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Original Articles

Bromodomain containing protein represses the Ras/Raf/MEK/ERK pathway to attenuate human hepatoma cell proliferation during HCV infection

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ABSTRACT

Hepatitis C virus (HCV) infection facilitates the development of hepatocellular carcinoma (HCC). Activation of Ras/Raf/MEK/ERK pathway is found in more than 30% human cancers. Here, we revealed a novel mechanism underlying the regulation of hepatoma cell proliferation mediated by HCV. On one hand, hepatoma cell proliferation is facilitated by HCV infection through a positive feedback regulatory cycle. HCV promotes hepatoma cell proliferation by activating the Ras/Raf/MEK/ERK pathway, which in turn facilitates HCV replication to further enhance hepatoma cell proliferation. On the other hand, hepatoma cell proliferation is attenuated by the bromodomain containing 7 (BRD7), a tumor suppressor, through a negative feedback regulatory mechanism. After activation, the Ras/Raf/MEK/ERK pathway stimulates BRD7 production, which in turn represses the Ras/Raf/MEK/ERK pathway, leading to the attenuation of hepatoma cell proliferation. However, HCV persistent infection attenuates BRD7 gene expression and facilitates the protein degradation to release the Ras/Raf/MEK/ERK signaling, which results in the facilitation of hepatoma cell proliferation. Therefore, we proposed that the balance between BRD7 function and Ras/Raf/MEK/ERK activity is important for determining the outcomes of HCV infection and HCC development.

Introduction

Hepatitis C virus (HCV) infection can cause hepatocellular carcinoma (HCC), a liver cancer with a global health burden [1–5]. HCV infects more than 170 million people worldwide, and most infections are difficult to be eradicated [6–8]. HCV is an enveloped virus with a single positive-stranded RNA genome, which encodes a large polyprotein that is post-translationally processed into 10 individual proteins by cellular and viral proteases [9,10]. Among the viral proteins, Core, E2, NS3, and NS5A were reported to be involved in HCV-induced hepatocarcinogenesis [11].

It is reported that activation of Ras/Raf/MEK/ERK pathway has occurred in more than 30% human cancers [12]. This pathway has a great diversity of biological functions, including cell proliferation, differentiation, apoptosis, cell cycle regulation, and drug resistance [13–15]. Dysregulation of the pathway leads to tumorigenesis, and therefore, its inhibitors would be potential candidates for cancer therapy [16,17]. There are three important kinases in the pathway, Raf, MEK, and ERK. Signals are transmitted by means of the sequential phosphorylation of the kinases. Once an extracellular stimulus occurs, the Grb2-SOS complex was brought in the proximity of Ras and induces Ras into an active GTP-bound state. Raf is recruited by activated Ras to the plasma membrane, then phosphorylated by itself or other kinases. Activated Raf phosphorylates MEK, which in turn activates the downstream effector ERK. Activation of ERK is able to induce the expression of many transcription factors involved in a wide range of cellular functions.

Bromodomain containing 7 (BRD7) is a member of bromodomain-containing proteins, which are involved in chromatin remodeling,

Abbreviations: BRCA1, breast cancer 1; BRD7, bromodomain containing 7; CCK8, cell counting kit 8; c-Fos, cellular Fos proto-oncogene; CHX, cycloheximide; c-Myc, cellular Myc proto-oncogene; CRC, colorectal cancer; EOC, epithelial ovarian carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRB2, growth factor receptor-bound protein 2; Ha-Ras, Harvey rat sarcoma viral oncogene; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Huh7.5.1, human hepatoma cell line; IFN/JAK/STAT pathway, interferon/Janus tyrosine kinase/signal transducers and activators of transcription pathway; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; NPC, nasopharyngeal carcinoma; OS, osteosarcoma; PBAF, polybromo-associated BRG1-associated factor; Ras/Raf/MEK/ERK pathway, rat sarcoma viral oncogene/serine/threonine protein kinase/mitogen-activated protein kinase kinase/extracellular-signal-regulated kinases pathway; U0126, a selective inhibitor of MEK1 and MEK2.

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cell cycle regulation, and transcription regulation [18,19]. BRD7 has been found as a subunit of the polybromo-associated BRG1-associated factor (PBAF) complex [20], and plays a tumor-suppressive role in nasopharyngeal carcinoma (NPC) [21], epithelial ovarian carcinoma (EOC) [22], osteosarcoma (OS) [23,24], and colorectal cancer (CRC) [25–27]. Moreover, BRD7 is required for gene transcriptions regulated by well-known tumor suppressors, p53 and BRCA1 [26,27].

We previously studied the mechanism underlying HCV regulates HCC development and demonstrated that activation of Ras/Raf/MEK/ERK pathway plays an important role in the regulation of HCC [28–30]. In this study, we further revealed a novel mechanism by which HCV regulates hepatoma cell proliferation. Our results demonstrated that HCV stimulates the Ras/Raf/MEK/ERK pathway through a positive feedback regulatory mechanism and attenuates the production of BRD7 by a negative feedback mechanism, which lead to the facilitation of human hepatoma cell proliferation and HCC development. Therefore, we proposed that the balance between the activity of Ras/Raf/MEK/ERK and the function of BRD7 is important for determining the outcomes of HCV infection and HCC development.

Materials and methods

Cells and viruses

Human hepatoma cell line Huh7.5.1 was kindly provided by Dr. Francis Chisari and cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. The cells were maintained at 37 °C in a 5% CO_2 incubator.

HCV genotype 2a strain JFH-1 was kindly provided by Dr. Takaji Wakita. Cells were infected with JFH-1 at a multiplicity of infection (MOI) of 0.1–1 for 7 days. Stock virus was made by collecting and filtering the supernatant of infected cell cultures [31,32], and the viral titer was measured with a commercial kit (HCV RNA qPCR diagnostic kit, KHB Company). Aliquots were stored at –80 °C until use.

Plasmids, small interfering RNAs, antibodies, and inhibitors

Plasmid pcDNA3.1-Ras (V12) encoding an activated Harvey-Ras (Ha-Ras) [33] was constructed previously by our group [34]. The plasmid pFlag-CMV-BRD7 was constructed by inserting the cDNA clone of BRD7 kindly provided by Dr. Jiahua Han into vector p3XFLAG-CMV-14. The primers used in plasmid construction are listed in Table 1.

Table 1
Primer used in this study.

Gene	Sequences
BRD7-forward (PC)*	5′-TTTGCGGCCGCGCCACCATGGGCAAGAAGCACAAGA-3′
BRD7-reverse (PC)*	5′-TTTGGTACCGAACTTCCACCAGGTCCACACTC-3′
BRD7-forward (RT)**	5′-TCTTGGGTCCCTCATACA-3′
BRD7-reverse (RT)**	5′-ACTCAGCAACATCCGTCT-3′
Cyclin D1-forward (RT)**	5′-GAACACGGCTCACGCTTAC-3′
Cyclin D1-reverse (RT)**	5′-CCAGACCCTCAGACTTGC-3′
c-Fos-forward (RT)**	5′-CAGGGCTGGCGTTGTGA-3′
c-Fos-reverse (RT)**	5′-CGGTTGCGGCATTTGG-3′
c-Myc-forward (RT)**	5′-TCCTGTCCGTCCAAGCA-3′
c-Myc-reverse (RT)**	5′-TACGCACAAGAGTTCCGTAG-3′
GAPDH-forward (RT)**	5′-AAGGCTGTGGGCAAGG-3′
GAPDH-reverse (RT)**	5′-TGGAGGAGTGGGTGTCG-3′

* PC, primers used for plasmid construction.
** RT, primers used for real-time PCR detection.

The shRNA of BRD7 (shBRD7) and control shRNA (shCtrl) were purchased from GenePharma. The target sequence of shBRD7 is 5′-GCGAATTTGAAAGAAGAAAAC-3′.

The antibody to HCV Core protein (ab2740) was purchased from Abcam. The antibody to Flag (F3165) was purchased from Sigma. The antibody to ERK (sc-153) was purchased from Santa Cruz. The antibody to p-ERK (#4370) was purchased from Cell Signaling. The antibody to BRD7 (51009-2-AP) was purchased from Proteintech. The antibody to GAPDH was purchased from CWBio.

U0126, an inhibitor of Ras/Raf/MEK/ERK pathway, was purchased from Tocris Bioscience, and dissolved in dimethyl sulfoxide (Sigma).

RNA extraction and real-time PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA extract was treated with DNase I (Promega) at 37 °C for 30 min. 1 mg of the RNA extract was used as a template for reverse transcription by murine leukemia virus reverse transcriptase (MLV RT) (Promega) with random primers at 37 °C for 60 min.

Real-time PCR was performed in a LightCycler 480 thermal cycler (Roche) under the following conditions: heat activation of the polymerase at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. The fluorescence was measured at the 72 °C step. A final melting curve step from 50 °C to 95 °C was performed to examine the quality of the detection primers. The primers used in real-time detection are listed in Table 1.

Cell transfection

Cells were seeded at a density of 8×10^4 cells per well in 24-well plates, and grew to approximately 80% confluence prior to transfection. Plasmids were transfected into cells with Lipofectamine 2000 reagent (Invitrogen). Assays were performed in triplicate, and results are expressed as mean luciferase activities ± standard deviations (SD).

Cell viability and proliferation assays

The cell viability was measured with Cell Counting Kit 8 (CCK8). In brief, cells were seeded in a 96-well plate and processed according to the experimental design. On the day of detection, 10 μl of CCK8 reagent was added into the cell supernatant, and the OD450nm value was detected 1 h later. There are 6 replicate wells for each sample, and the result was expressed as the mean value ± standard deviations (SD).

Cells were seeded in a 6-well plate and processed according to the experimental design. On the day of detection, cells were trypsinized *in situ* and dispersed thoroughly, then the plate was transferred to an inverted optical microscope (Olympus). Each plate well was observed and photographed with a 4× objective lens. The cell number on the photo was counted by the software named "ImageJ". Three independent visual fields were counted for each plate well, and the result was obtained as the mean cell number. Each sample well was normalized with the control well, and the final result was expressed as the fold of the control.

Cytoplasm and nucleus extractions

This experiment was performed with a commercial kit (NE-PER, Thermo). Briefly, cells were collected and incubated with reagent CER I and CER II for 1 min on ice. Then the samples were centrifuged at 16,000 *g* for 5 min, and the supernatant (cytoplasmic extract) was collected in a clean pre-chilled tube for further use. The insoluble fraction of each sample was suspended with ice-cold reagent NER. Each sample was vortexed for 15 s every 10 min, for a total of 40 min. After centrifugation at 16,000 *g* for 10 min, the supernatant (nuclear extract) was collected in a clean pre-chilled tube for further use.

Statistical analyses

All experiments were reproducible and repeated at least three times with similar results. Parallel samples were analyzed for normal distribution using Kolmogorov–Smirnov tests. Abnormal values were eliminated using a follow-up Grubbs test. Levene's test for equality of variances was performed, which provided information for Student's t-tests to distinguish the equality of means. Means were illustrated using histograms with error bars representing the SD; a p value of <0.05 was considered statistically significant.

Results

HCV facilitates the viability and proliferation of human hepatoma cells through activating the Ras/Raf/MEK/ERK pathway

Initially, the effects of HCV infection on the viability and proliferation of human hepatoma cells were evaluated in Huh7.5.1 cells infected with HCV. The cell viability was monitored by using CCK8 assay kits, in which OD450nm value representing cell viability. The

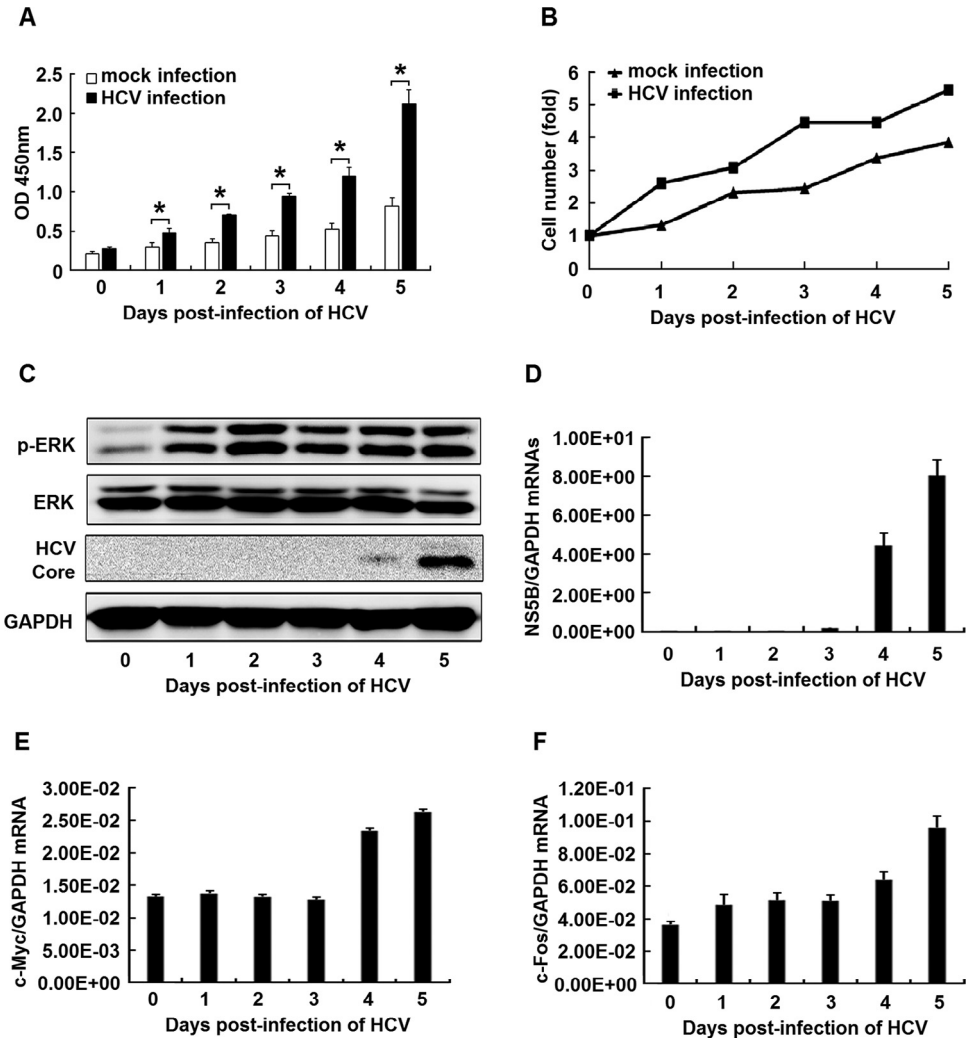

Fig. 1. HCV facilitates the viability and proliferation of hepatoma cells through activating the Ras/Raf/MEK/ERK pathway. (A–F) Huh7.5.1 cells were infected with HCV strain JFH-1 at MOI of 0.5 for 0, 1, 2, 3, 4, and 5 days, respectively. The viabilities of infected cells were measured by using Cell Counting Kit 8 (CCK8), and the values of OD450nm represent cell viability. There were 6 replicate wells for each sample, and the result was expressed as the mean value ± standard deviations (SD), $^*p < 0.05$ (A). The infected cells were trypsinized *in situ* and dispersed thoroughly, and the plates were then transferred to an inverted. The cell numbers were counted under optical microscope (Olympus) with the software ImageJ. Each sample was normalized, and the final result was presented as the fold of control (B). Extracts of the infected cells were prepared, and phosphorylated ERK (p-ERK), ERK, HCV Core, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies (C). Total mRNAs were extracted from infected cells. The levels of HCV NS5B mRNA along with GAPDH mRNA (D), the levels of c-Myc mRNA along with GAPDH mRNA (E), and the levels of c-Fos mRNA and GAPDH mRNA (E) were determined by real-time PCR analyses.

results showed that OD450nm values were higher in viral-infected cells as compared to mock-infected cells (Fig. 1A), suggesting that HCV enhances hepatoma cell viability. The numbers of cells were increased during HCV infection as compared to mock infection (Fig. 1B), indicating that HCV enhances hepatoma cell proliferation. Since the Ras/Raf/MEK/ERK pathway plays an important role in cell proliferation, we speculated that this pathway is involved in cell proliferation mediated by HCV. To confirm this speculation, we initially determined the effect of HCV on the phosphorylation of ERK. The level of phosphorylated ERK (p-ERK) was higher in HCV-infected cells as compared to mock-infected cells, whereas ERK and GAPDH were relatively unchanged by HCV (Fig. 1C), suggesting that HCV stimulates the activity of ERK, leading to activating the Ras/Raf/MEK/ERK pathway. Moreover, the effects of HCV infection on the expression of cMyc and c-Fos, two important components downstream of the pathway, were investigated. The relative mRNA levels of c-Myc (Fig. 1D), c-Fos (Fig. 1E), and HCV NS5B (Fig. 1F) were increased by HCV, suggesting that HCV activates the Ras/Raf/MEK/ERK pathway, which leads to upregulating c-Myc and c-Fos. Taken together, we demonstrated that HCV facilitates the viability and proliferation of human hepatoma cells through activating the Ras/Raf/MEK/ERK pathway.

HCV represses BRD7 expression and facilitates protein degradation

Since BRD7 was reported as a potential tumor suppressor, it is reasonable for us to postulate that BRD7 may play an inhibitory role in the regulation of HCV-associated HCC. The role of BRD7 in human hepatoma cell proliferation mediated by HCV was then evaluated. Initially, we investigated the effect of HCV on BRD7 expression in HCV-infected Huh7.5.1 cells. The level of BRD7 protein was reduced by HCV, whereas the level of HCV Core protein was significantly increased (Fig. 2A). Similarly, the relative level of HCV NS5B mRNA was significantly increased (Fig. 2B), whereas the relative level of BRD7 mRNA was significantly reduced by HCV (Fig. 2C). These results suggested that HCV represses BRD7 expression. Since BRD7 is a transcription factor, its subcellular localization was examined in Huh7.5.1 cells infected with HCV. The level of BRD7 protein was reduced by

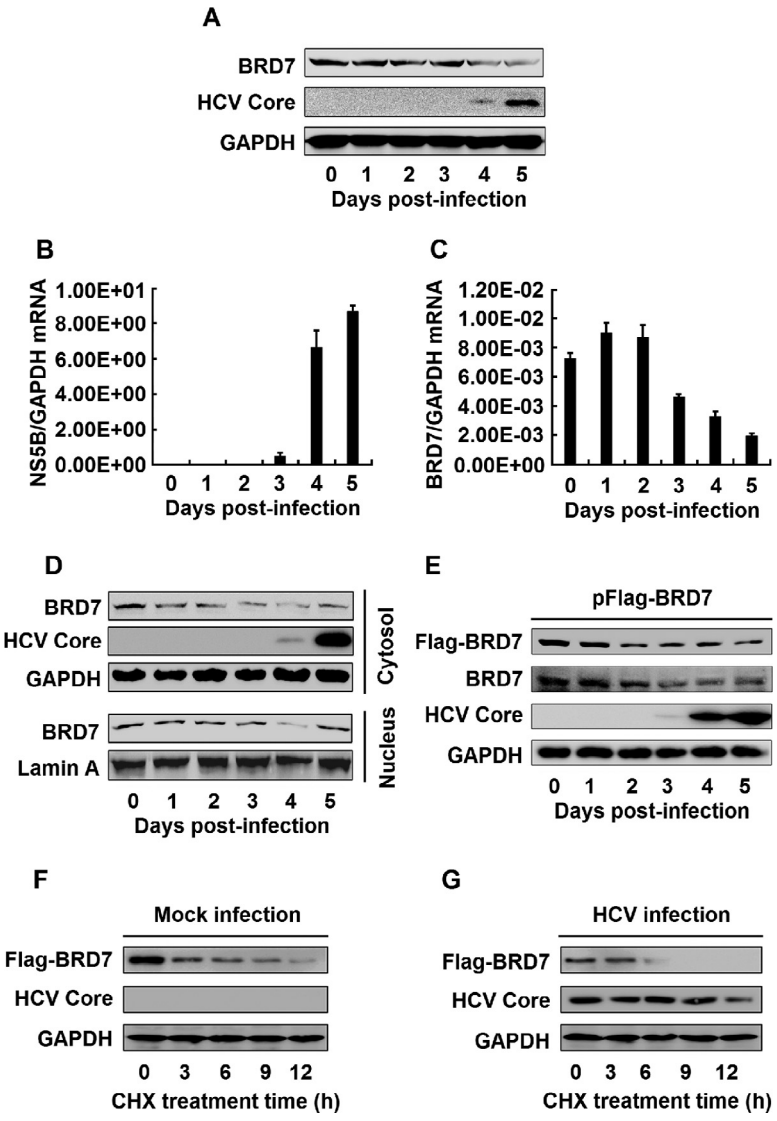

Fig. 2. HCV represses BRD7 expression and facilitates protein degradation. (A–D) Huh7.5.1 cells were infected with HCV strain JFH-1 at MOI of 0.5 for 0, 1, 2, 3, 4, and 5 days, respectively. Extracts of the infected cells were prepared, and BRD7, HCV Core, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies (A). Total mRNAs were extracted from infected cells. The levels of HCV NS5B mRNA and GAPDH mRNA (B), and the levels of BRD7 mRNA and GAPDH mRNA (C), were determined by real-time PCR analyses. Cytoplasm extracts and nucleus extracts were prepared from infected cells, the BRD7, HCV Core, and GAPDH proteins in the cytoplasm extracts (upper panel), and the BRD7 and Lamin A proteins in the nucleus extracts (lower panel), were determined by Western blot analyses using corresponding antibodies (D). (E) Huh7.5.1 cells were transfected with pFlag-CMV-BRD7 and infected with HCV strain JFH-1 at MOI of 0.5 for 0, 1, 2, 3, 4, and 5 days, respectively. Cell extracts were prepared from the treated cells, and the level of exogenous BRD7 (Flag-BRD7) and HCV Core, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies. (F) Huh7.5.1 cells were transfected with pFlag-CMV-BRD7 and treated with CHX for 0, 3, 6, 9, and 12 h, respectively. Cell extracts were prepared from treated cells, and the level of exogenous BRD7 (Flag-BRD7) and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies. (G) Huh7.5.1 cells were transfected with pFlag-CMV-BRD7, infected with HCV strain JFH-1 at MOI of 0.5, and then treated with CHX for 0, 3, 6, 9, and 12 h, respectively. Cell extracts were prepared from treated cells, and the level of exogenous BRD7 (Flag-BRD7), HCV Core, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies.

HCV in the cytoplasm (Fig. 2D, upper panel), but relatively unaffected by HCV in the nucleus (Fig. 2D, lower panel), suggesting that HCV represses BRD7 expression, but not nucleus translocation.

The mechanism by which HCV represses BRD7 expression was then investigated in Huh7.5.1 cells transfected with pFlag-CMV-BRD7 and infected with HCV. The level of exogenous BRD7 protein (Flag-BRD7) was reduced during HCV infection (Fig. 2E). Because exogenous BRD7 expression is under the control of CMV promoter, but not BRD7 promoter, our results suggested that the regulation of exogenous BRD7 expression mediated by HCV is not at transcription level, but at post-translational level. Therefore, we further investigated the effect of HCV on BRD7 protein stability. Cells were transfected with pFlag-CMV-BRD7, infected by HCV, and treated with cycloheximide (CHX), an inhibitor of protein biosynthesis. The level of BRD7 protein was gradually decreased in mock-infected cells (Fig. 2F), but dramatically reduced in HCV-infected cells (Fig. 2G), indicating that HCV facilitates the degradation of BRD7 protein. Taken together, HCV represses BRD7 gene expression and facilitates BRD7 protein degradation.

Activation of Ras/Raf/MEK/ERK pathway facilitates BRD7 expression

Since HCV facilitates Ras/Raf/MEK/ERK activity and also represses BRD7 expression, the correlation between Ras/Raf/MEK/ERK activation and BRD7 expression was determined in HCV-infected Huh7.5.1 cells. The level of BRD7 protein was reduced by

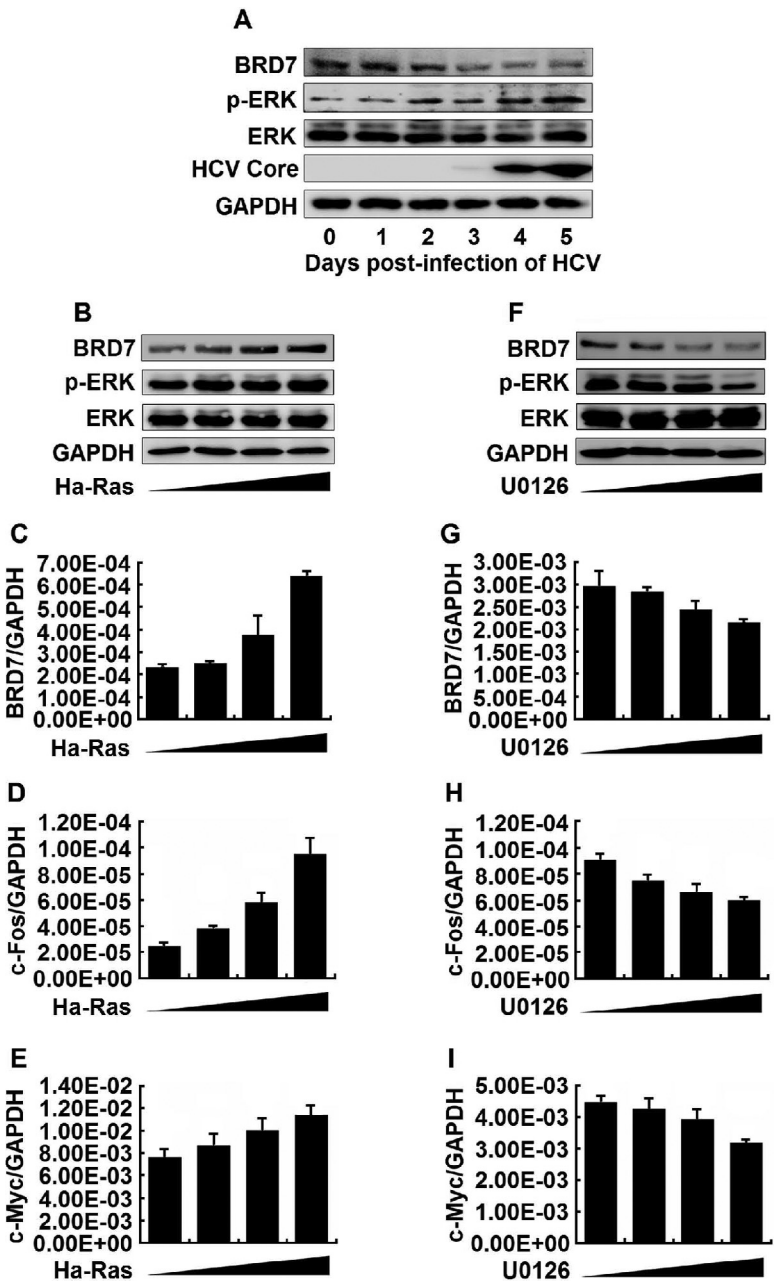

Fig. 3. Activation of Ras/Raf/MEK/ERK pathway facilitates BRD7 expression. (A) Huh7.5.1 cells were infected with HCV strain JFH-1 at MOI of 0.5 for 0, 1, 2, 3, 4, and 5 days, respectively. Cell extracts were prepared from infected cells, and the BRD7, p-ERK, ERK, HCV Core, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies. (B–E) Huh7.5.1 cells were transfected with pcDNA3.1-Ras(V12), which expresses an activated Ras (Ha-Ras), to activate the Ras/Raf/MEK/ERK pathway. Cell extracts were prepared from the transfected cells, and the BRD7, p-ERK, ERK, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies (B). Total mRNAs were extracted from infected cells. The levels of BRD7 mRNA and GAPDH mRNA (C), the levels of c-Fos mRNA and GAPDH mRNA (D), and the levels of c-Myc mRNA and GAPDH mRNA (E) were determined by real-time PCR analyses. (F to I) Huh7.5.1 cells were treated with U0126, a specific inhibitor of MEK1 and MEK2, to inhibit the Ras/Raf/MEK/ERK pathway. Cell extracts were prepared from the transfected cells, and the BRD7, p-ERK, ERK, and GAPDH proteins in the cell extracts were determined by Western blot analyses using corresponding antibodies (F). Total mRNAs were extracted from infected cells. The levels of BRD7 mRNA and GAPDH mRNA (G), the levels of c-Fos mRNA and GAPDH mRNA (H), and the levels of c-Myc mRNA and GAPDH mRNA (I), were determined by real-time PCR analyses.

HCV, whereas the levels of phosphorylated ERK (p-ERK) and HCV Core proteins were enhanced by HCV, but ERK and GAPDH proteins were relatively unchanged by HCV (Fig. 3A). These results indicated that the expression of BRD7 is negatively correlated with the activation of Ras/Raf/MEK/ERK during HCV infection.

The role of Ras/Raf/MEK/ERK pathway in the regulation of BRD7 was then investigated. On one hand, Huh7.5.1 cells were transfected with pcDNA3.1-Ras (V12), which expresses an activated Ras (Ha-Ras), to activate the Ras/Raf/MEK/ERK pathway. BRD7 and p-ERK proteins were upregulated by Ha-Ras, whereas EKR and GAPDH proteins were not affected by Ha-Ras (Fig. 3B), demonstrating that the activity of Ras/Raf/MEK/ERK is stimulated by Ha-Ras, which leads to facilitating BRD7 expression. Similarly, BRD7 mRNA was enhanced by Ha-Ras (Fig. 3C), suggesting that activation of Ras/Raf/MEK/ERK enhances BRD7 expression. Moreover, the mRNA levels of c-Fos (Fig. 3D) and c-Myc (Fig. 3E), two important components downstream of the Ras/Raf/MEK/ERK pathway, were upregulated by Ha-Ras, confirming that Ha-Ras is active and effective.

On the other hand, Huh7.5.1 cells were treated with U0126, a specific inhibitor of MEK1 and MEK2, to inhibit the Ras/Raf/MEK/ERK pathway. BRD7 and p-ERK proteins were downregulated by U0126, whereas EKR and GAPDH proteins were relatively unaffected by U0126 (Fig. 3F), demonstrating that the activity of Ras/Raf/MEK/ERK pathway is repressed by U0126, which results in attenuating BRD7 expression. Similarly, BRD7 mRNA was reduced by U0126 (Fig. 3G), suggesting that inhibition of Ras/Raf/MEK/ERK leads to the repression of BRD7 expression. Moreover, c-Fos mRNA (Fig. 3H) and c-Myc mRNA (Fig. 3I) were downregulated by U0126, confirming that repression of Ras/Raf/MEK/ERK attenuates c-Fos and c-Myc expression. Taken together, activation of Ras/Raf/MEK/ERK leads to upregulating BRD7 expression, whereas inhibition of the pathway results in downregulating BRD7 production, and therefore, the Ras/Raf/MEK/ERK pathway plays a stimulatory role in BRD7 expression.

BRD7 attenuates hepatoma cell viability and proliferation during HCV infection

Since HCV facilitates hepatoma cell viability and proliferation through activating Ras/Raf/MEK/ERK, and this pathway is required for the activation of BRD7, we speculated that BRD7 may play a role in regulating hepatoma cell viability and proliferations. Huh7.5.1 cells were transfected with pFlag-BRD7 and infected with HCV. The results indicated that cell viabilities were higher in viral-infected cells as compared to mock-infected cells, but HCV-mediated activations were reduced by BRD7 (Fig. 4A), demonstrating that BRD7 attenuates hepatoma cell viability. We also showed that cell numbers were higher during HCV infection as compared to mock infection, but HCV-mediated upregulations were downregulated by BRD7 (Fig. 4B), demonstrating that BRD7 represses hepatoma cell proliferation.

In addition, we revealed that the viability (Fig. 4C) and proliferation (Fig. 4D) of hepatoma cells were reduced by BRD7 in dose-dependent manners at 4 days post-transfection. Similarly, the viability (Fig. 4E) and proliferation (Fig. 4F) of hepatoma cells were enhanced by shBRD7 in dose-dependent fashions at 4 days post-transfection. Moreover, cell viability (Fig. 4G) and proliferation (Fig. 4H) were reduced by shBRD7 in time-dependent manners. Similarly, cell viability (Fig. 4I) and proliferation (Fig. 4J) were upregulated by shBRD7 in time-dependent fashion. These results demonstrated that over-expression of BRD7 downregulates hepatoma cell viability and proliferation, whereas knock-down of BRD7 upregulates hepatoma cell of viability and proliferation.

BRD7 represses hepatoma cell viability and proliferation through attenuating the Ras/Raf/MEK/ERK pathway

HCV facilitates cell proliferation through activating the Ras/Raf/MEK/ERK pathway, but BRD7 attenuates cell viability and proliferation during HCV infection. These findings intrigued us to explore the correlation between the function of BRD7 and the activity of Ras/Raf/MEK/ERK in Huh7.5.1 cells transfected with pFlag-CMV-BRD7 (Fig. 5A–C) or with shBRD7 (Fig. 5D–F). Phosphorylation of ERK (p-ERK) was attenuated by BRD7, but ERK and GAPPDH were relatively unaffected by BRD7 (Fig. 5A). Cyclin D1 mRNA, a cell cycle regulator downstream of the Ras/Raf/MEK/ERK pathway, was reduced by BRD7 (Fig. 5B), whereas BRD7 mRNA was increased, as expected (Fig. 5C). These results suggested that BRD7 attenuates the activity of Ras/Raf/MEK/ERK. Moreover, phosphorylation of ERK (p-ERK) was facilitated by shBRD7, whereas ERK and GAPPDH were not affected by shBRD7 (Fig. 5D). Cyclin D1 mRNA was upregulated by shBRD7 (Fig. 5E), whereas BRD7 mRNA was downregulated by shBRD7, as expected (Fig. 5F). These results indicated that the activity of ERK is facilitated by shBRD7. Therefore, over-expression of BRD7 downregulates the activity of Ras/Raf/MEK/ERK, whereas knock-down of BRD7 upregulates the activity of this pathway.

Moreover, Huh7.5.1 cells were co-transfected with pcDNA3.1-Ras (V12) and pFlag-BRD7. The results showed that cell viability (Fig. 5G) and proliferation (Fig. 5H) were upregulated by Ha-Ras, but such activations were attenuated by BRD7. Therefore, activation of Ras/Raf/MEK/ERK leads to the upregulation of cell viability and proliferation, and BRD7 downregulates cell viability and proliferation. Finally, Huh7.5.1 cells were treated with U0126 and transfected with pFlag-CMV-BRD7. The results revealed that cell viability (Fig. 5I) and proliferation (Fig. 5J) were downregulated by U0126, and further reduced by BRD7. Therefore, inhibition of Ras/Raf/MEK/ERK results in the downregulation of cell viability and proliferation, and over-expression of BRD7 leads to the reduction of cell viability and proliferation. Taken together, we demonstrated that BRD7 represses the viability and proliferation of hepatoma cells through attenuating the activity of Ras/Raf/MEK/ERK pathway.

Discussion

Although HCV infection has a profound impact on cell proliferation, the exact mechanism underlying such regulation is very complicated and remains largely elusive. We previously reported that during HCV infection, the Ras/Raf/MEK/ERK pathway is activated, which in turn attenuates the IFN/JAK/STAT signaling, resulting in the stimulation of HCV replication [30]. Since the Ras/Raf/MEK/ERK pathway plays an important role in the regulation of cell proliferation and differentiation, in this study, we further revealed the mechanism by which HCV regulates hepatoma cell proliferation and HCC development. Initially, the effect of HCV on cell proliferation was evaluated. After confirming that HCV enhances the proliferation of human hepatoma cells, we speculated that the Ras/Raf/MEK/ERK pathway may be involved in HCV-mediated cell proliferation. The results indeed revealed that HCV activates Ras/Raf/MEK/ERK pathway by stimulating ERK activity and activating c-Myc and c-Fos expression, and further demonstrated that HCV facilitates of human hepatoma cell proliferation through activating Ras/Raf/MEK/ERK signaling.

In the process of evaluating the mechanism underlying such regulation, we revealed that HCV represses BRD7 expression and facilitates protein degradation. Because HCV facilitates the Ras/Raf/MEK/ERK signaling and also attenuates BRD7 expression, the correlation between Ras/Raf/MEK/ERK activation and BRD7 expression was determined. Activation of Ras/Raf/MEK/ERK by Ha-Ras leads to the upregulation of BRD7, whereas inhibition of Ras/Raf/MEK/ERK by U0126 results in the downregulation of BRD7, demonstrating the Ras/Raf/MEK/ERK pathway plays a stimulatory role in BRD7 expression.

Since HCV facilitates hepatoma cell proliferation and also represses BRD7 expression, it is reasonable for us to postulate that BRD7 may play a role in the regulation of hepatoma cell proliferation mediated by HCV. To conform this speculation, we initially demonstrated that over-expression of BRD7 leads to the downregulation of cell proliferation, whereas knock-down of BRD7 results in the upregulation of cell proliferation. Therefore, BRD7 acts as a suppressor in the regulating hepatoma cell proliferation during HCV infection. These results suggested that HCV may induce HCC through repressing the tumor suppressor, BRD7. BRD7 is involved in the regulation of cell cycle, and has a close relationship with the well-known tumor suppressor p53 [26,35–37]. Although previous studies reported that BRD7 has tumor-suppressive roles in several cancers [21–24,38], some studies demonstrated that BRD7 has no correlation with many tumors [38–41]. Therefore, we at the first time revealed that BRD7 acts as a tumor suppressor in the regulation of hepatoma cell proliferation and perhaps HCC development. Furthermore, the effect of BRD7 on apoptosis was investigated, since

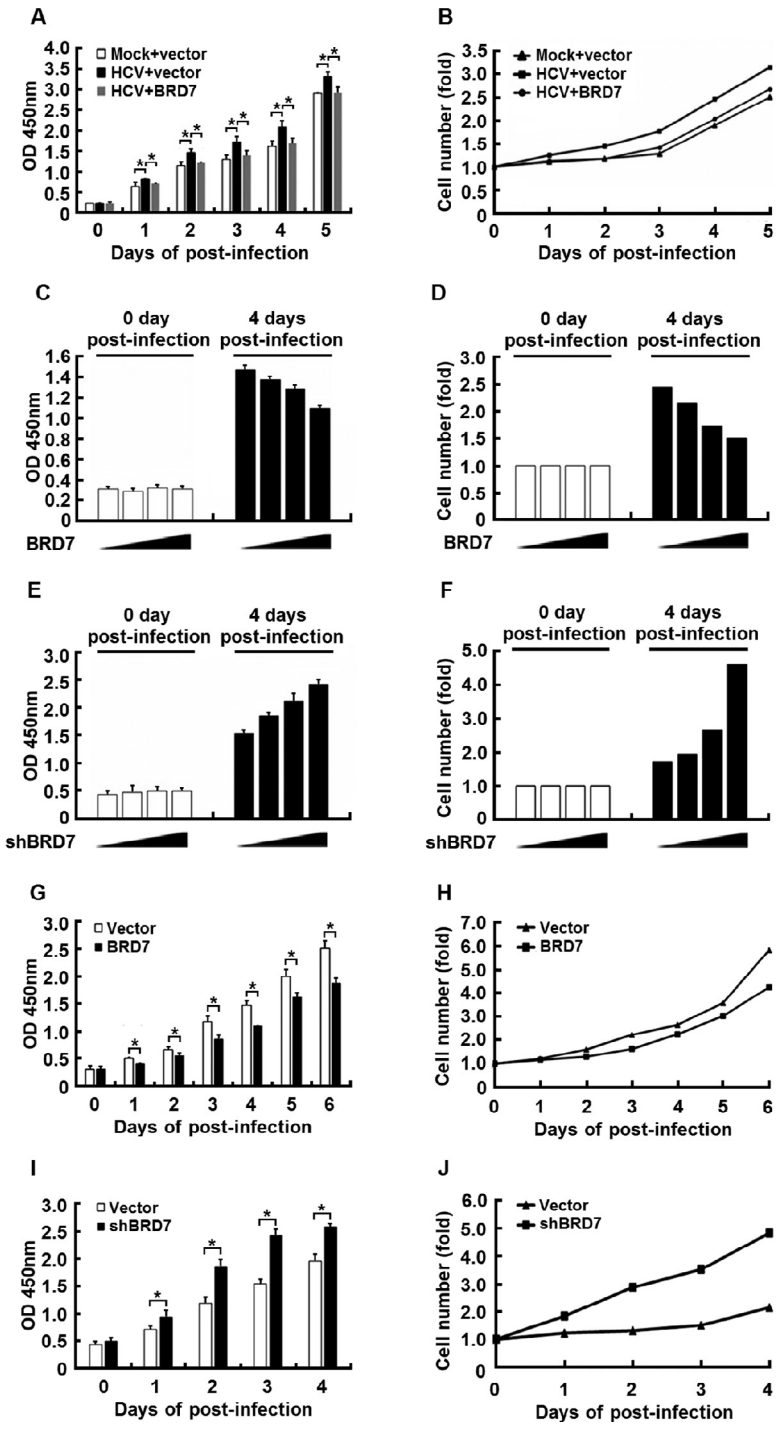

Fig. 4. BRD7 attenuates hepatoma cell viability and proliferation during HCV infection. (A and B) Huh7.5.1 cells were transfected with/without pFlag-CMV-BRD7 and infected with/without HCV strain JFH-1. (C–F) Huh7.5.1 cells were transfected with pFlag-BRD7 (C and D) or shBRD7 (E and F) at concentration of 0, 0.5, 1, and 2 μg, and then infected with HCV strain JFH-1 for 0 and 4 days. (G–J) Huh7.5.1 cells were transfected with pFlag-BRD7 (G and H) or shBRD7 (I and J) for 0, 1, 2, 3, 4, 5, and 6 days. The viabilities of treated cells were measured with Cell Counting Kit 8 (CCK8). The values of OD450nm represent cell viability. There are 6 replicate wells for each sample, and the result was expressed as the mean value ± standard deviations (SD). *$p < 0.05$ (A, C, E, G, and I). The treated cells were trypsinized *in situ* and dispersed thoroughly, and the plate was then transferred to an inverted optical microscope (Olympus). The cell numbers were counted with the software ImageJ. Each sample was normalized, and the final result was presented as the fold of control (B, D, F, H, and J).

it is a common feature of tumor suppressors to stimulate apoptosis. The results showed that the levels of apoptosis were increased in the presence of BRD7 (Fig. S1), indicating that BRD7 has effects on pro-apoptosis. Recent reports demonstrated that HCV could antagonize cell apoptosis [42,43]. Here, we offer a new approach for apoptosis antagonizing of HCV through repressing the BRD7 expression.

Because HCV facilitates hepatoma cell proliferation through activating Ras/Raf/MEK/ERK and this pathway can activate the expression of BRD7, which in turn attenuates cell proliferation, these

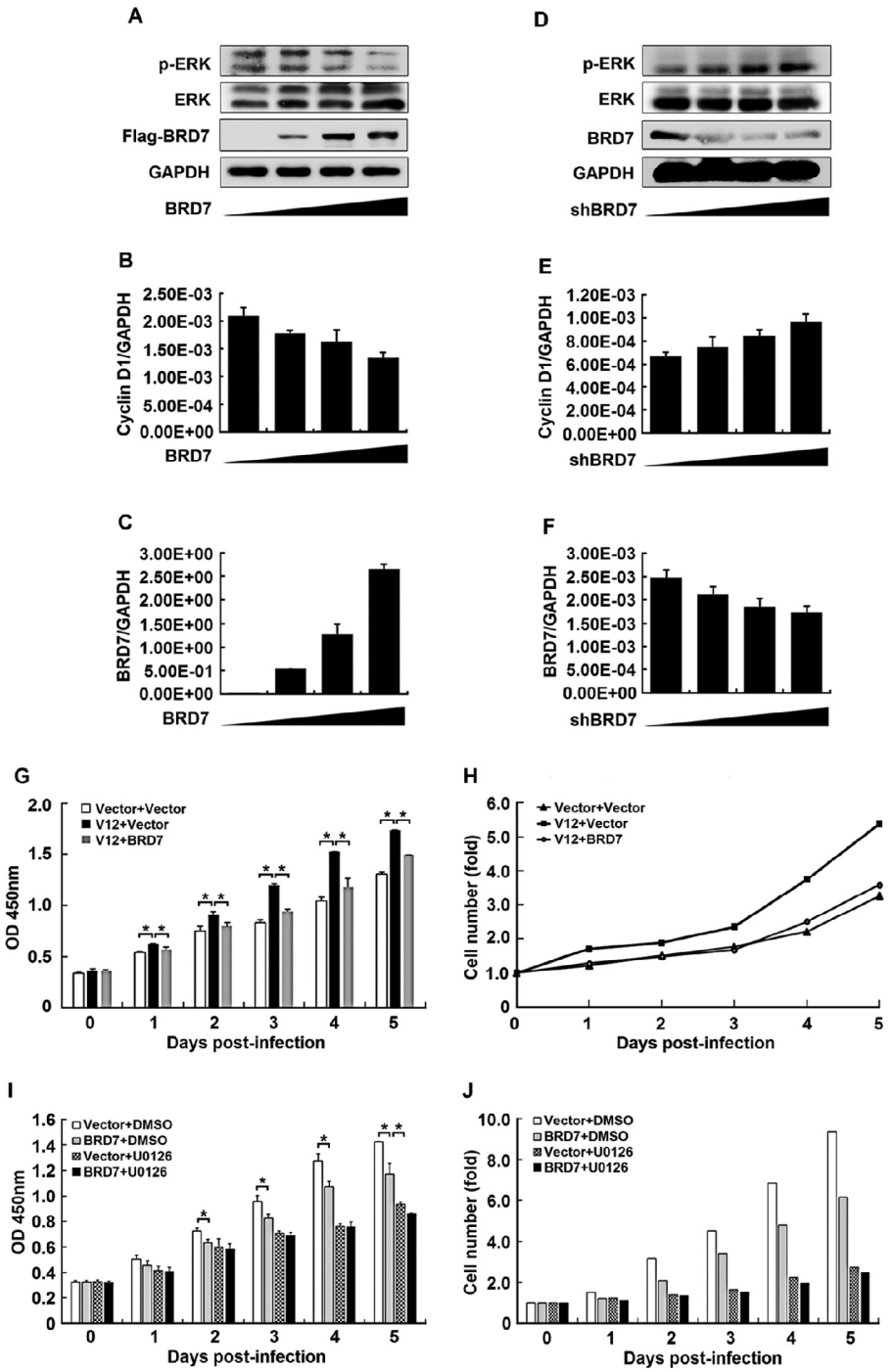

Fig. 5. BRD7 represses hepatoma cell viability and proliferation through attenuating the Ras/Raf/MEK/ERK pathway. (A–F) Huh7.5.1 cells were transfected with pFlag-CMV-BRD7 (A–C) or shBRD7 at concentration of 0, 0.5, 1, and 2 μg for 48 h (D–F). Cell extracts were prepared from the treated cells, and p-ERK, ERK, BRD7, and GAPDH in the cell extracts were determined by Western blot analyses using corresponding antibodies (A and D). Total mRNAs were extracted from the transfected cells. The levels of cyclin D1 mRNA and GAPDH mRNA (B and E), and the levels of BRD7 mRNA and GAPDH mRNA (C and F), were determined by real-time PCR analyses. (G–J) Huh7.5.1 cells were co-transfected with pcDNA3.1-Ras(V12) and pFlag-CMV-BRD7 for 0, 1, 2, 3, 4, and 5 days (G and H) or treated with U0126 for 0, 1, 2, 3, 4, and 5 days (I and J). The viabilities of infected cells were measured with Cell Counting Kit 8 (CCK8), and the values of OD450nm represent cell viability. There are 6 replicate wells for each sample, and the result was expressed as the mean value ± standard deviations (SD). $^{*}p < 0.05$ (G and I). The infected cells were trypsinized *in situ* and dispersed thoroughly, and the plates were then transferred to an inverted optical microscope (Olympus). The cell numbers were counted with the software ImageJ. Each sample was normalized, and the final result was presented as the fold of control (H and J).

results intrigued us to explore the correlation between the activity of Ras/Raf/MEK/ERK and the function of BRD7. Interestingly, overexpression of BRD7 downregulates Ras/Raf/MEK/ERK signaling, whereas knock-down of BRD7 upregulates this pathway, and demonstrated that BRD7 represses hepatoma cell proliferation through attenuating Ras/Raf/MEK/ERK. The Ras/Raf/MEK/ERK signaling is an important pathway with multiple functions, including regulation of proliferation, apoptosis, and cell cycle. Therefore, inhibition of Ras/Raf/MEK/ERK signaling mediated by BRD7 may provide an important mechanism underlying the regulation of caner development,

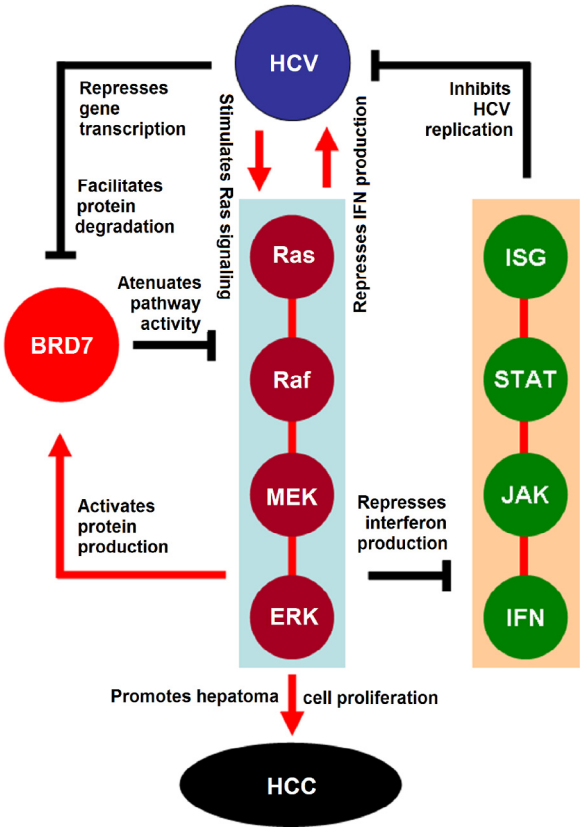

Fig. 6. A proposed mechanism by which BRD7 attenuates human hepatoma cell proliferation. On one hand, hepatoma cell proliferation is facilitated by a positive feedback regulatory mechanism during HCV infection. The virus promotes hepatoma cell proliferation by activating the Ras/Raf/MEK/ERK pathway, which in turn facilitates HCV replication by repressing the IFN/JAK/STAT signaling, leading to further facilitates hepatoma cell proliferation. On the other hand, hepatoma cell proliferation is attenuated by a negative feedback regulatory mechanism. After activation, the Ras/Raf/MEK/ERK pathway stimulates the tumor suppressor BRD7 production, which in turn represses the Ras/Raf/MEK/ERK signaling, leading to the attenuation of hepatoma cell proliferation. However, HCV persistent infection attenuates BRD7 gene expression and facilitates BRD7 protein degradation to release the Ras/Raf/MEK/ERK signaling, which results in the facilitation of hepatoma cell proliferation. Therefore, the balance between BRD7 function and Ras/Raf/MEK/ERK activity is important for determining the outcomes of HCV infection and HCC development. HCV, hepatitis C virus; BRD7, bromodomain containing 7; HCC, hepatocellular carcinoma; IFN, interferon; JAK, Janus tyrosine kinase; STAT, signal transducers and activators of transcription pathway; Ras, rat sarcoma viral oncogene protein; Raf, serine/threonine protein kinase; MEK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinases pathway.

although detailed mechanism underlying such regulation needs to be elucidated.

In conclusion, we proposed a novel and important mechanism underlying the regulation of HCC development mediated by HCV infection (Fig. 6). On one hand, hepatoma cell proliferation is facilitated by a positive feedback regulatory cycle during HCV infection. The virus promotes hepatoma cell proliferation by activating the Ras/Raf/MEK/ERK pathway, which in turn facilitates HCV replication by repressing the IFN/JAK/STAT signaling [30], leading to further facilitation of hepatoma cell proliferation. On the other hand, hepatoma cell proliferation is attenuated by a negative feedback regulatory mechanism. After activation, the Ras/Raf/MEK/ERK pathway stimulates the tumor suppressor BRD7, which in turn represses the Ras/Raf/MEK/ERK signaling, leading to the attenuation of hepatoma cell proliferation. However, HCV persistent infection attenuates BRD7 gene expression and facilitates the protein degradation to release the Ras/Raf/MEK/ERK signaling, which results in the facilitation of hepatoma cell proliferation. Therefore, the balance between BRD7 function and Ras/Raf/MEK/ERK activity is important for determining the outcomes of HCV infection and HCC development.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.11.027.

References

[1] J. Bruix, J.M. Barrera, X. Calvet, G. Ercilla, J. Costa, et al., Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis, Lancet 2 (1989) 1004–1006.
[2] S. Deuffic, T. Poynard, L. Buffat, A.J. Valleron, Trends in primary liver cancer, Lancet 351 (1998) 214–215.
[3] H.B. El-Serag, Hepatocellular carcinoma and hepatitis C in the United States, Hepatology 36 (2002) S74–S83.
[4] T. Stroffolini, P. Andreone, A. Andriulli, A. Ascione, A. Craxi, M. Chiaramonte, et al., Gross pathologic types of hepatocellular carcinoma in Italy, Oncology 56 (1999) 189–192.
[5] H. Yoshizawa, Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future, Oncology 62 (2002) 8–17.
[6] Global Burden of Hepatitis C Working Group, Global burden of disease (GBD) for hepatitis C, J. Clin. Pharmacol. 44 (2004) 20–29.
[7] F.X. Bosch, J. Ribes, R. Cleries, M. Diaz, Epidemiology of hepatocellular carcinoma, Clin. Liver Dis. 9 (2005) 191–211.
[8] M. Feitelson, Chronic hepatitis C virus infection and the pathogenesis of hepatocellular carcinoma, Arch. Immunol. Ther. Exp. (Warsz) 49 (2001) S65–S74.
[9] C.W. Kim, K.M. Chang, Hepatitis C virus: virology and life cycle, Clin Mol Hepatol 19 (2013) 17–25.
[10] M. Niepmann, Hepatitis C virus RNA translation, Curr. Top. Microbiol. Immunol. 369 (2013) 143–166.
[11] M.V. Lin, L.Y. King, R.T. Chung, Hepatitis C virus-associated cancer, Annu. Rev. Pathol. 10 (2015) 345–370.
[12] F.A. Scholl, P.A. Dumesic, P.A. Khavari, Effects of active MEK1 expression in vivo, Cancer Lett. 230 (2005) 1–5.
[13] P.E. Hoyle, P.W. Moye, L.S. Steelman, W.L. Blalock, R.A. Franklin, et al., Differential abilities of the Raf family of protein kinases to abrogate cytokine dependency and prevent apoptosis in murine hematopoietic cells by a MEK1-dependent mechanism, Leukemia 14 (2000) 642–656.
[14] J.A. McCubrey, L.S. Steelman, S.L. Abrams, J.T. Lee, F. Chang, et al., Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance, Adv. Enzyme Regul. 46 (2006) 249–279.
[15] S. Meloche, J. Pouyssegur, The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition, Oncogene 26 (2007) 3227–3239.
[16] B.B. Friday, A.A. Adjei, Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy, Clin. Cancer Res. 14 (2008) 342–346.
[17] P. Rusconi, E. Caiola, M. Broggini, RAS/RAF/MEK inhibitors in oncology, Curr. Med. Chem. 19 (2012) 1164–1176.
[18] P. Filippakopoulos, S. Picaud, M. Mangos, T. Keates, J.P. Lambert, et al., Histone recognition and large-scale structural analysis of the human bromodomain family, Cell 149 (2012) 214–231.

[19] J. Zhou, J. Ma, B.C. Zhang, X.L. Li, S.R. Shen, et al., BRD7, a novel bromodomain gene, inhibits G1-S progression by transcriptionally regulating some important molecules involved in ras/MEK/ERK and Rb/E2F pathways, J. Cell. Physiol. 200 (2004) 89–98.
[20] M.D. Kaeser, A. Aslanian, M.Q. Dong, J.R. Yates, B.M. Emerson, BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells, J. Biol. Chem. 283 (2008) 32254–32263.
[21] C. Peng, H.Y. Liu, M. Zhou, L.M. Zhang, X.L. Li, et al., BRD7 suppresses the growth of Nasopharyngeal Carcinoma cells (HNE1) through negatively regulating beta-catenin and ERK pathways, Mol. Cell. Biochem. 303 (2007) 141–149.
[22] Y.A. Park, J.W. Lee, H.S. Kim, Y.Y. Lee, T.J. Kim, et al., Tumor suppressive effects of bromodomain-containing protein 7 (BRD7) in epithelial ovarian carcinoma, Clin. Cancer Res. 20 (2004) 565–575.
[23] K. Hu, D. Liao, W. Wu, A.J. Han, H.J. Shi, et al., Targeting the anaphase-promoting complex/cyclosome (APC/C)- bromodomain containing 7 (BRD7) pathway for human osteosarcoma, Oncotarget 5 (2014) 3088–3100.
[24] Z. Xue, J. Zhao, L. Niu, G. An, Y. Guo, et al., Up-Regulation of MiR-300 Promotes Proliferation and Invasion of Osteosarcoma by Targeting BRD7, PLoS ONE 10 (2015) e0127682.
[25] W.J. Wu, K.S. Hu, D.L. Chen, Z.L. Zeng, H.Y. Luo, et al., Prognostic relevance of BRD7 expression in colorectal carcinoma, Eur. J. Clin. Invest. 43 (2013) 131–140.
[26] J. Drost, F. Mantovani, F. Tocco, R. Elkon, A. Comel, et al., BRD7 is a candidate tumour suppressor gene required for p53 function, Nat. Cell Biol. 12 (2010) 380–389.
[27] M.T. Harte, G.J. O'Brien, N.M. Ryan, J.J. Gorski, K.I. Savage, et al., BRD7, a subunit of SWI/SNF complexes, binds directly to BRCA1 and regulates BRCA1-dependent transcription, Cancer Res. 70 (2010) 2538–2547.
[28] Y.X. Li, Q. Zhang, Y. Liu, Z. Luo, L. Kang, J. Qu, et al., Hepatitis C Virus Activates Bcl-2 and MMP-2 Expression through Multiple Cellular Signaling Pathways, J. Virol. 86 (2012) 12531–12543.
[29] L.L. Lu, Q. Zhang, K.L. Wu, X. Chen, Y. Zheng, et al., Hepatitis C virus NS3 protein enhances cancer cell invasion by activating matrix metalloproteinase-9 and cyclooxygenase-2 through ERK/p38/NF-kappa B signal cascade, Cancer Lett. 356 (2015) 470–478.
[30] Q. Zhang, R. Gong, J. Qu, Y. Zhou, W. Liu, M. Chen, et al., Activation of the Ras/Raf/MEK pathway facilitates hepatitis C virus replication via attenuation of the interferon-JAK-STAT pathway, J. Virol. 86 (2012) 1544–1554.
[31] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, et al., Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.
[32] D.M. Tscherne, C.T. Jones, M.J. Evans, B.D. Lindenbach, J.A. McKeating, C.M. Rice, Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry, J. Virol. 80 (2006) 1734–1741.
[33] A. Balmain, I.B. Pragnell, Mouse skin carcinomas induced *in vivo* by chemical carcinogens have a transforming harvey-ras oncogene, Nature 303 (1983) 72–74.
[34] W. Wei, W.J. Huang, Y. Pan, F. Zhu, J.G. Wu, Functional switch of viral protein HBx on cell apoptosis, transformation, and tumorigenesis in association with oncoprotein Ras, Cancer Lett. 244 (1) (2006) 119–128.
[35] A.E. Burrows, A. Smogorzewska, S.J. Elledge, Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence, Proc. Natl. Acad. Sci. USA 107 (2010) 14280–14285.
[36] C. Peng, J. Zhou, H.Y. Liu, M. Zhou, L.L. Wang, et al., The transcriptional regulation role of BRD7 by binding to acetylated histone through bromodomain, J. Cell. Biochem. 97 (2006) 882–892.
[37] M. Zhou, H. Liu, X. Xu, H. Zhou, X. Li, et al., Identification of nuclear localization signal that governs nuclear import of BRD7 and its essential roles in inhibiting cell cycle progression, J. Cell. Biochem. 98 (2006) 920–930.
[38] Y.A. Park, J.W. Lee, J.J. Choi, H.K. Jeon, Y. Cho, et al., The interactions between MicroRNA-200c and BRD7 in endometrial carcinoma, Gynecol. Oncol. 124 (2012) 125–133.
[39] J. Penkert, B. Schlegelberger, D. Steinemann, D. Gadzicki, No evidence for breast cancer susceptibility associated with variants of BRD7, a component of p53 and BRCA1 pathways, Fam. Cancer 11 (2012) 601–606.
[40] F. Pern, N. Bogdanova, P. Schurmann, M. Lin, A. Ay, et al., Mutation analysis of BRCA1, BRCA2, PALB2 and BRD7 in a hospital-based series of German patients with triple-negative breast cancer, PLoS ONE 7 (2012) e47993.
[41] X.M. Zhang, X.Y. Wang, S.R. Sheng, J.R. Wang, J. Li, Expression of tumor related genes NGX6, NAG-7, BRD7 in gastric and colorectal cancer, World J. Gastroenterol. 9 (2003) 1729–1733.
[42] X. Jiang, T. Kanda, S. Wu, S. Nakamoto, T. Wakita, H. Shirasawa, et al., Hepatitis C virus nonstructural protein 5A inhibits thapsigargin-induced apoptosis, PLoS ONE 9 (2014) e113499.
[43] X. Jiang, T. Kanda, S. Wu, S. Nakamoto, M. Nakamura, R. Sasaki, et al., Hepatitis C virus nonstructural protein 5A inhibits MG132-induced apoptosis of hepatocytes in line with NF-kappaB-nuclear translocation, PLoS ONE 10 (2015) e0131973.