

Figure 3. Effect of TXAS on infectious HCV production. (A) siRNA-mediated knockdown of TXAS mRNA expression. (B) Effects of TXAS-specific siRNA on HCV-RNA levels in the HCVcc-producing cell-culture system. (C) Effects of control and TXAS-specific siRNA on the infectivity of HCVcc in medium obtained from HCVcc-producing cell cultures were assessed. (D) Effects of Ozagrel on HCV-RNA levels were assessed in HCVcc-producing cell cultures. (E) Effects of Ozagrel on the infectivity of HCVcc produced from the cell-culture system were assessed. **Differs from control, $P < .001$.

TXA₂ Receptor Is Not Required for TXAS-Dependent Regulation of Infectious HCV Production

TXA₂ exerts its physiologic functions through the TXA₂ receptor (TP) on plasma membranes.¹⁷ To examine the contribution of TXA₂/TP signaling, we investigated the effects of the TP agonist U-46619 in our system. Regardless of dose, however, U-46619 did not affect infectious HCVcc production in the culture system

(Supplementary Figure 7). Treating Huh-7-derived cell lines with the TP agonist did not increase the calcium ion concentration—a major downstream effect of TXA₂/TP signaling—even though the activity of U-46619 was confirmed in HEK293 cells¹⁷ (Supplementary Figure 8A). We also evaluated the activity of U-46619 in terms of TP-dependent activation of Rho and observed the Rho-dependent stress fiber formation induced with U-46619 in HEK293 cells (Supplementary Figure 8B). In addition,

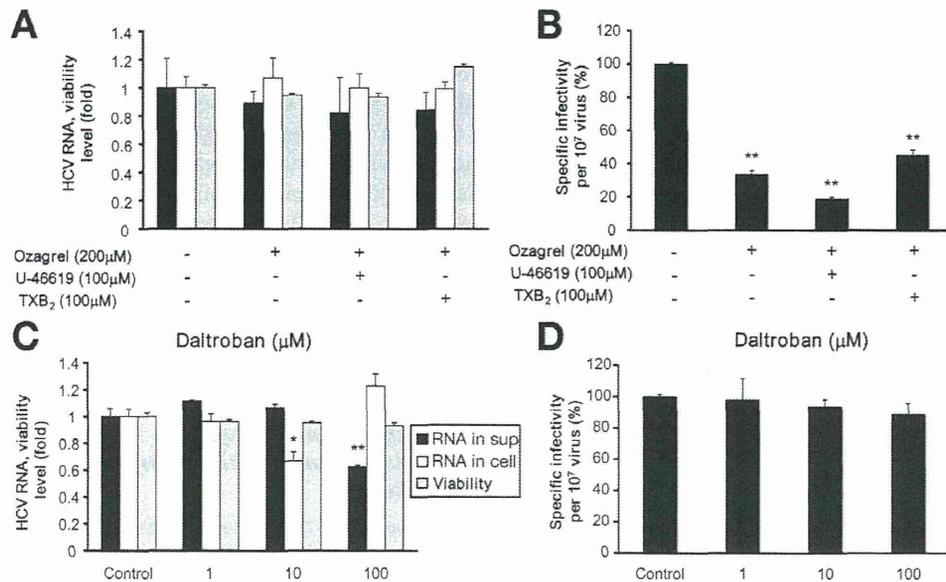


Figure 4. Role of TP in infectious HCVcc-producing cell cultures. (A) Effects of U-46619 and TXB₂ on HCV-RNA levels in HCVcc-producing cell cultures in the presence of Ozagrel were assessed. (B) The infectivity of HCVcc in culture medium from HCVcc-producing cells treated with U-46619 or TXB₂ in the presence of Ozagrel was assessed. (C) Effect of daltroban on HCV-RNA levels in HCVcc-producing cell cultures. (D) The infectivity of HCVcc in culture medium from HCVcc-producing cells treated with daltroban. *Differs from control, $P < .01$; **differs from control, $P < .001$.

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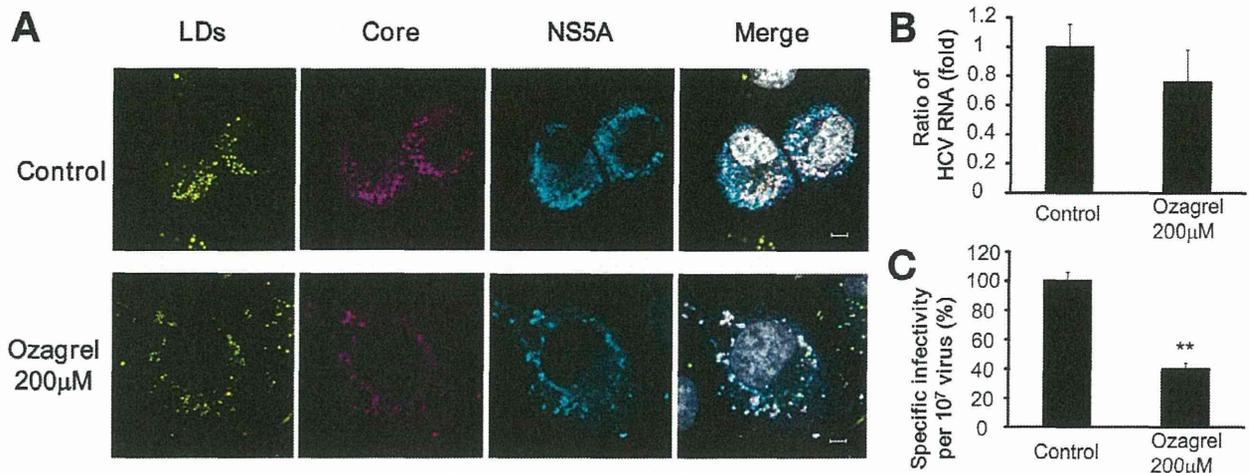


Figure 5. Core and NS5A near LDs and the quantity and infectivity of intracellular HCVcc. (A) HCV core (magenta) and NS5A (cyan) around LDs (yellow) in HCVcc-producing cells treated with the indicated reagents were observed using immunofluorescence analysis. Nuclei were stained with 4',6-diamidino-2-phenylindole (gray). Scale bars, 5 μ m. (B) Levels of intracellular HCV RNA obtained from the cells treated with Ozagrel. (C) The infectivity of intracellular HCV from cells treated with Ozagrel. Averages of triplicate samples from 2 independent experiments \pm SD are shown. **Differs from control, $P < .001$.

the level of TP mRNA was, if any, quite low in human hepatocyte-derived cells and primary human hepatocytes, although only a small amount of the mRNA was detected in HuS-E/2 cells (Supplementary Figure 3B). These data suggest that TXA₂/TP signal transduction is deficient in Huh-7-derived cell lines.

To determine whether TP on the Huh-7 cells was saturated with endogenous TXA₂ ligand, we examined the effects of U-46619 in the presence of Ozagrel. U-46619, however, did not rescue the Ozagrel-mediated suppression of infectious HCVcc production (Figure 4A and B). In addition, the TP antagonist daltroban did not affect infectious HCVcc production (Figure 4C and D). These data indicate that TP-mediated signaling is not involved in TXAS-dependent regulation of infectious HCVcc production. In addition, we examined whether TXB₂—a stable metabolite of TXA₂ that does not activate TP—could be used to replace TXAS during infectious HCVcc production. TXB₂ did not counteract the effects of Ozagrel (Figure 4A and B), and did not by itself affect the HCV lifecycle (Supplementary Figure 7). These data suggest that TXA₂ or an unidentified metabolite of TXA₂ acts as a TP-independent regulator of infectious HCV production (see Discussion section).

TXAS-Derived Signaling Contributes to HCV Infectivity

A previous study showed that infectious HCV is produced near LDs, to which HCV proteins are recruited.⁶ As shown in Figure 5A, inhibiting TXAS did not markedly affect the locations of the viral proteins core and NS5A around LDs, suggesting that TXAS-derived signaling does not contribute to the recruitment of HCV proteins to the LDs. Next, to examine whether TXAS-mediated signaling drives the egression of infectious HCVcc from the cells, we analyzed intracellular HCVcc in cells treated with Ozagrel. Levels of intracellular HCVcc RNA in Huh-7 cells treated

with or without Ozagrel were equivalent (Figure 5B). Nevertheless, the infectivity of intracellular HCVcc from the cells treated with Ozagrel was decreased markedly, as was that of HCVcc in the medium (Figure 5C). This result indicated that TXAS-derived signaling is not involved in the release of infectious HCV particles. Taken together, it seems likely that TXAS-derived signaling plays a role in infectious particle formation in the cells.

Inhibition of TXAS Changes the Physicochemical Properties of HCVcc

We next analyzed HCVcc produced from cells treated with Ozagrel using sucrose density gradient ultracentrifugation. As reported previously,¹¹ 2 types of fractions containing either highly infectious, low-density HCVcc (peak fraction, 6) or less infectious, high-density HCVcc (peak fraction, 5) were obtained using samples derived from cells without treatment with Ozagrel, indicating that infectious HCVcc was present mainly in fraction 6 (Figure 6, white bars, upper and lower panels). On the other hand, analyzing Ozagrel-treated cells showed decreased levels of HCV RNA in fraction 6 (Figure 6, solid lines, upper and lower panels). Of note, the amount of HCV RNA in fraction 5 remained similar with or without Ozagrel treatment (Figure 6, lower panel). These results suggest that inhibition of TXAS-mediated signaling changes the physicochemical characteristics of HCVcc, resulting in altered infectivity.

A TXAS Inhibitor and IP Agonists Inhibit Early HCV Expansion in bbHCV-Infected Chimeric Mice

Finally, we examined the in vivo anti-HCV effects of a TXAS inhibitor using bbHCV and urokinase plasminogen activator/severe combined immunodeficiency mice bearing transplanted human hepatocytes. The IP

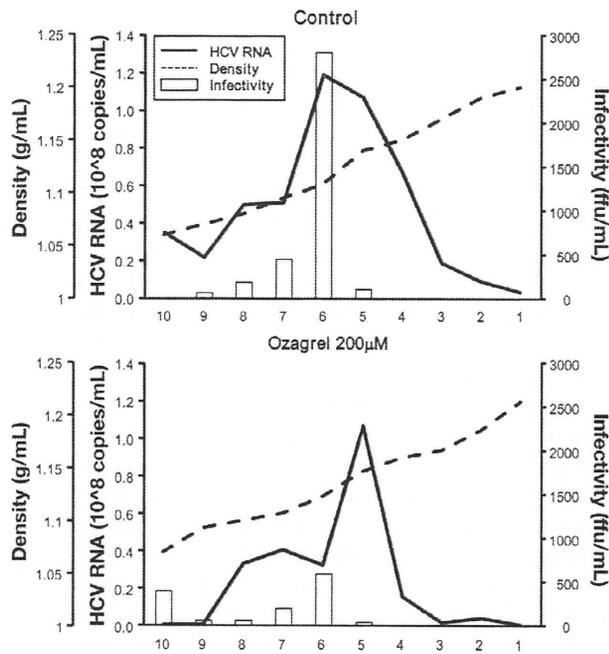


Figure 6. Buoyant density of HCVcc produced from cells treated with Ozagrel. Lower and upper panels show the results of HCVcc from the cells with and without Ozagrel treatment, respectively. HCV RNA (solid line), fraction density (dotted line), and HCV infectivity (white bars) in each fraction collected after ultracentrifugation are shown. Representative results from 2 independent experiments are shown.

agonist Beraprost also was tested because PGI₂ produces effects opposite of TXA₂ during several physiologic processes, including vascular constriction in human beings.²⁵ Both drugs delayed the increase in serum levels of HCV

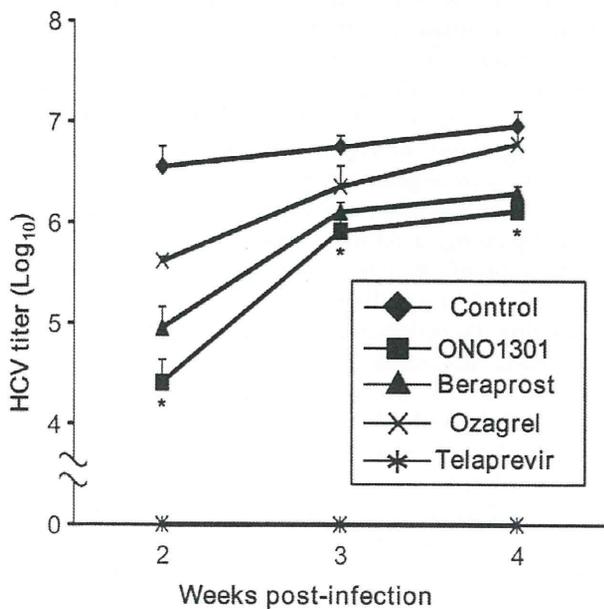


Figure 7. Effects of ONO1301, Beraprost, Ozagrel, and Telaprevir on the expansion of bbHCV-infected urokinase plasminogen activator/severe combined immunodeficiency mice bearing human hepatocytes. Data are presented as means ± SD for 6 (control, diamonds), 4 (ONO1301, squares; Ozagrel, crosses; Telaprevir, asterisk), and 3 (Beraprost, triangles) samples. *Differs from control, *P* < .05.

RNA (Figure 7). Of note, even 4 weeks after treatment, Beraprost reduced serum HCV-RNA levels to less than a quarter of those observed in control mice (Figure 7). Our results indicate that these drugs may inhibit HCV proliferation in vivo, and that inhibition of TXAS-derived signaling and activation of IP-mediated PGI₂ signaling can control HCV proliferation. Although we examined the effects of the PGIS and IP agonist on the HCV lifecycle using the HCVcc-producing cell-culture system, Beraprost did not result in any notable changes (Supplementary Figure 9). To examine whether the Huh-7 cells respond to the IP agonist, we assessed cAMP signaling using a plasmid-bearing cAMP-responsive element in a promoter upstream of the luciferase gene; cAMP signaling is a major intracellular signaling pathway that is activated by IP agonists. The IP agonist, however, did not activate cAMP signaling in Huh-7 cells even though the pathway was activated in HuS-E/2 cells.

As another candidate anti-HCV drug, we examined the effect of ONO1301, possessing both TXAS-inhibitor and IP-agonist activities, in the humanized chimeric mouse. ONO1301 produced the most robust suppression of HCV infections (Figure 7). The effects of ONO1301 also were studied in the HCVcc-producing cell cultures, as with Ozagrel. ONO1301 suppressed infectious HCV production (Supplementary Figure 10), although ONO1301 did not activate cAMP signaling in Huh-7 cells (Supplementary Figure 11). A slight decrease of HCVcc egression, however, caused by the treatment with ONO1301 at high concentrations, might be of note. These results further supported our conclusion that TXAS-mediated signaling contributes to infectious HCV production, although the functional role of PGI₂ in this process still is unknown.

Discussion

In this study, we showed that TXAS is involved in the development of infectious HCV. Administration of a TXAS inhibitor inhibited early stages of HCV proliferation postinfection in a chimeric mouse model. These results suggest that TXAS-mediated infectious HCV production is a potential target for novel anti-HCV therapies.

We first found that inhibiting COX1 and TXAS decreased the infectivity of HCVcc in the culture medium without any significant effects on viral genome replication and particle egression (Figures 2 and 3). In addition, we showed that inhibition of TXAS did not affect the release of infectious HCVcc from the cells (Figure 5). Thus, we concluded that TXAS probably regulates HCV particle maturation and the development of infectivity. Knock-down of apolipoprotein E, heat shock protein 70, and annexin A2 expression was shown previously to inhibit infectious HCVcc production.²⁶ Of note, decreased expression of these host factors reduced the production of HCVcc in the culture medium as well as intracellular HCVcc levels in HCVcc-producing cells, suggesting that TXAS is playing a different role in HCVcc production.

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Moreover, our results suggest that TXAS is a host factor that contributes only to the development of HCV infectivity.

A previous study reported that infectious HCVcc is produced near LDs.¹¹ The HCV proteins core and NSSA are located on and nearby LDs, respectively, suggesting a role in the production of infectious HCVcc.¹¹ Because LD localization of these proteins was not affected by the TXAS inhibitor (Figure 5A), TXAS probably is required after these viral proteins are recruited to LDs.

Several studies have shown that the buoyant density of infectious HCVcc differs slightly from that of noninfectious HCVcc.^{11,27,28} Infectious and noninfectious HCVcc are found in lower- and higher-density fractions, respectively. In the present study, inhibition of TXAS reduced the amount of HCVcc in the lower-density fraction (fraction 6) containing the infectious particles, whereas levels of primarily noninfectious HCVcc particles in the higher-density fraction (fraction 5) were not affected (Figure 6). These results mirrored previously reported data about NSSA mutant HCVcc, which do not produce infectious particles.¹¹ These results suggest that TXAS might be required to produce infectious HCVcc near the LDs.

Studies with treatment with methyl- β -cyclodextrin or lipoprotein lipase have shown that changing the physicochemical properties of HCVcc,²⁸⁻³⁰ in which the peak density fraction containing HCVcc shifted higher, diminished the infectivity of the particles.^{29,30} Because TXAS inhibitors did not show the apparent shift to a higher density (Figure 6), future molecular analyses of HCVcc particles should be required to reveal the underlying structural mechanisms for HCV infectivity.

Prostanoids play various physiologic functions, including regulatory roles in muscle and blood vessels.¹⁷ Although inhibition of TXAS decreased the infectivity of HCVcc (Figure 3), the identity of the relevant prostanoid and how that product functions in the development of infectious HCV are currently unclear. Because the treatment of PGH₂, a substrate of TXAS, caused the increase of infectious HCV production (Supplementary Figure 6), it seemed likely that the decrease of HCV infectivity is not due to an accumulation of PGH₂ caused by TXAS inhibition. Although we analyzed the total fatty acids in the HCV-infected Huh-7.5 cells treated with and without Ozagrel, the compositions of fatty acids, including arachidonic acid, were not largely different from each other (Supplementary Figure 12, the arachidonic acid is shown as C20:4 ω -6), suggesting that the effect of Ozagrel is not caused by major changes of fatty acid composition.

The activity of the TXAS product TXA₂ was not examined directly because its half-life is quite short.³¹ Usually, TXA₂ activity is measured using stable agonists and antagonists of TP. We showed, however, that TP-mediated signaling is not related to the processes examined in the current study (Figure 4). It seemed likely that TXA₂ itself or an unidentified metabolite of TXA₂ mediates the development of HCV infectivity in a

TP-independent manner. PGI₂ and the PGD₂ metabolite 15d-PGJ₂ have been identified as ligands of peroxisome proliferator activated receptor δ and γ , respectively.^{32,33} Therefore, the TXAS product may act as a ligand of various nuclear receptors to regulate infectious HCV production. In primary human hepatocytes and liver of chimeric mice transplanted with human hepatocytes, the expression of human TP mRNA was not observed (Supplementary Figures 3A and 13), although it was detected in human liver tissue, consisting of many cell types (Supplementary Figure 13). It may be true, therefore, that the TP gene is not largely expressed in human hepatocytes in the liver as with Huh-7 cells. Taken together, it is probable that infectious HCV is produced in a TP-independent manner in human liver infected with HCV. Further studies regarding the TP-independent roles of TXAS products in hepatocytes may be required to elucidate the mechanisms of infectious HCV formation.

Recently, various drugs targeting viral proteins were developed, resulting in more HCV-specific therapeutic profiles than those of conventional drugs.³⁴ Monotherapy with an HCV-specific drug, however, sometimes fails to clear the HCV infection because of rapidly emerging resistant variants.³⁵ We found that a TXAS inhibitor and IP agonists suppressed early stage expansion of bbHCV postinfection of chimeric mice bearing human hepatocytes (Figure 7). These results clearly indicate that the TXAS inhibitor and IP agonist are novel candidates for anti-HCV drugs. In this experiment, the effects of an IP agonist and the TXAS inhibitor were compared because TXA₂ and IP agonists have opposite clinical effects.²⁰ This implies that the IP agonist may have suppressed the effects of TXAS in the bbHCV-infected transplanted human hepatocytes. Contrary to our expectations, however, neither siRNA-mediated knockdown of PGIS expression nor treatment with the IP agonist Beraprost affected HCV genome replication, particle egression, or HCVcc infectivity (Supplementary Figure 9). The responsiveness of Huh-7 cells to the IP agonist then was examined by monitoring activation of cAMP signaling, a pathway that normally is activated downstream of IP. The results show that Huh-7 cells were deficient in signaling from IP to intracellular cAMP production (Supplementary Figure 11). Although the therapeutic mechanism of action for the IP agonist in chimeric mice has not been clarified yet, the IP agonist may signal through IP to counteract TXA₂ signaling and suppress the effects of endogenous TXAS products on the formation of infectious HCV. In this in vivo experiment, the effect of drugs waned over time, especially in the case of Ozagrel. We examined the effect of Ozagrel on the secondary HCV propagation in the mice inoculated with the sera of the HCV infected mice treated with Ozagrel in the first drug treatment. The results showed that HCV proliferated in the secondarily infected chimeric mice irrespective of the treatment of Ozagrel (Supplementary Figure 14), suggesting that HCV proliferating in the chimeric mice with the first treatment

acquired the resistance against Ozagrel. We analyzed partial genomic sequences of the drug-resistant HCVs by the direct sequencing method. We found that 68 base substitutions, 10 of which were accompanied with amino acid substitution, were present in such HCV genomes, compared with those in the mice untreated with the drug (Supplementary Figure 15). This indicated that the HCV proliferating in the chimeric mouse treated with Ozagrel included a large number of base substitutions in the genome. Further study of such drug-resistant HCV, for example, the reverse genetic analysis using a recombinant HCV system, will help to reveal the molecular mechanisms of the medicinal effects of this drug and the infectious HCV production. Furthermore, these results show the need to find the optimum dose of TXAS inhibitor for effective therapy and to use this drug as one option with different action mechanisms for multidrug therapy.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.05.014>.

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Transcript Profiling: The microarray data in this study was named "HuSE2, 2Dvs3D," and was registered in ArrayExpress. Accession number: E-MTAB-1491.

Nucleic acid sequences: Sequencing data in this study were named "Bankit1626925 Seq1" and "Bankit1626925 Seq3", and registered with GenBank. Accession numbers: KF006982 and KF006984, respectively.

Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Preparation of Subcellular Fraction and Protein Detection With Western Blotting

Subcellular fractions of HuS-E/2 cells and patient's tissues were prepared with the ProteoExtract Subcellular proteome Extraction Kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Five micrograms of total protein of each fraction or whole-cell lysate of each cell was analyzed by Western blotting. Western blotting was performed as described previously.¹

Collection of Total RNA and Cell Lysate From HCV-Infected Patients' Tissue

Total RNA from patients' tissue was collected with RNeasy mini (Qiagen, Hilden, Germany). In brief, frozen tissues were homogenized in lysis buffer with a Power Masher (Nippi, Tokyo, Japan). Homogenized samples were used for RNA purification according to the manufacturer's protocol. Cell lysate from tissues were collected with RIPA buffer (Thermo Scientific, Waltham, MA) or the ProteoExtract Subcellular proteome Extraction Kit according to the manufacturer's protocol.

cAMP Reporter Assay

Huh-7-derived and HuS-E/2 cells were transfected with pCRE-Luc (Agilent Technologies, Santa Clara, CA) using Fugene6 (Roche) and Effectene (Qiagen), respectively, essentially according to the manufacturers' protocols. Six hours and 2 days post-transfection of Huh-7-derived and HuS-E/2 cells, respectively, the culture medium was replaced with fresh medium containing one of the reagents. One and 3 day(s) post-transfection of Huh-7-derived and HuS-E/2 cells, respectively, luciferase activity in the cells was measured using a luciferase activity detection reagent (Promega, Madison, WI) and a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

Calcium Ion Quantification

HEK293, Huh-7-derived, and HuS-E/2 cells were treated with the calcium ionophore A23187 (Sigma-Aldrich) and the TP agonist U-46619 for 1 day. Calcium ion concentrations were quantified using a calcium assay kit (Cayman Chemical) according to the manufacturer's protocols.

Actin Polymerization Assay

Activation of actin polymerization via TP was measured with fluorescein isothiocyanate-phalloidin (Sigma-Aldrich). After culture in lipid-free fresh medium, cells were stimulated with 10 $\mu\text{mol/L}$ U46619 containing medium for 30, 60, and 180 seconds. Then, samples were stained with 10 $\mu\text{g/mL}$ fluorescein isothiocyanate phalloidin. Fluorescent intensity at 520 nm was measured.

Fatty Acid Analysis

Fatty acid analysis of HCV-infected Huh-7.5 cells treated with or without Ozagrel was performed by Toray Research Center, Inc, Tokyo, Japan using gas chromatography. Total fatty acid samples were extracted from the cells according to the Bligh-Dyer² method.

Secondary Infection Experiments in Chimeric Mice Transplanted Human Hepatocytes

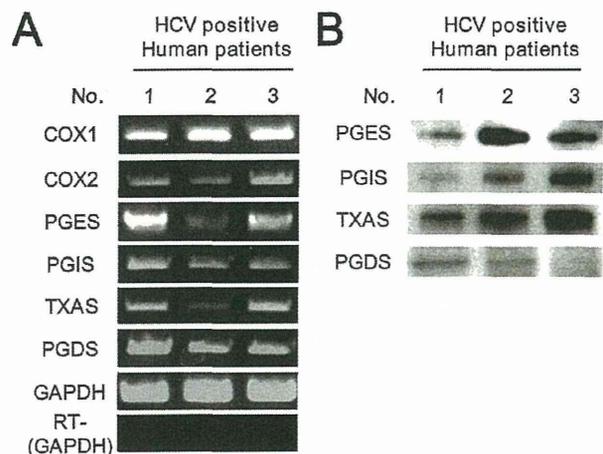
The chimeric mice were inoculated intravenously with patient serum including 1.0×10^5 genome titer of bbHCV (genotype 1b) as the first infection. Ozagrel was administered orally twice each day (300 $\mu\text{g/day}$) 1 week after the inoculation. The serum samples from those mice were collected at 5 weeks after starting the drug treatments, and used as inocula in the secondary infection experiment. Naive chimeric mice were inoculated with the collected chimeric mice serum including 1.0×10^5 genome titer of HCV. Administration of Ozagrel was started simultaneously. HCV-RNA levels in the blood of the chimeric mice at 1, 2, and 3 weeks after infection in secondary infection experiments were evaluated by qRT-PCR.

Determination of Nucleotide Sequence of HCV Genome After Treatment With Ozagrel

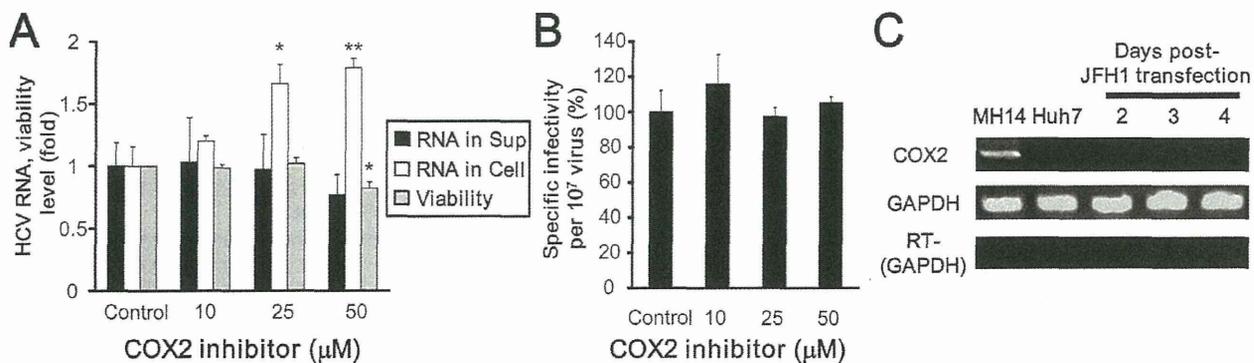
Chimeric mice were inoculated secondarily with sera from HCV-infected chimeric mice with or without Ozagrel treatment. Sera of these chimeric mice treated with or without Ozagrel were collected 5 weeks after the inoculation and the start of the treatment. HCV genome sequences of these samples were determined by the direct sequencing method according to the protocol described previously.³ HCV genomic sequences obtained from sera of mice with 2 different types of treatment were compared with each other. Mice with 2 different types of treatments were as follows: first, mice inoculated secondarily with sera from the first chimeric mouse without treatment were not treated with the drug (BankIt1626925 Seq3 in GenBank). Second, mice inoculated secondarily with sera from the first chimeric mouse with treatment were treated with the drug (BankIt1626925 Seq1 in GenBank). These sequencing data were registered with GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Supplementary References

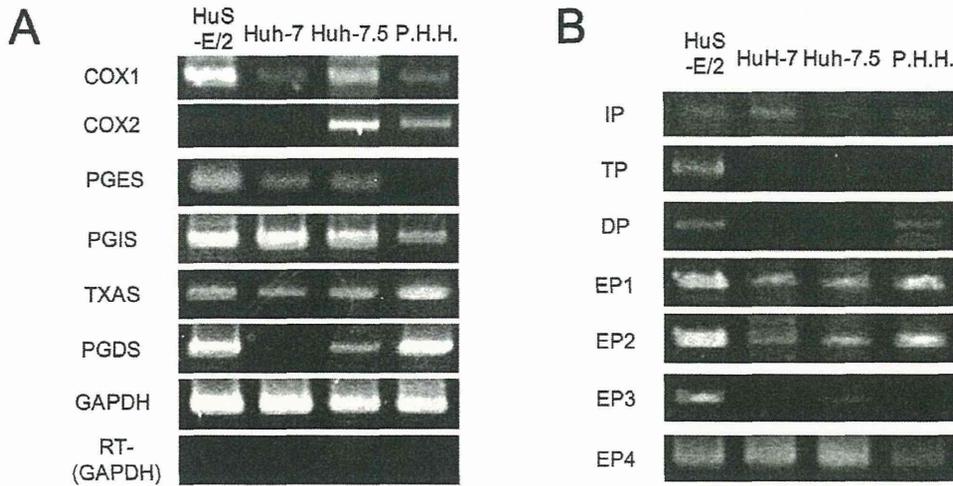
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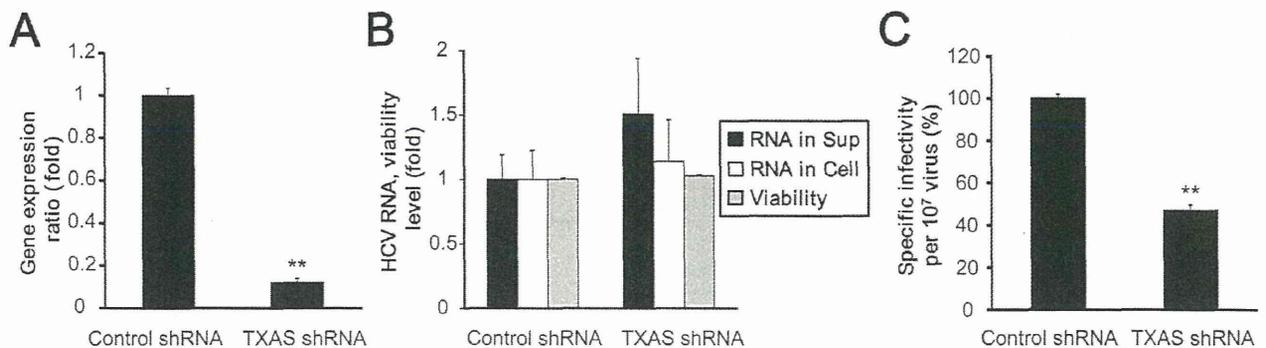
Supplementary Figure 1. Protein and mRNA levels of PG synthases in HCV-infected patient tissue. (A and B) mRNA expression and protein levels of PG synthases in HCV-infected patient tissue. Representative results from 2 independent experiments are shown.



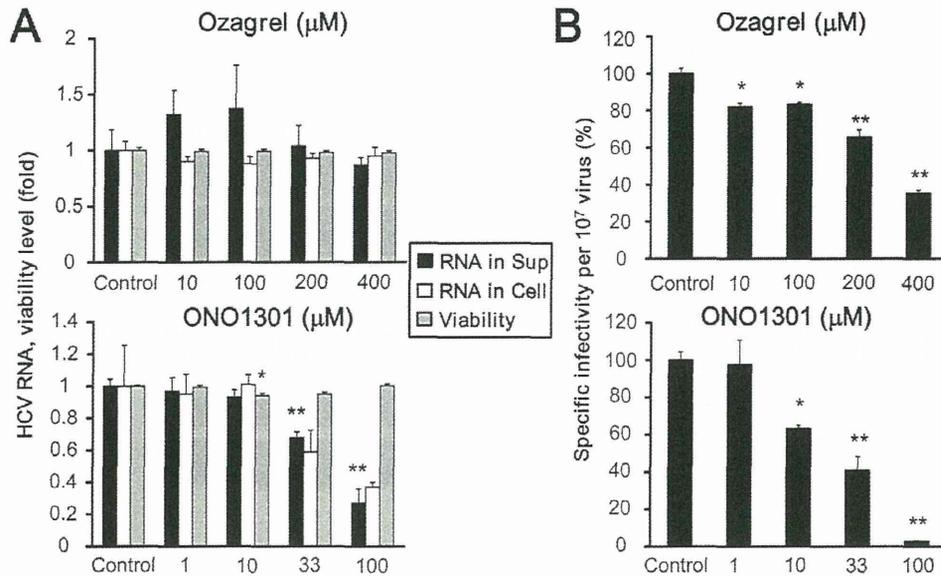
Supplementary Figure 2. Effects of COX2 inhibitor 1 on infectious HCV production. (A) Effects of COX2 inhibitor 1 on HCV-RNA levels in the HCVcc-producing cell-culture system. Levels of HCV RNA in medium (*black bars*) and cells (*white bars*) treated with or without COX2 inhibitor 1 were assessed with qRT-PCRs and plotted as amounts relative to results observed with control cells (control). Mean cell viability \pm SD for each sample condition also is plotted (*gray bars*). (B) Effects of COX2 inhibitor 1 on the infectivity of HCVcc produced using the cell-culture system. (C) Expression of COX2 mRNA in MH14 (positive control), Huh-7, and JFH1-transfected Huh-7 cells. *Differs from control, $P < .01$; **differs from control, $P < .001$.



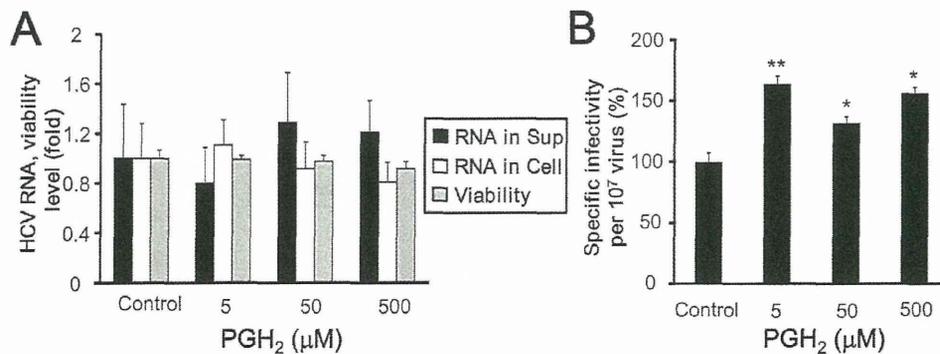
Supplementary Figure 3. Expression of PG synthase and PG-receptor mRNA in immortalized and primary hepatocyte cell lines. (A and B) mRNA expression levels of various PG synthases and PG receptors in HuS-E/2 cells, Huh-7 cells, Huh-7.5 cells, and primary human hepatocytes were analyzed in RT-PCRs. Representative results from 2 independent experiments are shown.



Supplementary Figure 4. Effects of short hairpin RNA (shRNA)-mediated knockdown of TXAS mRNA levels on infectious HCV production. (A) Knockdown of TXAS mRNA levels using shRNA. (B) Effects of TXAS-specific shRNA on HCV-RNA levels in the HCVcc-producing cell culture system. Levels of HCV RNA in medium (black bars) and cells (white bars) treated with control or TXAS-specific shRNA were assessed with qRT-PCRs and plotted as amounts relative to results observed with control shRNA-treated cells (control). Mean cell viability \pm SD for each sample condition also is plotted (gray bars). (C) Effects of TXAS-specific shRNA on the infectivity of HCVcc produced using the cell-culture system. **Differs from control, $P < .001$.



Supplementary Figure 5. Effects of Ozagrel and ONO1301 on the infectivity of HCVcc produced from J6/JFH1-transfected Huh-7.5 cells. (A) Effects of Ozagrel (upper panel) and ONO1301 (lower panel) on HCV-RNA levels in HCVcc-producing cell cultures. Levels of HCV RNA in the medium (black bars) and cells (white bars) treated with Ozagrel or ONO1301 cells were assessed in qRT-PCRs and plotted as the amount relative to results from untreated cells (control). Mean cell viability \pm SD for each sample condition also is plotted (gray bars). (B) Effects of Ozagrel (upper panel) and ONO1301 (lower panel) on the infectivity of HCVcc produced in the cell-culture system. *Differs from control, $P < .01$; **differs from control, $P < .001$.



Supplementary Figure 6. Effects of PGH₂ on infectious HCV production. (A) Effects of PGH₂ on HCV-RNA levels in the HCVcc-producing cell-culture system. Levels of HCV RNA in medium (black bars) and cells (white bars) treated with or without PGH₂ were assessed with qRT-PCRs and plotted as amounts relative to results observed with control cells (control). Mean cell viability \pm SD for each sample condition also is plotted (gray bars). (B) Effects of PGH₂ on the infectivity of HCVcc produced using the cell culture system. *Differs from control, $P < .01$; **differs from control, $P < .001$.