.25% trypsin/EDTA and HBV DNA was

detected by qPCR.

Cytokine array analysis

Human Cytokine Antibody Array Membrane (Abcam, Cambridge, UK) was used to detect

cytokines according to the manufacturer's protocol. iPSC-derived LSECs were suspended in NPC

maintenance medium and seeded at the density of 40,000 cells/cm^2 in the upper chamber of trans-

well. Control wells contained NPC maintenance medium only. HepG2-NTCP maintenance

medium was added to the lower chamber and changed to fresh medium every day. The samples

were prepared from the lower chamber at day 5, and the data were analyzed by ImageJ (National

Institutes of Health) quantitatively.

Knockdown of EGFR

Sequences of siRNAs used for EGFR knockdown were: si-EGFR: 5'-

GGAACUGGAUAUUCUGAAA TT-3' and 5'-GAUCUUUCCUUCUUAAAGA TT-3'.

Cells were transfected with siRNAs at a final concentration of 30 nM using Lipofectamine

RNAiMAX Reagent (Life Technologies, California, US). The Mission siRNA Universal Negative

control were purchased from Sigma- Aldrich (St. Louis, MO, USA). Cells were used for

experiments 48 h post-transfection.

Immunocytostaining

Cultured cells were fixed in 4% PFA and permeabilized with 0.25% tritonX-100. The cells were

blocked with SuperBlock™ Blocking Buffer (Thermo Fisher Scientific, Waltham, USA). Then,

they were incubated with primary and secondary antibodies. Antibodies are listed in

Supplementary information.

Cross-linking assay

In cross-linking assays, the cells were incubated with the cross-linker BS^3 at 4°C for 30 minutes,

and the cross-linking reaction was terminated using 20 mM of glycine (final concentration) as

previously described[11]. Cell surface lysates were prepared by using Pierce Cell Surface Protein

Isolation Kit (Thermo Fisher Scientific, Waltham, USA).   Lysates were cleared by centrifugation

prior to addition of gel sample buffer. Samples were then separated on SDS-PAGE, and

transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h in

SuperBlock™ Blocking Buffer (Thermo Fisher Scientific, Waltham, USA), and then incubated

overnight with the primary antibody. Secondary anti-mouse or anti-rabbit antibodies linked to

horseradish peroxidase were used to detect immunoreactive proteins utilizing the enhanced

chemoluminescence reaction (Bio-Rad, California, USA).

Statistical analysis

Data are expressed as mean ± SEM and analyzed by Student's t-test, one-way ANOVA, or two-

way ANOVA. The statistical significance was determined at $P<0.05$.

Results

Development of an HBV infection system using iPSC-derived hepatocytes

I prepared iPS-hepatoblasts according to the 3-step-induction protocol by using Activin

A (ActA), bone morphogenetic protein 4 (BMP4), and hepatocyte growth factor (HGF), followed

by induction to iPS -hepatocytes by Oncostatin M (OSM) (Si-Tayeb et al., 2010). HBV infection

was limited in iPS-hepatoblasts. As iPS-hepatocytes after OSM induction are more mature than iPS-

hepatoblast, the HBV infection in iPS-hepatocytes was expected to be enhanced. In fact, iPS-

hepatocytes exhibited higher levels of HBsAg and HBV DNA than iPS-hepatoblasts. However, the

cccDNA level was not increased (Fig. 4), showing the limitation of iPSC-hepatocytes in HBV

infection.

Development of a co-culture system of iPSC-derived hepatocytes with NPCs

We previously showed that NPCs promoted maturation of hepatocytes *in vitro* (Koui et

al., 2017). In order to test if HBV infection is enhanced by NPCs, I isolated and cultured LSEC and

HCS progenitors from E14.5 mouse fetal livers as Stab2$^+$ and Ngfr$^+$ cells, respectively. They

exhibited distinctive morphology and expressed unique sets of genes specific to each cell type (Fig.

5). They were then co-cultured with hiPSC-derived hepatocytes by using a Transwell cell culture

insert (Fig. 6A). HBV infection was evaluated by recombinant HBV expressing NanoLuc (NL) (HBV/NL) (Nishitsuji et al., 2015) (Fig. 6B), in which HBV s gene is replaced with nanoluciferase (Fig. 6C). HBV/NL provides a convenient assay system to evaluate HBV infection by luciferase activity. Interestingly, LSECs significantly enhanced NL activity after 5 days of virus infection, whereas HSCs did not enhance it (Fig. 6C). The results indicated that the co-culture system of iPSC-derived hepatocytes with NPCs would be useful for the studies on the mechanisms of the infection and replication of HBV.

As we have already established culture systems to generate LSECs and HSCs from hiPSCs and shown that they promote hepatic maturation of hiPSC-derived hepatocytes *in vitro* (Koui et al., 2017), I applied those hiPSC-derived NPCs to HBV infection (Fig. 7A) After induction of hepatic endodermal cells from iPSCs, they were co-cultured with hiPSC-derived LSECs or HSCs in trans-well cell culture inserts and infected with HBV/NL virus or wild-type HBV (Fig. 7B). Similar to fetal mouse LSECs, hiPSC-derived LSECs also significantly enhanced HBV infection in the trans-well co-culture system as shown by NL activity, HBsAg, cccDNA, and HBV DNA (Fig. 7C, D). These data demonstrated that the co-culture system of hiPSC-derived liver cells would be useful for studies on the mechanism of HBV infection and relationships between HBV genotypes and genetic backgrounds of individuals.

Mechanism of the enhancement of HBV infection by LSECs

To reveal how hiPSC-derived LSECs enhance the HBV infection and replication, I utilized the HepG2-NTCP hepatoma cell line overexpressing NTCP, which provides a simplified system to evaluate HBV infection (Nishitsuji et al., 2015) (Fig. 8A, B). In the trans-well co-culture system, hiPS-derived LSECs significantly enhanced NL activity, HBsAg, cccDNA, and HBV DNA (Fig.8C, D) showing that the simplified culture system can be used to study HBV infection and replication. Because the co-culture system using trans-well avoids a direct cell-cell contact, the positive effect of LSECs on HBV infection should be mediated by a soluble factor(s). I therefore performed cytokine array analysis of LSEC culture medium to find a factor responsible for enhancing HBV infection. Among 42 cytokines I analyzed, EGF was found to be most in the supernatant of iPSC-derived LSECs (Fig. 9). In contrast, no detectable EGF was found in the supernatant from iPS-derived HSCs, indicating the possibility that EGF from LSECs regulated the HBV infection in the co-culture model.

Modulation of HBV infection by EGF

It was recently reported that the EGFR activation by EGF plays a positive role for NTCP-mediated HBV internalization (Iwamoto et al., 2019). To confirm the enhancement of HBV infection by EGF, I performed an HBV infection assay using HepG2-NTCP at 2 ng/mL, 10 ng/ml and 50 ng/ml of EGF. HBV infection was enhanced by EGF at 2 ng/mL. Unexpectedly, however, HBV infection was suppressed at a higher concentration of EGF (Fig. 10), and this dose-dependent response was detected in few hours of post infection of HBV. The effect of EGF was blocked by neutralizing anti-EGF antibody (Fig. 10). I also found that HBV infection was suppressed in HepG2-NTCP when co-cultured with a high density of hiPSC-derived LSECs (data not shown), suggesting that EGF at a high dose suppressed HBV infection. This EGF-dependent modulation of HBV internalization occurred before 24 hours of post-infection (Fig. 11). These results were confirmed by using primary human hepatocytes and iPSC-derived hepatocytes. In primary human hepatocytes, 2 ng/mL of EGF enhanced HBV infection in the FBS-free condition and the infection was gradually reduced over 5 ng/mL of EGF (Fig. 12). The EGF dose response curve was in a rather gradual manner. This is likely to reflect a variation of cell density and maturation stage of iPS-derived cells, which cannot be precisely controlled (data not shown). Nevertheless, dose-dependent regulation was reproducibly observed (Fig. 13). These results suggested that modulation of the HBV infection by EGF was dose-dependent.

Activated EGFR enhance HBV attachment

To reveal whether EGF affected HBV infection at the cell surface or intracellular pathways, I examined the attachment of HBV to HepG2-NTCP cells. When the cells were pretreated with EGF at 37˚C for 30 min, EGF at 2 ng/ml enhanced the binding of HBV to the cells, whereas the enhancement by 50 ng/ml EGF was less than that by 2 ng/ml (Fig. 14A). By contrast, EGF enhanced the HBV binding equally well when pretreated at 4ºC to prevent internalization (Fig. 14B). From the western blot analysis using cross-linker BS^3, the cell surface EGFR existed as a monomeric form without EGF stimulation, and both monomer and dimer forms were found in the presence of EGF; at a high dose of EGF, less EGFR was detected compared to a low dose of EGF (Fig. 15). To reveal the mechanisms of the differential response to 2 and 50 ng/mL of EGF at 37ºC, I performed immunocytochemical analysis and found that few EGFR was colocalized with a lysosome maker, LAMP2, whereas EGFR was mostly present on the cell membrane at 4˚C compared with 37 ˚C (Fig. 16). As the receptor internalization was suppressed at 4ºC (Björkelund et al., 2013), the results suggested that the effect of EGF on HBV binding to the cell surface was independent from EGF doses, and that the dose-dependent effect of EGF on HBV infection was caused by an event post binding to cells.

The results show that EGF stimulation enhances HBV binding on the cell surface. However, as EGFR exists as a monomer and dimer depending on the activation status, it remained unknown whether EGF affected either form or both forms. In fact, HepG2-NTCP cells showed predominantly the monomer form without EGF stimulation, and both monomer and dimer forms were found in the presence of EGF (Fig. 15). Gefitinib was reported as an EGFR kinase inhibitor that blocks EGFR phosphorylation, and stabilizes dimerized EGFR (Björkelund et al., 2011). The EGFR dimers formed in the presence of gefitinib were shown to exhibit an ectodomain structure different from the EGF-stimulated dimers (Lu et al., 2012). I found that gefitinib reversed the enhanced HBV attachment by EGF stimulation, but had no effect on HBV attachment to the cells without EGF stimulation (Fig. 17). The results indicated that the activated EGFR dimers enhanced HBV binding to HepG2-NTCP. Knockdown of EGFR expression also cancelled the enhanced HBV attachment by EGF in HepG2-NTCP cells (Fig. 18). Interestingly, EGF enhanced HBV binding to HepG2 and knocking down EGFR also suppressed EGF-mediated enhancement of HBV binding to HepG2 without NTCP (Fig. 19). These results provide evidence that the up-regulation of HBV attachment is due to the EGF-induced EGFR dimer, which is independent to NTCP expression, and that monomer EGFR is not involved in HBV binding.

Endocytosis of EGFR and HBV

Previous studies showed that the internalization of EGFR was EGF dose-dependent *via* different endocytosis pathways (Bakker et al., 2017; Sigismund et al., 2008). Ligand-bound EGFR form an asymmetric dimer on the cell membrane, which is followed by activation of its tyrosine kinase (Fig. 3). At a low dose of EGF stimulation, EGFR is internalized *via* clathrin-mediated endocytosis (CME) and then recycled back to the cell surface. On the other hand, the activated EGFR by a high dose of EGF induces a higher level of phosphorylation at the C-terminus of EGFR, resulting in the internalization through clathrin-independent endocytosis (CIE). The highly phosphorylated EGFR is ubiquitinylated and transported to the late endosome, and subsequently to the lysosome to be degraded. In addition to such canonical mechanisms of ligand-induced receptor activation, the non-canonical unliganded EGFR monomers can also get internalized *via* a tyrosine kinase independent mechanism (Tanaka et al., 2018) (Fig. 3). I assumed that the HBV internalization is closely related with this EGF dose dependent modulation.

Therefore, I hypothesized that HBV was internalized with EGFR *via* the tyrosine kinase-independent (TK-independent) CME pathway without EGF binding, TK-dependent CME pathway at a low dose of EGF, whereas HBV was internalized *via* the TK-dependent CIE pathway at a high dose of EGF and then degraded in the lysosome. To test this possibility, I evaluated the effect of specific inhibitors to block each pathway, CME and CIE. As EGFR knockdown suppressed HBV

internalization with or without EGF, EGFR was indispensable for the HBV internalization (Fig.

20A). However, gefitinib, an EGFR tyrosine kinase inhibitor, inhibited HBV infection in the

presence of 2 ng/mL of EGF, but had no effect on HBV infection in the absence of EGF, indicating

that the unliganded EGFR monomer internalize *via* TK-independent CME (Fig. 20B, 21).

Ikarugamycin (IKA) (Elkin et al., 2016) is a CME inhibitor and blocked HBV infection at 0 ng/ml

and 2 ng/ml of EGF, whereas it had no effect on HBV infection at 50 ng/ml of EGF (Fig. 22).

Immunocytochemistry showed IKA blocked the internalization of both EGFR and NTCP (Fig. 23).

On the other hand, the suppression of HBV infection by the high dose of EGF was reversed by the

treatment with filipin (FLP), a CIE inhibitor (Sigismund et al., 2008) (Fig. 24).

Immunocytochemical analysis showed the colocalization of EGFR and LAMP2, a lysosome marker,

by stimulation with EGF at a high dose. And the colocalization was inhibited by FLP (Fig. 25),

suggesting that HBV was degraded *via* the CIE pathway. I investigated this possibility by inhibiting

lysosomal functions. As expected, HBV infection was clearly increased in the presence of

chloroquine (CQ), a lysosomal inhibitor, at 50 ng/ml of EGF (Lin et al., 2019) (Fig. 26). The

colocalization of EGFR and LAMP2 was only observed at a high dose but not at a low dose (Fig.

27). Taken together, these results strongly suggested that HBV is internalized by the pathways

shared with EGFR endocytosis: TK-independent CME, TK-dependent CME and CIE, and that HBV

is degraded in the lysosomes at a high dose of EGF.

Possible binding of EGFR to HBV via a domain distinct from PreS1

It was unclear how EGF-activated EGFR enhanced HBV attachment on the cell surface.

Because NTCP is an HBV entry receptor and PreS1 binds to NTCP, exogenous PreS1 peptide has

been used as a competitive inhibitor to prevent HBV infection *in vivo* and *in vitro* (Meier et al.,

2013). Fluorescence-conjugated PreS1 peptide was also used as a substitute of HBV binding for

imaging. However, PreS1-NTCP binding seems not to be the only interaction site between HBV

and hepatocyte. Immunocytochemical analysis showed the colocalization of EGFR and NTCP

without EGF stimulation, however, they were not colocalized after EGF stimulation (Fig. 28). In

contrast to the co-internalization of HBV with EGFR in the lysosome, PreS1 was colocalized with

NTCP. Furthermore, competitive activity of PreS1 to HBV attachment and infection was lower in

cells stimulated with 50 ng/ml EGF (Fig. 29), suggesting the possibility that activated EGFR dimer

by a high dose EGF interacts with HBV surface protein other than PreS1 domain.

Discussion

In order to study HBV infection and replication, primary cultures of human hepatocytes have been used as an assay system *in vitro*. Nevertheless, as the supply of fresh human hepatocytes is limited, hepatocytes derived from iPSCs should be a stable source for studies on HBV infection. Hepatocytes derived from iPSCs will also provide a tool to study the genetic relationship between HBV genotype and susceptive individuals. However, the hepatocytes derived from iPSCs are immature and HBV infection efficiency is limited. This is the major problem to use hepatocytes from iPSCs and I have approached this problem from liver development. During liver development, immature hepatocytes proliferate and differentiate into mature hepatocytes through interactions with hepatic NPCs such as LSECs and HSCs. NPCs secrete factors such as HGF, EGF, BMP and FGF that play a central role in liver development. In fact, we previously showed that HSCs support maturation of iPSC-derived hepatocytes (Koui et al., 2017). Accordingly, I established a trans-well co-culture system of iPSC-derived hepatocytes with NPCs to improve the hepatic maturation by a paracrine mechanism. I found in this study that both mouse embryonic LSECs and iPSC-derived LSECs, but not HSCs, enhanced the HBV infection by providing EGF, showing the paracrine regulation for HBV infection by LSECs. NTCP has been known as the main receptor that specifically binds HBV, and EGFR was recently shown to be a cofactor of NTCP-mediated entry

of HBV into hepatocytes, however the origin of EGF remained unknown. In this study I show that

LSEC is the main cell source of EGF in NCP. In the liver, the circulating HBV passes through the

space of Dissé, a gap between LSECs and hepatocyte basolateral membrane. As NTCP is

exclusively sorted to the basolateral membrane of hepatocytes (Schulze et al., 2012) and binds HBV,

it is very reasonable that EGF secreted from LSECs modulates NTCP-mediated entry of HBV in

hepatocytes.

Modulation of HBV attachment to hepatocytes by EGF

Another important finding in this study is that EGF modulates HBV infection in a dose-

dependent manner, i.e. EGF enhances HBV infection at a low dose, whereas it suppresses HBV

infection at a high dose. HBV infection initiates by the attachment to hepatocytes via the low

affinity interaction with heparin sulfate proteoglycan (HSPG) on the cell surface, followed by

high-affinity binding to sodium-taurocholate co-transporting polypeptide (NTCP) (Yan et al.,

2012). However, it has not yet been confirmed that HBV virions directly bind to NTCP. The

PreS1 domain is a short peptide of the HBV envelope L protein (L-HBsAg) on HBV surface and

binds to NTCP in vitro (Meier et al., 2013). It was reported that BNCs, the hollow bio-

nanocapsules displaying L-HBsAg in a manner similar to HBV, bound to human hepatic cells

lacking NTCP, suggesting that there may be another HBV binding receptor (Somiya et al., 2016).

HBV binds to NTCP on hepatocytes without EGF, but it was recently reported that EGF

stimulation triggers internalization of EGFR, surface NTCP protein and PreS1 peptide (Iwamoto

et al., 2019). Thus, EGFR is considered as a cofactor for NTCP-dependent entry of HBV. Unlike

the previous study showing that EGF only enhances HBV internalization (Iwamoto et al., 2019), I

show in this study that EGF stimulation also enhanced HBV attachment to HepG2 without NTCP.

The enhancing effect was cancelled by knockdown of EGFR by siRNA (Fig.19), indicating that

activated EGFR can bind HBV at some degree. Furthermore, gefitinib binding changes the

ectodomain of dimer EGFR (Fig18, Lu et al., 2012), and cancelled the EGF-induced HBV binding

in HepG2-NTCP cells, similar to knockdown of EGFR (Fig.19). As EGF induces dimerization of

EGFR, my results suggest that a dimeric but not a monomeric EGFR binds HBV.

Endocytosis of HBV and EGFR

Binding of EGF to EGFR induces dimerization and also activation of the receptor

tyrosine kinase, leading to internalization. At a low dose of EGF stimulation, EGFR is internalized

via clathrin-mediated endocytosis (CME) and then recycled back to the cell surface. On the other

hand, the activated EGFR by a high dose of EGF is internalized through clathrin-independent

endocytosis (CIE), which is initiated by a higher level of phosphorylation at the C-terminus of

EGFR. Ubiquitin is added to the highly phosphorylated EGFR, which is transported to the late

endosome and subsequently to the lysosome to be degraded. In previous reports (Lin et al., 2019;

Macovei et al., 2013), it was described that HBV was internalized and then transferred into the

lysosome, a "dead end" in the infection process. Rab proteins such as Rab5 and Rab7 were shown

to be involved in HBV infection (Macovei et al., 2013) . Rab5 is responsible for the transfer of

vesicles from the plasma membrane to early endosomes, and Rab7 is involved in the traffic to the

late endosomes and lysosome. Besides, other than the facilitated internalization of HBV by EGF

stimulation, I found HBV could get internalized without EGF stimulation when cells were

incubated with HBV *in vitro*. Using tyrosine kinase inhibitor, gefitinib, I found that EGFR

internalization and HBV infection could be only inhibited in the presence of EGF, providing that

HBV infection could also via TK-independent CME pathway.

Specific inhibitors of CME and CIE revealed that they were internalized *via* CME when

stimulated with a low dose of EGF, whereas they were transported to the lysosome via CIE to be

degraded. Dose-dependent two distinct traffic pathways of EGFR after stimulation are the basis of

dose-dependent modulation of HBV infection by EGF. Based on the hypothesis, a CME inhibitor

and a CIE stimulator might be a good candidate to prevent HBV infection. Moreover, it is worthy

to note that a high dose of EGF is used to induce mature hepatocytes from iPSCs in some protocol

including our previous studies (Kido et al., 2015) and those hepatocytes might exhibit limited

HBV susceptibility.

In this study, I traced the fate of HBV, NTCP and EGFR after EGF stimulation. It was

reported that NTCP traffics on microtubules between the cell surface and endocytic vesicles, and

the knockdown of EGFR resulted in loss of microtubule-based motility of these vesicles in vitro.

NTCP was also found to undergo cellular redistribution upon stimulation of cells with EGF,

consistent with the model of NTCP and EGF-EGFR internalization (Wang et al., 2016). However,

EGFR and NTCP follow different endocytic routes following internalization (Fig. 28). Previous

studies showed that only EGFR, but not NTCP is degraded while EGF-EGFR complexes traffic to

lysosome upon the stimulation of EGF (Wang et al., 2016). Thus, EGFR is indispensable for

NTCP internalization, but EGFR and NTCP are trafficked to different routes intracellularly. As

the route of HBV has not yet shown directly, the interaction between EGFR, NTCP, and HBV

after internalization still need to be elucidated.

Conclusions

In this study, I have developed a co-culture system of human iPSC-hepatocytes with NPCs and found that mouse fetal LSEC and human-iPSC-LSEC enhance HBV infection. Furthermore, HepG2-NTCP cells can be used as an alternative to study the role of LSEC for HBV infection. Analysis of the conditioned medium of LSEC revealed EGF as a factor that enhances HBV infection. While EGFR is known to be a co-receptor of NTCP for EGF entry, I found that EGF modulates HBV infection in a dose dependent manner; EGF at a low dose enhances HBV infection whereas it at a high dose suppresses. I show that EGF-stimulated EGFR has higher affinity toward HBV, and that HBV is endocytosed *via* CME and CIE pathway at a low and high dose of EGF, respectively, providing an explanation of dual effects of EGF on HBV infection. As LSEC faces hepatocytes, EGF secreted from LSEC may play a role for HBV infection *in vivo.* The *in vitro* system of HBV infection using iPSC-derived liver cells provides a useful to study HBV infection *in vitro*.

Acknowledgements

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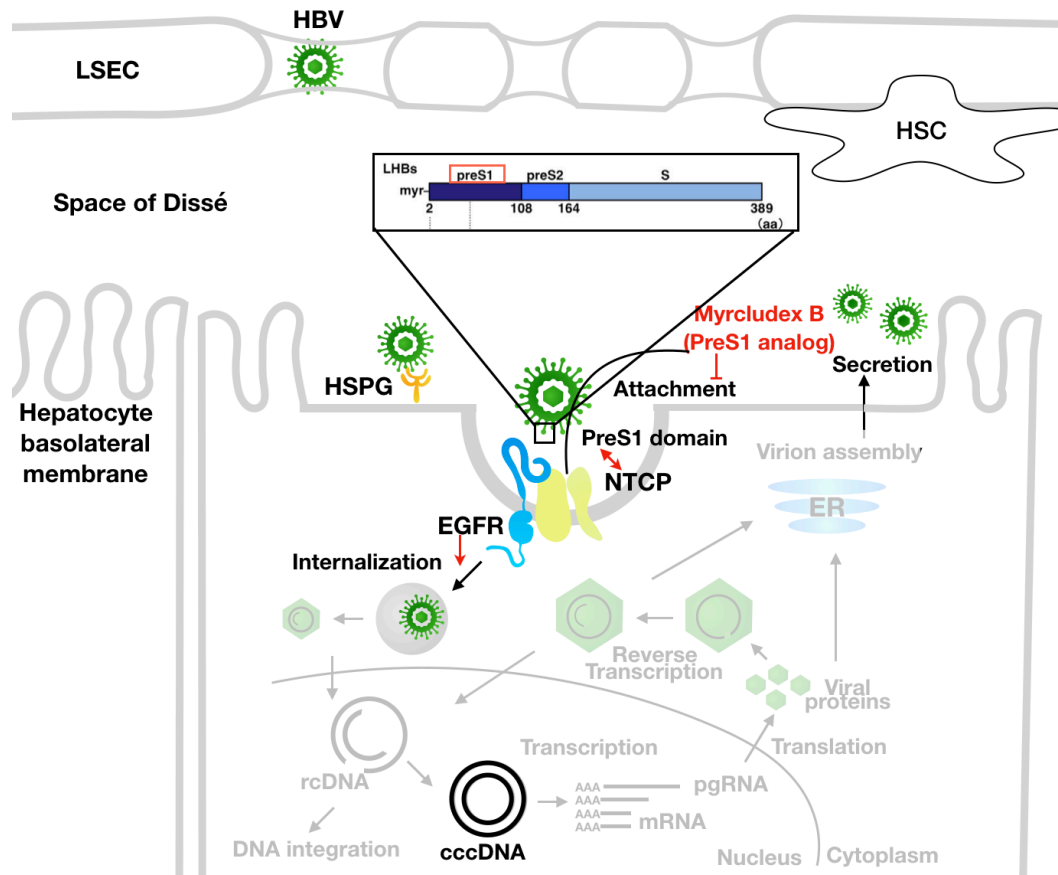
Fig. 1. HBV infection and life cycle

In the liver, the circulating HBV passes through the fenestrae of LSECs and reaches the space of Dissé; initiates the cell attachment with HSPG on the cell surface. NTCP, which is exclusively sorted to the basolateral membrane, has an interaction with PreS1 domain of Large envelope protein on HBV. EGFR, associates with NTCP, facilitates HBV internalization by EGF stimulation. After virus entry, it establishes a nuclear pool of episomal DNA into the form of cccDNA, which is copied into RNA that is transported to the cytoplasm where it reverse-transcribes into DNA and packed in a virus particle. Exogenous PreS1 peptide can work as a competitive inhibitor and affect HBV infection.
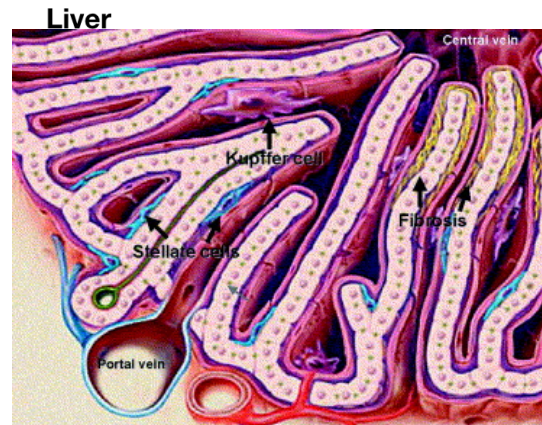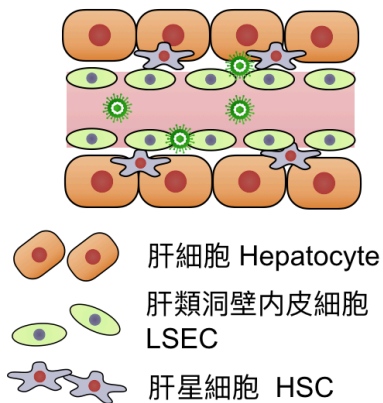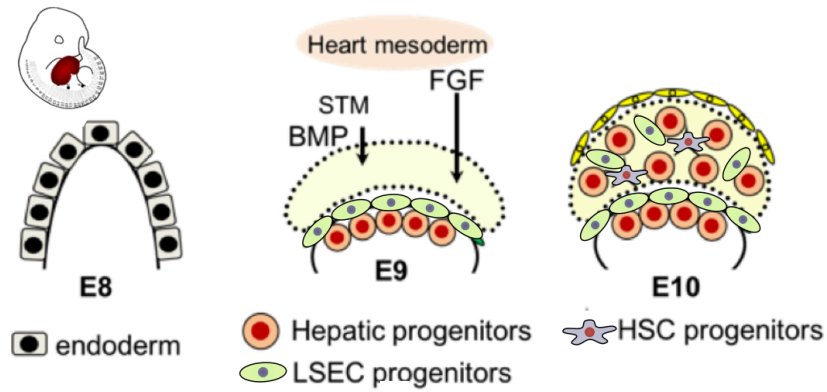
Fig. 2. The development of liver

Liver development begins at mouse embryonic day 9. The cells of the ventral foregut endoderm

are induced to the hepatoblast stage by FGF and BMP signaling from the heart and septum

transversum mesenchyme (STM). Following induction, hepatoblasts proliferate and migrate into

the STM to form the liver bud with non-parenchymal cells, such as endothelial progenitor cells

and hepatic mesenchymal cells. Finally, they differentiate into mature hepatocytes and

cholangiocytes through interactions with LSECs and HSCs. (Castillon et al., 2003).

EGFR internalization is EGF dose dependent
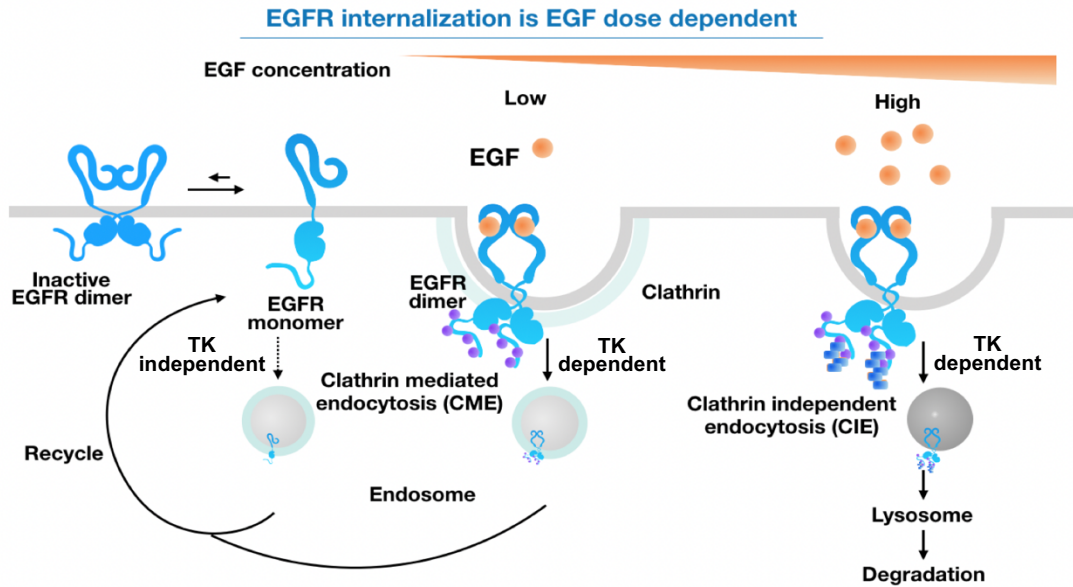
Fig. 3. EGFR endocytosis depends on the dose of EGF

EGFR is one of the best-characterized receptor tyrosine kinases (RTKs) and activated by several ligands, of which EGF is most extensively studied. In general, EGFR is present as the monomeric inactive form in the absence of EGF and is activated by EGF to form the dimer form. A primary mechanism of internalization of EGFR is clathrin-mediated endocytosis (CME); the receptor is removed from the surface as clathrin-coated pits and routed to the early endosome, then recycled back to the cell surface at a low dose of EGF stimulation. On the other hand, once the receptor is activated by a high dose of EGF, EGFR is internalized through clathrin-independent endocytosis (CIE), which is regulated by the phosphorylation level at the C-terminal and ubiquitinylated; after late endosomal vesicles are trafficked to lysosome to be degradated.
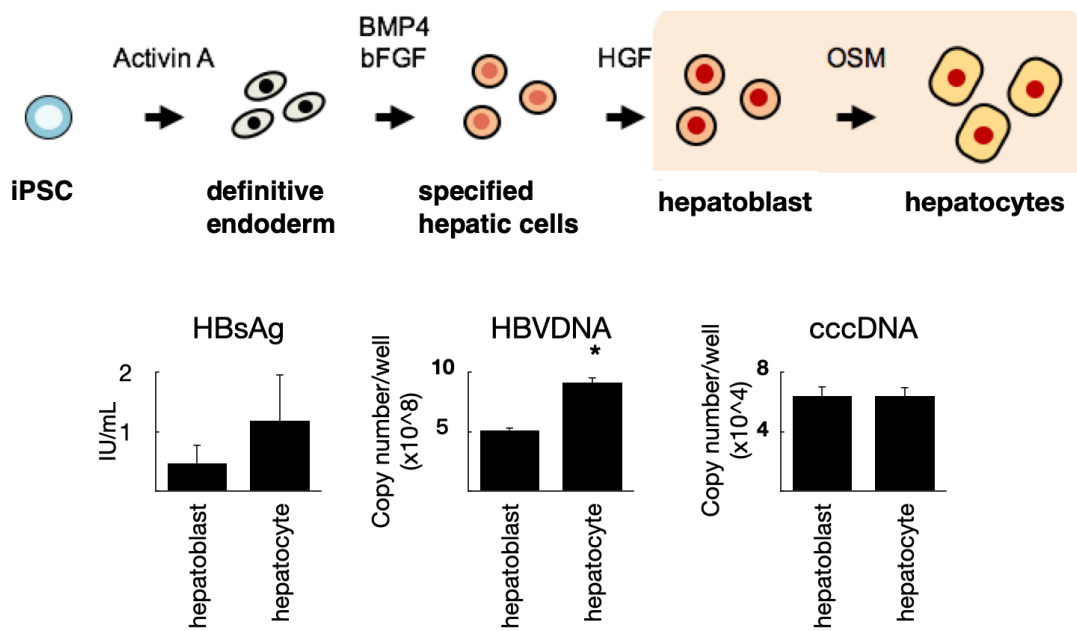
Fig. 4. Development of an HBV infection system using iPSC-derived hepatocytes

(A) procedure to induce iPS-hepatocytes. iPSCs were maintained in mono-layer culture on

Matrigel. To initiate definitive endoderm differentiation, iPSCs were cultured in RPMI media

containing B27 supplements and 100 ng/mL activin A. After 5 days of culture under $20\%O_2$, cells

were next moved to $4\%O_2/5\%CO_2$ in RPMI/B27 media supplemented with 20 ng/ML BMP4 and

20 ng/mL FGF2 for 5 days. The specified hepatic cells in RPMI/B27 supplemented with 20 ng/mL

HGF under $4\% O_2/5\% CO_2$ were incubated for 5 days. For the final stage, hepatoblasts were

differentiated in HCM supplemented with 20 ng/mL OSM.

(B) Levels of HBsAg, HBV DNA and cccDNA in iPSC-derived hepatoblasts and iPSC-derived

hepatocytes. The results are shown as the mean ± SEM of 4 independent experiments. **p < 0.01.
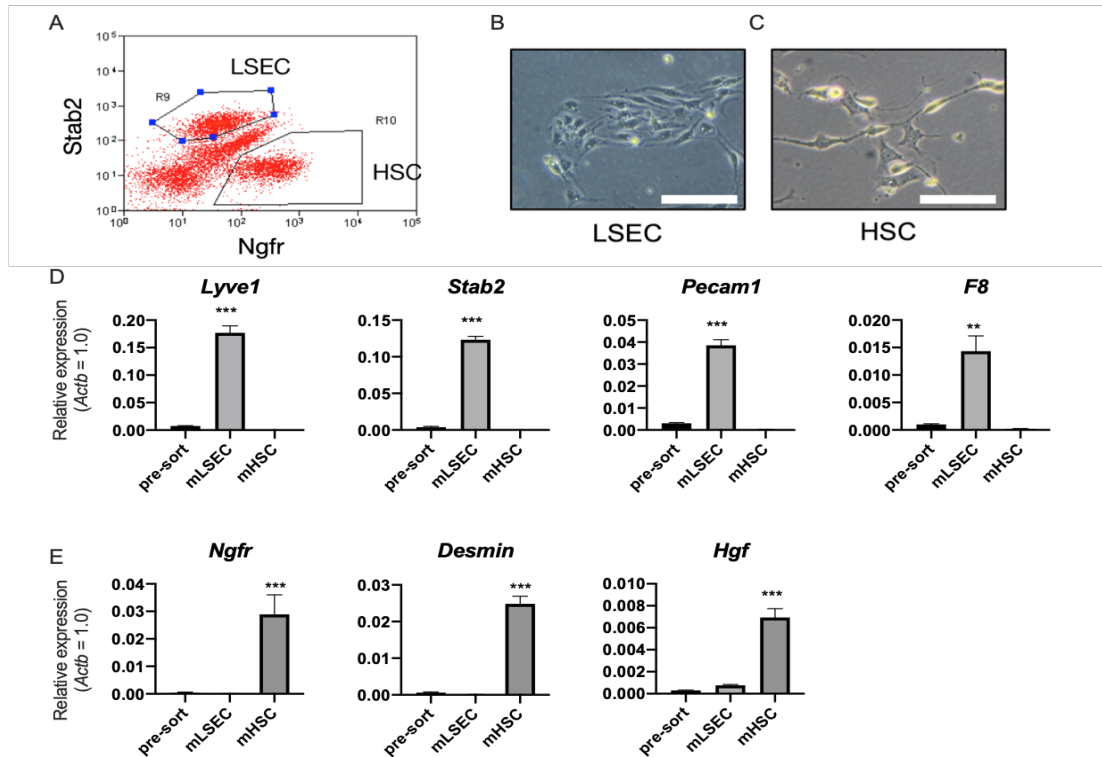
Fig. 5. Isolation and characterization of fetal mouse LSECs and HSCs.

(A) Flow cytometric analysis of fetal mouse liver cells at E14.5.

(B) Primary culture of Stab2$^+$ cells. Scale bar, 100 μm.

(C) Primary culture of Ngfr$^+$ cells. Scale bar, 100 μm.

(D) Expression levels of LSEC markers in pre-sorting cells (pre-sort), Stab2$^+$ cells (LSECs), and

Ngfr$^+$ cells (HSCs). The results are shown as the mean ± SEM of 3 independent experiments. n =

3 in each group. ***p < 0.001.

(E) Expression levels of HSC markers in pre-sorting cells (pre-sort), Stab2$^+$ cells (LSECs) and

Ngfr$^+$ (HSCs) cells. The results are shown as the mean ± SEM of 3 independent experiments. ***p

< 0.001.
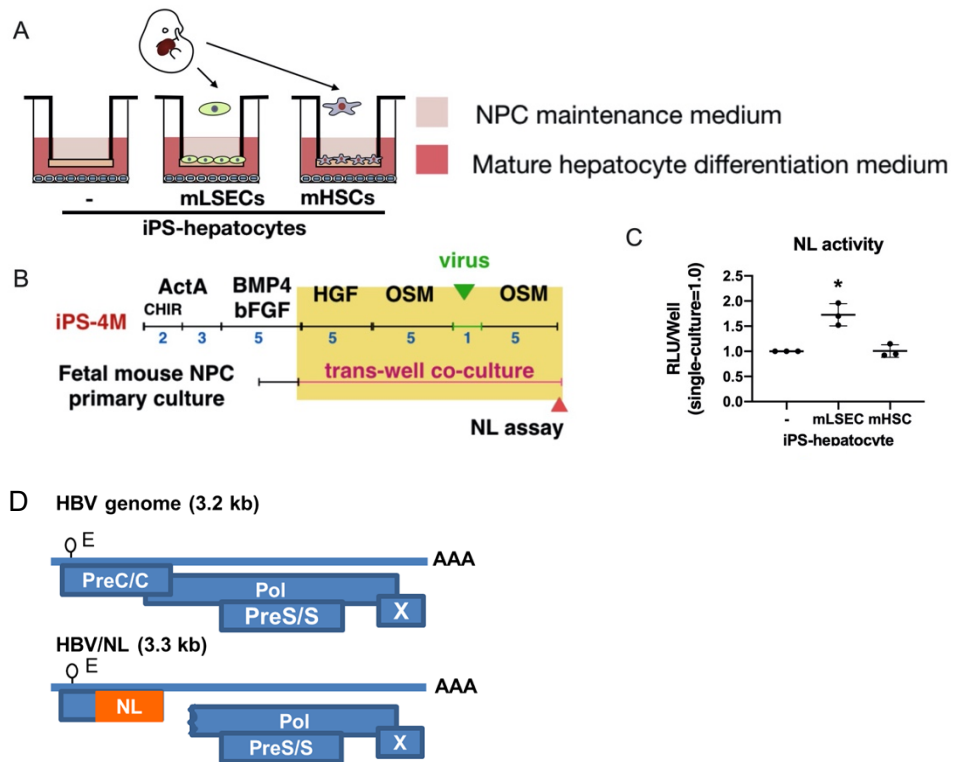
Fig. 6. Enhancement of HBV infection to iPSC-derived hepatocytes by fetal mouse LSECs

(A) Trans-well co-culture system of iPSC-derived hepatocytes with fetal mouse NPCs.

(B) Schematic representation of the HBV-NL infection assay in iPSC-derived hepatocytes co-cultured with fetal mouse NPCs.

(C) Relative HBV-NL activities in iPSC-derived hepatocytes (-), iPSC-derived hepatocytes co-cultured with fetal mouse LSECs (LSEC), and fetal mouse HSCs (HSC). The results are shown as the mean ± SEM of 3 independent experiments. **$p < 0.01$.

(D) Pregenomic structure of the reporter hepatitis B viruses (HBVs) and relative NanoLuc (NL)

activity in cells infected by the viruses. (a) Pregenomes of wild-type and reporter HBV/NL are

shown. The indicated sizes (kb) are of the pregenomes. A stretch of "A"s indicates a poly A tail of

the putative pregenomic RNA. A lariat rope with "E" indicates an encapsidation signal and "X" on

that indicates defect of encapsidation. The NL gene is inserted into the genome so as to be translated

from its own initiator methionine. (Nishitsuji et al., 2015)
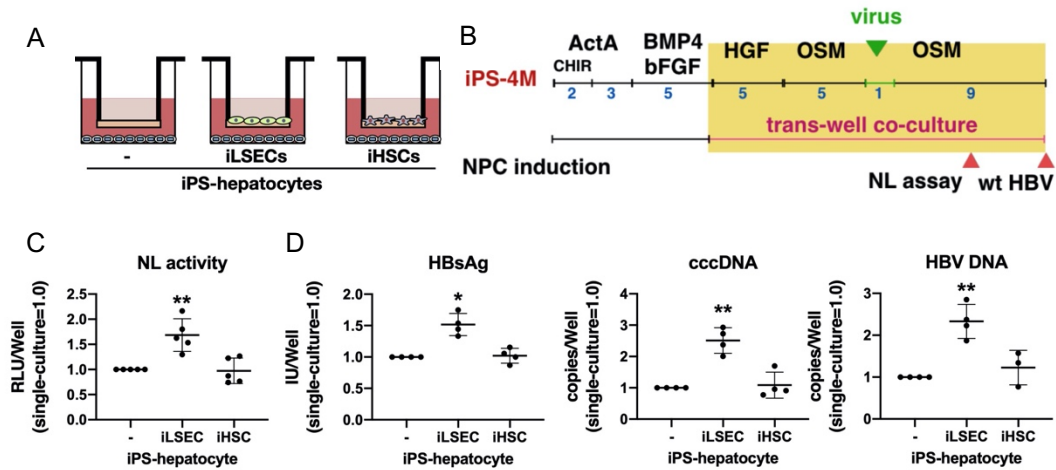
Fig. 7. Enhancement of HBV infection to iPSC-derived hepatocytes by iPSC-derived LSECs

(A) Trans-well co-culture system of iPSC-derived hepatocytes with iPSC-derived NPCs.

(B) Schematic representation of the HBV-NL and wild type HBV infection assay in iPSC-derived hepatocytes co-cultured with iPSC-derived NPCs.

(C) Relative HBV-NL activities in iPSC-derived hepatocytes (-), iPSC-derived hepatocytes co-cultured with iPSC-derived LSECs (iLSEC), and iPSC-derived HSCs (iHSC). The results are shown as the mean ± SEM of 5 independent experiments. **$p < 0.01$.

(D) Levels of HBsAg, cccDNA and HBV DNA in iPSC-derived hepatocytes (-), iPSC-derived hepatocytes co-cultured with iPSC-derived LSECs (iLSEC), and iPSC-derived HSCs (iHSC). The results are shown as the mean ± SEM of 4 independent experiments. **$p < 0.01$.
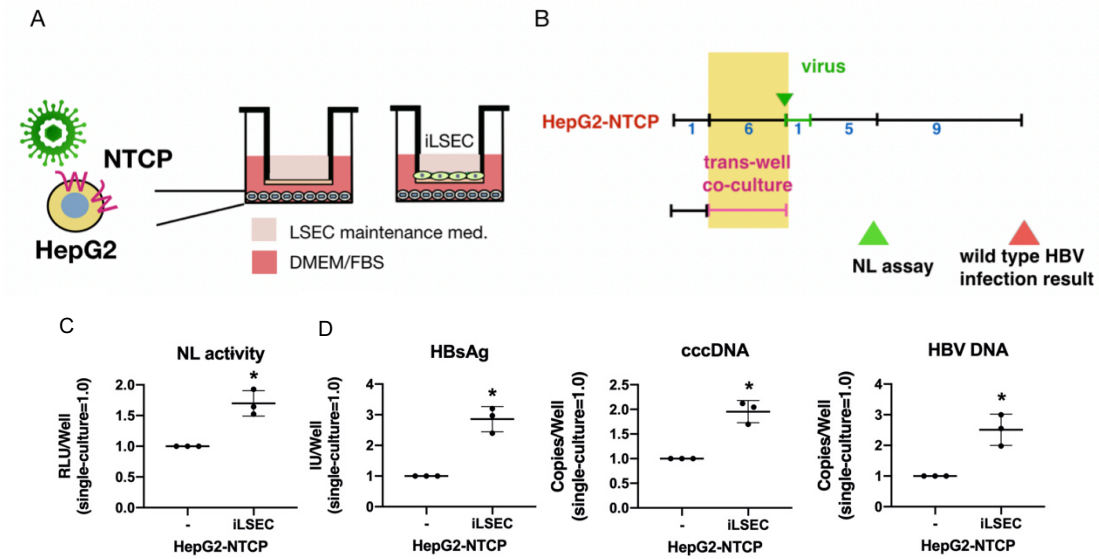
Fig. 8. HBV infection to HepG2-NTCP cells co-cultured with iPSC-derived LSECs in the trans-well system.

(A) Experimental design of the trans-well co-culture system of HepG2-NTCP cells with iPSC-derived LSECs.

(B) Experimental design of HBV infection using HBV/NL and wild type HBV in the trans-well co-culture system

(C) Relative HBV-NL activities in HepG2-NTCP cells (-) and HepG2-NTCP cells co-cultured with iPSC-derived LSECs (iLSEC). The result is shown as the mean ± SEM of 3 independent experiments. **p < 0.01.

(D) Levels of HBsAg, cccDNA and HBV DNA in HepG2-NTCP cells (-) and HepG2-NTCP cells co-cultured with iPSC-derived LSECs (iLSEC). The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05.
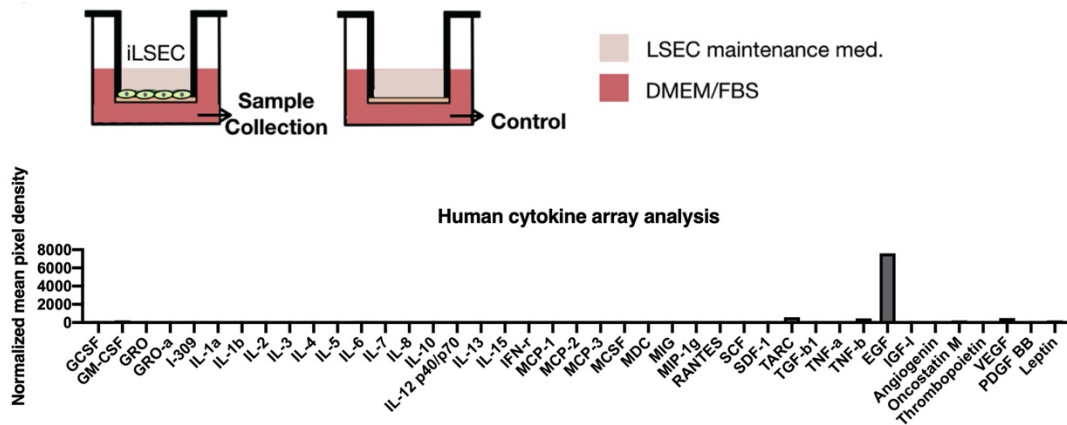
Fig. 9. Detection of EGF in the supernatant of iPSC-derived LSECs by human cytokine array analysis

iPS-derived LSECs were suspended in NPC maintenance medium and seeded at the density of 40,000 cells/cm^2 in the upper chamber of trans-well. HepG2-NTCP maintenance medium was added to the lower chamber and changed to fresh medium every day. The samples were prepared from the lower chamber at day 5. Human cytokine antibody array membrane was used to detect cytokines and the data were analyzed by ImageJ quantitatively. Trans-well inserts without iPSC-derived LSECs was used as the control. Normalized mean pixel density = mean pixel density on sample array x positive control on sample array/ positive control on control array - mean pixel density on control array.
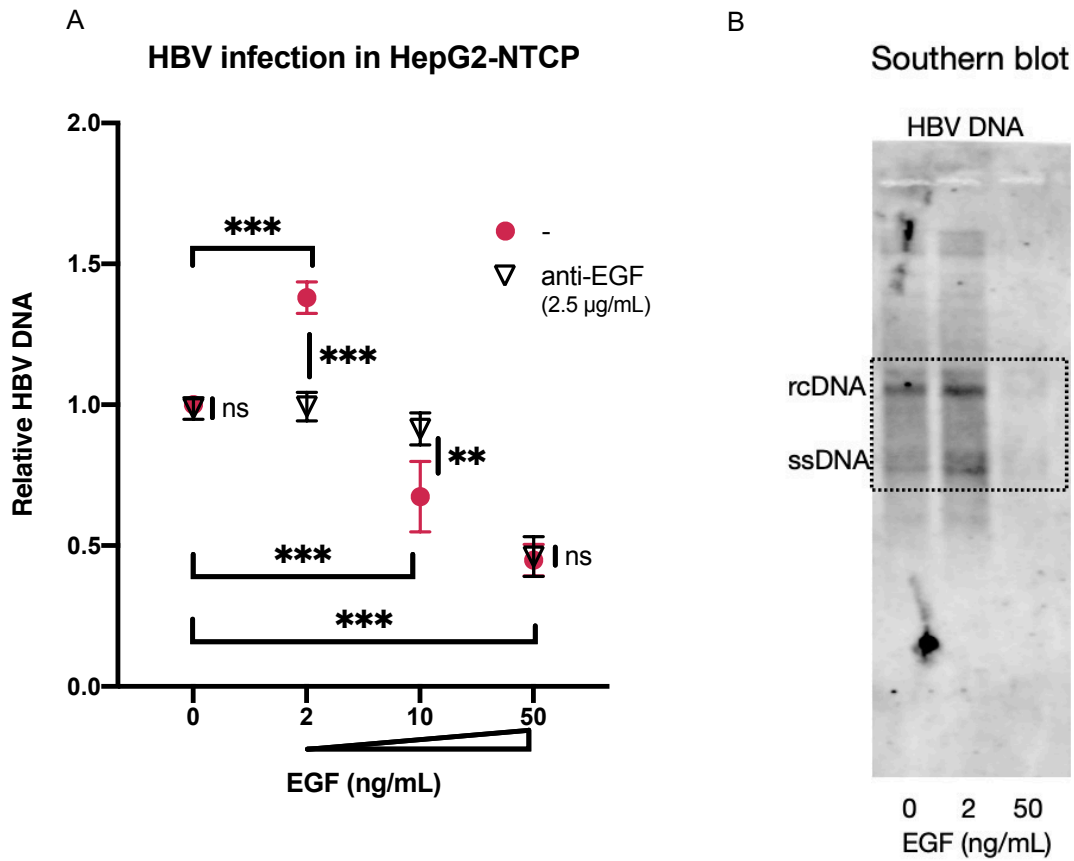
Fig. 10. Modulation of HBV infection by EGF in HepG2-NTCP cells.

(A) Relative HBV DNA levels in HepG2-NTCP cells at 2 ng/mL, 10 ng/ml and 50 ng/ml of EGF with or without 2.5 μg/mL anti-EGF antibody. The result is shown as the mean ± SEM of 3 independent experiments. **p < 0.01, ***p < 0.001.

(B) Southern blot analysis of HBV DNA fractions obtained from HepG2-NTCP cells infected with HBV at post-infection day7.

HBV internalization in HepG2-NTCP
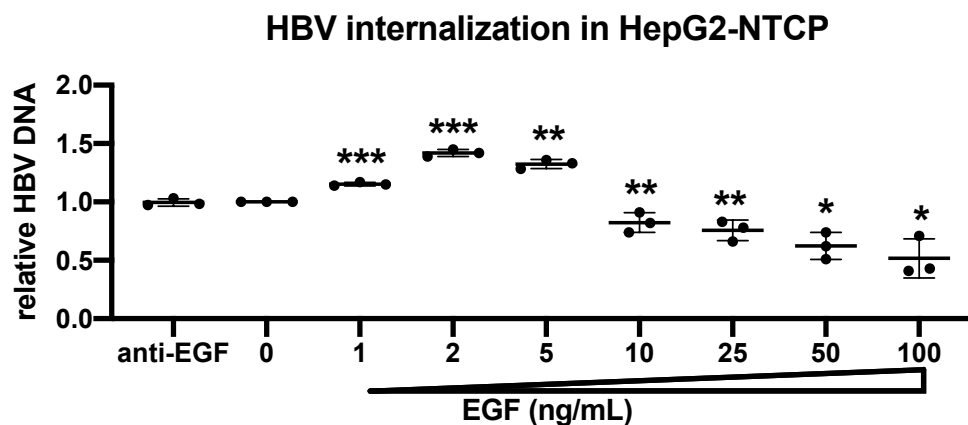
Fig. 11. Modulation of HBV infection by EGF

Relative HBV DNA levels in HepG2-NTCP cells at 0 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/ml, 25 ng/mL, 50 ng/mL and 100 ng/ml of EGF in HBV internalization assay. The result is shown as the mean ± SEM of 3 independent experiments. The result is shown as the mean ± SEM of 3 independent experiments. *p<0.05, **p < 0.01, ***p < 0.001.
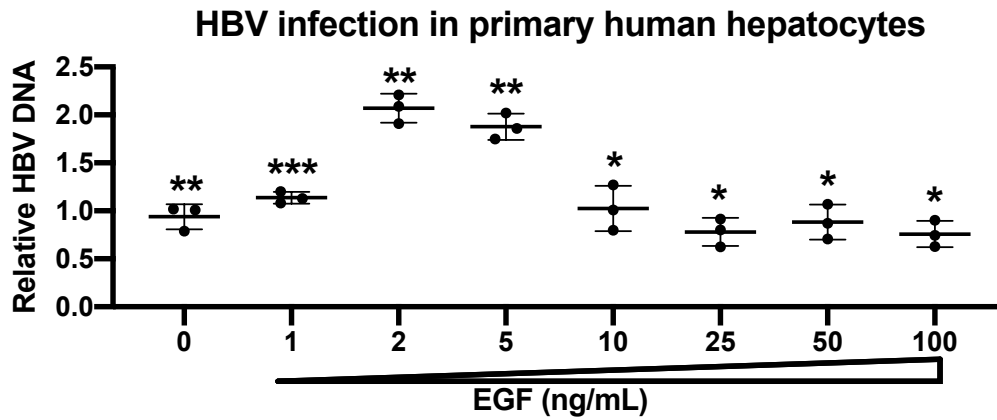
Fig. 12. Modulation of HBV infection by EGF in primary human hepatocytes.

Relative HBV DNA levels in primary human hepatocytes at 0 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/ml, 25 ng/mL, 50 ng/mL and 100 ng/ml of EGF. The result is shown as the mean ± SEM of 3 samples in one experiment. *p<0.05, **p < 0.01, ***p < 0.001..

HBV infection in iPS-hepatocytes
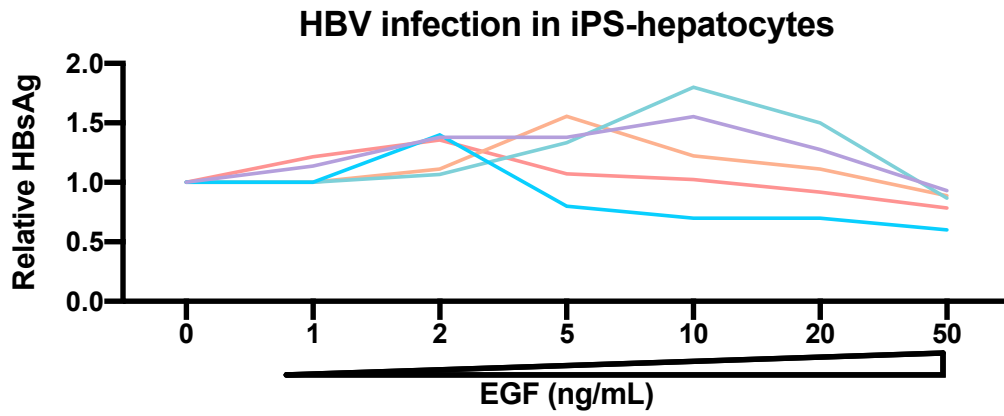
Fig. 13. Modulation of HBV infection by EGF in iPS-hepatocytes.

Relative HBsAg level in iPS-hepatocytes at 0 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/ml, 20 ng/mL, and 50 ng/ml of EGF in HBV infection assay. The results of 5 independent experiments are shown.
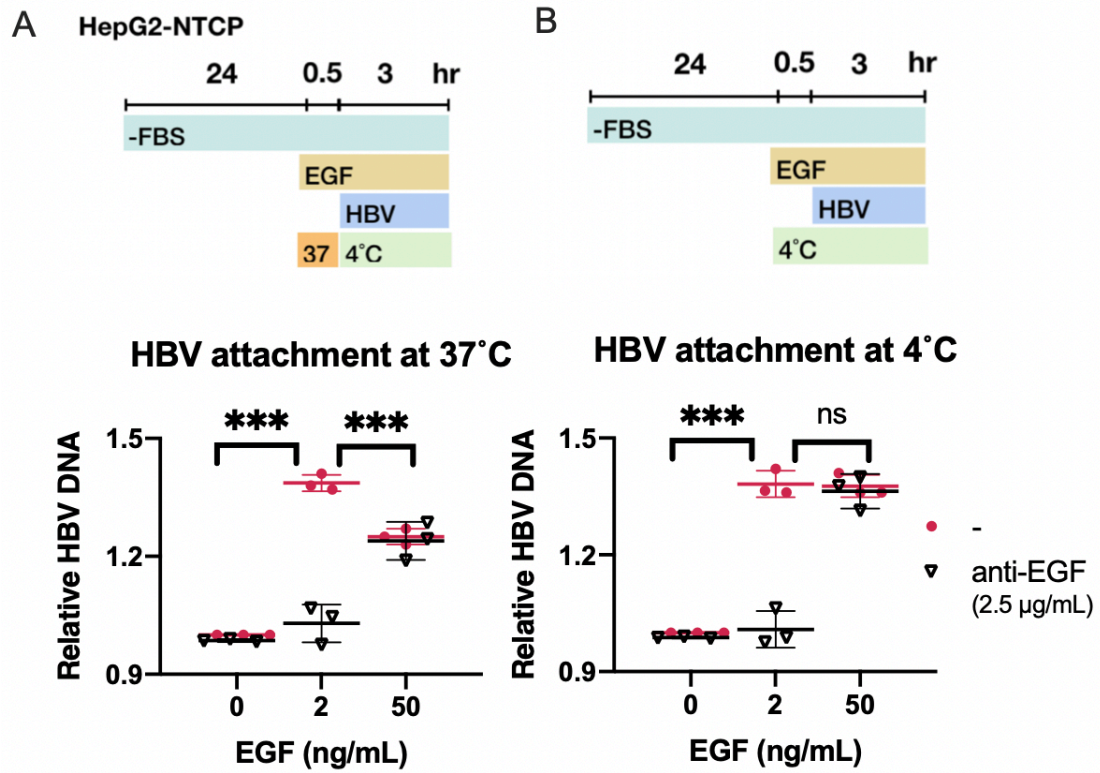
Fig. 14. Attachment of HBV by stimulation of EGF

(A, B) Relative HBV attachment in HepG2-NTCP cells treated with 2 ng/ml and 50 ng/ml of EGF

at 37˚C (A) and 4˚C (B). The results are shown as the mean ± SEM of 3 independent experiments.

*p < 0.05. **p < 0.01.
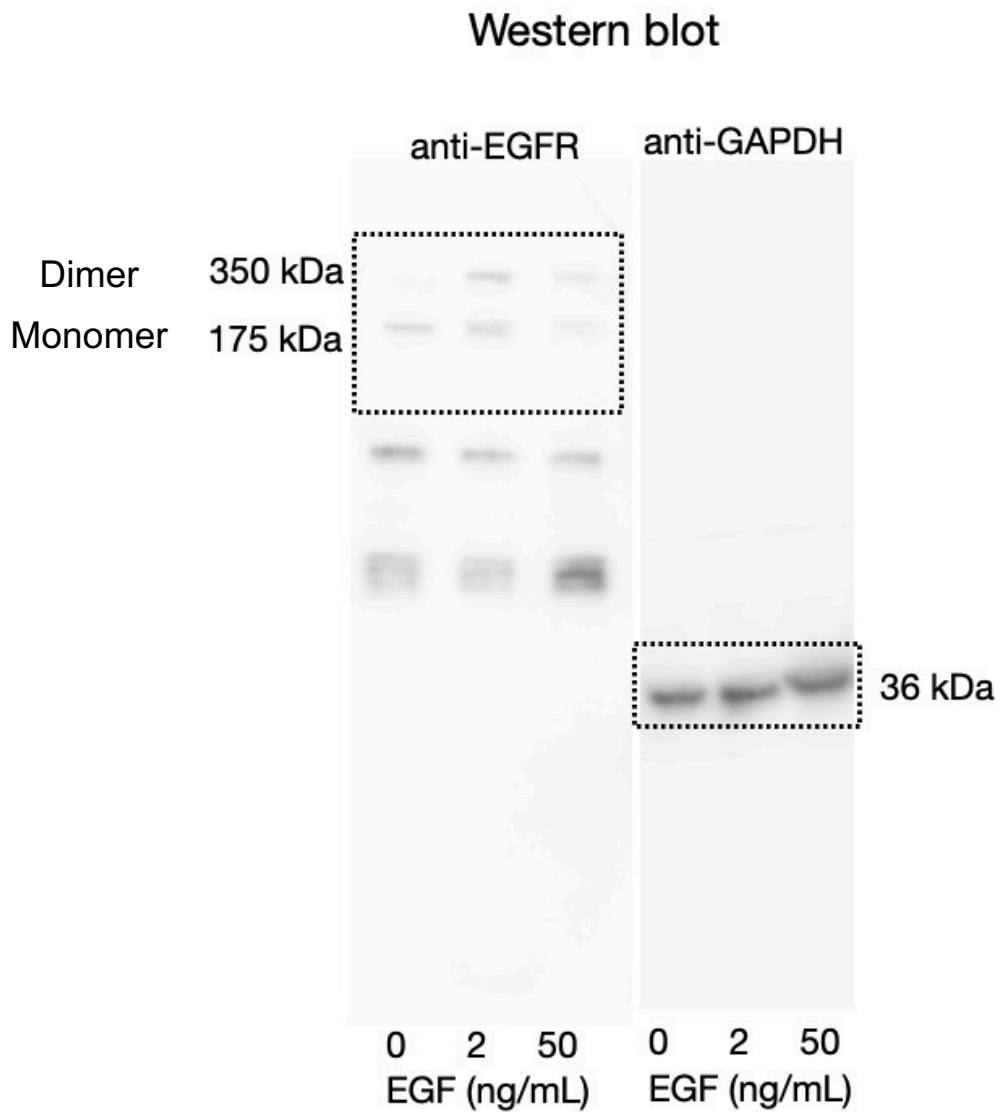
Western blot

Fig. 15. EGFR expression on cell surface is modulated by EGF

Western blot analysis of cell surface protein treated with cross-linker BS^3 at 0 ng/mL, 2 ng/mL and

50 ng/mL. monomer: 175 kDa, dimer: 350 kDa.
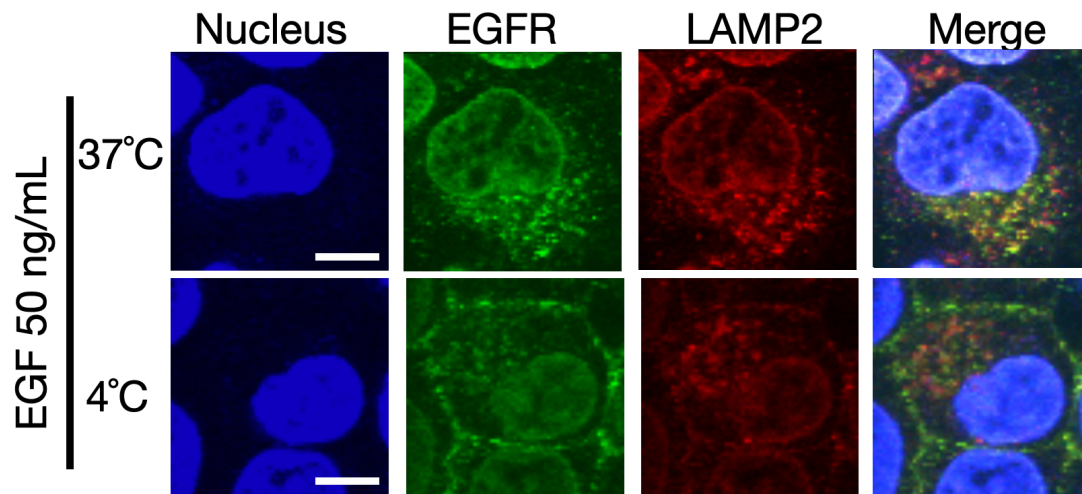
Fig. 16. Localization of EGFR and LAMP2

Immunofluorescence staining for EGFR (green) and LAMP2 (red) in HepG2-NTCP cells at 50 ng/ml of EGF at 37°C and 4°C. Scale bar, 10 μm. EGFR and LAMP2 are colocalized at 4°C but not at 37°C.
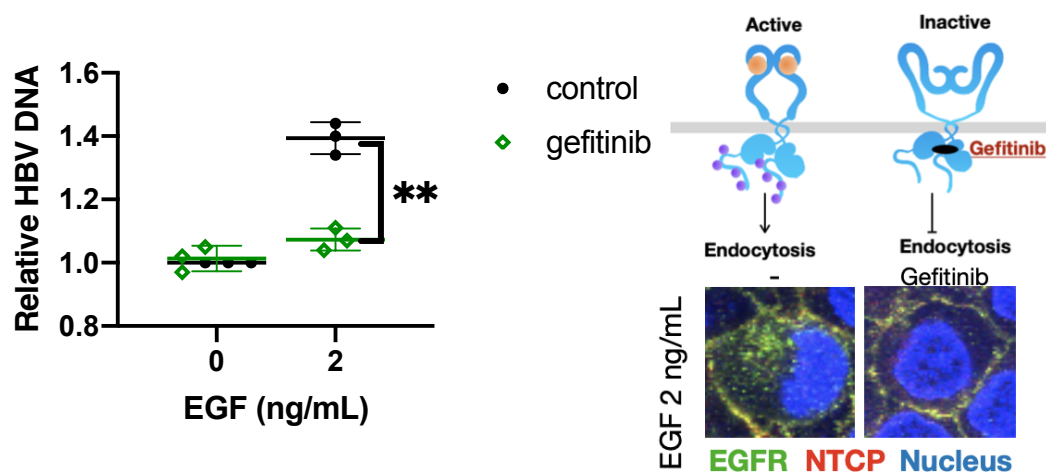
Fig. 17. The extracellular HBV attachment is increased by the formation of active dimer EGFR

Relative HBV attachment in HepG2-NTCP cells treated with or without 10 μM gefitinib and 2 ng/mL EGF. The results are shown as the mean ± SEM of 3 independent experiments. **$p < 0.01$.

Immunofluorescence staining for EGFR (green) and NTCP (red) in Hepg2-NTCP cells treated with/without gefitinib at 2 ng/ml of EGF at 37°C and 4°C.
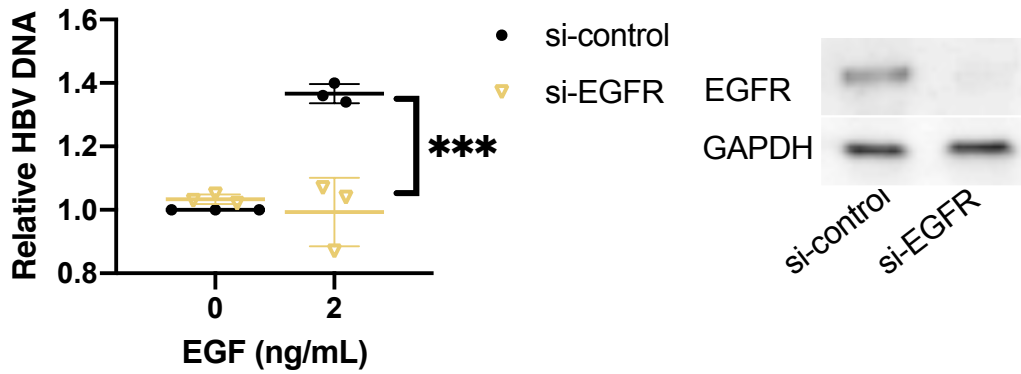
Fig. 18. Knockdown of EGFR expression cancelled the enhanced HBV attachment by EGF in HepG2-NTCP

Relative HBV attachment in EGFR knock down HepG2-NTCP cells treated with or without 2 ng/mL EGF. The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05.

N.S., not significant.
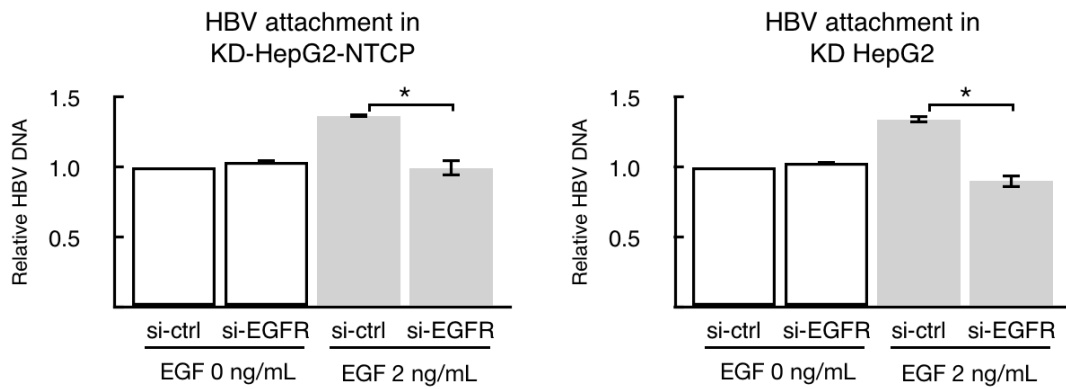
Fig. 19. Knockdown of EGFR expression cancelled the enhanced HBV attachment by EGF in HepG2-NTCP and HepG2 cells

Relative HBV attachment in EGFR knock down HepG2-NTCP and HepG2 cells treated with or without 2 ng/mL EGF. The results are shown as the mean ± SEM of 3 independent experiments.

*p < 0.05. N.S., not significant.
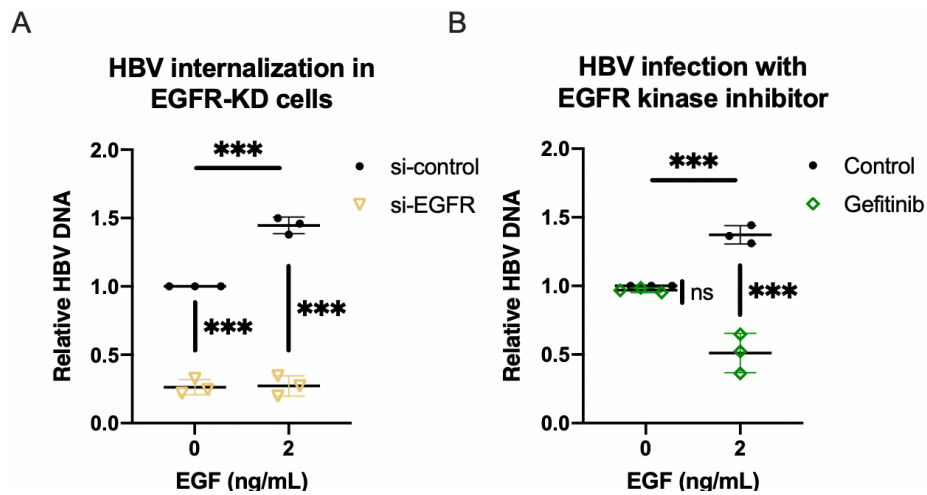
Fig. 20. HBV is internalized *via* the TK-independent CME pathways without EGF stimulation

(A) Relative HBV internalization in EGFR knockdown HepG2-NTCP cells pretreated with 2 ng/ml of EGF. The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05. **p < 0.01. ***p < 0.001. N.S., not significant.
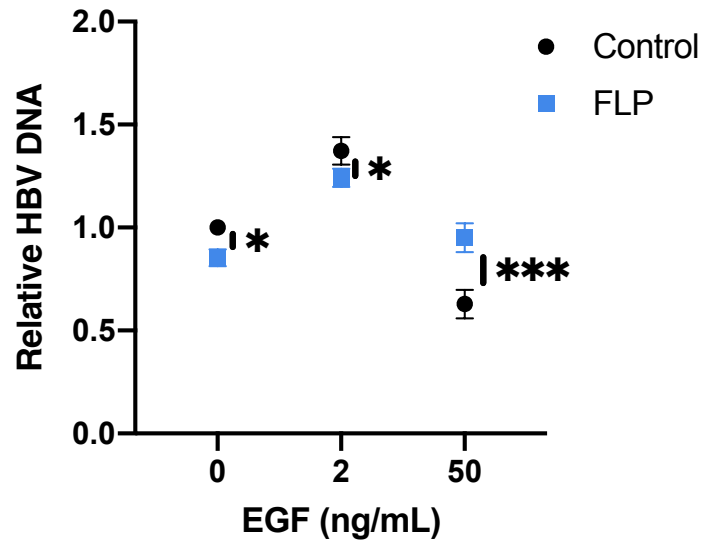
(B) Relative HBV infection levels in HepG2-NTCP cells (-), HepG2-NTCP cells treated with 10 μM gefitinib. The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05. **p < 0.01. ***p < 0.001. N.S., not significant.
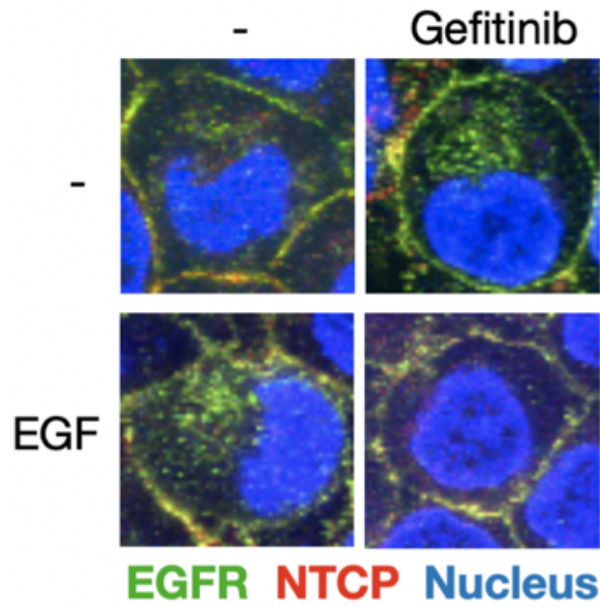
Fig. 21. Gefitinib inhibits EGFR endocytosis in the presence of EGF

Immunofluorescence staining for EGFR (green) and NTCP (red) in HepG2-NTCP cells treated with

or without 2 ng/ml of EGF and 10 µM gefitinib.
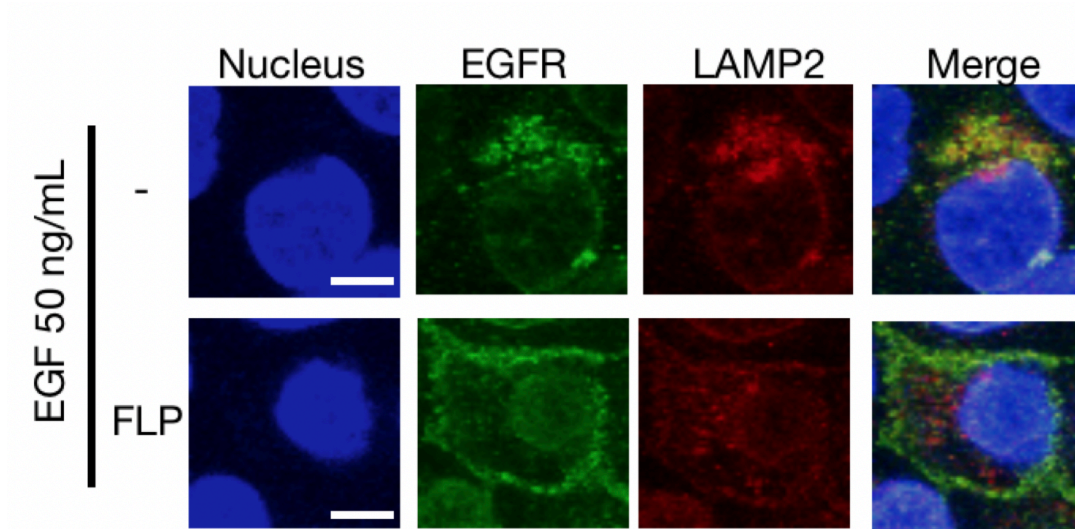
HBV infection with CME inhibitor
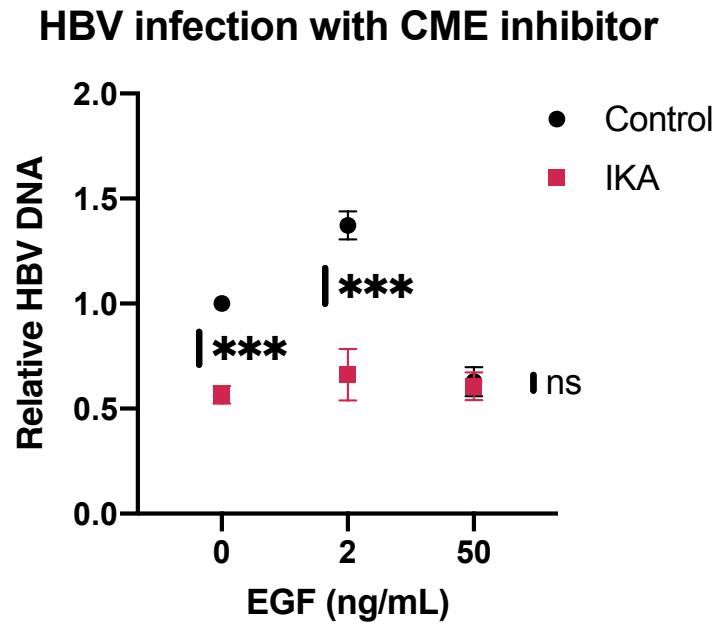
Fig. 22. HBV is internalized *via* the CME pathways at low dose of EGF

(A) Relative HBV infection levels in HepG2-NTCP cells (-), HepG2-NTCP cells treated with ikarugamycin (IKA). The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05. **p < 0.01. ***p < 0.001. N.S., not significant.
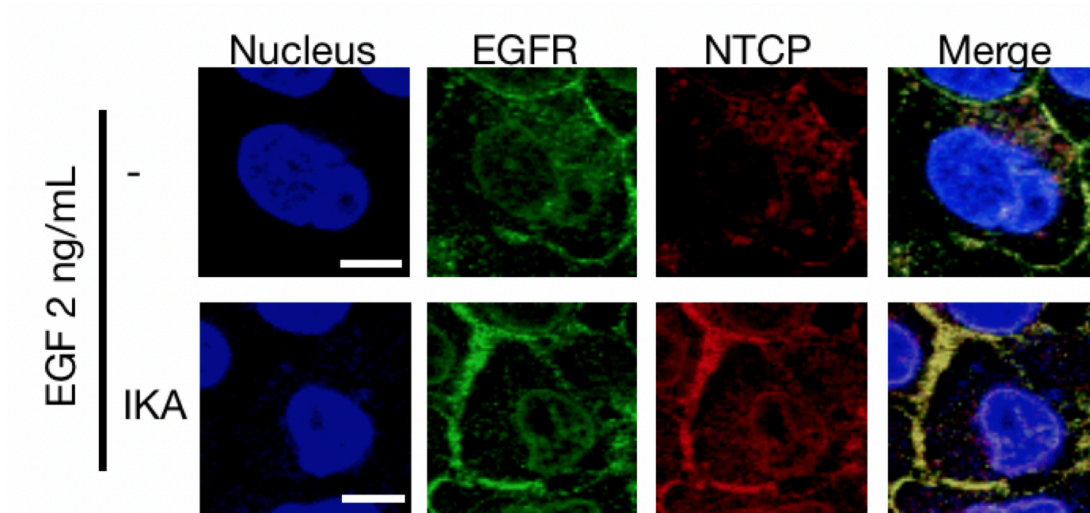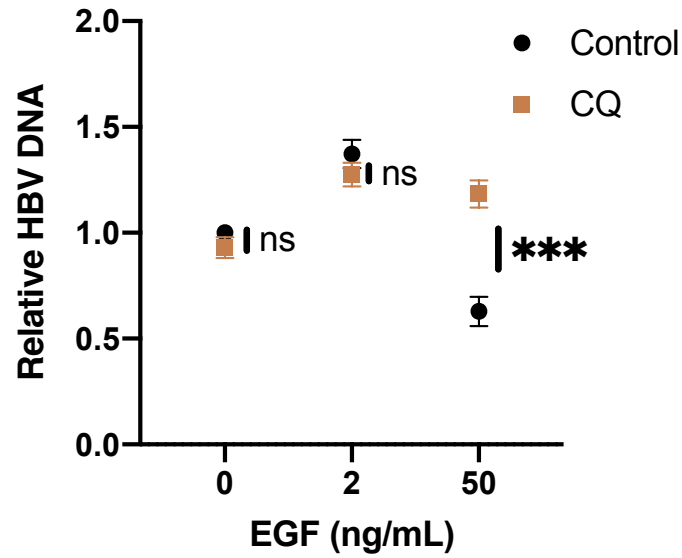
Fig. 23. IKA inhibits EGFR internalization at a low dose of EGF

Immunofluorescence staining for EGFR (green) and NTCP (red) in HepG2-NTCP cells at 2 ng/ml

of EGF treated with or without 2 μM IKA. Scale bar, 10 μm.

Fig. 24. HBV is internalized *via* the CIE pathway at a high dose of EGF

Relative HBV infection levels in HepG2-NTCP cells (-), HepG2-NTCP cells treated with filipin

(FLP). The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05. **p <

0.01. ***p < 0.001. N.S., not significant.

Fig. 25. FLP inhibits EGFR internalization at a high dose of EGF

Immunofluorescence staining for EGFR (green) and LAMP2 (red) in HepG2-NTCP cells at 2 ng/ml

of EGF treated with or without 1 µM FLP. Scale bar, 10 µm.
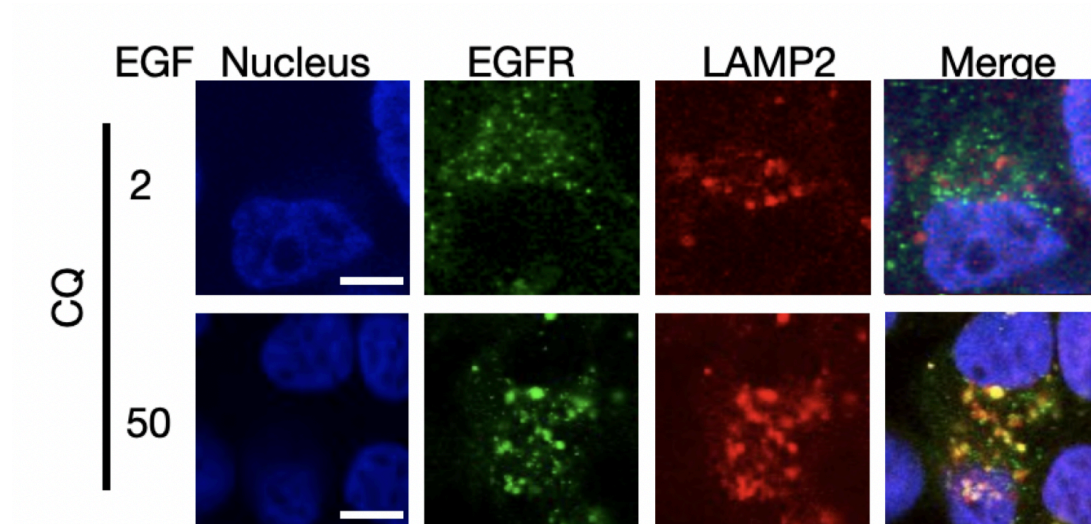
HBV infection with lysosome inhibitor

Fig. 26. HBV is degraded in the lysosome at a high dose of EGF

Relative HBV levels in HepG2-NTCP cells (-), HepG2-NTCP cells treated with chloroquine (CQ).

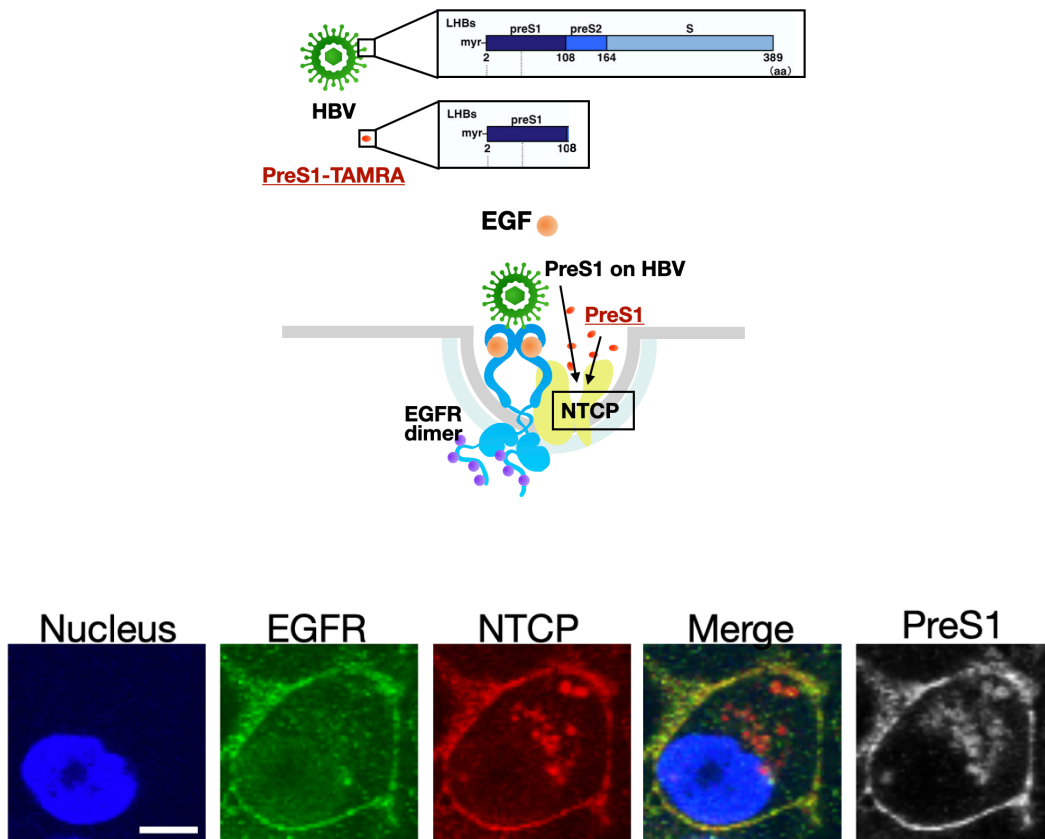The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05. **p < 0.01.

***p < 0.001. N.S., not significant.

**Fig. 27. CQ inhibits EGFR degradation at a high dose of EGF**

Immunofluorescence staining for EGFR (green) and LAMP2 (red) in HepG2-NTCP cells at 2 ng/ml

and 50 ng/mL of EGF treated with 25 µM CQ. Scale bar, 10 µm.

Fig. 28. NTCP and EGFR are not colocalized after internalization

Immunofluorescence staining for EGFR (green) and NTCP (red) in HepG2-NTCP cells treated with

PreS1-TAMRA (white) and HBV at 2 ng/ml of EGF treated. Scale bar, 10 µm.
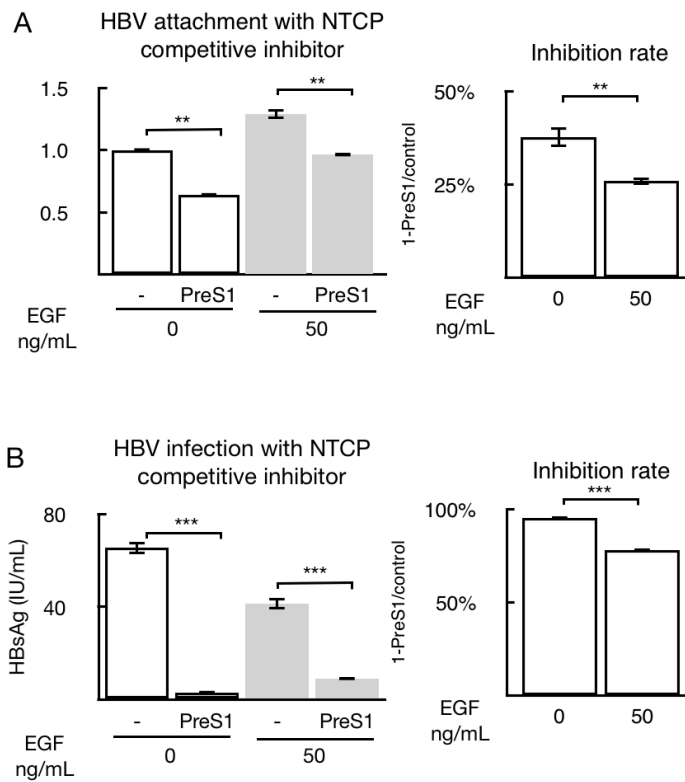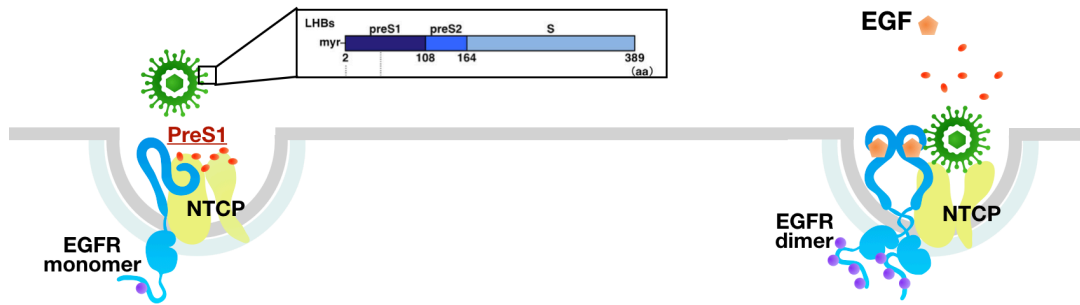
Fig. 29. Competition between HBV and PreS1 in cells with or without EGF stimulation

Relative HBV attachment (A), relative HBV infection (B) in HepG2-NTCP cells treated with or without 1μM PreS1 and 50 ng/mL EGF. Inhibition rate = (1- PreS1/control) x100%. The results are shown as the mean ± SEM of 3 independent experiments. **p < 0.01. ***p < 0.001.
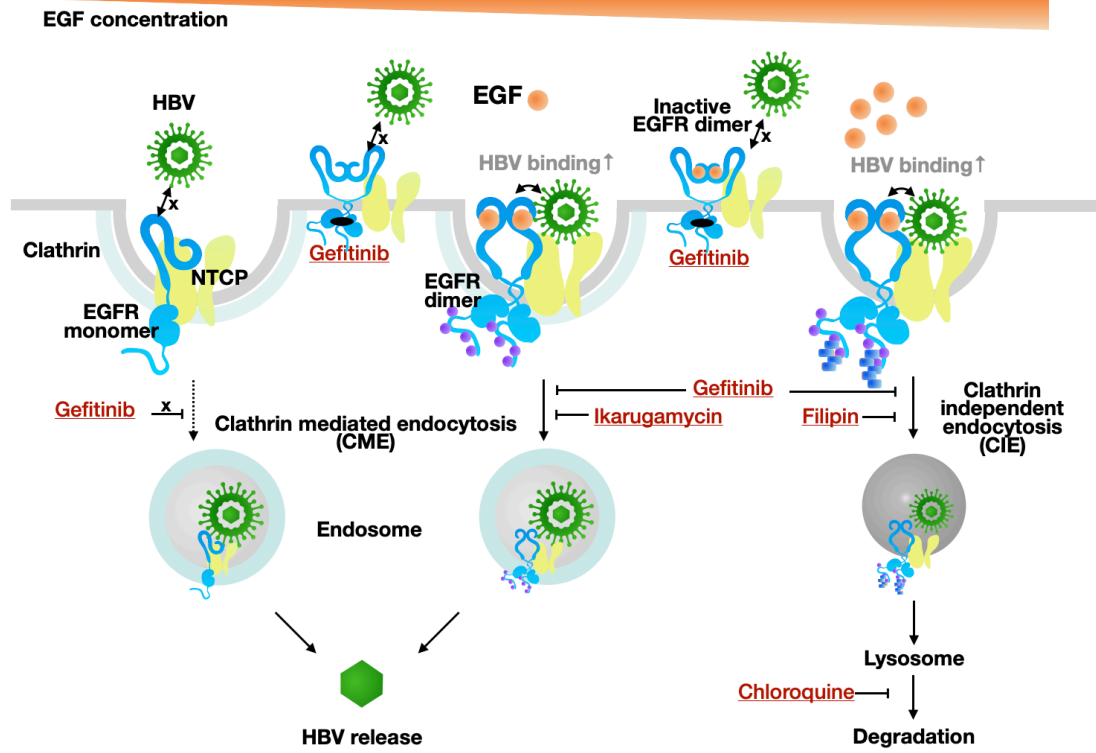
Fig. 30. Summary

Table 1. List of quantitative PCR primers for mouse genes

gene	Left Primer	Right Primer
Stab2	TGTCCAGACGGCTACATCAA	CCAGGGATATCCAGGACGTA
Lyve1	CCTCCAGCCAAAAGTTCAAA	TCCAACACGGGGTAAAATGT
Pecam1	CTGGTGCTCTATGCAAGCCT	AGTTGCTGCCCATTCATCAC
F8	TCATGTATAGCCTGGATGGGA	GATGAGTCCACATTGCCAAA
Ngfr	GTGTGCGAGGACACTGAGC	GGGGGTAGACCTTGTGATCC
Desmin	GTGAAGATGGCCTTGGATGT	CTCGGAAGTTGAGAGCAGAGA
Hgf	CCTGACACCACTTGGGAGTA	CTTCTCCTTGGCCTTGAATG
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC

Table 2. List of quantitative PCR primers and probes for HBV detection

	Primer/Probe
HBSF2	CTTCATCCTGCTGCTATGCCT
HBSR2	AAAGCCCAGGATGATGGGAT
cccDNA F7	TCCCCGTCTGTGCCTTCTC
cccDNA R7	GCACAGCTTGGAGGCTTGA
cccDNA P7	FAM- CCGTGTGCACTTCG

Table 3. List of primary and secondary antibodies used for immunocytochemistry analysis and FCM analysis of mouse and human cells

Primary Antibody	Supplier
LAMP2	abcam (ab25631)
EGFR	cell signaling (D38B1)
Myc	abcam (ab206486)
Stabilin2	Nonaka et al.
LNGFR	miltenyibiotec (REA648)

Secondary Antibody	Supplier
Alexa Fluor 488 anti-Rabbit IgG	Invitrogen (A32790)
Alexa Fluor 555 anti-Mouse IgG	Invitrogen (A21424)
Alexa Fluor 647 anti-Mouse IgG	Invitrogen (A28181)