改変 HBV ゲノムを作成した（pUC19/HBV-SEAP）。また、昨年作成した CMV-GFP-pA のカセットを挿入した改変 HBV ゲノム保有プラスミドも準備した（pUC19/HBV-GFP）。これらの改変で失われたウイルス遺伝子を補充するために，パッケージングシグナルであるイプシロンの機能を欠損させた HBV ゲノムを準備した（pUC19/HBV-⊿ε）。このイプシロン欠損ゲノムは粒子内に取り込まれないことを昨年までに確認している。加えて、HBV の POL 遺伝子を強制発現するプラスミドも準備した。

細胞としては、ヒト由来の初代培養肝細胞（PXB cell）、培養細胞株の HuH7、HepG2 を利用した。培養細胞株へは一過性のトランスフェクションを行い、SLC10A1 分子を発現させた。また、レンチウイルス感染系を用いて、SLC10A1 の定常発現細胞を作成した。

（倫理面への配慮）

本研究で使用する細胞は，いずれもインフォームドコンセントのもとに倫理審査を得て採取され，市販されているヒト肝細胞であるため、倫理的な問題点はない。

培養細胞に関しては，所内のバイオセーフティ委員会の承認を得て、実験を実施した。

C．研究結果

新規に作成した改変 HBV である pUC19/HBV-SEAP と pUC19/HBV-GFP を pUC19/HBV-⊿ε と組み合わせることでウイルス粒子を作成した。培養細胞株には、SLC10A1 をトランスフェクションし、遺伝子発現を確認した。その細胞に作成したそれぞれの組み換えウイルスを感染させ、隔日で培養上清を回収し、HBs 抗原を測定した。その結果、pUC19/HBV-SEAP 由来のウイルスでは、HBs 抗原のレベルは低値であり、感染効率が低かったが、SEAP の値は確認でき、感染を簡易にモニターすることは可能であった。pUC19/HBV-GFP においては、GFP で確認したところ、数個の細胞で感染が確認できたが、感染のレベルは低値であった。細胞を初代培養肝細胞に変更することで感染の効率は 5 倍程度上昇した。

蛍光遺伝子保有 HBV（複製能なし）の発現系を利用して効率的にウイルス産生が行われる条件を検討した。粒子形成に不足している HBV 遺伝子をトランスに供給する必要があるが、強制的に Pol 遺伝子を発現するコンストラクトを併せて供給した場合は複製効率が改善した。また、複製能が有るコンストラクトを Pol のトランス供給用として使用することでも改善が認められた。このことから、複製の効率化には Pol 濃度が重要な因子の一つであることが示唆された。

SLC10A1 の恒常発現細胞の樹立を目指して、レンチウイルスベクターで遺伝子導入した細胞をクローニングし、複数の株を取得した。それらの SLC10A1 発現量をウエスタンブロットで確認した。感染効率の異なる細胞株を選択するために HBV 感染をさせて、感染効率の

確認を進めた。

D. 考察

既報のように、SLC10A1 を細胞に導入することで、感染性が得られることを確認した。今回作製した改変 HBV においても同様の経路で感染することが確認でき、改変 HBV の有用性が伺えた。感染効率を改善する必要があり、ゲノムサイズの最適化、トランスに供給するウイルスタンパク質の量について、来年度以降に検討する必要がある。感染感受性細胞の取得も進めることができ、クローンの中から感染感受性の異なるものを複数樹立することで必要な受容体分子の探索が可能となると考えられる。

E. 結論

改変 HBV を感染させることに成功した。様々な種類の感染感受性もしくは、非感受性細胞を取得することで、差分解析をすることが可能となる。

F. 健康危険情報

特に無し.

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（B 型肝炎創薬実用化等研究事業）
分担研究報告書

HBV 感染を阻害する低分子化合物のスクリーニング

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研究要旨：微生物や植物の二次代謝産物、およびその誘導体を中心に収集した化合物ライブラリーを用いて新規抗 HBV 薬を探索する。この探索においては、HBV の感染初期過程を高感度に検出できる感染モデル系を利用し、従来の治療薬（インターフェロンや核酸アナログ）とは作用点が異なる新たなタイプの HBV 治療薬の開発を目的とする。研究前期（H24-25）は微生物生合成遺伝子改変技術やフラクションライブラリー、表現型スクリーニング基盤を利用し、新規天然化合物の創製を行う。研究中期（H26-）から HBV 様擬粒子を用いたスクリーニングを実施し、阻害活性を示す化合物をバイオプローブとしたケミカルバイオロジー研究を展開する。これら一連の研究を通じて、HBV 治療に資する候補化合物の創製と新規創薬標的（HBV 受容体など）の提示を目指す。

A. 研究目的

HBV 感染阻害物質の探索を実施するためには、多様性に富んだケミカルライブラリーが必要である。またそのようなライブラリー構築には、①質の高い化合物群と②構造や活性情報が充実したデータベースやデータマイニング技術、などの創薬基盤の整備が不可欠である。本年度は、微生物生合成遺伝子改変技術やフラクションライブラリーを用い、天然化合物の収集を加速する。また細胞表現型を利用して薬剤の作用機序解析を実施し、HBV 感染阻害薬探索に資する化合物ライブラリーの整備・拡充を行う。

B. 研究方法

化合物ライブラリーの整備を目的とし、①天然化合物の網羅的な収集と②細胞形態変化を指標とした作用機序解明を計画した。①では、生合成遺伝子改変微生物やフラクションライブラリー、天然化合物データベース NPPlot を用いて新規天然化合物を探索した。②では細胞形態変化データベース「モルフォベース」を利用し、①で取得した天然化合物の作用機序を検証した。また計画に先行して HBV 様擬粒子を用いたパイロットスクリーニングを実施した（実施場所：国立国際医療研究センター）。NanoLuc 遺伝子を導入した組換え HBV 粒子を PXB 細胞に感染させ、理研天然化合物バンク NPDepo から提供された標準化合物ライブラリー（作用既知薬剤 80 種）のウイルス感染阻害活性を評価した。

（倫理面への配慮）

遺伝子組み換え生物等の使用に際しては、理研の定める細則や指針を遵守した。

C. 研究結果

①天然化合物の網羅的な収集

微生物代謝産物を系統的に収集したフラクションライブラリーと天然化合物データベース NPPlot を用いた新規天然化合物探索を実施し、ピロリジジノン系新規代謝産物ピロリジラクトン（Nogawa et al）や新規キノマイシン類縁体（Lim et al）など、様々な新規物質を取得した。

② ピロリジラクトンの作用機序解析

細胞形態変化データベース「モルフォベース」を用い、ピロリジラクトンの作用メ

カニズムを解析した。ピロリジラクトンはトリプシン活性を選択的に阻害する新たなタイプのプロテアソーム阻害剤であった（Futamura et al）。

③ HBV 擬粒子を用いたスクリーニング

NPDepoから提供された標準化合物についてウイルス感染阻害活性を評価した。その結果、タンパク質合成やDNA合成阻害剤、微小管重合阻害剤、抗ウイルス物質を含む11化合物で顕著な活性が見られた。HBV の生活環を考えると、ヒットが予想される化合物群であり、本評価系がワークしていることが示唆された。

D. 考察

研究前期にHBV感染阻害薬の探索源に資する化合物を3万種類以上整えることをマイルストーンとしていた。今年度までに様々な新規微生物代謝産物を見い出すと共に植物エキスのフラクションライブラリー作製も順調に進み、計画通りにサンプルの準備を整えた。さらにHBV擬粒子を用いた評価系の検証を実施し、大規模スクリーニングを開始するめどをつけた。

E. 結論

生合成遺伝子改変微生物やフラクションライブラリーからの新規代謝産物取得、微生物培養物や植物エキスのフラクションライブラリー作製により、化合物ライブラリーを整備・拡充し、抗HBV薬のスクリーニング体制を整えた。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得：なし
2. 実用新案登録：なし
3. その他：なし

Ⅲ　研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表レイアウト

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Ⅳ　研究成果の刊行物・別刷り

Biochemical and Biophysical Research Communications 430 (2013) 592–597

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PML tumor suppressor protein is required for HCV production

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ARTICLE INFO

Article history:
Received 8 November 2012
Available online 5 December 2012

Keywords:
Hepatitis C virus
PML
INI1
DDX5
Tumor suppressor
Lipid droplet

ABSTRACT

PML tumor suppressor protein, which forms discrete nuclear structures termed PML-nuclear bodies, has been associated with several cellular functions, including cell proliferation, apoptosis and antiviral defense. Recently, it was reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML. However, role(s) of PML in HCV life cycle is unknown. To test whether or not PML affects HCV life cycle, we examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, stably expressing short hairpin RNA targeted to PML. In this context, the level of secreted HCV core and the infectivity in the supernatants from PML knockdown cells was remarkably reduced, whereas the level of HCV RNA in the PML knockdown cells was not significantly affected in spite of very effective knockdown of PML. In fact, we showed that PML is unrelated to HCV RNA replication using the subgenomic HCV-JFH1 replicon RNA, JRN/3-5B. Furthermore, the infectivity of HCV-like particle in the culture supernatants was significantly reduced in PML knockdown JRN/3-5B cells expressing core to NS2 coding region of HCV-JFH1 genome using the *trans*-packaging system. Finally, we also demonstrated that INI1 and DDX5, the PML-related proteins, are involved in HCV production. Taken together, these findings suggest that PML is required for HCV production.

1. Introduction

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1,2]. HCV core protein forms a viral capsid and is essential for infectious virion production. The core protein is targeted to lipid droplets. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [3].

In addition, HCV core has been reported to facilitate cellular transformation as well as development of hepatocellular carcinoma in HCV core-transgenic mice [4]. Interactions of core with tumor suppressor proteins such as p53 and DDX3 may lead to enhanced cellular proliferation [4]. Indeed, HCV core interacts with promyelocytic leukemia (PML) protein and inhibits the PML tumor suppressor pathway through interfering with the PML-mediated apoptosis-inducing function [5]. PML forms discrete nuclear structures termed PML-nuclear bodies (PML-NBs) and associates with several cellular functions, including cell proliferation, apoptosis and antiviral defense [6,7]. In acute promyelocytic leukemia (APL) patient, the PML gene is fused with the retinoic acid receptor-α (RARα) gene, thus resulting in expression of an oncogenic PML-RARα fusion protein [6,7]. Conversely, treatment of APL patient with arsenic trioxide leads to reformation of PML-NBs and results in disease remission [6,7], indicating that PML is a target of arsenic trioxide. Interestingly, we have recently demonstrated that arsenic trioxide strongly inhibited HCV infection and HCV RNA replication without cell toxicity [8]. However, the role of PML in HCV life cycle yet remains unclear. To investigate the possible involvement of PML in HCV life cycle, we examined the accumulation of HCV RNA as well as the release of HCV core into culture

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http://dx.doi.org/10.1016/j.bbrc.2012.11.108

supernatants from cells rendered defective for PML by RNA interference. The results provide evidence that PML is required for HCV production.

2. Materials and methods

2.1. Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The three HuH-7-derived cell lines: RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [9] could infect and effectively replicate [10–13], OR6c cells is cured cells of OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter [14] or OR6c JRN/3-5B cells harboring the subgenome HCV-JFH1 RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [13].

2.2. RNA interference

Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to DDX5 in a lentiviral vector: 5′-GATCCCCCTCTAATGTGGAGTGCGACTTCAAGAGAGTCGCACTCCACA TTAGAGTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAACTCTAATGT GGAGTGCGACTCTCTTGAAGTCGCACTCCACATTAGAGGGG-3′ (antisense). The oligonucleotides above were annealed and subcloned into the *Bgl*II-*Hin*dIII site, downstream from an RNA polymerase III promoter of pSUPER [15], to generate pSUPER-DDX5i. To construct pLV-DDX5i, the *Bam*HI-*Sal*I fragments of the pSUPER-DDX5i were subcloned into the *Bam*HI-*Sal*I site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells [16]. We previously described pLV-PMLi [8] and pLV-INI1i [17], respectively.

2.3. Lentiviral vector production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [18,19]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [18,19] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pLV-PMLi into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

2.4. HCV infection experiments

The supernatants was collected from cell culture-generated HCV-JFH1-infected RSc cells at 5 days post-infection and stored at −80 °C after filtering through a 0.45 μm filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus or J6/JFH1 [20], RSc cells (5×10^4 cells/well) were plated onto 6-well plates and cultured for 24 h (hrs). We then infected the cells at a multiplicity of infection (MOI) of 0.05. The culture supernatants were collected at the indicated time post-infection and the levels of the core protein were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of intracellular HCV RNA. The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection.

2.5. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [14]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: PML, 5′-GAGGAGTTCCAGTTTCT GCG-3′ (forward), 5′-GCGCCTGGCAGATGGGGCAC-3′ (reverse); DDX5, 5′-ATGTCGGGTTATTCGAGTGA-3′ (forward), 5′-TTTCTCC CCAGGGTTTCCAA-3′ (reverse); INI1, 5′-ATGATGATGATGGCGCTG AG-3′ (forward), 5′-TCGGAACATACGGAGGTAGT-3′ (reverse); β-actin, 5′-TGACGGGGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAG CCGCGCTCGGT-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGT CTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse).

2.6. Western blot analysis

Cells were lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-HCV core (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan) or anti-β-actin antibody (Sigma).

2.7. WST-1 assay

RSc or OR6c JRN/3-5B cells (1×10^3 cells/well) were plated onto 96-well plates and cultured. The cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The absorbance was read using a microplate reader at 440 nm with a reference wavelength of 690 nm.

2.8. Renilla luciferase (RL) assay

OR6c JRN/3-5B cells (1.5×10^4 cells/well) were plated onto 24-well plates and cultured for 72 h, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI, USA). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

2.9. RNA synthesis and transfection

Plasmid pJRN/3-5B was linearized by digestion with *Xba*I and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [13]. *In vitro* transcribed RNA was transfected into OR6c cells by electroporation as described previously [14].

2.10. Immunofluorescence and confocal microscopic analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Nonidet P-40 in PBS at room temperature, and incubated with anti-PML antibody (PM001, MBL) and anti-HCV core at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37 °C for 30 min. They were then stained with anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 647-conjugated anti-rabbit antibody (Molecular Probes, Invitrogen) at a 1:300 dilution in PBS containing BSA at 37 °C for 30 min. Lipid droplets and nuclei were stained with BODIPY 493/503 (Molecular Probes, Invitrogen) and DAPI (4′, 6′-diamidino-2-phenylindole), respectively. Following extensive washing in PBS, the cells were mounted on slides using a mounting media of SlowFade Gold antifade reagent (Invitrogen) added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

3. Results

3.1. PML is involved in the propagation of HCV

To investigate the potential role(s) of PML in HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown PML in HuH-7-derived RSc cured cells that HCV-JFH1 [9] could infect and effectively replicate [10–13]. Real-time RT-PCR analysis for PML demonstrated a very effective knockdown of PML in RSc cells transduced with lentiviral vector expressing shRNA targeted to PML (Fig. 1A). To test the cell toxicity of shRNA, we examined WST-1 assay. In spite of very effective knockdown of PML, we demonstrated that the shRNA targeted to PML did not affect the cell viabilities (Fig. 1B). We next examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in PML knockdown RSc cells 24, 48, or 72 h after HCV-JFH1 infection at an MOI of 0.05. The results showed that the level of HCV RNA in PML knockdown cells was not affected until 72 h post-infection (Fig. 1C), while the release of HCV core protein into the culture supernatants was significantly suppressed in PML knockdown cells at 48 or 72 h post-infection (Fig. 1D). Consistent with this finding, the infectivity of HCV in the culture supernatants was also significantly suppressed in the PML knockdown cells at 48 or 72 h post-infection (Fig. 1E). We also obtained similar results using siRNA specific for human PML (siGENOME SMRT pool M-006547-01-0005, Dharmacon, Thermo Fisher Scientific, Waltham, MA) (data not shown). These results suggested that PML is associated with propagation of HCV.

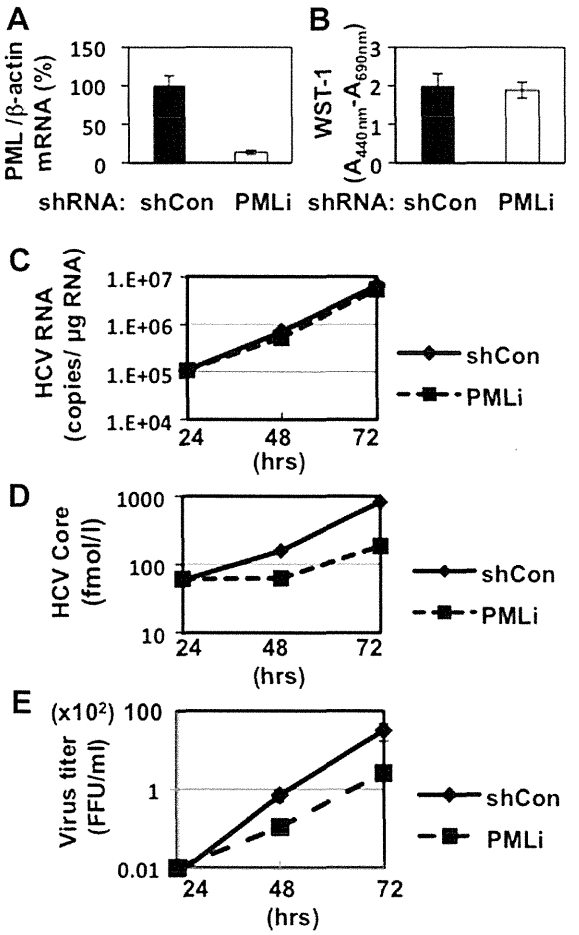

Fig. 1. PML is required for infectious HCV production. (A) Inhibition of PML mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for PML was performed as well as for β-actin mRNA. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon) which was assigned as 100%. (B) WST-1 assay of the PML knockdown (PMLi) or the control (shCon) RSc cells. (C) The levels of intracellular genome-length HCV-JFH1 RNA in the PML knockdown or the control cells at 24, 48 or 72 h post-infection at an MOI of 0.05 were monitored by real-time LightCycler RT-PCR. (D) The levels of HCV core in the culture supernatants from the PML knockdown or the control RSc cells 24, 48 or 72 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. All experiments were done in triplicate.

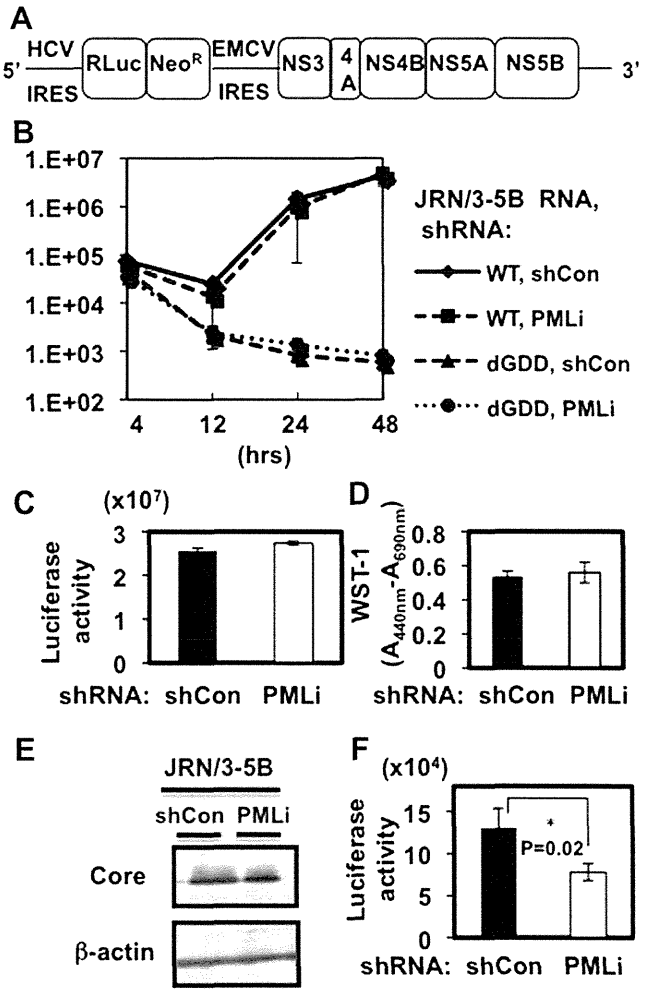

Fig. 2. PML is unrelated to the HCV RNA replication. Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase (RL) gene. *Renilla* luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. (B) The transient replication of subgenomic HCV-JFH1 replicon in the PML knockdown (PMLi) or the control OR6c cells (shCon) after electroporation of *in vitro* transcribed JRN/3-5B RNA (10 μg) was monitored by RL assay at the indicated time. The results of *Renilla* luciferase activity are shown. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the subgenomic HCV replicon with the deletion of GDD was used as a negative control. (C) The level of HCV RNA replication in PML knockdown (PMLi) or the control (shCon) OR6c JRN/3-5B cells was monitored by RL assay. The results shown are means from three independent experiments. (D) WST-1 assay of the PML knockdown or the control JRN/3-5B cells. (E) The level of HCV core protein in OR6c JRN/3-5B cells by expression of HCV core to NS2 coding region of HCV-JFH1 using mouse retroviral vector. pCX4bsr-JFH1-myc-C-NS2 and pMDG2 were cotransfected into Plat-E cells, mouse retroviral packaging cells. Mouse retroviral vector was obtained from their culture supernatants and transduced into OR6c JRN/3-5B PML knockdown or the control cells. The results of Western blot analysis of cellular lysates with anti-HCV core or an anti β-actin antibody are shown. (F) The level of HCV RNA replication in RSc cells 72 h after inoculation of HCV-like particles produced using *trans*-packaging system was monitored by RL assay. Asterisk indicates significant difference compared to the control. $^{*}P = 0.02$.

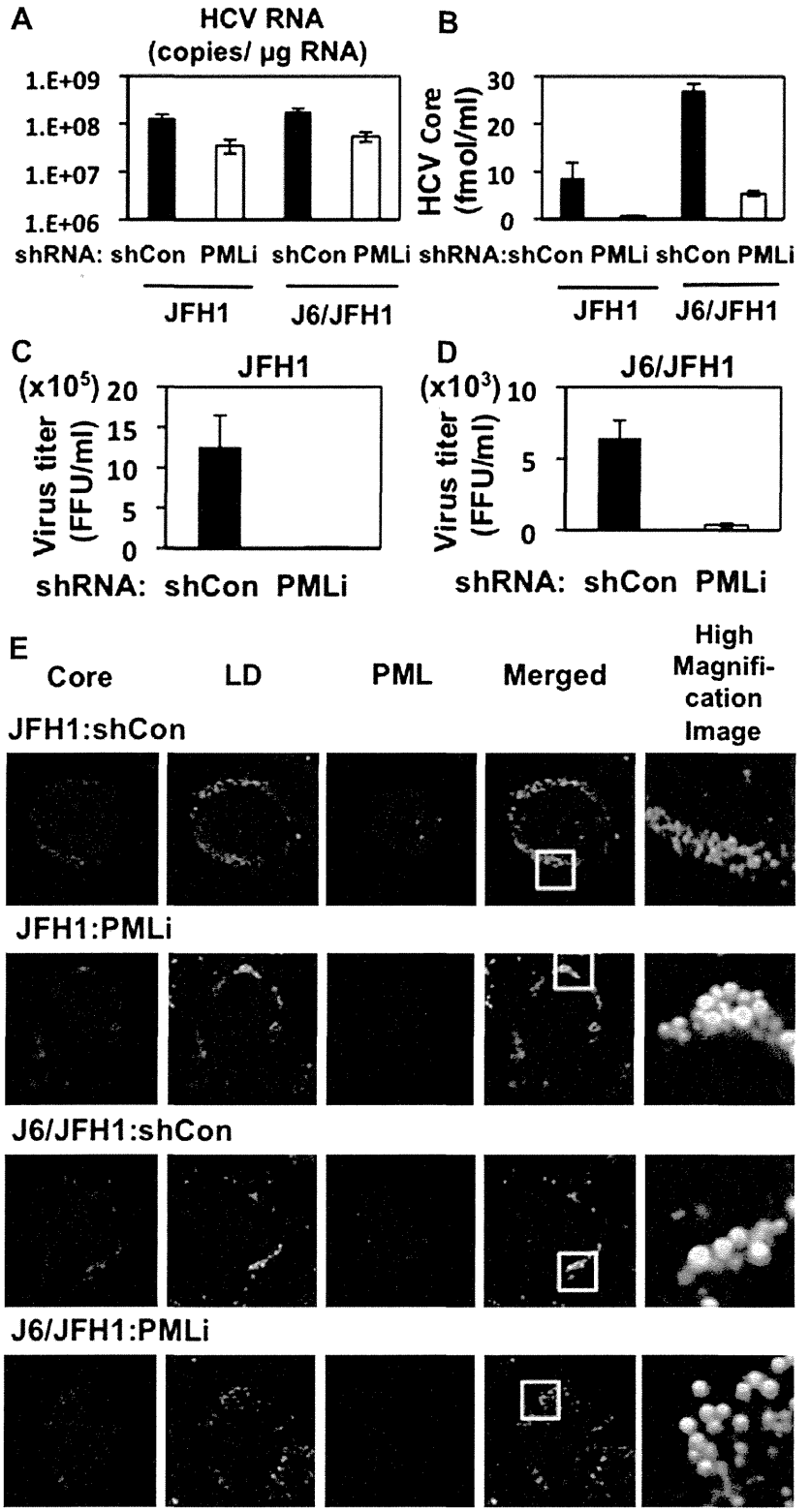

Fig. 3. PML is dispensable for the localization of HCV core to lipid droplet. (A) The levels of intracellular HCV RNA in PML knockdown or the control RSc cells 96 h after inoculation of HCV-JFH1 or HCV-J6/JFH1 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments are shown (A–C). (B) The levels of HCV core in the culture supernatants from the PML knockdown RSc cells at 96 h post-infection were determined by ELISA. (C, D) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. (E) HCV core localizes to lipid droplet (LD) in the PML knockdown (PMLi) or the control (shCon) cells after infection with either HCV-JFH1 or HCV-J6/JFH1. Cells were fixed 72 h post-infection and were then examined by confocal laser scanning microscopy.

3.2. PML is unrelated to HCV RNA replication

To examine whether or not PML is involved in HCV RNA replication, we used the subgenomic replicon RNA of HCV-JFH1, JRN/3-5B, encoding *Renilla* luciferase gene for monitoring the HCV RNA replication (Fig. 2A). *In vitro* transcribed JRN/3-5B RNA was transfected into the PML knockdown OR6c cells by electroporation and we examined the luciferase activity. Consequently, the

luciferase activity in the PML knockdown cells was similar to that of the control cells (Fig. 2B), indicating that shRNA targeted to PML could not affect the transient HCV RNA replication. As well, the level of HCV RNA in PML knockdown HuH-7-derived OR6c JRN/3-5B cells harboring the subgenomic replicon RNA of HCV-JFH1 and the cell growth was not affected (Fig. 2C and D), suggesting that PML is unrelated to the HCV RNA replication. To further confirm whether or not PML is involved in HCV production, we used *trans*-packaging system [21,22], that HCV subgenomic replicon was efficiently encapsidated into infectious virus-like particles by expression of HCV core to NS2 coding region. In fact, infectious HCV-like particles were produced and released into the culture medium from PML knockdown JRN/3-5B cells stably expressing core to NS2 coding region of HCV-JFH1 genome by mouse retroviral vector (Fig. 2E). We could monitor the HCV RNA replication by *Renilla* luciferase assay in target naïve RSc cells after the inoculation of infectious HCV-like particles. Consequently, the release of infectious HCV-like particles into the culture supernatants was significantly suppressed in PML knockdown cells at 72 h post-infection (Fig. 2F). Thus, we conclude that PML is associated with HCV production.

3.3. PML is required for the late step in the HCV-JFH1 life cycle

To avoid the possibility of specific finding when we only used HCV-JFH1, we examined another strain of HCV-J6JFH1 [20]. For this, we analyzed the level of HCV core and the infectivity in the culture supernatant as well as the level of HCV RNA in the PML knockdown RSc cells 96 h after inoculation of HCV-J6/JFH1. In this context, the level of HCV RNA in PML knockdown cells was only somewhat decreased (Fig. 3A), while the level of core and the infectivity in the culture supernatants was remarkably reduced (Fig. 3B–D), indicating that PML is required for infectious HCV-J6/JFH1 production as well as HCV-JFH1.

Since lipid droplets have been shown to be involved in an important cytoplasmic organelle for HCV production [3], we performed immunofluorescence and confocal microscopic analyses to determine whether or not HCV core misses localization into lipid droplets in the PML knockdown cells. We found that the core protein was targeted into lipid droplets even in PML knockdown RSc cells as well as in the control RSc cells after infection with either HCV-JFH1 or HCV-J6/JFH1 (Fig. 3E). This suggests that PML plays a role in the late step after the core is targeted into lipid droplet in the HCV life cycle. Importantly, HCV did not disrupt the formation of PML-NBs in response to HCV infection (Fig. 3E) unlike HIV-1 and other DNA viruses [6,7,23].

3.4. INI1 and DDX5, PML-related proteins, are involved in HCV production

Finally, we established the INI1 or DDX5, PML-related protein [23,24], knockdown RSc or OR6c JRN/3-5B cells by lentiviral vector expressing shRNA target to INI1 [17] or DDX5 to examine potential role of INI1 and DDX5 in HCV life cycle. Consequently, we found that the release of HCV core or the infectivity of HCV into the culture supernatants was significantly suppressed in the INI1 or DDX5 knockdown RSc cells 96 h after HCV-JFH1 infection, while the RNA replication in the knockdown cells was only somewhat decreased in spite of the very effective knockdown of INI1 or DDX5 mRNA without growth inhibition (Fig. 4A–F), suggesting that INI1 and DDX5 are involved in HCV life cycle. To confirm whether or not these proteins are involved in HCV RNA replication, we examined the luciferase assay in the INI1 or DDX5 knockdown OR6c JRN/3-5B cells. In this context, the shRNA target to INI1 or DDX5 did not affect the luciferase activity and the cell growth in these knockdown cells (Fig. 4G and H), suggesting that both INI1 and DDX5 are required for HCV production like PML.

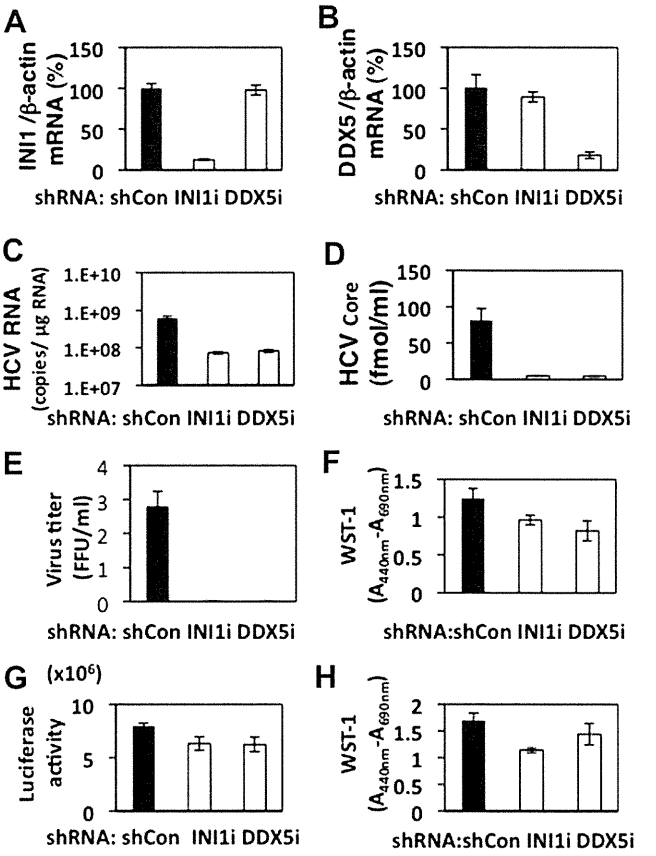

Fig. 4. INI1 and DDX5, PML-related proteins, are required for HCV production. (A, B) Inhibition of INI1 and DDX5 mRNA expressions by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for INI1 and DDX5 was performed as well as for β-actin mRNA in triplicate. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (Con) which was assigned as 100%. (C) The levels of intracellular genome-length HCV-JFH1 RNA in each knockdown cells at 96 h post-infection at an MOI of 0.05 were monitored by real-time LightCycler RT-PCR. (D) The levels of HCV core in the culture supernatants from the INI1 (INI1i) or DDX5 knockdown (DDX5i) RSc cells 96 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. Virus titer is shown as ($x10^7$) FFU/ml. (F) WST-1 assay of each knockdown RSc cells at 96 h post-infection. (G) The HCV RNA replication level in INI1 and DDX5 knockdown OR6c JRN/3-5B cells was monitored by RL assay. (H) WST-1 assay of each knockdown OR6c JRN/3-5B cells. All results shown are means from three independent experiments.

4. Discussion

So far, the PML tumor suppressor protein, which forms PML-NBs, has been implicated in host antiviral defenses [6,7]. In fact, PML is induced by interferon after viral infection and suppresses some viral replication [6,7]. In contrast, PML-NBs are often disrupted or sequestered in the cytoplasm by infection with several DNA or RNA viruses to protect from the antiviral function of PML [6,7,23]. In case of HCV, Herzer et al. recently reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML isoform IV by over-expression studies [5]. However, we did not observe such colocalization of HCV core with PML and HCV did not affect the formation of PML-NBs in response to HCV-JFH1 infection (Fig. 3E). Interestingly, Watashi et al., previously demonstrated the HCV core modulates the retinoid signaling pathway through sequestration of

Sp110b, PML-related potent transcriptional corepressor of retinoic acid receptor, in the cytoplasm from nucleus [25].

In contrast, we have demonstrated that PML is required for infectious HCV production (Fig. 1). However, the molecular mechanism(s) how PML regulates HCV production yet remains unclear. At least, PML seems to be unrelated to the HCV RNA replication (Fig. 2). In this regard, several host factors including apolipoprotein E, components of ESCRT system, and PA28γ have been implicated in infectious HCV production [13,26,27]. Indeed, PA28γ, a proteasome activator, interacts with HCV core and affects nuclear retention and stability of the core protein. Importantly, PA28γ participates in the propagation of infectious HCV by regulation of degradation of the core protein [27]. Intriguingly, Zannini reported that PA28γ interacts with PML and Chk2 and affects PML-NBs number [28]. Accordingly, we demonstrated that ATM and Chk2, which phosphorylates PML and regulates the PML function, are involved in HCV life cycle [11]. In addition, other PML-related proteins such as INI1 and DDX5 seem to be involved in HCV production (Fig. 4). Indeed, INI1, also known as hSNF5, is incorporated into HIV-1 virion and is required for efficient HIV-1 production [29]. On the other hand, cytoplasmic PML may be involved in HCV production, since endoplasmic reticulum (ER) and lipid droplets are important cytoplasmic organelle for the HCV life cycle. In this regard, Giorgi et al. recently reported that cytoplasmic PML specifically enriches at ER [30], suggesting that cytoplasmic PML may be associated with HCV production. Altogether, the PML pathway seems to be involved in infectious HCV production.

Acknowledgments

We thank Drs. Didier Trono, Reuven Agami, Richard Iggo, Toshio Kitamura, Kenichi Abe and Apath LLC for the VSV-G-pseudotyped HIV-1-based vector system pCMVΔR8.91, pMDG2, pSUPER, pRDI292, Plat-E cells, pJRN/3-5B and pJFH1. We also thank Mr. Takashi Nakamura and Ms. Keiko Takeshita for their technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS), by a Grant-in-Aid for Research on Hepatitis from the Ministry of Health, Labor, and Welfare of Japan, and by the Viral Hepatitis Research Foundation of Japan. M. K. was supported by a Research Fellowship from JSPS for Young Scientists.

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GASTROENTEROLOGY 2013;145:658–667

BASIC AND TRANSLATIONAL—LIVER

Thromboxane A_2 Synthase Inhibitors Prevent Production of Infectious Hepatitis C Virus in Mice With Humanized Livers

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BACKGROUND & AIMS: A 3-dimensional (3D) culture system for immortalized human hepatocytes (HuS-E/2 cells) recently was shown to support the lifecycle of blood-borne hepatitis C virus (HCV). We used this system to identify proteins that are active during the HCV lifecycle under 3D culture conditions. **METHODS:** We compared gene expression profiles of HuS-E/2 cells cultured under 2-dimensional and 3D conditions. We identified signaling pathways that were activated differentially in the cells, and analyzed their functions in the HCV lifecycle using a recombinant HCV-producing cell-culture system, with small interfering RNAs and chemical reagents. We investigated the effects of anti-HCV reagents that altered these signaling pathways in mice with humanized livers (carrying human hepatocytes). **RESULTS:** Microarray analysis showed that cells cultured under 2-dimensional vs 3D conditions expressed different levels of messenger RNAs encoding prostaglandin synthases. Small interfering RNA–mediated knockdown of thromboxane A2 synthase (TXAS) and incubation of hepatocytes with a TXAS inhibitor showed that this enzyme is required for production of infectious HCV, but does not affect replication of the HCV genome or particle release. The TXAS inhibitor and a prostaglandin I2 receptor agonist, which has effects that are opposite those of thromboxane A_2, reduced serum levels of HCV and inhibited the infection of human hepatocytes by blood-borne HCV in mice. **CONCLUSIONS: An inhibitor of the prostaglandin synthase TXAS inhibits production of infectious HCV particles in cultured hepatocytes and HCV infection of hepatocytes in mice with humanized livers. It therefore might be therapeutic for HCV infection.**

Keywords: Infectious Virus Particle; Lipid Mediator; Antiviral Drug.

Approximately 170 million people worldwide are infected with hepatitis C virus (HCV),[1] with the majority suffering from chronic hepatitis, cirrhosis, and/or hepatocellular carcinoma.[2] HCV currently is treated using a combination of polyethylene glycol–conjugated interferon and ribavirin, although no more than 60% of individuals adequately respond.[3] Recently, inhibitors of HCV nonstructural proteins have been developed as direct-acting antiviral agents to treat HCV effectively.[4–6] However, HCV often acquires resistance against treatment with direct-acting antiviral agents in cases of monotherapy.[7] Current efforts therefore are focused on better understanding the lifecycle of HCV to find the cellular target of novel anti-HCV drugs to use the options for multi-drug therapy.

A cell-culture system that allows the production of recombinant infectious HCV (HCVcc) recently was developed using a cloned HCV genome and the hepatocellular carcinoma–derived Huh-7 cell line.[8–10] Experiments using the culture system have provided novel insights on the HCV lifecycle such as finding the production of infectious HCV particles near lipid droplets (LDs) and endoplasmic reticulum–derived LD-associated membranes.[11] Huh-7 cells, however, only allow the proliferation of recombinant HCV, and not blood-borne HCV (bbHCV).

To study the lifecycle of bbHCV, we cloned immortalized human hepatocyte HuS-E/2 cells, which permitted some degree of bbHCV infection.[12] Integrating hollow fibers into the 3-dimensional (3D) culture system resulted in efficient continuous proliferation of infected HCV production from the cells.[13] By using the improved system, we previously compared the gene expression profiles of HuS-E/2 cells under the 2-dimensional (2D) and 3D culture conditions using microarray analysis. This allowed us to identify signaling pathways that contribute to the proliferation of HCV, for example, peroxisome proliferator–activated

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; AAC, arachidonic acid cascade; bbHCV, blood-borne hepatitis C virus; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; HCV, hepatitis C virus; HCVcc, hepatitis C virus from cell culture; IP, prostaglandin I_2 receptor; LD, lipid droplet; mRNA, messenger RNA; PG, prostaglandin; PGIS, prostaglandin I_2 synthase; RT-PCR, reverse-transcription polymerase chain reaction; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; TP, thromboxane A_2 receptor; TXA_2, thromboxane A_2; TXAS, thromboxane A_2 synthase; TXB_2, thromboxane B_2.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2013.05.014

receptor α signaling, which enhances HCV replication.[14] This result was confirmed by other groups,[15,16] corroborating that our strategy can uncover cellular events that support the proliferation of HCV. We therefore hypothesized that leveraging the in vitro systems described earlier may help elucidate the molecular mechanisms underlying the HCV lifecycle.

Prostanoids are metabolites of the arachidonic acid cascade (AAC) that possess various physiologic activities.[17] These metabolites include prostaglandin (PG) E_2, D_2, I_2, and F_2, and thromboxane A_2 (TXA_2).[17] Although several studies have shown that PG signaling contributes to liver regeneration,[18,19] the physiologic functions of these lipid mediators in human hepatocytes still are unknown. Interestingly, one report showed that PGE_2 might support HCV genome replication in cells bearing self-replicating HCV subgenomic replicon RNA.[20] Whether prostanoids are involved in the HCV lifecycle, however, has not been precisely investigated.

In this study, we provide evidence that TXA_2 synthase (TXAS) is involved in the formation of infectious HCV, by cell culture system, and that a TXAS inhibitor and PGI_2 receptor (IP) agonist that has opposite physiological effects to TXA_2 can be used as a novel anti-HCV drug by using chimeric mice bearing transplanted human hepatocytes.[21] This report shows the contribution of the AAC to HCV infectivity and the potency of a prostanoid as an antiviral agent.

Materials and Methods

Cell Culture

The human hepatocellular carcinoma-derived Huh-7 and Huh-7.5 cell lines were cultured as described previously.[22] HuS-E/2 cells are immortalized human hepatocytes transduced with *E6* and *E7* genes of human papilloma virus 18 and human telomerase reverse-transcription gene as described previously.[12] The 2D and 3D culture conditions for HuS-E/2 cells were as described previously.[12]

Reagents and Antibodies

FR122047, PGH_2, ONO1301, daltroban, and dibutyryl cyclic adenosine monophosphate (cAMP) sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Cyclooxygenase (COX)-2 inhibitor 1 and Ozagrel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U-46619 was purchased from Cayman Chemical (Ann Arbor, MI). Beraprost was a generous gift from Toray, Co (Tokyo, Japan). FR122047, PGH_2, ONO1301, Daltroban, COX-2 inhibitor 1, Ozagrel, Beraprost, and calcium ionophore were dissolved in dimethyl sulfoxide. U-46619 and thromboxane B_2 (TXB_2) were dissolved in methyl acetate. Dibutyryl cAMP was dissolved in water. The effect of each reagent on cell viability was analyzed using a Cell Proliferation Kit 2 (Roche, Basel, Switzerland) based on the manufacturer's instructions. An antibody specific for core protein (antibody 32-1) was a gift from Dr Michinori Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Rabbit polyclonal anti-NS5A protein CL1 antibody and anti-HCV protein antibody in human serum were described previously.[11]

Microarray Analysis

Total RNA purified from HuS-E/2 cells cultured under 2D or 3D conditions in the absence of HCV infection was analyzed with a 3D-Gene Human Chip 25k (Toray, Co) to compare gene expression profiles as described previously.[14] The accession number of the results is listed as "E-MTAB-1491" in ArrayExpress.

Production of HCVcc and Sample Preparation

HCVcc was produced from the Huh-7 or Huh-7.5 cells transfected with in vitro synthesized Jikei Fulminant Hepatitis (JFH) 1^{E2FL} or J6/JFH1 RNA as described previously.[11] The transfected cells and culture medium were harvested at 4 days post-transfection. For $JFH1^{E2FL}$ RNA-transfected Huh-7 cells treated with TXAS-specific small interfering RNA (siRNA), cells and culture medium were harvested 3 days after transfection. Culture medium including HCVcc was concentrated and used for infection experiments as described previously.[11] Concentrated culture medium from JFH1 RNA-transfected Huh-7 cells was fractionated as described previously.[11] The infectivity titer in each fraction was analyzed by focus-formation assay, which was determined by the average number of HCV-positive foci.

Reverse-Transcription Polymerase Chain Reaction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from the cells and medium using Sepasol I Super and Sepasol II (Nacalai Tesque, Kyoto, Japan), respectively, according to the manufacturer's instructions. By using 200 ng of total RNA as a template, we performed reverse-transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) with a 1-step RNA PCR kit and a 1-step SYBR Primescript RT-PCR kit 2 (Takara, Shiga, Japan), respectively, according to the manufacturer's instructions. Information on both experiments is shown in Supplementary Tables 1 and 2.

Infection of HCVcc

Infection experiments of HCVcc and detection of infected Huh-7.5 cells by indirect immunofluorescence analysis were performed mainly as described previously.[11] The number of infection-positive cells detected in 4×10^4 target cells 1 day after infection with HCVcc including 10^7 copies of RNase-resistant HCV genome was defined as the specific infectivity in the infection experiments in our protocol.

Indirect Immunofluorescence Analysis

HCV proteins were examined in cells using a Leica SP2 confocal microscope (Leica, Heidelberg, Germany), and infected cells were counted using a BioZero fluorescence microscope (Keyence, Tokyo, Japan).

Preparation of Intracellular HCV Particles

Intracellular HCV particles were prepared as described previously.[23]

Pharmacologic Test in Chimeric Mice Bearing Transplanted Human Hepatocytes

All mouse studies were conducted at Hiroshima University (Hiroshima, Japan) in accordance with the guidelines of the local committee for animal experiments. Chimeric mice

transplanted with human hepatocytes were generated as described previously.[21] The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences (Hiroshima University). The reagents were first administered 1 week after the chimeric mice were infected with a 1.0×10^5 titer of bbHCV. ONO1301 was injected subcutaneously at a dose of 200 μg/mouse. Beraprost and Ozagrel were administrated orally at a dose of 10 and 300 μg/mouse, respectively. For a positive control experiment, telaprevir was administered as described previously.[24] All reagents were administrated twice each day. Serum samples were collected at 2, 3, and 4 weeks after starting the treatments. HCV-RNA levels in the samples were evaluated in qRT-PCR.

Statistical Analyses of Data

The significance of differences in the means was determined by the Student *t* test (Figures 1-6, and Supplementary Figures 1-13) or the Wilcoxon signed-rank test (Figure 7 and Supplementary Figure 14).

Results

Expression of PG Synthase messenger RNA in HuS-E/2 Cells Cultured Under 3D Conditions

To identify signaling pathways that contribute to HCV proliferation under the 3D culture conditions, we compared the gene expression profiles of 2D- and 3D-cultured HuS-E/2 cells as described previously.[14] We found that the expression of 984 genes was up-regulated more than 2 times in both of 2 types of 3D-cultured HuS-E/2 cells, and expression of 1491 genes was down-regulated less than half the time. For the two 3D conditions, we identified the expression of a set of genes encoding enzymes of the AAC. The expression levels of messenger RNAs (mRNAs) for AAC enzymes, COX1, PGD_2 synthase, and TXAS increased in HuS-E/2 cells cultured under 3D conditions (Figure 1*A*), whereas those for PGE_2 and PGI_2 synthases (PGIS) decreased (Figure 1*A*). These results were confirmed by qRT-PCR analysis (Figure 1*A*, gray bars). The relative protein levels of those enzymes in 2D- and 3D-cultured HuS-E/2 cells reflected the quantitative difference of those mRNAs, except PGD_2 synthase (PGDS) (Figure 1*B*). The expression of those genes and the production of those proteins also were observed in the liver tissues from patients with hepatitis C, suggesting the functional roles of those products in the human liver (Supplementary Figure 1).

The AAC Contributes to Infectious HCV Production

To assess whether the AAC plays a role in the HCV lifecycle, the contributions of the AAC rate-limiting enzymes COX1 and COX2 were examined using the JFH1 cell culture system. We first investigated the role of COX1, which is known to be a constitutively expressed gene in general, by adding the COX1 inhibitor FR122047 to JFH1-RNA-transfected cell cultures. Even at higher concentrations, FR122047 did not markedly affect the amount of HCV RNA in the medium or cells (Figure 2*A*, black and white bars) with a little cytotoxicity at 10 μmol/L. Nevertheless, FR122047 dose-dependently decreased the infectivity of HCVcc in the culture medium (Figure 2*B* and *C*). Because the infection experiment using a lower titer of HCVcc from inhibitor-treated cells showed that the inhibitor affected the number of foci, but not the number of cells in a focus (Figure 2*C*), the treatment of COX1 inhibitor seemed to decrease the focus-forming ability of HCVcc. Next, the contribution of inducible COX2 was examined using COX2 inhibitor 1.

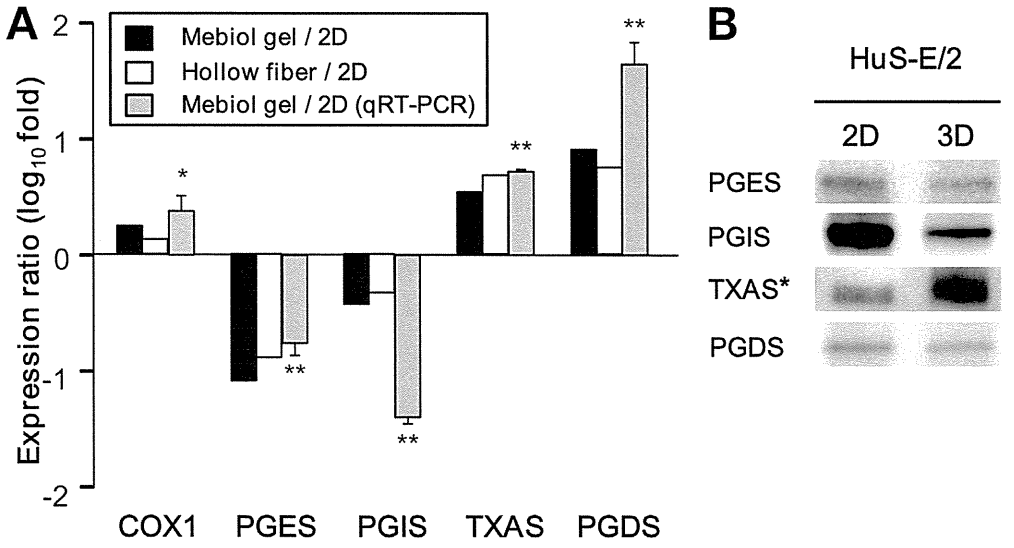

Figure 1. PG synthase mRNA expression under 3D culture conditions. (*A*) Results of microarray analysis. Black and white bars represent mRNA expression levels in HuS-E/2 cells cultured with Mebiol gel and hollow fibers, respectively, relative to levels observed in cells cultured under 2D conditions. Gray bars represent mRNA expression levels in HuS-E/2 cells cultured with Mebiol gel (Mebiol, Hiratuka, Japan) by quantifying with qRT-PCRs. qRT-PCR data show the averages from quadruplicate samples in 2 independent experiments ± SD. *Differs from control, $P < .01$; **differs from control, $P < .001$. (*B*) Protein levels of TXAS and various PG synthases in 2D-cultured and 3D-cultured HuS-E/2 cells. PG synthases except for TXAS were detected in whole-cell lysate. Asterisks show the result in membrane fraction.

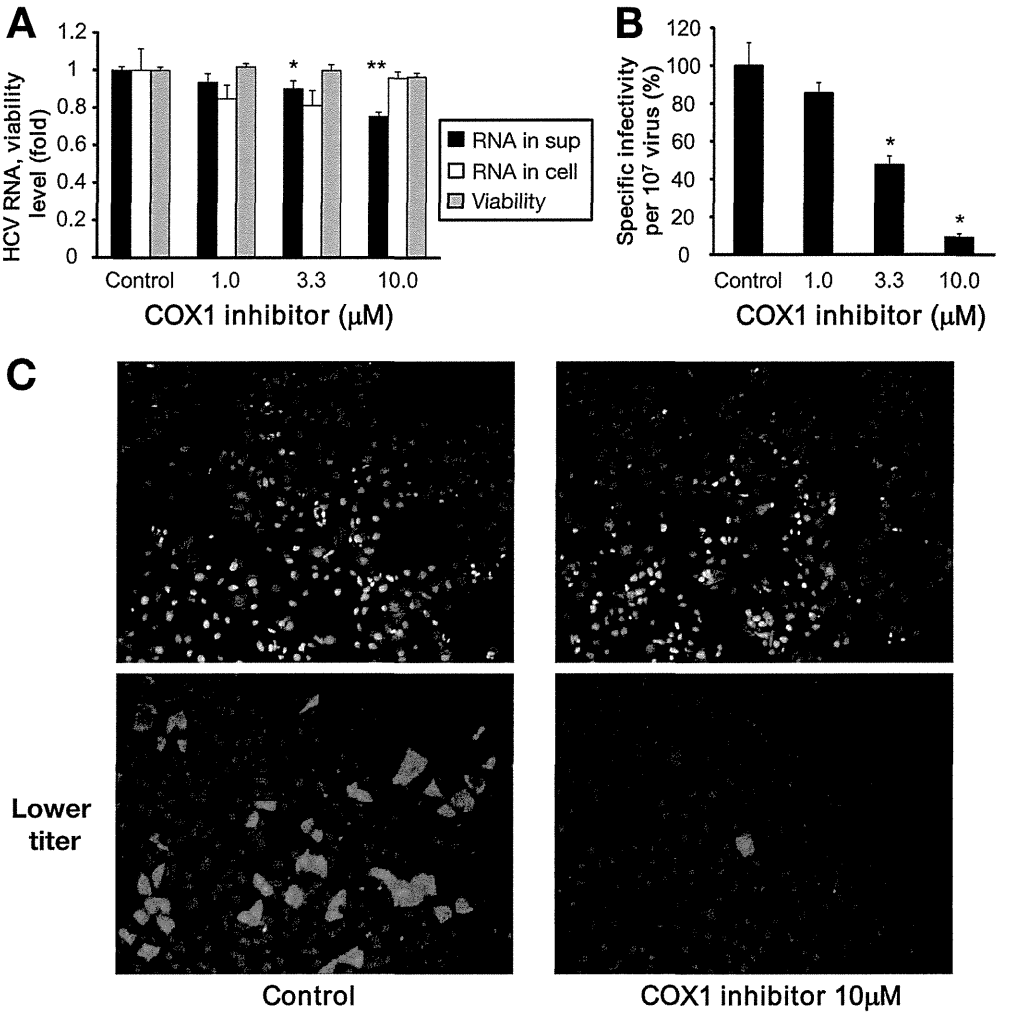

Figure 2. Effects of FR122047 on HCVcc-producing Huh-7 cells. (*A*) Effects of FR122047 on HCV-RNA levels in cultured HCVcc-producing cells. HCV RNA was collected from the medium (black bars) and cells (white bars), which were treated with FR122047 at the indicated concentrations. Mean cell viability ± SD for each sample condition also is plotted (gray bars). Averages from quadruplicate samples in 2 independent experiments ± SD are shown. (*B*) Effects of FR122047 on the infectivity of HCVcc produced using this cell-culture system. (*C*) FR122047 reduces infectious HCVcc in the culture medium. Huh-7.5 cells infected with HCVcc from the culture medium of cells treated with (*right panel*) and without (*left panel*) FR122047 at the indicated concentrations were stained with anti-HCV antibodies (magenta) and the nuclear stain 4′,6-diamidino-2-phenylindole (cyan). *Lower panels*: infected cells at a lower titer of inoculums. *Differs from control, $P < .01$; **differs from control, $P < .001$.

The inhibitor, however, did not affect the infectivity of HCVcc in the medium (Supplementary Figure 2*A* and *B*), probably because of the lack of *COX2* gene expression in Huh-7 cells (Supplementary Figures 2*C* and 3*A*). These data suggest that COX1 and the AAC play a role in infectious HCVcc production without significant effects on HCV genome replication or particle release from the cells.

TXAS Plays a Key Role in Infectious HCV Production

To further examine the contribution of the AAC to infectious HCVcc production, we focused on TXAS, because, similar to COX1 mRNA, TXAS mRNA levels increased in HuS-E/2 cells cultured under 3D conditions. Although PGD_2 synthase mRNA levels also increased, this synthase was unlikely to contribute to these processes because we did not detect PGD_2 synthase mRNA in Huh-7 cells in the JFH1 cell culture system (Supplementary Figure 3*A*). By using siRNA- and short hairpin RNA–mediated suppression of mRNA expression, we found that reducing TXAS mRNA levels in HCVcc-producing Huh-7 cells did not significantly affect the amount of HCV RNA in the medium or cells (Figure 3*B* and Supplementary Figure 4*B*, black and white bars), whereas HCVcc in the medium was less infectious, as was observed when the cells were treated with FR122047 (Figure 3*C* and Supplementary Figure 4*C*). Treatment with the TXAS inhibitor Ozagrel also dose-dependently suppressed infectious HCVcc production without significantly affecting HCV-RNA levels in the medium or cells (Figure 3*D* and *E*). Similar effects of Ozagrel were observed in another HCV cell culture system using Huh-7.5 cells and chimeric recombinant J6/JFH1 HCV, which encoded different structural proteins from JFH1[9] (Supplementary Figure 5), indicating that our results were not specific to the JFH1 cell culture system. Furthermore, treatment with PGH_2, a product of COX1 and a substrate of TXAS, was shown to increase the infectivity of HCVcc without effect on the HCV replication and egression despite the short half-life of PGH_2 (Supplementary Figure 6). These data suggest that the AAC, in particular TXAS activity and probably TXA_2 produced from PGH_2 by TXAS activity, contributes to infectious HCV production.