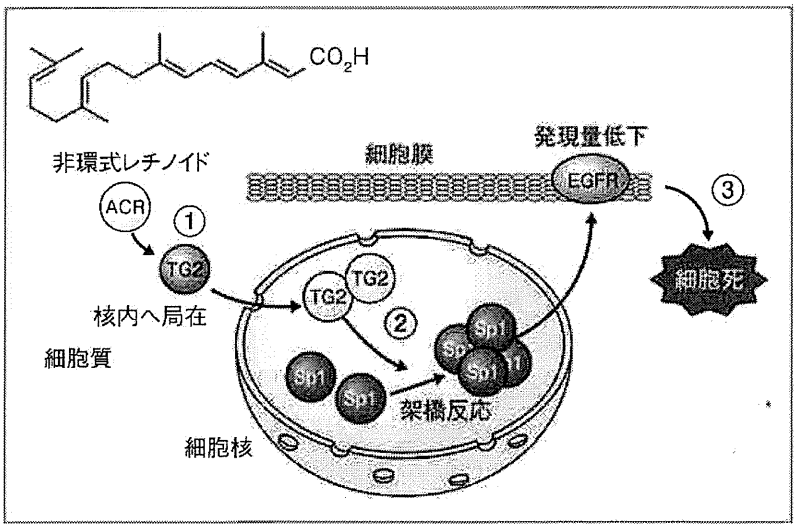

図1　TG2を介したアポトーシス誘導機構[6,10]

非環式レチノイドは，①通常は肝癌細胞の細胞質に存在するTG2が核内へ局在し，②核内TG2が遺伝子発現に欠かせない転写因子“Sp1”をのり付けするかのように結合させ(架橋反応)，Sp1の機能が失われることがわかった．さらに，③Sp1が機能不全になることにより上皮細胞増殖因子受容体“EGFR”の発現が低下し，肝癌細胞が死に至ることも明らかとなった．

制作用である．培養細胞の実験では正常肝細胞株Hcでは非環式レチノイドによる細胞死誘導作用がみられず，肝癌細胞株JHH7，Huh7，HepG2でのみ細胞死誘導作用が認められた[6]．Field cancerization[7]という概念によって一次肝細胞癌がみられた時点ではすでに肝内に高癌化状態にあるか，あるいはすでに癌化した顕微鏡レベルの細胞集団(クローン)が存在するといわれており，非環式レチノイドは選択的にこのような二次発癌の“芽”を肝から除出し，あるいは肝癌細胞の細胞死を誘導すると考えられている．

本稿では，より効果的な発癌予防法(薬)を開発するために著者らのグループで行われた非環式レチノイドの癌特異的増殖抑制作用のメカニズムの解明および癌治療の標的となる分子の同定などの基礎研究について最新の知見を紹介する．

Genomic制御：蛋白質架橋酵素トランスグルタミナーゼによる細胞死の制御

著者らのグループは，アルコール性肝障害，アルコール性脂肪性肝炎(ASH)および非アルコール性脂肪性肝炎(NASH)などの肝疾患の病態形成過程では蛋白質架橋酵素トランスグルタミナーゼ(Transglutaminase：TG)を介する転写因子Sp1の架橋不活性化によって肝細胞の生存シグナルにたいへん重要な役割をもつ肝細胞増殖因子受容体c-Metの発現が低下し，肝細胞が死に至るという肝細胞死経路を見出した[8,9]．TGはすべての組織・細胞において発現され，蛋白質架橋反応とその逆反応のみならず，蛋白質のpolyamine反応，Gln残基のアミド反応，蛋白質と脂肪間のエステル結合反応，GTP水解反応，細胞接着反応，さらに蛋白質ジスルフィド結合異性化反応など，多彩な機能を発揮する多機能性酵素ファミリーの代表である[10]．ヒトでは9種類のTG遺伝子が同定され，このうち古くから細胞死にかかわるのはTG2である．TG2は生体構造の構築や安定化を行う一方，細胞の増殖・分化，アポトーシスに働き，多様な生命現象，さらに動脈硬化，脳神経変性疾患，肝疾患など，幅広く病態形成に関連している．

著者らは，古くからレチノイドがもっとも強いTG2発現誘導剤であることが知られていたことからTG2を介する細胞死経路に着目し，非環式レチノイドが肝癌細胞死誘導時にどのような働きをするのかを調べた[6]．非環式レチノイド処理した肝癌細胞株JHH7では，TG2の発現ならびに核局在が誘導され，アンチセンスTG2を細胞内に発現させて内在性TG2の機能を抑制させると非環式

レチノイドによる細胞死を部分的に抑制した．そこで，非環式レチノイド処理により変化する細胞死や肝機能障害関連遺伝子を調べたところ，肝癌細胞の異常増殖に必須な上皮細胞増殖因子受容体(epidermal growth factor receptor：EGFR)の発現が顕著に低下していることを見出した．EGFRプロモーター配列を上流に挿入したルシフェラーゼ発現プラスミドをJHH7細胞に導入し，EGFRの遺伝子発現が転写因子Sp1の架橋不活性化によって調節されるかどうかについて調べたところ，Sp1の過剰発現は非環式レチノイドによるEGFRのトランス活性化活性(transactivation activity)の抑制が緩和されたことがわかった．Sp1は非常に広範囲の遺伝子発現にかかわる基本転写因子のひとつであり，Sp1欠損マウスは胎性致死を示すことから，生体の維持に重要な因子であることがわかっている．またRNA干渉を用いてJHH7細胞のEGFRあるいはSp1遺伝子発現をノックダウンすると，細胞死が誘導されることがわかった．つぎに，非環式レチノイドの刺激によってTG2が細胞核に局在し，Sp1を架橋し，その活性が消失することで，Sp1が調節しているEGFRの発現が低下し，肝癌細胞死が起こるという一連の過程が実際に生体内で起きているのかどうかを，ヒト肝癌細胞を移植したマウスモデルとN-diethylnitrosamine(DEN)を用いた化学誘導性肝発癌ラットモデルの肝組織を用いて検討した．著者らが開発したTG2の作用で生成する不活性型Sp1架橋体を特異的に認識する抗体を用いた実験では，非環式レチノイドを投与することによってTG2が細胞核に蓄積することや，Sp1架橋体が生成されることがわかった．これらの結果は細胞レベルでみられた一連の現象が生体内でも起こっていることを示唆する結果である(図1)．

Non-genomic制御：レチノイド核内受容体RXRαの異常過リン酸化の抑制

レチノイドには核内受容体を介さない生物活性(non-genomic action)も知られている．著者らは岐阜大学との共同研究により非環式レチノイドによるレチノイド核内受容体であるRXRαの過リン酸化反応の抑制とそれによるRXRαの転写因子活性の回復が肝癌(幹)細胞の異常増殖抑制，さらには細胞死誘導に大切であることを報告した[11,12]．肝の発癌過程にはレチノイドの代謝異常が深くかかわっていることが古くから知られている[1]．肝細胞癌患者癌部ではレチノイドが欠乏状態にあること，またはRXRαが過リン酸化によって不活性化し，ユビキチン化による分解を免れて蓄積し，細胞増殖制御遺伝子の発現が損なわれており，これが癌化の一原因と考えられた[11]．特異的リン酸化酵素阻害剤を用いた検索から，ヒト肝癌細胞ではmitogen-activated protein(MAP)キナーゼファミリーのひとつであるextracellular signal-regulated kinase(Erk)がRXRαのセリン(S)残基とスレオニン(T)残基をリン酸化させることがわかった[11]．非環式レチノイド処理した肝癌細胞株Huh7ではErkの活性が有意に抑制され，RXRαのリン酸化も抑制されたことが判明した．それに対して，RXRの内在性リガンドである9-シスレチノイン酸(9cRA)ではHuh7細胞内RXRαのリン酸化抑制も，Erk活性の抑制も認められなかった．また，非環式レチノイドはErkの上流にあるRasの活性も抑制したことを見出した．そこでつぎに，Huh7細胞内のRXR-response elementを介した転写活性をluciferase reporter assayで検討したところ，非環式レチノイドは単独ではRXRの転写活性を誘導しなかったが，9cRAの存在下では相乗的に転写活性を誘導することが証明された．さらに，非環式レチノイドと9cRAは相乗的にHuh7細胞の増殖を抑制することがわかった．また，非リン酸化型のRXRαを肝癌細胞に導入すると9cRA単独でも非環式レチノイドの存在下と同様の効果が認められた[12]．これらの結果より，非環式レチノイドはRXRαのリガンドとしての作用(genomic action)に加えて肝癌細胞の過剰RXRαリン酸化を抑制することでRXRαの働きを正常化し，TG2などの異常細胞増殖抑制因子の発現を回復させ，正常肝細胞には影響を与えずして肝癌(幹)細胞に選択的な細胞死を引き起こすことが示唆された(図2)．

また著者らは，非環式レチノイドが血管内皮細胞増殖因子(vascular endothelial growth factor：VEGF)/MAPキナーゼ経路阻害によって腫瘍血

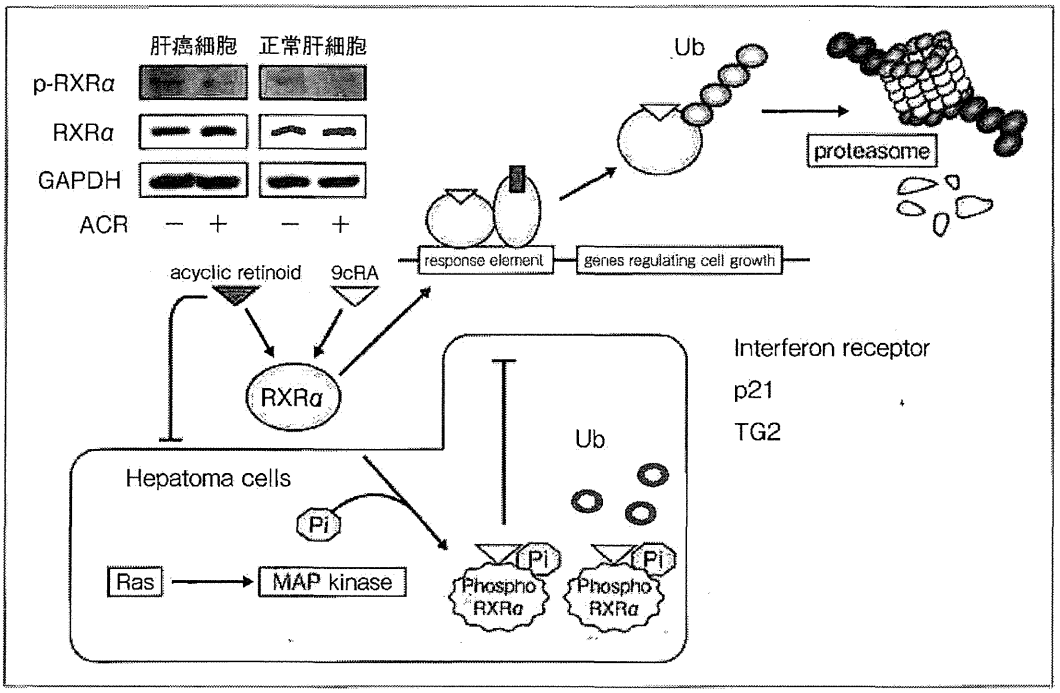

図 2　レチノイド核内受容体RXRαの異常リン酸化の抑制[6,11,12]

非環式レチノイドは肝癌細胞の過剰RXRαリン酸化(p-RXRα)を抑制することでRXRαの働きを正常化し，TG2などの異常細胞増殖抑制因子の発現を回復させ，正常肝細胞には影響を与えずして肝癌(幹)細胞に選択的な細胞死を引き起こす．

管新生選択的に抑制することを見出した[13]．さらにほかのグループの研究によって，非環式レチノイドは活性酵素の働きを抑える抗酸化作用と[14,15]，線維芽細胞増殖因子受容体(FGFR)3[16]，C-X-C chemokine receptor type 4(CXCR-4)[17]，血小板由来増殖因子(PDGF)-C[18]などの標的遺伝子の発現制御によってヒト免疫不全症ウイルス(HIV)感染や肝線維化・肝発癌の抑制などの多様なnon-genomicとgenomicな作用を示すことがわかってきた．

サイドメモ　オミックス解析

オミックス(omics)解析はギリシャ語の“すべて・完全”などを意味する接尾辞(ome)に“学問”を意味する接尾辞(ics)を合成した言葉で，生物の分子全体を包括的に解析する学問である．測定の対象によってゲノミクス(遺伝子)，トランスクリプトミクス(遺伝子の発現)，プロテオミクス(蛋白質)，メタボロミクス(代謝物)とよばれる．これらのオミックス解析の結果得られる大量の情報を分子ネットワーク/パスウェイに結びつけ，人間の病気とその治療法を分子レベルのシステムとして理解することによって新規創薬標的分子の網羅的探索が行われている．

網羅的オミックス解析の展開

近年，生命現象を包括的に研究するオミックス(Omics)解析(「サイドメモ」参照)に基づく創薬標的の網羅的探索が盛んに行われている．細胞や生体内代謝物質の総体を対象として包括的に観測することは，メタボローム解析(Metabolomic analysis)とよばれる．病態などの表現形質発現に直結する特異的な脂質，糖，アミノ酸，核酸関連物質などの代謝産物群を体系的あるいは網羅的に解析し，癌の早期診断や予後診断，および有効な治療薬・治療法の開発につながる新規マーカー候補となる代謝物・代謝経路を同定する．著者らは抽出操作を必要としない核磁気共鳴スペクトル(NMR)法とイオン性代謝物を網羅的に測定しうるキャピラリー電気泳動-飛行時間型質量分析(CE-TOFMS)系などのメタボローム解析系を用いて，非環式レチノイドの肝細胞癌に対する選択的増殖抑制作用の分子機構の解明に着目した[19]．

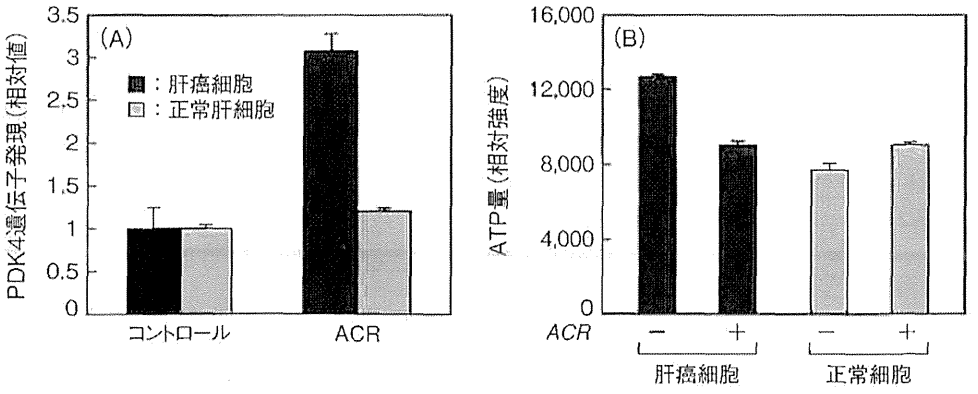

図 3　メタボローム解析を用いた非環式レチノイドの標的分子探索[19]
非環式レチノイドはPDK4依存性シグナル伝達経路(A)によって肝癌細胞選択的にエネルギー代謝(ATP合成)を阻害する(B).

その結果，肝癌細胞株JHH7および正常肝細胞株Hcから229種類の代謝物質が同定された．これらの物質は，解糖系/糖新生，ペントースリン酸経路，クエン酸回路，尿素回路，プリン代謝経路，ピリミジン代謝経路，ニコチン酸・ニコチンアミド代謝経路および各種アミノ酸代謝経路とかかわることがわかった．JHH7細胞とHc細胞の代謝プロファイリングを比較したところ，71種類の代謝物質が有意に異なることが明らかになった．そのうち49種類の代謝物質がJHH7細胞で非環式レチノイド処理によって有意に抑制された．非環式レチノイドによって癌特異的に変動する代謝経路のパスウェイ解析にはIngenuity Pathway Analysis (IPA)プログラムを使用した．査読ありの文献から得られた細胞・生体内代謝経路と関連した代謝物質の変動比率をIPAデータベースの代謝物質数を母集団としてFisher's正確確率検定によって算出し，“tRNA Charging”“Purine Nucleotides *De Novo* Biosynthesis Ⅱ”“Pyrimidine Ribonucleotides *De Novo* Biosynthesis”などのアミノ酸代謝やヌクレオチド合成と関連するパスウェイを抽出した．非環式レチノイドが，これらの経路から癌細胞の活発な増殖のために必要な蛋白質や核酸の供給を阻害するのではないかと考えた．

興味深いことに，細胞のエネルギー源であるATPの産生はJHH7細胞でHc細胞より約1.6倍高く，非環式レチノイド処理によってほぼHc細胞と同じレベルに抑制された．その分子メカニズムを解明するために，癌細胞のエネルギー代謝とかかわる遺伝子の発現を調べた．その結果，エネルギー代謝調節鍵酵素pyruvate dehydrogenase kinase 4(PDK4)の遺伝子発現が非環式レチノイドによって肝癌細胞選択的に誘導されることが見出された．非環式レチノイド処理によってJHH7細胞ではPDK4遺伝子発現が約3倍に誘導されるのに対して，Hc細胞ではまったく変化しなかった(図3)．PDK4は脂質，糖代謝調節の鍵酵素として知られ，ピルビン酸からアセチルCoAへの合成を阻害することで，細胞内でのエネルギー源を糖質から脂質へと変換し代謝を調節する．癌治療においてPDK4の役割には不明なことが多いが，乳癌細胞のPDK4遺伝子を過剰発現させることによって細胞内のATPレベルが減少し，デノボ脂質生成および細胞増殖が抑制されることが報告されている[20]．著者らはRNA干渉を用いてJHH7細胞のPDK4遺伝子発現をノックダウンすると，非環式レチノイドによって減少された細胞内のATPレベルがレスキューされたことを見出した．アメリカ国立生物工学情報センター(National Center for Biotechnology Information：NCBI)のGEO(Gene Expression Omnibus)データベースに登録されたマイクロアレイデータを用いて臨床検体におけるPDK4遺伝子の発現を調べたところ，肝細胞癌患者の非癌部肝組織(n=243)に比べて肝癌組織(n=268)でのPDK4遺伝子発現が有意に減少していることがわかった．PDK4遺伝子発現の低下が肝細胞癌の発癌とかかわることが示唆された．これらの結果より，非環式レチノイドは

肝癌細胞選択的にエネルギー代謝を阻害し，細胞増殖を抑制しており，その分子メカニズムのひとつはPDK4依存性シグナル伝達経路に関連することがわかった．

おわりに

非環式レチノイドは正常肝細胞には影響せず，肝癌(幹)細胞選択的に異常増殖を抑制し，世界初の肝細胞癌再発予防剤として大きく期待されている．その作用機構として，TG2を介する転写因子Sp1の架橋不活性化によって異常細胞増殖シグナルの抑制というgenomicな作用とRXRαの過リン酸化反応の抑制とそれによるRXRαの転写因子活性の回復というnon-genomicな作用の二重の作用を有することがわかった．さらに，網羅的オミックス解析を用いてPDK4依存的に肝癌細胞のエネルギー代謝を阻害するという新しい非環式レチノイドの標的経路も見出された．これらの標的分子についてさらなる研究が進み，“癌の芽(クローン)”を完全除出する治療法が開発されることを願っている．

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*　　*　　*

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Dysregulation of Retinoic Acid Receptor Diminishes Hepatocyte Permissiveness to Hepatitis B Virus Infection through Modulation of Sodium Taurocholate Cotransporting Polypeptide (NTCP) Expression*

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Senko Tsukuda[‡§], Koichi Watashi[‡1], Masashi Iwamoto[‡], Ryosuke Suzuki[‡], Hideki Aizaki[‡], Maiko Okada[¶], Masaya Sugiyama[‖], Soichi Kojima[§], Yasuhito Tanaka[**], Masashi Mizokami[‖], Jisu Li[‡‡], Shuping Tong[‡‡], and Takaji Wakita[‡]

From the [‡]Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, the [§]Micro-signaling Regulation Technology Unit, RIKEN Center for Life Science Technologies, Wako 351-0198, Japan, the [¶]Department of Translational Oncology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan, the [‖]Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa 272-8516, Japan, the [**]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medicinal Sciences, Nagoya 467-8601, Japan, and the [‡‡]Liver Research Center Rhode Island Hospital, Warren Alpert School of Medicine, Brown University, Providence, Rhode Island 02912

Background: Host factors regulating hepatitis B virus (HBV) entry receptors are not well defined.
Results: Chemical screening identified that retinoic acid receptor (RAR) regulates sodium taurocholate cotransporting polypeptide (NTCP) expression and supports HBV infection.
Conclusion: RAR regulates NTCP expression and thereby supports HBV infection.
Significance: RAR regulation of NTCP can be a target for preventing HBV infection.

Sodium taurocholate cotransporting polypeptide (NTCP) is an entry receptor for hepatitis B virus (HBV) and is regarded as one of the determinants that confer HBV permissiveness to host cells. However, how host factors regulate the ability of NTCP to support HBV infection is largely unknown. We aimed to identify the host signaling that regulated NTCP expression and thereby permissiveness to HBV. Here, a cell-based chemical screening method identified that Ro41-5253 decreased host susceptibility to HBV infection. Pretreatment with Ro41-5253 inhibited the viral entry process without affecting HBV replication. Intriguingly, Ro41-5253 reduced expression of both NTCP mRNA and protein. We found that retinoic acid receptor (RAR) regulated the promoter activity of the human *NTCP* (h*NTCP*) gene and that Ro41-5253 repressed the hNTCP promoter by antagonizing RAR. RAR recruited to the h*NTCP* promoter region, and nucleotides −112 to −96 of the h*NTCP* was suggested to be critical for RAR-mediated transcriptional activation. HBV susceptibility was decreased in pharmacologically RAR-inactivated cells. CD2665 showed a stronger anti-HBV potential and disrupted the spread of HBV infection that was achieved by continuous reproduction of the whole HBV life cycle. In addition, this mechanism was significant for drug development, as antagonization of RAR blocked infection of multiple HBV genotypes and also a clinically relevant HBV mutant that was resistant to nucleoside analogs. Thus, RAR is crucial for regulating NTCP expression that determines permissiveness to HBV infection. This is the first demonstration showing host regulation of NTCP to support HBV infection.

Hepatitis B virus (HBV)[2] infection is a major public health problem, as the virus chronically infects ~240 million people worldwide (1–3). Chronic HBV infection elevates the risk for developing liver cirrhosis and hepatocellular carcinoma (4–6). Currently, two classes of antiviral agents are available to combat chronic HBV infection. First, interferon (IFN)-based drugs, including IFNα and pegylated-IFNα, modulate host immune function and/or directly inhibit HBV replication in hepatocytes (7, 8). However, the antiviral efficacy of IFN-based drugs is restricted to less than 40% (9, 10). Second, nucleos(t)ide analogs, including lamivudine (LMV), adefovir, entecavir (ETV), tenofovir, and telbivudine suppress HBV by inhibiting the viral reverse transcriptase (11, 12). Although they can provide significant clinical improvement, long term therapy with nucleos(t)ide analogs often results in the selection of drug-resistant mutations in the target gene, which limits the treatment outcome. For example, in patients treated with ETV, at least three mutations can arise in the reverse transcriptase sequence of the

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[1] To whom correspondence should be addressed: Dept. of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan. Tel.: 81-3-5285-1111; Fax: 81-3-5285-1161; E-mail: kwatashi@nih.go.jp.

[2] The abbreviations used are: HBV, hepatitis B virus; NTCP, sodium taurocholate cotransporting polypeptide; RAR, retinoic acid receptor; LMV, lamivudine; ETV, entecavir; HB, HBV surface protein; SLC10A1, solute carrier protein 10A1; hNTCP, human NTCP; ATRA, all-*trans*-retinoic acid; SHP, small heterodimer partner; ASBT, apical sodium-dependent bile salt transporter; RARE, RAR-responsive element; RXR, retinoid X receptor; SEAP, secreted alkaline phosphatase; FXR, farnesoid X receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nt, nucleotide; cccDNA, covalently closed circular DNA.

polymerase L180M and M204V plus either one of Thr-184, Ser-202, or Met-250 codon changes to acquire drug resistance (13). Therefore, development of new anti-HBV agents targeting other molecules requires elucidation of the molecular mechanisms underlying the HBV life cycle.

HBV infection of hepatocytes involves multiple steps. The initial viral attachment to the host cell surface starts with a low affinity binding involving heparan sulfate proteoglycans, and the following viral entry is mediated by a specific interaction between HBV and its host receptor(s) (14). Recently, sodium taurocholate cotransporting polypeptide (NTCP) was reported as a functional receptor for HBV (15). NTCP interacts with HBV large surface protein (HBs) to mediate viral attachment and the subsequent entry step. NTCP, also known as solute carrier protein 10A1 (SLC10A1), is physiologically a sodium-dependent transporter for bile salts located on the basolateral membrane of hepatocytes (16). In the liver, hepatocytes take up bile salts from the portal blood and secrete them into bile for enterohepatic circulation, and NTCP-mediated uptake of bile salts into hepatocytes occurs largely in a sodium-dependent manner. Although NTCP is abundant in freshly isolated primary hepatocytes, it is weakly or no longer expressed in most cell lines such as HepG2 and Huh-7, and these cells rarely support HBV infection (17, 18). In contrast, primary human hepatocytes, primary tupaia hepatocyte, and differentiated HepaRG cells, which are susceptible to HBV infection, express significant levels of NTCP (19). Thus, elucidation of the regulatory mechanisms for *NTCP* gene expression is important for understanding the HBV susceptibility of host cells as well as for developing a new anti-HBV strategy. HBV entry inhibitors are expected to be useful for preventing *de novo* infection after liver transplantation, for post-exposure prophylaxis, or for vertical transmission by short term treatment (20, 21).

In this study, we used a HepaRG-based HBV infection system to screen for small molecules capable of decreasing HBV infection. We found that pretreatment of host cells with Ro41-5253 reduced HBV infection. Ro41-5253 reduced NTCP expression by repressing the promoter activity of the human *NTCP* (h*NTCP*) gene. Retinoic acid receptor (RAR) played a crucial role in regulating the promoter activity of h*NTCP*, and Ro41-5253 antagonized RAR to reduce *NTCP* transcription and consequently HBV infection. This and other RAR inhibitors showed anti-HBV activity against different genotypes and an HBV nucleoside analog-resistant mutant and moreover inhibited the spread of HBV. This study clarified one of the mechanisms for gene regulation of NTCP to support HBV permissiveness, and it also suggests a novel concept whereby manipulation of this regulation machinery can be useful for preventing HBV infection.

EXPERIMENTAL PROCEDURES

Reagents—Heparin was obtained from Mochida Pharmaceutical. Lamivudine, cyclosporin A, all-*trans*-retinoic acid (ATRA), and TO901317 were obtained from Sigma. Entecavir was obtained from Santa Cruz Biotechnology. Ro41-5253 was obtained from Enzo Life Sciences. PreS1-lipopeptide and FITC-labeled preS1 were synthesized by CS Bio. IL-1β was purchased from PeproTech. CD2665, BMS195614, BMS493, and MM11253 were purchased from Tocris Bioscience.

Cell Culture—HepaRG cells (BIOPREDIC) and primary human hepatocytes (Phoenixbio) were cultured as described previously (19). HepG2 and HepAD38 cells (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center) (22) were cultured with DMEM/F-12 + GlutaMAX (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, and 5 μg/ml insulin. HuS-E/2 cells (kindly provided by Dr. Kunitada Shimotohno at National Center for Global Health and Medicine) were cultured as described previously (23).

Plasmid Construction—phNTCP-Gluc, pTK-Rluc was purchased from GeneCopoeia and Promega, respectively. pRARE-Fluc was generated as described (25). For constructing phNTCP-Gluc carrying a mutation in a putative RARE (nt −491 to −479), the DNA fragments were amplified by PCR using phNTCP-Gluc as a template with the following primer sets: F1, 5′-CAGATCTTGGAATTCCCAAAATC-3′ and 5′-GAGGGGATGTGTCCATTGAAATGTTAATGGGAGCT-GAGAGGATGCCAGTATCCTCCCT-3′ and primer sets 5′-CTCTCAGCTCCCATTAACATTTCAATGGACACATCC-CCTCCTGGAGGCCAGTGACATT-3′ and R6, 5′-CTCGGT-ACCAAGCTTTCCTTGTT-3′. The resultant products were further amplified by PCR with F1 and R6 and then inserted into the EcoRI/HindIII sites of phNTCP-Gluc to generate phNTCP Mut(−491 to −479)-Gluc. Other promoter mutants were prepared by the same method using the following primer sets: F1, 5′-GTGGGTTATCATTTGTTTCCCGAAAACATTAG-AGTGAAAGGAGCTGGGTGTTGCCTTTGG-3′ and 5′-TCCTTTCACTCTAATGTTTTCGGGAAACAAATGATA-ACCCACTGGACATGGGGAGGGCAC-3′; R6 for −368 to −356; F1 and 5′-AATCTAGGTCCAGCCTATTTAAGTCC-CTAAATTTCCTTTTCCCAGCTCCGCTCTTGATTCCTT-3′, 5′-CTGGGAAAAGGAAATTTAGGGACTTAAATAGG-CTGGACCTAGATTCAGGTGGGCCCTGGGCAG-3′, and R6 for −274 to −258; F1 and 5′-TTCTGGGCTTATTTCTA-TATTTTGCAATCCACTGAGTGTGCCTCATGGGCATT-CATTC-3′, 5′-CACACTCAGTGGATTGCAAAATATAGA-AATAAGCCCAGAAGCAGCAAAGTGACAAGGG-3′, and R6 for −179 to −167; F1 and 5′-AGCTCTCCCAAGCTCAA-AGATAAATGCTAGTTTCCTGGGTGCTACTTGTACTC-CTCCCTTGTC-3′, 5′-GTAGCACCCAGGAAACTAGCAT-TTATCTTTGAGCTTGGGAGAGCTAGGGCAGGCAGAT-AAGGT-3′, and R6 for −112 to −96, respectively. For constructing the hNTCP promoter carrying these five mutations (5-Mut), five DNA segments were amplified using the primers as follows: segment 1, F1 and 5′-GAGGGGATGTGTCCATG-ACC-3′; segment 2, 5′-AGCTCCTTTCACTCTCATGGGT-3′ and 5′-TCCTTTTCCCAGCTCCGC-3′; segment 3, 5′-GAG-CTGGGAAAAGGAGCTGC-3′ and 5′-CCACTGAGTGTG-CCTCATGG-3′; segment 4, 5′-AGGCACACTCAGTGGA-GGG-3′ and 5′-CTGGGTGCTACTTGTACTCCTCC-3′; and segment 5, 5′-CAAGTAGCACCCAGGAATCCA-3′ and R6. For producing a deletion construct for the h*NTCP* promoter, phNTCP (−53 to +108)-Gluc, DNA fragment was amplified using the primer sets 5′-GGTGAATTCTGTTCCTCTTTGG-GGCGACAGC-3′ and 5′-GGTGGTAAGCTTTCCTTGTTC-

TCCGGCTGACTCC-3′ and then inserted into the EcoRI and HindIII sites of phNTCP-Gluc.

HBV Preparation and Infection—HBV was prepared and infected as described (19). HBV used in this study was mainly derived from HepAD38 cells (22). For Fig. 8, *A–E*, we used concentrated (~200-fold) media of HepG2 cells transfected with an expression plasmid for either HBV genotypes A, B, C, D or genotype C carrying mutations at L180M, S202G, and M204V (HBV/Aeus, HBV/Bj35s, HBV/C-AT, HBV/D-IND60, or HBV/C-AT(L180M/S202G/M204V)) (24) and infected into the cells at 2000 GEq/cell in the presence of 4% PEG8000 at 37 °C for 16 h as described previously (19). HBV for Fig. 8*F* (genotype C) was purchased from Phoenixbio.

Real Time PCR and RT-PCR—Real time PCR for detecting HBV DNAs and cccDNA was performed as described (19). RT-PCR detection of mRNAs for *NTCP, ASBT, SHP*, and *GAPDH* was performed with one-step RNA PCR kit (TaKaRa) following the manufacturer's protocol with primer set 5′-AGGGAGGAGGTGGCAATCAAGAGTGG-3′ and 5′-CCGGCTGAAGAACATTGAGGCACTGG-3′ for *NTCP*, 5′-GTTGGCCTTGGTGATGTTCT-3′ and 5′-CGACCCAATAGGCCAAGATA-3′ for *ASBT*, 5′-CAGCTATGTGCACCTCATCG-3′ and 5′-CCAGAAGGACTCCAGACAGC-3′ for *SHP*, and 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′ for *GAPDH*, respectively.

Immunofluorescence Analysis—Immunofluorescence was conducted essentially as described (25) using an anti-HBc antibody (DAKO, catalog no. B0586) at a dilution of 1:1000.

Detection of HBs and HBe Antigens—HBs and HBe antigens were detected by ELISA and chemiluminescence immunoassay, respectively, as described (19).

MTT Assay—The MTT cell viability assay was performed as described previously (19).

Southern Blot Analysis—Isolation of cellular DNA and Southern blot analysis to detect HBV DNAs were performed as described previously (19).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (26, 27). Anti-NTCP (Abcam) (1:2000 dilution), anti-RARα (Santa Cruz Biotechnology) (1:6000 dilution), anti-RARβ (Sigma) (1:6000 dilution), anti-RARγ (Abcam) (1:2000 dilution), anti-RXRα (Santa Cruz Biotechnology) (1:8000 dilution), and anti-actin (Sigma) (1:5000 dilution) antibodies were used for primary antibodies.

Flow Cytometry—1×10^6 primary human hepatocytes were incubated for 30 min with a 1:50 dilution of anti-NTCP antibody (Abcam) and then washed and incubated with a dye-labeled secondary antibody (Alexa Fluor 488, Invitrogen) at 1:500 dilution in the dark. Staining and washing were carried out at 4 °C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide. The signals were analyzed with Cell Sorter SH8000 (Sony).

FITC-preS1 Peptide-binding Assay—Attachment of preS1 peptide with host cells was examined by preS1 binding assay essentially as described previously (28). HepaRG cells treated with or without Ro41-5253 (28) for 24 h or unlabeled preS1 peptide for 30 min were incubated with 40 nM FITC-labeled preS1 peptide (FITC-preS1) at 37 °C for 30 min. After washing the cells twice with culture medium and once with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde. Then the cells were treated with 4% Block Ace (DS Pharma Biomedical) containing DAPI for 30 min.

Reporter Assay—HuS-E/2 cells were transfected with phNTCP-Gluc (GeneCopoeia), a reporter plasmid carrying the *NTCP* promoter sequence upstream of the Gaussia luciferase (Gluc) gene, and pSEAP (GeneCopoeia), expressing the secreted alkaline phosphatase (SEAP) gene, together with or without expression plasmids for RARα, RARβ, RARγ, with RXRα using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cells were stimulated with the indicated compounds for a further 24 h. The activities for Gluc as well as for SEAP were measured using a Secrete-Pair Dual-Luminescence assay kit (GeneCopoeia) according to the manufacturer's protocol, and Gluc values normalized by SEAP are shown.

pRARE-Fluc, carrying three tandem repeats of RAR-binding elements upstream of firefly luciferase (Fluc), and pTK-Rluc (Promega), which carries herpes simplex virus thymidine kinase promoter expressing *Renilla* luciferase (Rluc) (25), were used in dual-luciferase assays for detecting Fluc and Rluc. Fluc and Rluc were measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol, and Fluc activities normalized by Rluc are shown.

For evaluating HBV transcription in Fig. 2*B*, we used a reporter construct carrying HBV enhancer I, II, and core promoter (nt 1039–1788) ("Enh I + II") and that carrying enhancer II and core promoter (nt 1413–1788) ("Enh II"). These were constructed by inserting the corresponding sequences derived from a genotype D HBV in HepG2.2.15 cells into pGL4.28 vector (Promega). pGL3 promoter vector (Promega), which carries SV40 promoter ("SV40") was used as a control.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed using a Pierce-agarose ChIP kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Huh7-25 cells transfected with phNTCP-Gluc together with or without expression plasmids for FLAG-tagged RARa and for RXRa were treated with 5 mg/ml actinomycin D for 2 h. The cells were then washed and treated with or without 2 mM ATRA for 60 min. Formaldehyde cross-linked cells were lysed, digested with micrococcal nuclease, and immunoprecipitated with anti-FLAG antibody (Sigma) or normal IgG. Input samples were also recovered without immunoprecipitation. DNA recovered from the immunoprecipitated or the input samples was amplified with primers 5′-CCCAGGGCCCACCTGAATCTA-3′ and 5′-TAGATTCAGGTGGGCCCTGGG-3′ for detection of NTCP.

RESULTS

Anti-HBV Activity of Ro41-5253—We searched for small molecules capable of decreasing HBV infection in a cell-based chemical screening method using HBV-susceptible HepaRG cells (29). As a chemical library, we used a set of compounds for which bioactivity was already characterized (19). HepaRG cells were pretreated with compounds and then further incubated with HBV inoculum in the presence of compounds for 16 h (Fig. 1*A*). After removing free HBV and compounds by washing, the cells were cultured for an additional 12 days without compounds. For robust screening, HBV infection was monitored by

F1

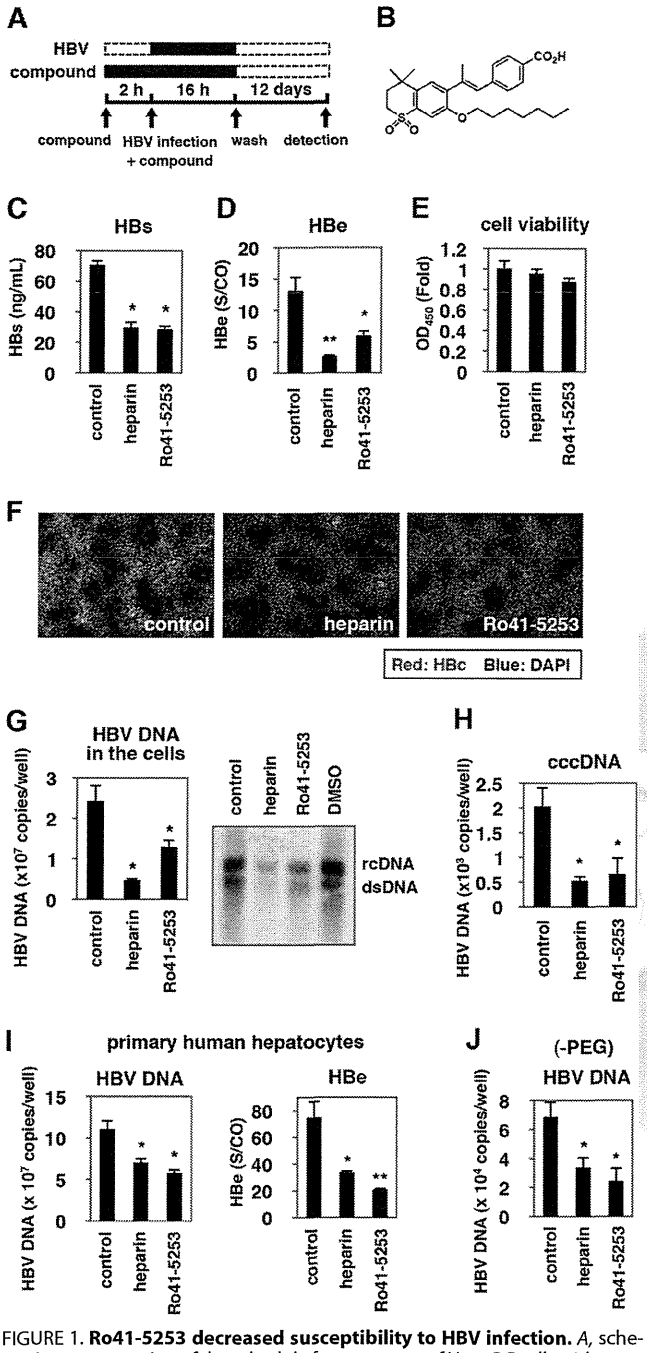

FIGURE 1. **Ro41-5253 decreased susceptibility to HBV infection.** *A*, schematic representation of the schedule for treatment of HepaRG cells with compounds and infection with HBV. HepaRG cells were pretreated with compounds for 2 h and then inoculated with HBV in the presence of compounds for 16 h. After washing out the free HBV and compounds, cells were cultured in the absence of compounds for an additional 12 days followed by quantification of secreted HBs protein. *Black* and *dashed bars* indicate the interval for treatment and without treatment, respectively. *B*, chemical structure of Ro41-5253. *C–E*, HepaRG cells were treated with or without 10 μM Ro41-5253 or 50 units/ml heparin according to the protocol shown in *A*, and HBs (*C*) and HBe (*D*) antigens in the culture supernatant were measured. Cell viability was also examined by MTT assay (*E*). *F–H*, HBc protein (*F*), HBV DNAs (*G*), and cccDNA (*H*) in the cells according to the protocol shown in *A* were detected by immunofluorescence, real time PCR, and Southern blot analysis. *Red* and *blue* in *F* show the detection of HBc protein and nuclear staining, respectively. *I* and *J*, primary human hepatocytes were treated with the indicated compounds and infected with HBV in the presence (*I*) or absence (*J*) of PEG8000 according to the protocol shown in *A*. The levels of HBV DNA in the cells (*I* and *J*) and HBe antigen in the culture supernatant (*I*) were quantified. The data show the means of three independent experiments. Standard deviations are also shown as *error bars*. Statistical significance was determined using Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

ELISA quantification of HBs antigen secreted from the infected cells at 12 days postinfection. This screening revealed that HBs was significantly reduced by treatment with Ro41-5253 (Fig. 1*B*) as well as heparin, a competitive viral attachment inhibitor that served as a positive control (Fig. 1*C*) (14). HBe in the medium (Fig. 1*D*) as well as intracellular HBc protein (Fig. 1*F*), HBV replicative (Fig. 1*G*), and cccDNA (Fig. 1*H*) were consistently decreased by treatment with Ro41-5253, without serious cytotoxicity (Fig. 1*E*). This effect of Ro41-5253 was not limited to infection of HepaRG cells because we observed a similar anti-HBV effect in primary human hepatocytes (Fig. 1*I*). The anti-HBV effect of Ro41-5253 on HBV infection of primary human hepatocytes was also observed in the absence of PEG8000 (Fig. 1*J*), which is frequently used to enhance HBV infectivity *in vitro* (14, 29). These data suggest that Ro41-5253 treatment decreases hepatocyte susceptibility to HBV infection.

Reduced HBV Entry in Ro41-5253-treated Cells—Ro41-5253 decreased HBs secretion from infected cells in a dose-dependent manner without significant cytotoxicity (Fig. 2*A*). We next investigated which step in the HBV life cycle was blocked by Ro41-5253. The HBV life cycle can be divided into two phases as follows: 1) the early phase of infection, including attachment, internalization, nuclear import, and cccDNA formation, and 2) the following late phase representing HBV replication that includes transcription, pregenomic RNA encapsidation, reverse transcription, envelopment, and virus release (19, 20, 30–34). LMV and ETV, inhibitors of reverse transcriptase, dramatically decreased HBV DNA in HepAD38 cells (Fig. 2*B*, *left panel*), which can replicate HBV DNA but are resistant to infection (22). However, LMV and ETV did not show a significant effect in HepaRG-based infection (Fig. 1*A*), in contrast to the anti-HBV effect of CsA, an HBV entry inhibitor (Fig. 2*C*) (19, 35), suggesting that this infection assay could be used to evaluate the early phase of infection without the replication process, including the reverse transcription. Ro41-5253 was suggested to inhibit the early phase of infection prior to genome replication as an anti-HBV activity was evident in Fig. 2*C* but not in Fig. 2*B*. Moreover, Ro41-5253 had little effect on HBV transcription, which was monitored by a luciferase activity driven from the HBV enhancer I, II, and the core promoter (Fig. 2*B*, *middle panel*), and by the HBV RNA level in HepG2.2.15 cells, persistently producing HBV (Fig. 2*B*, *right panel*) (36). We then examined whether Ro41-5253 pretreatment affected viral attachment to host cells. To this end, HepaRG cells were exposed to HBV at 4 °C for 3 h, which allowed HBV attachment but not subsequent internalization (19) (Fig. 2*D*). After washing out free viruses, cell surface HBV DNA was extracted and quantified to evaluate HBV cell attachment (Fig. 2*D*). Pretreatment with Ro41-5253 significantly reduced HBV DNA attached to the cell surface, as did heparin (Fig. 2*D*). In a preS1 binding assay, where FITC-labeled preS1 lipopeptide was used as a marker for HBV attachment to the cell surface, Ro41-5253-

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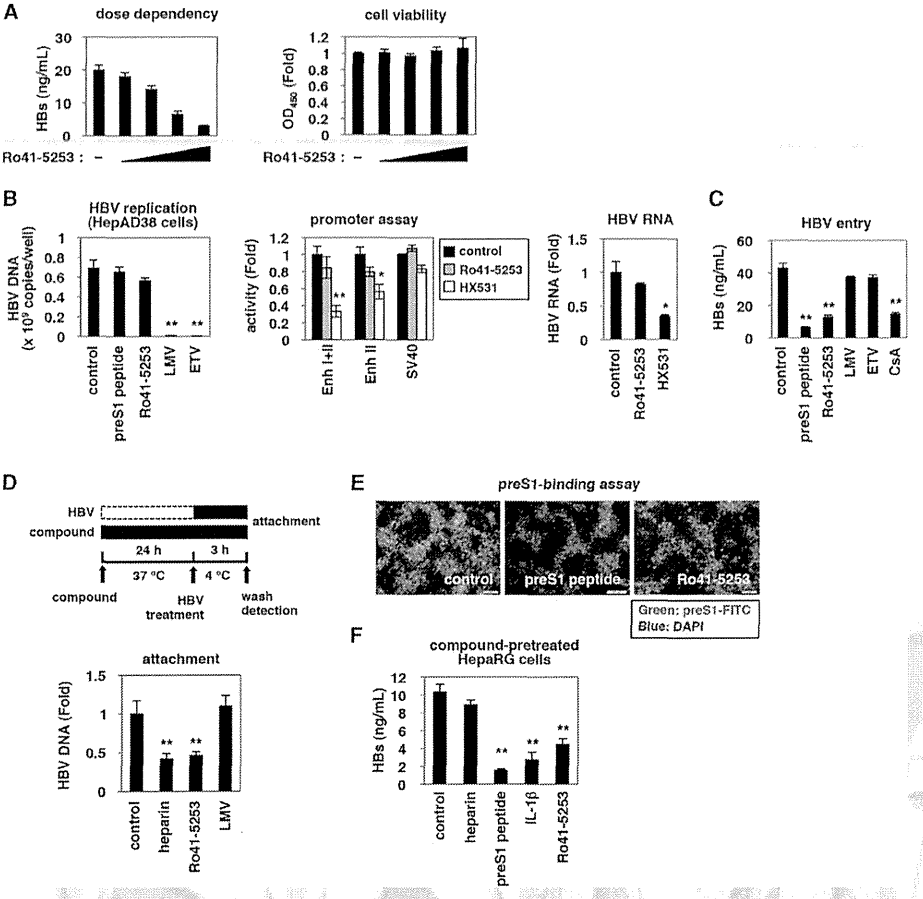

FIGURE 2. **Ro41-5253 decreased HBV entry.** *A*, HepaRG cells were treated with or without various concentrations (2.5, 5, 10, and 20 μM) of Ro41-5253 followed by HBV infection according to the protocol shown in Fig. 1*A*. Secreted HBs was detected by ELISA (*left panel*). Cell viability was also determined by ELISA (*right panel*). *B, left panel*, nucleocapsid-associated HBV DNA in HepAD38 cells treated with the indicated compounds (200 nM preS1 peptide, 20 μM Ro41-5253, 1 μM lamivudine, or 1 μM entecavir) for 6 days without tetracycline was quantified by real time PCR. *Middle panel*, HepG2 cells transfected with the reporter plasmids carrying HBV Enhancer (*Enh*) I + II, HBV Enhancer II, or SV40 promoter ("Experimental Procedures") were treated with or without Ro41-5253 or HX531 as a positive control to measure the luciferase activity. *Right panel*, HepG2.2.15 cells were treated with or without Ro41-5253 or HX531 for 6 days, and intracellular HBV RNA was quantified by real time RT-PCR. *C*, HepaRG cells were treated with or without indicated compounds (200 nM preS1 peptide, 20 μM Ro41-5253, 1 μM lamivudine, 1 μM entecavir, or 4 μM CsA) followed by HBV infection according to the protocol shown in Fig. 1*A*. *D, upper scheme* shows the experimental procedure for examining cell surface-bound HBV. The cells were pretreated with compounds (50 units/ml heparin, 20 μM Ro41-5253, or 1 μM lamivudine) at 37 °C for 24 h and then treated with HBV at 4 °C for 3 h to allow HBV attachment but not internalization into the cells. After removing free virus, cell surface HBV DNA was extracted and quantified by real time PCR. *E*, HepaRG cells pretreated with the indicated compounds (1 μM unconjugated preS1 peptide, 20 μM Ro41-5253) for 24 h were treated with 40 nM FITC-conjugated pre-S1 peptide (*FITC-preS1*) in the presence of compounds at 37 °C for 30 min. *Green* and *blue signals* show FITC-preS1 and nuclear staining, respectively. *F*, HepaRG cells pretreated with the indicated compounds (50 units/ml heparin, 200 nM preS1 peptide, 100 ng/ml IL-1β, or 20 μM Ro41-5253) for 24 h were used for the HBV infection assay, where HBV was inoculated for 16 h in the absence of the compounds. Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

treated cells showed a reduced FITC fluorescence measuring viral attachment (Fig. 2*E*). Thus, Ro41-5253 primarily decreased the entry step, especially viral attachment. Next, to examine whether Ro41-5253 targeted HBV particles or host cells, HepaRG cells pretreated with compounds were examined for susceptibility to HBV infection in the absence of compounds (Fig. 2*F*). As a positive control, HBV infection was blocked by pretreatment of cells with an NTCP-binding lipopeptide, preS1(2–48)^{myr} (preS1 peptide) (15), but not by heparin, which binds HBV particles instead (Fig. 2*F*, *2nd* and *3rd lanes*) (14). HBV infection was also diminished in HepaRG cells pretreated with IL-1β, which induced an innate immune response (Fig. 2*F*, *4th lane*) (37). In this experiment, Ro41-5253-pretreated HepaRG cells were less susceptible to HBV infection (Fig. 2*F*, *5th lane*), suggesting that the activity of Ro41-5253 in host cells contributed to the inhibition of HBV entry.

Ro41-5253 Down-regulated NTCP—Next, we examined how treatment of hepatocytes with Ro41-5253 decreased HBV susceptibility. Recently, NTCP was reported to be essential for HBV entry (15). Intriguingly, we found that Ro41-5253 decreased the level of NTCP protein in HepaRG cells (Fig. 3*A*). Flow cytometry showed that NTCP protein on the cell surface was consistently down-regulated following treatment with Ro41-5253 (Fig. 3*B*, compare *red* and *blue*). Semi-quantitative RT-PCR revealed that mRNA levels for *NTCP*, but not apical sodium-dependent bile salt transporter (ASBT, also known as NTCP2 or SLC10A2), another SLC10 family transporter, were reduced by Ro41-5253 in HepaRG cells (Fig. 3*C*). Thus, Ro41-5253 could reduce NTCP expression. When endogenous NTCP and RAR was knocked down by siRNA, the anti-HBV effect of Ro41-5253 was significantly diminished (Fig. 3*D*), suggesting that the inhibitory activity of Ro41-5253 to HBV infec-

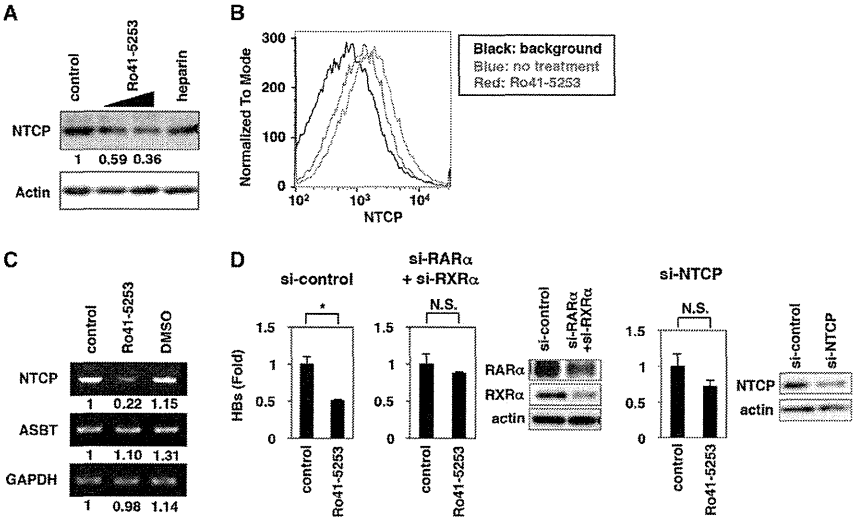

FIGURE 3. **Ro41-5253 reduced NTCP expression.** *A*, HepaRG cells were treated or untreated with 10 and 20 μM Ro41-5253 or 50 units/ml heparin for 12 h, and the levels of NTCP (*upper panel*) and actin (*lower panel*) were examined by Western blot analysis. The relative intensities for the bands of NTCP measured by densitometry are shown below the *upper panel*. *B*, flow cytometric determination of NTCP protein level on the cell surface of primary human hepatocytes treated with 20 μM Ro41-5253 (*red*) for 24 h or left untreated (*blue*). The *black line* indicates the background signal corresponding to the cells untreated with the primary antibody. *C*, RT-PCR determination of the mRNA levels for *NTCP* (*upper panel*), *ASBT* (*middle panel*), and *GAPDH* (*lower panel*) in cells treated with 20 μM Ro41-5253 or 0.1% DMSO for 12 h or left untreated. The relative intensities for the bands measured by densitometry are shown *below the panels*. *D*, HepaRG cells were treated with siRNA against RARa (*si-RARa*) plus that against RXRa (*si-RXRa*), that against NTCP (*si-NTCP*), and a randomized siRNA (*si-control*) for 3 days and then were re-treated with siRNAs for 3 days. The cells were pretreated with or without Ro41-5253 for 24 h and then infected with HBV for 16 h. HBs antigen produced from the infected cells were measured at 12 days postinfection. Statistical significance was determined using Student's *t* test (*, $p < 0.05$; *NS*, not significant).

tion was, at least in part, mediated by targeting NTCP. These data suggest that Ro41-5253 down-regulated NTCP, which probably contributed to the anti-HBV activity of Ro41-5253.

Retinoic Acid Receptor Regulated NTCP Promoter Activity—To determine the mechanism for Ro41-5253-induced down-regulation of NTCP, we used a reporter construct inserting nucleotides (nt) −1143 to +108 of the human *NTCP* (h*NTCP*) promoter upstream of the Gluc gene (Fig. 4*A*, *upper panel*). Ro41-5253 dose-dependently decreased the luciferase activity driven from this promoter, although the effect was modest and showed up to ~40% reduction (Fig. 4*A*, *left panel*). Ro41-5253 had little effect on the herpes simplex virus thymidine kinase promoter (Fig. 4*A*, *right panel*), suggesting that Ro41-5253 specifically repressed hNTCP promoter activity. As reported previously (38), Ro41-5253 specifically inhibited RAR-mediated transcription (Fig. 4, *B* and *C*). RARα, RARβ, and RARγ are members of the nuclear hormone receptor superfamily, which are ligand-activated transcription factors that regulate the transcription of specific downstream genes by binding to the RAR-responsive element (RARE) predominantly in the form of a heterodimer with RXR. We therefore asked whether RAR could regulate the h*NTCP* promoter. As shown in Fig. 4*D*, hNTCP promoter activity was stimulated by overexpression of either RARα, RARβ, or RARγ together with RXRα, and transcription augmented by RAR could be repressed by Ro41-5253 (Fig. 4*D*). Knockdown of endogenous RARα, RXRα, or both dramatically impaired the activity of the h*NTCP* promoter (Fig. 4*E*). These results suggest that RAR/RXR is involved in the transcriptional regulation of the h*NTCP* gene. Consistently, an RAR agonist, ATRA, induced *NTCP* mRNA expression (Fig. 4*F*).

Importantly, endogenous expression of RARα was more abundant in differentiated HepaRG cells, which are susceptible to HBV infection, than that in undifferentiated HepaRG and HepG2 cells, which are not susceptible (Fig. 4*G*) (29). This expression pattern was consistent with the expression of NTCP and with HBV susceptibility, suggesting the significance of RAR in regulating NTCP expression.

Promoter Analysis of hNTCP—We next examined whether RAR regulation of the h*NTCP* promoter is direct or indirect. From the analyses so far using the rat *Ntcp* (r*Ntcp*) promoter, one of the major regulators for rNtcp expression is farnesoid X receptor (FXR), which is a nuclear receptor recognizing bile acids (39). FXR, which is activated upon intracellular bile acids, indirectly regulates r*Ntcp* expression; FXR induces its downstream small heterodimer partner (Shp), another nuclear receptor, and Shp recruits to the r*Ntcp* promoter to repress the promoter activity (39). Then we examined whether RAR affected the expression of human SHP. As shown in Fig. 5*A*, although an FXR agonist GW4064 remarkably induced SHP expression as reported (39), RAR did not have a remarkable effect on the SHP level in HepaRG cells (Fig. 5*A*). To assess the direct involvement of RAR in h*NTCP* regulation, the ChIP assay showed that RAR was associated with the h*NTCP* promoter both in the presence and absence of ATRA (Fig. 5*B*), consistent with the characteristic that RAR/RXR binds to RARE regardless of ligand stimulation (40). The Genomatix software predicts that the h*NTCP* promoter possesses five putative RAREs in nt −1143 to +108 (Fig. 5*C*). Introduction of mutations in all of these five elements lost the promoter activation by RAR/RXR overexpression (Fig. 5*C*, *5-Mut*). Although the promoters mutated in the motif nt −491 to −479, −368 to −356, −274 to −258, or −179 to −167 were activated by ectopic expression of RAR/RXR and this activation was cancelled by Ro41-5253 treatment, the h*NTCP* promoter with

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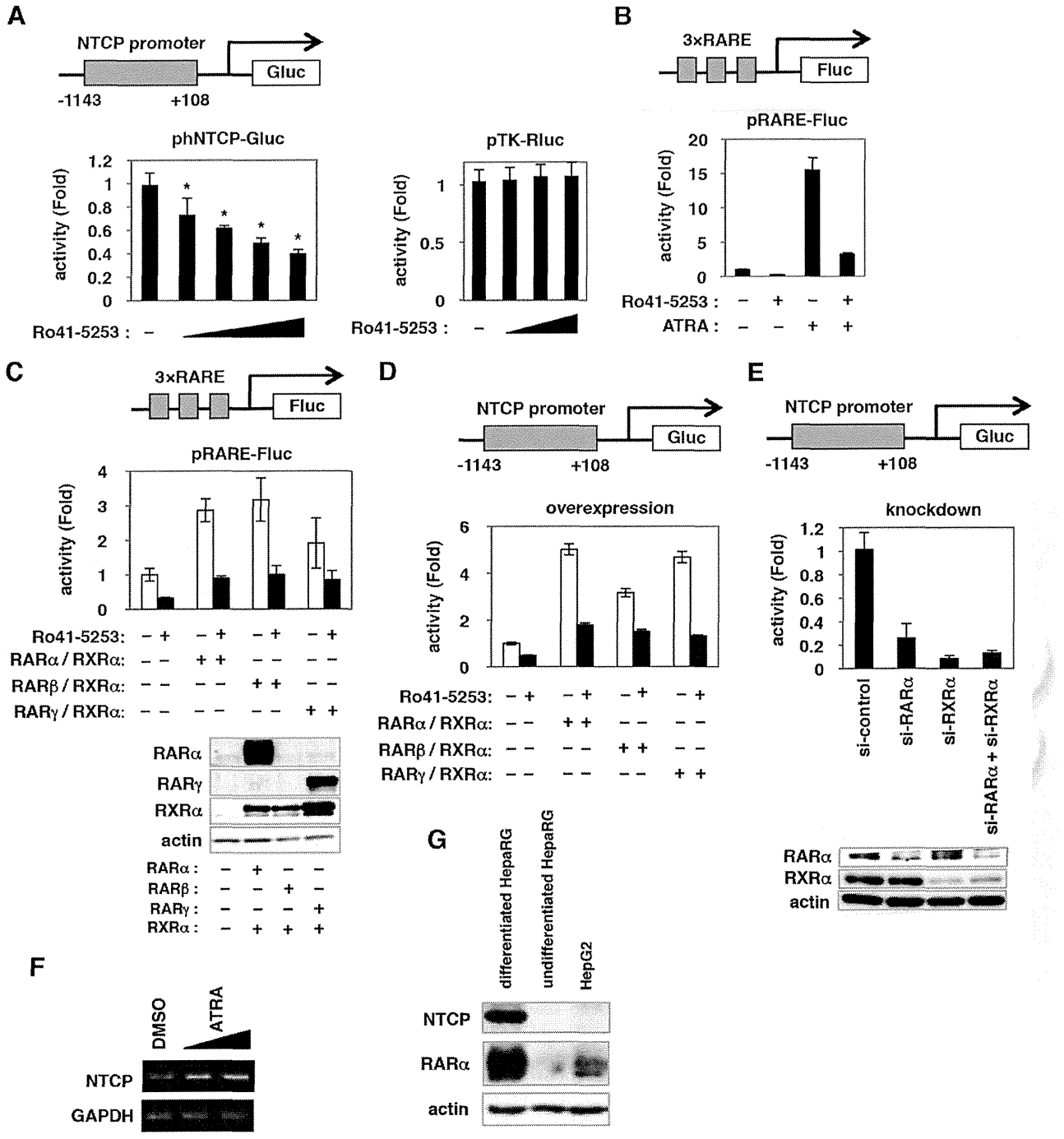

FIGURE 4. **RAR could regulate h*NTCP* promoter activity.** *A, left panel,* HuS-E/2 cells were transfected for 6 h with an hNTCP reporter construct with −1143/+108 of the h*NTCP* promoter region cloned upstream of the Gluc gene (*upper panel, phNTCP-Gluc*), together with an internal control plasmid expressing SEAP (pSEAP). Cells were treated or untreated with various concentrations of Ro41-5253 (5–40 μM) for 48 h. The Gluc and SEAP activities were determined, and the Gluc values normalized by SEAP are shown. *Right panel,* HuS-E/2 cells transfected with a reporter construct carrying the herpes simplex virus thymidine kinase promoter (*pTK-Rluc*) were examined for luciferase activity in the presence or absence of Ro41-5253 (10–40 μM). *B,* HuS-E/2 cells transfected with a Fluc-encoding reporter plasmid carrying three tandem repeats of RARE (*upper panel, pRARE-Fluc*), and Rluc-encoding reporter plasmid driven from herpes simplex virus thymidine kinase promoter (*pTK-Rluc*) were treated with or without 20 μM Ro41-5253 in the presence or absence of an RAR agonist, ATRA, 1 μM for 24 h. Relative values for Fluc normalized by Rluc are shown. *C,* HuS-E/2 cells transfected with pRARE-Fluc and pTK-Rluc with or without expression plasmids for RARs (RARα, RARβ, or RARγ) and RXRα were treated with (*black*) or without (*white*) Ro41-5253 for 48 h. Relative values for Fluc/Rluc are shown. *D,* HuS-E/2 cells were cotransfected with phNTCP-Gluc and pSEAP with or without the expression plasmids for RARs (RARα, RARβ, or RARγ) and RXRα, followed by 24 h of treatment or no treatment with 20 μM Ro41-5253. Relative Gluc/SEAP values are shown. *E,* phNTCP-Gluc and pSEAP were transfected into HuS-E/2 cells together with siRNAs against RARα (*si-RARα*), RXRα (*si-RXRα*), si-RARα plus si-RXRα, or randomized siRNA (*si-control*) for 48 h. Relative Gluc/SEAP values are indicated. Endogenous RARα, RXRα, and actin proteins were detected by Western blot analysis (*lower panels*). *F,* mRNA levels for *NTCP* and *GAPDH* were detected in differentiated HepaRG cells treated with or without ATRA (0.5 and 1 μM) for 24 h. *G,* protein levels for endogenous NTCP (*upper panel*), RARα (*middle panel*), and actin (*lower panel,* as an internal control) were determined by Western blot analysis of differentiated HepaRG, undifferentiated HepaRG, and HepG2 cells. Statistical significance was determined using Student's *t* test (*, $p < 0.05$).

Retinoids Reduced HBV Susceptibility by Down-regulating NTCP

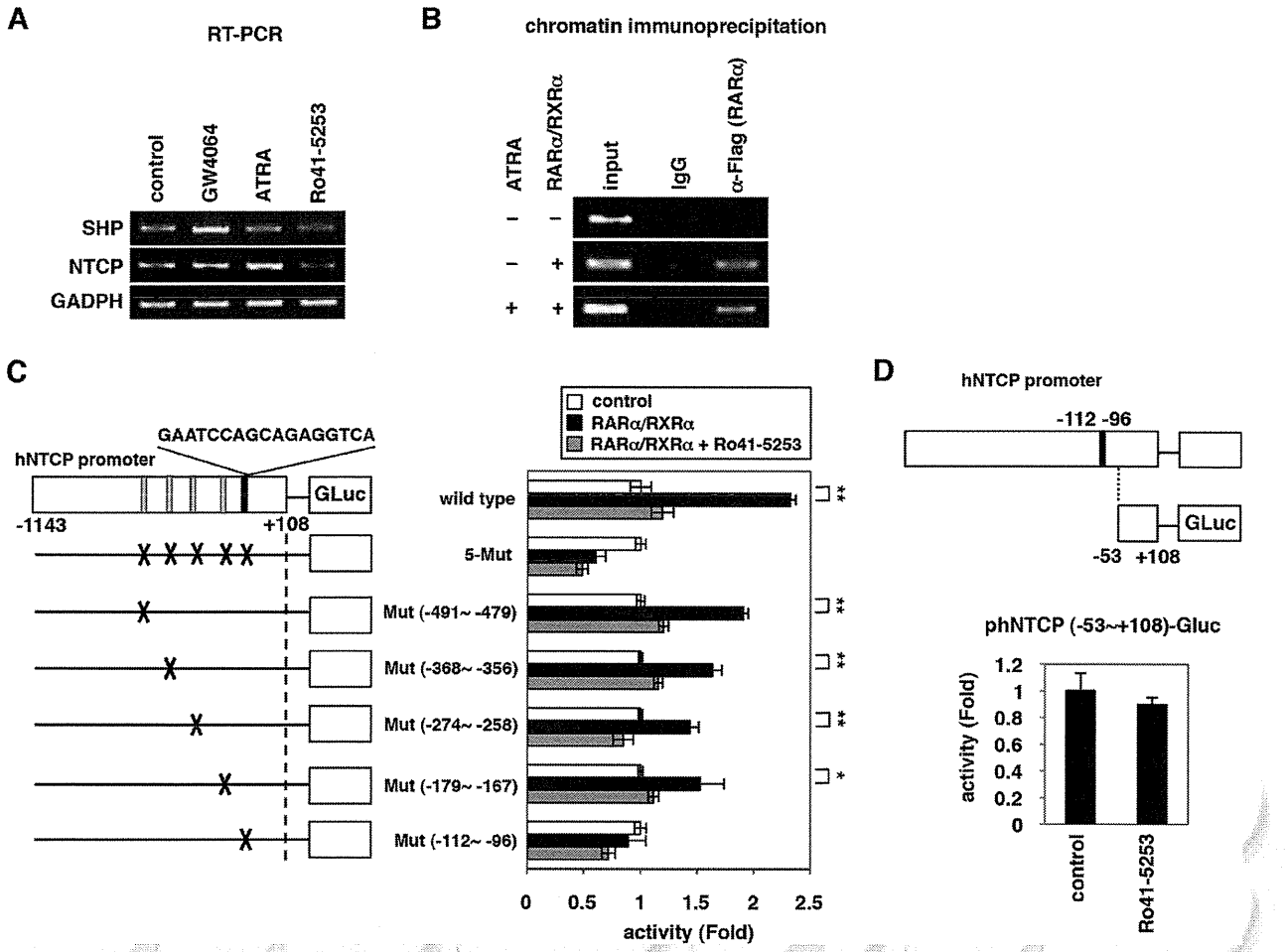

FIGURE 5. **RAR directly regulated the activity of hNTCP promoter.** *A*, HepaRG cells were treated with or without ATRA, Ro41-5253, or a positive control GW4064, which is an FXR agonist, for 24 h. mRNAs for SHP as well as *NTCP* and *GAPDH* were detected by RT-PCR. *B*, ChIP assay was performed as described under "Experimental Procedures" with Huh7-25 cells transfected with or without an expression plasmid for FLAG-tagged RARa plus that for RXRa in the presence or absence of ATRA stimulation. *C, left panel*, schematic representation of h*NTCP* promoter and the reporter constructs used in this study. h*NTCP* promoter has five putative RAREs (nt −491 to −479, −368 to −356, −274 to −258, −179 to −167 (*gray regions*), and −112 to −96 (*black regions*, GAATCCAGCAGAGGTCA)) in nt −1143 to +108 of h*NTCP*. The mutant constructs possessing mutations within each putative RAREs and in all of five elements (*5-Mut*) as well as the wild type construct are shown. *Right panel*, relative luciferase activities upon overexpression with or without RARa plus RXRa in the presence or absence of Ro41-5253. *D*, deletion reporter construct carrying the region nt −53 to +108 of the h*NTCP* upstream of the Gluc gene was used for the reporter assay in the presence or absence of Ro41-5253.

mutations in nt −112 to −96 had no significant response by RAR/RXR (Fig. 5*C*). These data suggest that the nt −112 to −96 region is responsible for RAR-mediated transcriptional activation of h*NTCP*.

HBV Susceptibility was Decreased in RAR-inactivated Cells—We further investigated the impact of RAR antagonization on HBV infectivity. BMS195614, BMS493, and MM11253, which repressed RAR-mediated transcription (Fig. 6*A*), all decreased the susceptibility of HepaRG cells to HBV infection (Fig. 6*B*) without significant cytotoxicity (Fig. 6*C*). These data confirmed that HBV infection was restricted in RAR-inactivated cells. Among these, CD2665, a synthetic retinoid that is known to inhibit RAR-mediated transcription (Fig. 7*A*), had more potent anti-HBV activity than Ro41-5253 (Fig. 7*B*), which was accompanied by the inhibition of the hNTCP promoter (Fig. 7*C*) and down-regulation of NTCP protein (Fig. 7*D*).

CD2665 Showed a Pan-genotypic Anti-HBV Effect—We then examined the effect of CD2665 on the infection of primary human hepatocytes with different HBV genotypes. CD2665 significantly reduced the infection of HBV genotypes A, B, C, and D, as revealed by quantification of HBs and HBe antigens in the culture supernatant of infected cells (Fig. 8, *A–D*). Additionally, this RAR inhibitor decreased the infection of the ETV- and LMV-resistant HBV genotype C clone carrying mutations in L180M, S202G, and M204V (Fig. 8, *E* and *F*). Thus, CD2665 showed pan-genotypic anti-HBV effects and was also effective on an HBV isolate with resistance to nucleoside analogs.

We further investigated whether RAR inhibitors could prevent HBV spread. It was recently reported that HBV infection in freshly isolated primary human hepatocytes could spread during long term culture through production of infectious virions and reinfection of surrounding cells (41). As shown in Fig. 8*G*, the percentage of HBV-positive cells increased up to 30 days postinfection without compound treatment (Fig. 8*G*, *panels a–d*). However, such HBV spread was clearly interrupted by treatment with Ro41-5263 and CD2665 as well as preS1 peptide

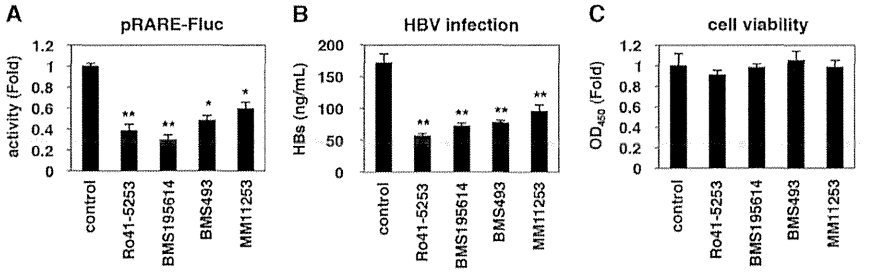

FIGURE 6. **HBV susceptibility was decreased in RAR-inactivated cells.** *A*, HuS-E/2 cells were transfected with the pRARE-Fluc and pTK-Rluc for 6 h followed by treatment with or without the indicated compounds at 20 μM for 48 h. Relative Fluc values normalized by Rluc are shown. *B* and *C*, HepaRG cells treated with or without the indicated compounds 20 μM were subjected to the HBV infection assay according to the scheme in Fig. 1*A*. HBs antigen in the culture supernatant was determined by ELISA (*B*). Cell viability was also quantified by MTT assay (*C*). Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

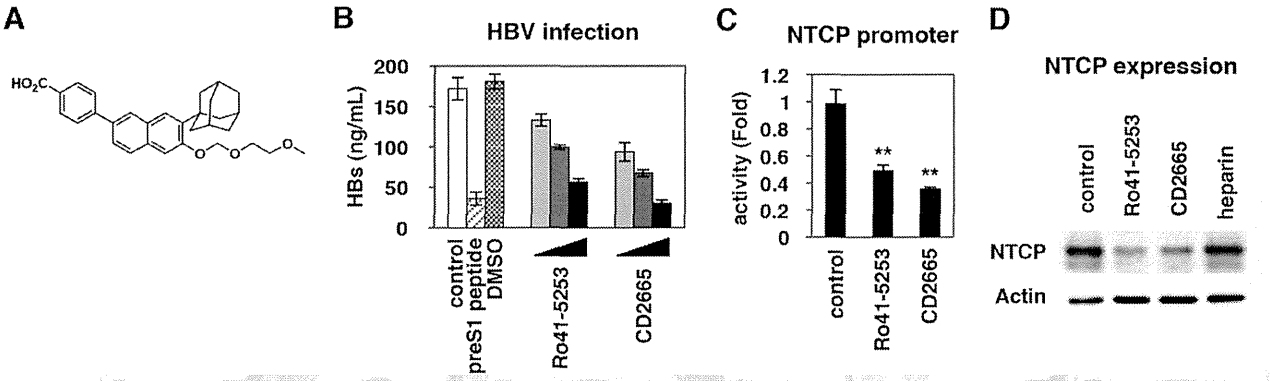

FIGURE 7. **CD2665 had a stronger anti-HBV activity than Ro41-5253.** *A*, chemical structure of CD2665. *B*, HepaRG cells treated with or without 1 μM preS1 peptide, 0.1% DMSO, or various concentrations of Ro41-5253 or CD2665 (5, 10, and 20 μM) were subjected to HBV infection according to the protocol shown in Fig. 1*A*. HBV infection was detected by quantifying the HBs secretion into the culture supernatant by ELISA. The efficiency of HBV infection was monitored by ELISA detection of secreted HBs. *C*, HuS-E/2 cells transfected with phNTCP-Gluc and pSEAP were treated with the indicated compounds at 20 μM for 24 h. Relative Gluc/SEAP values are shown. *D*, NTCP (*upper panel*) and actin proteins as an internal control (*lower panel*) were examined by Western blot analysis of HepaRG cells treated with or without the indicated compounds at 20 μM. Statistical significance was determined using Student's *t* test (**, $p < 0.01$).

(Fig. 8*G*, *panels e–p*). The rise of HBs antigen in the culture supernatant along with the culture time up to 30 days was remarkably inhibited by continuous treatment with Ro41-5253 and CD2665 as well as preS1 peptide without serious cytotoxicity (Fig. 8*G*, *right graph*). Thus, continuous RAR inactivation could inhibit the spread of HBV by interrupting *de novo* infection.

DISCUSSION

In this study, we screened a chemical library using a HepaRG-based HBV infection system and found that pretreatment with Ro41-5253 decreased HBV infection by blocking viral entry. HBV entry follows multiple steps starting with low affinity viral attachment to the cell surface followed by specific binding to entry receptor(s), including NTCP. NTCP is reported to be essential for HBV entry (42). So far, we and other groups have reported that NTCP-binding agents, including cyclosporin A and its derivatives, as well as bile acids, including ursodeoxycholic acid and taurocholic acid, inhibited HBV entry by interrupting the interaction between NTCP and HBV large surface protein (19, 35). Ro41-5253 was distinct from these agents and was found to decrease host susceptibility to HBV infection by modulating the expression levels of NTCP. These results suggest that the regulatory circuit for NTCP expression is one of the determinants for susceptibility to HBV infection. We previously showed that the cell surface NTCP protein expression correlated with susceptibility to HBV infection (43). We therefore screened for compounds inhibiting h*NTCP* promoter activity to identify HBV entry inhibitors (data not shown) (44). Intriguingly, all of the compounds identified as repressors of the h*NTCP* promoter were inhibitors of RAR-mediated transcription. This strongly suggests that RAR plays a crucial role in regulating the activity of the h*NTCP* promoter (Fig. 9). We consistently found that RAR was abundantly expressed in differentiated HepaRG cells susceptible to HBV infection, in contrast to the low expression of RAR in undifferentiated HepaRG and HepG2 cells, which were not susceptible to HBV (Fig. 4*E*). RARE is also found in the HBV enhancer I region (45). RAR is likely to have multiple roles in regulating the HBV life cycle. F9

So far, only transcriptional regulation of rat Ntcp has been extensively analyzed (39, 46, 47). However, the transcription of h*NTCP* was shown to be differently regulated mainly because of sequence divergence in the promoter region (48), and transcriptional regulation of hNTCP remains poorly understood. Hepatocyte nuclear factor (HNF)1α and HNF4α, which positively regulated the rat *Ntcp* promoter, had little effect on h*NTCP* promoter activity (48). HNF3β bound to the promoter region and inhibited promoter activities of both h*NTCP* and rat *Ntcp*. CCAAT/enhancer-binding protein also bound and regulated the h*NTCP* promoter (44, 48). A previous study, which

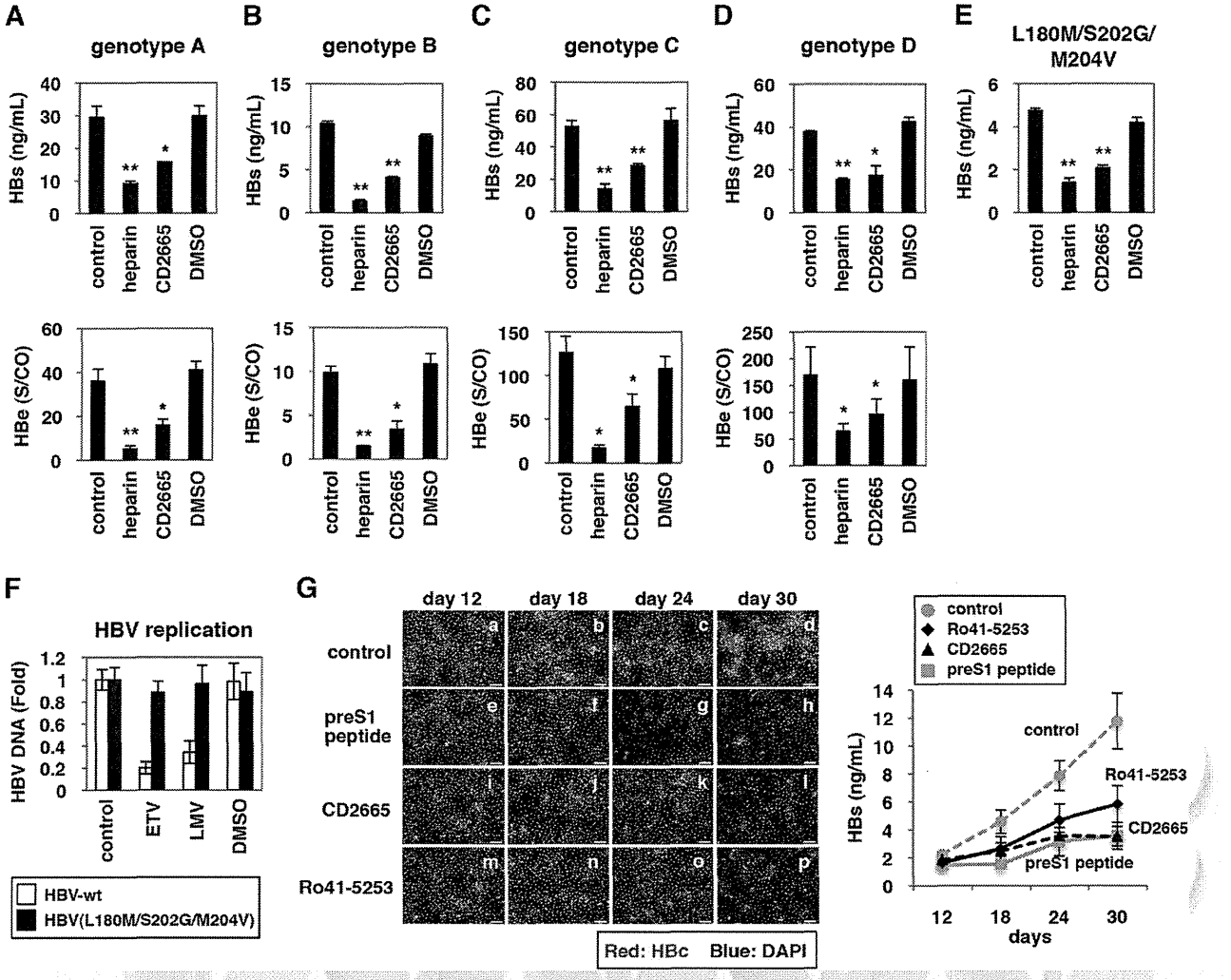

FIGURE 8. **CD2665 showed a pan-genotypic anti-HBV activity.** *A–E*, primary human hepatocytes were pretreated with or without compounds (50 units/ml heparin, 20 μM CD2665, or 0.1% DMSO) and inoculated with different genotypes of HBV according to the scheme show in Fig. 1*A*. HBs (*A–E*) and HBe (*A–D*) antigen secreted into the culture supernatant was quantified by ELISA. Genotypes A (*A*), B (*B*), C (*C*), D (*D*), and an HBV carrying mutations (L180M/S202G/M204V) (*E*) were used as inoculum. *F*, HBV(L180M/S202G/M204V) was resistant to nucleoside analogs. HepG2 cells transfected with the expression plasmid for HBV/C-AT (*white*) or HBV/C-AT(L180M/S202G/M204V) (*black*) were treated with or without 1 μM ETV, 1 μM LMV, or 0.1% DMSO for 72 h. The cells were lysed, and the nucleocapsid-associated HBV DNAs were recovered. Relative values for HBV DNAs are indicated. *G*, continuous RAR inactivation could inhibit HBV spread. Freshly isolated primary human hepatocytes were pretreated with or without indicated compounds (1 μM preS1 peptide, 10 μM Ro41-5253, or 10 μM CD2665) and inoculated with HBV at day 0. After removing free viruses, primary human hepatocytes were cultured in the medium supplemented with the indicated compounds for up to 30 days postinfection. At 12, 18, 24, and 30 days postinfection, HBc protein in the cells (*left panels, red*) and HBs antigen secreted into the culture supernatant (*right graph*) were detected by immunofluorescence and ELISA, respectively. *Red* and *blue* signals in the *left panels* show the detection of HBc protein and nucleus, respectively. Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

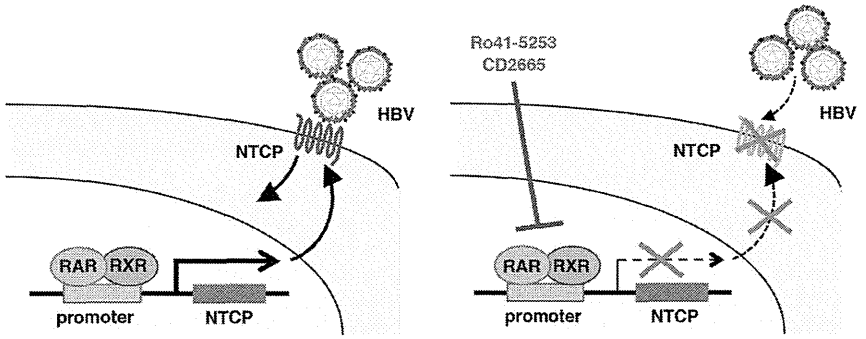

FIGURE 9. **Schematic representation of the mechanism for RAR involvement in the regulation of NTCP expression and HBV infection.** *Left panel*, RAR/RXR recruits to the promoter region of *NTCP* and regulates the transcription. The expression of NTCP in the plasma membrane supports HBV infection. *Right panel*, RAR antagonists, including Ro41-5253 and CD2665, repress the transcription of *NTCP* via RAR antagonization, which decreases the expression level of NTCP in the plasma membrane and abolishes the entry of HBV into host cells.

was mainly based on reporter assays using a construct of the region from −188 to +83 of the h*NTCP* promoter, concluded that RAR did not affect h*NTCP* transcription (48). By using a reporter carrying a longer promoter region, our study is the first to implicate RARs in the regulation of h*NTCP* gene expression (Fig. 9). The turnover of NTCP protein was reported to be rapid, with a half-life of much less than 24 h (49). Consequently, reduction in the *NTCP* transcription by RAR inhibition could rapidly decrease the NTCP protein level and affect HBV susceptibility.

NTCP plays a major role in the hepatic influx of conjugated bile salts from portal circulation. Because NTCP knock-out mice are so far unavailable, it is not known whether loss of NTCP function can cause any physiological defect *in vivo*. However, no serious diseases are reported in individuals carrying single nucleotide polymorphisms that significantly decrease the transporter activity of NTCP (50, 51), suggesting that NTCP function may be redundant with other proteins. Organic anion transporting polypeptides are also known to be involved in bile acid transport. Moreover, an inhibition assay using Myrcludex-B showed that the IC_{50} value for HBV infection was ~0.1 nM (52), although that for NTCP transporter function was 4 nM (28), suggesting that HBV infection could be inhibited without fully inactivating the NTCP transporter (53). HBV entry inhibitors are expected to be useful for preventing *de novo* infection upon post-exposure prophylaxis or vertical transmission where serious toxicity might be avoided with a short term treatment (54). For drug development studies against HIV, down-regulation of the HIV coreceptor CCR5 by ribozymes could inhibit HIV infection both *in vitro* and *in vivo* (55). Disruption of CCR5 by zinc finger nucleases could reduce permissiveness to HIV infection and was effective in decreasing viral load *in vivo* (56). Thus, interventions to regulate viral permissiveness could become a method for eliminating viral infection (55). Our findings suggest that the regulatory mechanisms of NTCP expression could serve as targets for the development of anti-HBV agents. High throughput screening with a reporter assay using an *NTCP* promoter-driven reporter, as exemplified by this study, will be useful for identifying more anti-HBV drugs.

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VIRAL HEPATITIS

Hepatic stellate cells that coexpress LRAT and CRBP-1 partially contribute to portal fibrogenesis in patients with human viral hepatitis

Keisuke Nagatsuma[1,2], Hiroshi Hano[2], Kazuhiro Murakami[3], Daisuke Shindo[4], Yoshihiro Matsumoto[1], Jimi Mitobe[1,2], Ken Tanaka[1], Masaya Saito[5], Haruka Maehashi[4], Mamiko Owada[2], Masahiro Ikegami[2], Akihito Tsubota[6], Toshifumi Ohkusa[7], Yoshio Aizawa[8], Ichiro Takagi[1], Hisao Tajiri[1] and Tomokazu Matsuura[1,4]

1 Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan
2 Department of Pathology, The Jikei University School of Medicine, Tokyo, Japan
3 Division of Clinical Pathology, Tohoku Welfare Pension Hospital, Sendai, Japan
4 Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, Japan
5 Kaijo Bldg. Clinic, Tokyo, Japan
6 Institute of Clinical Medicine and Research (ICMR), The Jikei University School of Medicine, Chiba, Japan
7 Division of Gastroenterology and Hepatology, Kashiwa Hospital, The Jikei University School of Medicine, Chiba, Japan
8 Division of Gastroenterology and Hepatology, Katsushika Medical Centre, The Jikei University School of Medicine, Tokyo, Japan

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Correspondence
Tomokazu Matsuura, MD., PhD., Department of Laboratory Medicine, The Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan
Tel: +81 3 3433 1111 (ext 3210)
Fax: +81 3 3437 5560
e-mail: matsuurat@jikei.ac.jp

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Abstract

Background & Aims: Precisely what type of cells mainly contributes to portal fibrosis, especially in chronic viral hepatitis, such as hepatic stellate cells (HSCs) in the parenchyma or myofibroblasts in the portal area, still remains unclear. It is necessary to clarify the characteristics of cells that contribute to portal fibrosis in order to determine the mechanism of portal fibrogenesis and to develop a therapeutic target for portal fibrosis. This study was undertaken to examine whether LRAT+/CRBP-1+ HSCs contribute to portal fibrosis on viral hepatitis. *Methods:* Antibodies to lecithin:retinol acyltransferase (LRAT), cellular retinol-binding protein-1 (CRBP-1) and widely ascertained antibodies to HSCs (alpha-smooth muscle actin, neurotrophin-3) and endothelial cells (CD31) were used for immunohistochemical studies to assess the distribution of cells that contribute to the development of portal fibrosis with the aid of fluorescence microscopy. A quantitative analysis of LRAT+/CRBP-1+ HSCs was performed. *Results:* The number of LRAT+/CRBP-1+ HSCs was increased in fibrotic liver in comparison with normal liver in the portal area and fibrous septa. The number of double positive cells was less than 20% of all cells/field in maximum. *Conclusion:* This study provides evidence that functional HSCs coexpressing both LRAT and CRBP-1 that continue to maintain the ability to store vitamin A contribute in part to the development of portal fibrogenesis in addition to parenchymal fibrogenesis in patients with viral hepatitis.

Hepatic stellate cells (HSCs), also referred to as Ito cells or fat-storing cells, have been regarded as essential cells for liver fibrogenesis. In a normal liver, HSCs store 80–90% of the hepatic retinoid in characteristic lipid droplets as *fat-storing cells*. When activated in the presence of liver damage, HSCs release cytokines, primarily TGF-β, and transform into myofibroblasts lacking fat droplets. They then produce excessive extracellular matrix and disrupt the liver cytoarchitecture, eventually leading to cirrhosis and liver failure (1).

Hepatic myofibroblasts are transdifferentiated from heterogeneous cell populations in response to a variety of fibrogenic stimuli. Recently, there have been reports that the cellular origin of fibrogenic myofibroblasts is HSCs, portal fibroblasts (6), other mesenchymal cells (6, 7), bone marrow cells (2–5), epithelial-like cells such as hepatocytes or cholangiocytes (8–11) and endothelial cells (12). Although these reports are credible, it is unclear whether this cellular transformation occurs in actual human liver diseases. The current consensus is that hepatic myofibroblasts, myofibroblasts generated in parenchymal injury appear to originate from HSCs and myofibroblasts generated in portal injury may originate from portal fibroblasts (13). However, most of these results were obtained using cultured cells or rodent models. There are few reports that examined the origin

of myofibroblasts in actual human liver diseases, especially in portal injury. Therefore, it is important to determine which type of cells contributes to portal fibrosis in human diseased livers.

One of the candidates that can serve as a marker of quiescent HSCs, which store retinoids in the space of Disse, is lecithin:retinol acyltransferase (LRAT). LRAT activity is strongly expressed in the liver, retinal pigment epithelial (RPE) cells, intestinal mucosa, basal keratinocytes, testis, lungs, *etc.* LRAT has been defined to play the following roles: storing systemic retinoid in the liver, incorporating retinol into the retina and adjusting its concentration in the retinal pigment epithelium to maintain visual function, adjusting the regional concentration of retinoid to differentiate the epithelium in the skin and lungs and regulating the concentration of retinoid to maintain a level optimal for maturation of spermatozoa in the testis (14–20).

Lecithin retinol acyltransferase is also the physiological retinol esterification enzyme which stores retinoid in the liver. Retinyl esters are biosynthesized by removing the fatty acid at position sn-1 of lecithin, using cellular retinol-binding protein-1 (CRBP-1)-bound retinol as a substrate. The expression of LRAT in the liver is regulated by the vitamin A status. Whereas hepatic LRAT activity is low in the livers of vitamin A deficient rats, the decreased LRAT activity is rapidly elevated by repletion with retinol, retinoic acid or RAR-agonists (21–23). Furthermore, LRAT activity is higher in the non-parenchymal cell fraction than in the hepatocyte fraction, and it is estimated that in the liver, the LRAT activity is specifically distributed in HSCs (15). We previously generated antimouse and antihuman LRAT antibodies using peptides of the amino acid sequence of mouse hepatic LRAT and human RPE LRAT, respectively, and reported that these proteins were expressed in quiescent HSCs and endothelial cells of rodent and human normal livers (24).

Cellular retinol-binding protein-1, one of retinol-binding proteins, is present in a variety of tissues, and is most highly expressed in the liver, kidneys and proximal epididymis (25, 26). CRBP-1 is highly expressed in the liver: hepatocytes account for more than 90% of hepatic CRBP-1, while the concentration of the protein (per protein unit) in HSCs is 22 times greater than in hepatocytes (27). In the liver, CRBP-1 mediates retinol esterification to retinyl esters (28). CRBP1-bound retinol is also the substrate of LRAT (29) and the interaction between LRAT and CRBP-1 is required for this enzymatic reaction (30). In an immunohistochemical study, it was shown that quiescent rat HSCs express CRBP-1 (31–33) and quiescent HSCs and myofibroblasts in human normal and diseased livers also express CRBP-1, in addition to hepatocytes and cholangiocytes express CRBP-1 (34, 35).

As described above, both LRAT and CRBP-1 are key molecules involved in the retinoid metabolism in the liver, and in this work we regarded cells coexpressing LRAT and CRBP-1 as functional HSCs that have the ability to store vitamin A.

In this study, the *in situ* distribution of functional HSCs that expressed both LRAT and CRBP-1 was examined in human normal and pathological livers, particularly livers of patients with hepatitis C and B, in order to clarify the contribution of HSCs to portal fibrosis.

Materials and methods

Human liver specimens

Human liver samples were obtained from 24 patients. They corresponded either to percutaneous liver biopsies (n = 20) or large surgical specimens (n = 4). Four corresponded to histologically normal livers and 20 to pathological specimens (Table. 1) with various fibrotic stages. Non-tumourous areas in specimens resected for liver metastasis of colon cancer and in specimens of haemangioma of the liver were studied as histologically 'normal' livers. The stage of fibrosis and the grade of inflammatory activity were classified according to the METAVIR score (36). This work was performed with the permission of the Ethics Committee for Biomedical Research of the Jikei University School of Medicine.

Table 1. Pathological specimens

	Age	Sex	Fibrosis*	Activity†	Aetiology
1	43	F	F0	A0	Metastatic liver tumor
2	60	F	F0	A0	Metastatic liver tumor
3	35	F	F0	A0	Liver hemangioma
4	83	F	F0	A0	Metastatic liver tumor
5	55	M	F1	A1	HCV
6	63	F	F1	A2	HCV
7	52	F	F1	A1	HCV
8	25	M	F1	A1	HBV
9	51	F	F1	A2	HBV
10	58	F	F2	A3	HCV
11	51	F	F2	A2	HCV
12	62	M	F2	A2	HBV
13	40	M	F2	A2	HBV
14	63	M	F2	A1	HCV
15	55	M	F3	A1	HCV
16	36	M	F3	A3	HBV
17	40	M	F3	A2	HBV
18	28	M	F3	A2	HBV
19	64	F	F3	A2	HCV
20	39	M	F4	A2	HCV
21	47	M	F4	A3	HCV
22	72	M	F4	A2	HCV
23	57	M	F4	A1	HBV
24	70	M	F4	A2	HCV

HBV, hepatitis B virus; HCV, hepatitis C virus.

*F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; F4, cirrhosis.

†A0, no activity; A1, mild activity; A2, moderate activity; A3, severe activity. No. 1-4: non-tumourous area in the livers with metastasis or haemangioma.

Tissue sampling and processing

A portion of the fresh tissue samples was routinely utilized to prepare 10% buffered neutral formalin-fixed and paraffin-embedded sections for liver pathology diagnosis; these samples were also used for this study. Three micrometer-thick paraffin sections of each sample were stained with haematoxylin-eosin, Masson's trichrome and silver impregnation for the diagnostic purposes.

Antibodies, immunostaining and observation

An antibody specific for human RPE LRAT (Immuno-Biological Laboratories, Gunma, Japan), a rabbit polyclonal antibody against human cellular retinol-binding protein-1 (CRBP-1) (FL-135) (sc-30106; Santa Cruz Biotechnology, Inc., California, CA, USA), a mouse monoclonal antibody against human smooth muscle actin (SMA) (IgG2a) (Clone 1A4; Dako A/S, Denmark), a rabbit polyclonal antibody against neurotrophin-3 (NT-3) (N-20) (sc-547; Santa Cruz Biotechnology, Inc., California, CA, USA) and a mouse monoclonal antibody against CD31 (IgG1, kappa) (Clone JC70A; Dako A/S, Denmark) were used for the immunohistochemical studies.

The formalin-fixed specimens were embedded in paraffin, and 3 μm-thick sections were cut for immunohistochemical examination. After deparaffinization, the sections of human liver were microwave-treated in 10 mM citrate buffer (pH 6.0) for 10 min at 95°C to activate the antigens, then samples were allowed to cool at room temperature for 15 min. The sections were then rinsed with PBS, and endogeneous peroxidase was inhibited by 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After blocking the samples with 5% goat whole serum (Immuno-Biological Laboratories)/PBS, the sections were incubated with antibodies diluted in PBS against human LRAT (1:20), human CRBP-1 (1:50), human SMA (1:500) and human NT-3 (1:50) for 60 min at room temperature. The sections were then rinsed in PBS, and the epitopes were detected with the Envision + system by horseradish peroxidase detection of antirabbit or antimouse (Dako, Carpinteria, CA, USA) for 60 min at room temperature. The immunoreaction was visualized by using 0.5% 3, 3′-diam-

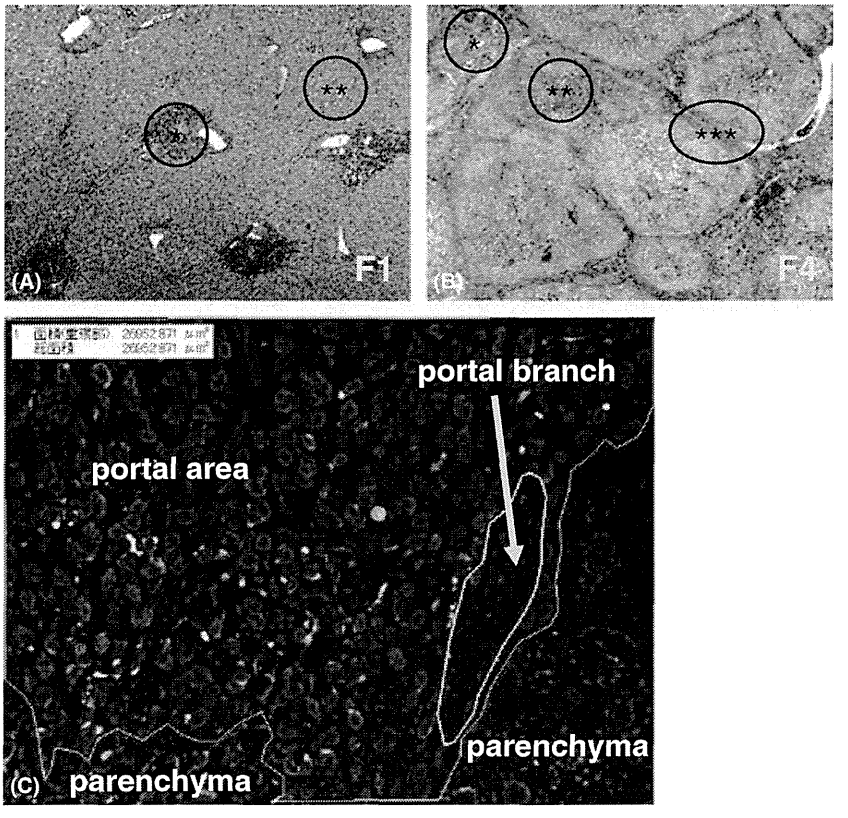

Fig. 1. (A, B): Masson-Trichrome staining of the normal and cirrhotic human liver sections. A total of 3 random fields each in the parenchyma, portal area and fibrous septa (only F3, F4) were used for the quantitative analysis of each case. *portal area, **parenchyma, ***fibrous septa (C) Double immunofluorescence staining for LRAT (green labelling) and CRBP-1 (red labelling) of human liver sections. Each area was automatically measured using a VH analyzer, using the Keyence software program for quantitative analysis, according to the formula: all field - (parenchyma area + portal branch area) = portal area.