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Differences in antiproliferative effect of STAT3 inhibition in HCC cells with versus without HBV expression

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ABSTRACT

Chronic infection with hepatitis B virus (HBV) plays an important role in the etiology of hepatocellular carcinoma (HCC). Signal transducer and activator of transcription 3 (STAT3) inactivation could inhibit the tumor growth of HCC. In this study, differential antiproliferative effect of STAT3 inhibition was observed with HBV-related HCC cells being more resistant than non-HBV-related HCC cells. Resistance of HBV-related HCC cells to STAT3 inhibition was positively correlated to the expression of HBV. Enhanced ERK activation after STAT3 blockade was detected in HBV-related HCC cells but not in non-HBV-related HCC cells. Combined ERK and STAT3 inhibition eliminates the discrepancy between the two types of HCC cells. Moderate reduced HBV expression was found after STAT3 inhibition. These findings disclose a discrepancy in cellular response to STAT3 inhibition between non-HBV-related and HBV-related HCC cells and underscore the complexity of antiproliferative effect of STAT3 inactivation in HBV-related HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring solid tumors and the leading cause of cancer-associated death in the world [1]. HCC carcinogenesis is a complex multistep process, comprising of progressive alterations in numerous genes and disruptions in multiple signaling pathways, which can affect cell proliferation, survival, differentiation, invasion and metastasis [2,3]. HCC often develops in a background of chronic inflammation, especially in response to viral hepatitis. Infection with hepatitis B virus (HBV) increases the risk of developing HCC by 5–15-fold [1]. It is well known that HBV has direct effects on hepatocarcinogenesis by insertion of viral DNA into the host genome, and indirect effects via synthesis of viral proteins that interact with proteins in the host hepatocyte, thus dysregulating various intracellular signaling pathways [4,5].

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family. When activated, STAT3 phosphorylates at a critical tyrosine residue, resulting in its dimerization, nuclear translocation and binding to its target DNA sequence, thus affecting target gene transcription levels. As a transcription factor, STAT3 induces a number of genes and is known to have anti-apoptotic, proliferative, angiogenic and metastatic effects [6]. Constitutive STAT3 activation is often detected in many human cancers including HCC, and is associated with a poor prognosis [7,8]. The growth of HCC cells was suppressed when STAT3 was directed targeted by inhibition of its expression or blocking its activation [9,10]. Some agents inhibited cell growth and induced apoptosis in HCC and the effects were also mediated through inhibition of the STAT3 signaling [11–13]. As such, STAT3 inhibition is regarded as a potential target for HCC therapy. Since HBV can cause further chaos in cellular signaling pathways in HCC, it is necessary to investigate the effect of HBV expression on the efficacy of STAT3 inhibition.

In this study, we demonstrated that HBV expression endowed HCC cells with resistance to STAT3 inactivation on proliferation and that abnormal ERK activation was responsible for the insensitivity of HBV-related HCC cells. Our results are significant as they reveal relative resistance to the antiproliferative effect of STAT3 inhibition

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in HBV-related cells and preliminarily explore the molecular mechanisms that account for this discrepancy.

2. Materials and methods

2.1. Reagents

The STAT3 inhibitor NSC 74859 (Merck KgaA, Germany), the MEK/ERK inhibitor U0126 (Cell Signaling Technology, USA), and the NF-κB inhibitor BAY11-7082 (Santa Cruz Biotechnology, USA) were all dissolved in dimethyl sulfoxide and stored at −20 °C. Prior to each experiment, each reagent was diluted to their working concentrations with fresh media.

2.2. Cell culture

Huh7, HepG2 and HepG2.2.15 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (Gibco, USA) with 10% fetal bovine serum (Gibco). The antibiotic G418 (Sigma–Aldrich, USA) was added to the culture media for HepG2.2.15 cells, at a final concentration of 400 μg/ml.

2.3. Plasmid and siRNA transfection

A plasmid pHBV1.2 carrying 129% length of HBV genome (subtype ayw) was provided by Betty L. Slagle (Baylor College of Medicine, Houston, TX) and the control plasmid pGEM-7Zf(+) was obtained from Promega (USA). STAT3 siRNA and negative control siRNA (NC siRNA) were purchased from Santa Cruz Biotechnology. HBS siRNA targeting HBV S gene was used to suppress HBV expression. HBS siRNA duplexes were synthesized and purified by Sangon Biotech (China) and the sequences were as follows: 5′-GUCUGUACAACAUCUUGAGTT-3′ (sense); 5′-CUCAAGAUGUUGUACAGACTT-3′ (antisense). Cells were seeded at approximate 60% confluency in plate one day before transfection. Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's instructions.

2.4. Cell viability assay and cell counting

Two methods were used to assess the cell proliferation rate. Cell viability was determined by the Cell Counting Kit-8 (CCK-8; Dojindo, Japan), according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plate at a density of 5×10^3 cells/well. After overnight incubation, the cells were treated with siRNA duplexes or the designated pathway inhibitors. At the end of the treatment period, the cells were exposed to cell counting solution and were incubated for an additional 1.5 h. Optical density values were read at 450 nm using an absorbance microplate reader. Three separate experiments were performed and each experiment had five replicates. The viable cell number was determined using a cell counter (Beckman, USA). After the desired treatment period, the cells were harvested and counted by trypan blue exclusion. The resulting data were expressed as percentages of the treated samples relative to their respective controls.

2.5. Dual luciferase reporter assay

HepG2.2.15 cells were prepared in a 6-well plate one day before co-transfection with 200 μg pNFκB-TA-luc (Beyotime, China) and 10 μg pRL-TK (Promega). At 24 h post-transfection, the cells were treated with NSC 74859 or BAY11-7082 for a further 24 h. Luciferase activity was assayed using the Dual-Glo Luciferase Assay System (Promega) and normalized to Renilla luciferase activity.

2.6. Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol regent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using oligo (dT) primers and M-MLV reverse transcriptase (Promega). Real-time PCR was carried out using SYBR Premix DimerEraser (Takara Biotechnology, China). The expression level of GAPDH was used to normalize the relative abundance of HBV expression. HBV core antigen (HBc) gene-specific primers were used for the detection of HBV. The following primer sequences were used: HBc sense: 5′-AGTGTGGATTCGCACTCC-3′; HBc antisense: 5′-GAGTTCTTCTTCTAGGGGACC-3′; GAPDH sense: 5′-ATGACATCAAGAAGGTGGTG-3′; GAPDH antisense: 5′-CATACCAGGAAATGAGCTTG-3′.

2.7. Western blotting

Cells were homogenized in cell lysis buffer containing protease inhibitor cocktail (Sigma). Sample lysates with 50 μg total protein per lane were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% bovine serum albumin in 0.05% Tween-TBS. Blots were then probed with the desired primary and secondary antibodies. The target proteins were visualized using an ECL chemiluminescence detection system (GE Healthcare, USA). GAPDH was used as loading control protein. The following primary antibodies were used: anti-STAT3, anti-p-STAT3 (Tyr705), anti-ERK, anti-p-ERK, anti-HBc, anti-GAPDH (Santa Cruz Biotechnology), anti-p-JNK and anti-p-Akt (Ser473) (Cell Signaling Technology).

2.8. Data analysis

Data were expressed as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using the Student's t-test. A P-value <0.05 was considered statistically significant.

3. Results

3.1. HBV-related HCC cells are relatively resistant to STAT3 inhibition on cell proliferation

To investigate the effect of STAT3 inactivation on cell proliferation in HCC cells with and without HBV expression, we compared cell viability in HepG2.2.15 (a stable HBV expressing cell line) and HepG2 (parental cell line) cells after treatment with various concentrations of the STAT3 inhibitor NSC 74859. HepG2 cell viability significantly decreased in a dose-dependent manner by NSC 74859. However, HepG2.2.15 cells were relatively resistant to the anti-proliferative effect of STAT3 inhibition (Fig. 1A). Direct counting of viable cells revealed that NSC 74859 reduced cell numbers in a time-dependent manner, and that HepG2.2.15 counts cells dropped less than HepG2 (Fig. 1B). In order to confirm the effect of NSC 74859 due to its inhibition of STAT3 rather than a non-specific effect of the compound, we carried out experiments using STAT3-specific siRNA. Cell viability assay (Fig. 1C) and cell counting (Fig. 1D) revealed that HepG2.2.15 cells appeared to be relatively resistant than HepG2 cells to the antiproliferative effect of STAT3 siRNA transfection. We wondered whether there was such a phenomenon in HCC cells transiently transfected with HBV plasmid. We therefore evaluated viable cell number after STAT3 inhibition in Huh7 cells that had been transfected with pHBV1.2 or control plasmid. Cell numbers dropped less in pHBV1.2 transfectants compared with control transfectants (Fig. 1E). Based on our data, it is apparent that the inactivation of STAT3 decreases cell

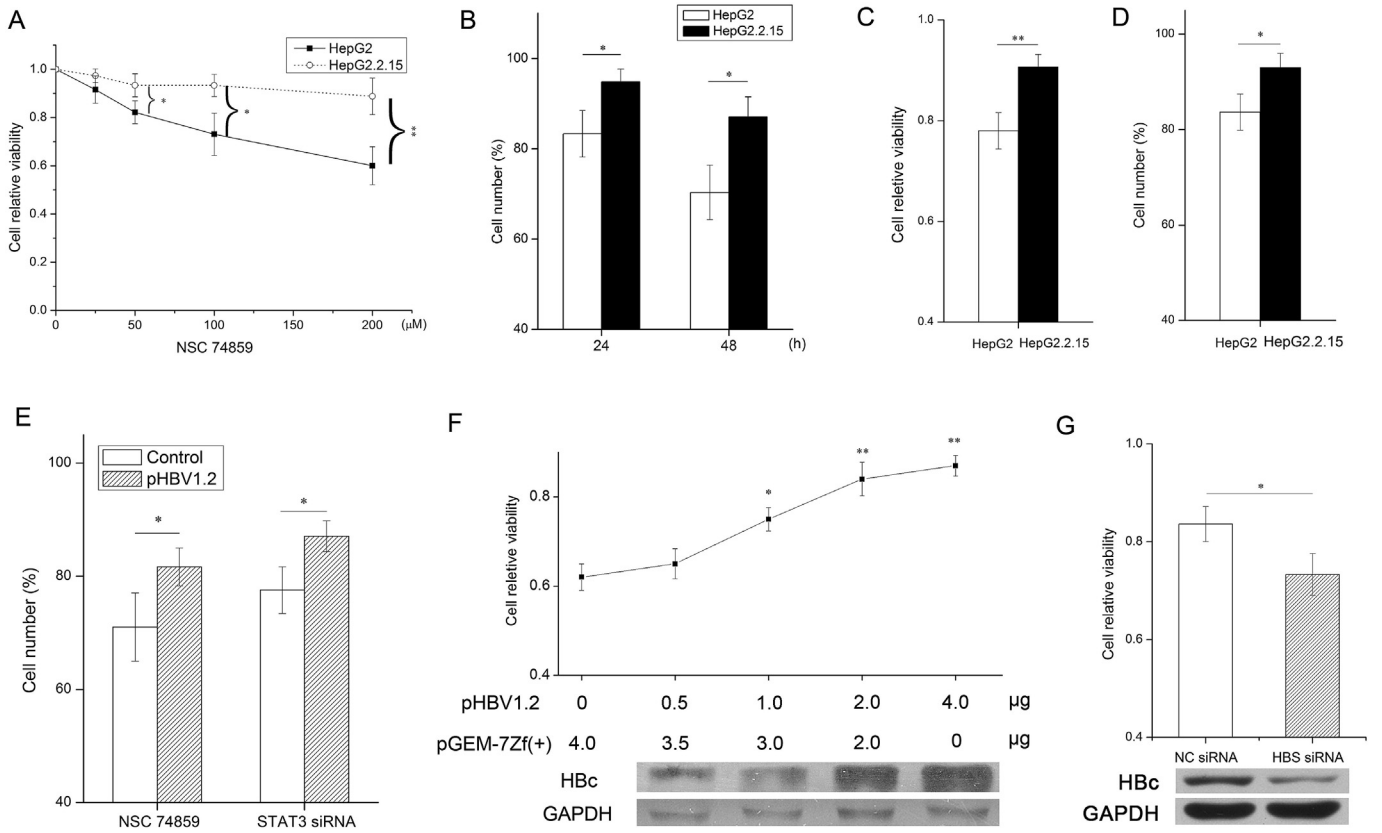

Fig. 1. Cell proliferation in HBV-related and non-HBV-related HCC cells following STAT3 inhibition. (A) Cell viability was measured 24 h after treatment with the indicated concentration of NSC 74859. (B) Cell numbers were counted after indicated time periods of exposure to 100 μM NSC 74859. (C&D) Relative cell viabilities and cell numbers of HepG2 or HepG2.2.15 cells at 48 h post transfection with STAT3 siRNA versus NC siRNA. (E) Huh7 cells were transiently transfected with pGEM-7Zf(+) or pHBV1.2 plasmid for 24 h, and then were treated with NSC 74859 or STAT3 siRNA and their respective controls for 48 h. Relative cell numbers were determined. (F) Huh7 cells were transfected with the indicated amount of pHBV1.2 plasmid. After transfection for 24 h, the cells were cultured with or without NSC 74859 for another 24 h and then measured for cell viability. (G) HepG2.2.15 cells were transfected with HBS siRNA or NC siRNA for 24 h followed by 48 h treatment with or without NSC 74859. Cell viability of the cells was measured. $^{*}P < 0.05$; $^{**}P < 0.01$.

proliferation in HCC cells; however, this effect is attenuated in HBV-related cells.

3.2. Resistance of HBV-related HCC cells to STAT3 inhibition is positively correlated to the expression of HBV

To further investigate the effect of HBV expression in resistance to STAT3 inhibition, we modulated HBV expression in both transiently and persistently HBV expressing cells. Increasing amounts of the pHBV1.2 plasmid or HBS siRNA were transfected into Huh7 or HepG2.2.15 cells. Results showed that the reduction of cell viability after STAT3 inactivation was attenuated by HBV in a dose-dependent manner in Huh7 cells (Fig. 1F). Similar results were found in HepG2.2.15 cells. The sensitivity to STAT3 inhibition was accompanied by a decrease in HBV expression (Fig. 1G). These results imply that HBV expression endows HCC cells with resistance to the antiproliferative effect of STAT3 inhibition and the resistance is positively correlated to the expression of HBV.

3.3. ERK activation is enhanced in HBV-related cells following STAT3 inhibition

HBV infection is known to promote STAT3 activation [14,15]. We detected an increased level of phosphorylated STAT3 protein in HepG2.2.12 cells compared with HepG2 cells, which confirmed the enhanced STAT3 activation associated with HBV expression (Fig. 2A). It has been reported that NSC 74859 only inhibits the proliferation of cells where STAT3 is persistently activated [16]. It was thus inferred that HBV expression would be expected to make cells more susceptible to STAT3 inhibition. However, we observed the opposite phenomenon. We speculated that a signaling pathway associated with cell proliferation may be activated in HBV-related cells to compensate for the effect of STAT3 blockade. Thus, we assessed the phosphorylation status of JNK, Akt, ERK, STAT3 and NF-κB activity. NF-κB activity (Fig. 2B) and the level of phosphorylated JNK and Akt (Fig. 2C) did not change in HepG2.2.15 cells treated with NSC 74859. As expected, a downregulation of phosphorylated STAT3 was observed, though unexpectedly an elevated level of phosphorylated ERK was also observed while total ERK protein levels were not affected (Fig. 2C). Interestingly, the level of p-ERK in HepG2 cells was not changed by NSC 74859 (Fig. 2D). In agreement with the pharmacological blockade of STAT3, STAT3 siRNA induced similar results (Fig. 2E). Elevated p-ERK expression following STAT3 inhibition was also detected in transiently HBV-transfected Huh7 cells while not in controls (Fig. 2F).

3.4. Combined ERK and STAT3 inhibition eliminates the discrepancy between the two types of HCC cells

To address whether enhanced ERK activation was responsible for the discrepancy between non-HBV-related and HBV-related HCC cells, we treated HepG2 and HepG2.2.15 cells with NSC 74859 alone or in combination with the MEK/ERK inhibitor U0126. These two inhibitors elicited overlapping biological activities on

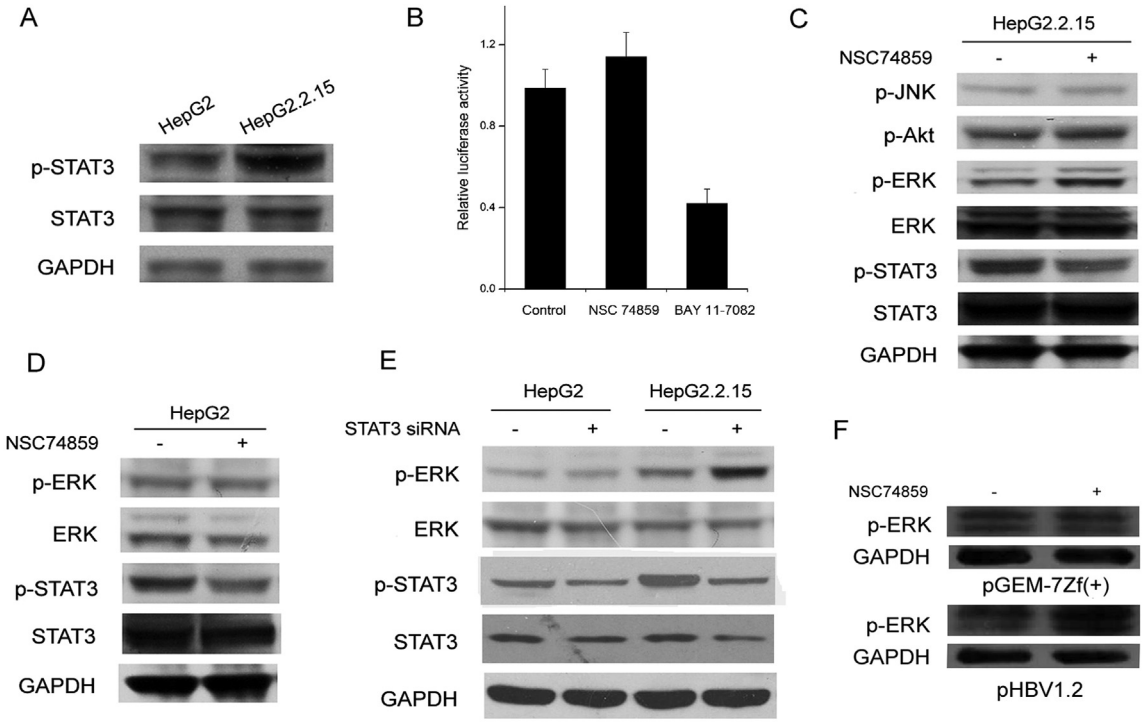

Fig. 2. Enhanced ERK activation in HBV-related HCC cells following STAT3 inhibition. (A) Expression of p-STAT3 in HepG2 and HepG2.2.15 cells. (B) HepG2.2.15 cells were co-transfected with pNFκB-TA-luc and pRL-TK. After the initial 24 h, the cells were incubated with or without NSC 74859 for 24 h. Cells were then detected for luciferase activity. BAY11-7082 was used as positive control. (C) Protein expression of p-JNK, p-Akt, p-ERK and ERK, p-STAT3 and STAT3 in HepG2.2.15 cells treated with or without 100 μM NSC 74859 for 48 h. (D) Protein expression of p-ERK, ERK, p-STAT3 and STAT3 in HepG2 cells treated with or without NSC 74859. (E) Protein expression of p-ERK, ERK, p-STAT3 and STAT3 in HepG2 and HepG2.2.15 cells transfected with 50 nM NC siRNA or STAT3 siRNA for 48 h. (F) After transiently transfected with pGEM-7Zf(+) or pHBV1.2 plasmid, Huh7 cells were then treated with or without NSC 74859. Protein expression of p-ERK was detected.

cell proliferation. The decrease in cell viability in HepG2.2.15 cells was comparable to HepG2 cells treated with both compounds (Fig. 3A). We then verified that U0126 induced a similar decrease in cell viability in HepG2 and HepG2.2.15 cells at various concentrations (Fig. 3B). Cell counting also revealed that combined ERK and STAT3 inhibition abolished the discrepancy between HBV and control transfectants (Fig. 3C).

3.5. *STAT3 inhibition moderately reduces HBV expression*

Previous studies have found that HBV expression can activate ERK [17,18], in accordance with our data (Fig. 2E). To determine whether the increased ERK activation in HBV-related HCC cells was due to the probable upregulated expression of HBV itself, HepG2.2.15 cells were treated with STAT3 inhibitors and the expression of HBV was assessed. Moderate reductions in the HBc mRNA (Fig. 4A) and protein (Fig. 4B) levels were detected after treatment with STAT3 siRNA or NSC 74859 respectively. These results suggest that the elevated ERK activation after STAT3 inhibition was not secondary to the HBV expression itself.

4. Discussion

STAT3 is known to upregulate a series of genes that contribute to carcinogenesis. Growing evidence has demonstrated that STAT3 is a potential therapeutic target for various tumors including HCC [19–21]. HBV infection is the main cause of HCC and dysregulates intracellular signaling pathways [4,5]. It was reported that 54.4% of new cases of HCC worldwide in 2002 could be attributed to HBV infection [22]. Previous studies have indicated that HBV may alter susceptibility of HCC to anticancer agents [23–25]. Thus, we wonder if HBV can affect the cellular response of HCC cells to STAT3 inhibition. Uncontrolled cell proliferation is the basis of tumor formation; therefore, we observed the effect of STAT3 inhibition on cell proliferation.

In the present study, we demonstrated that STAT3 inhibition decreased cell proliferation in HCC cells in a dose- and time-dependent manner. To our knowledge, we firstly described the differences in antiproliferative effect of STAT3 inhibition in HCC cells with versus without HBV expression. HBV-related HCC cells were resistant to the blockade of STAT3 signaling on cell proliferation compared with non-HBV-related cells, and this insensitivity of HBV-related HCC cells was positively correlated to the expression of HBV. It is well established that ERK signaling plays an important role in cell proliferation [26]. Further exploration of why HBV-related HCC cells were resistant to STAT3 inhibition indicated the involvement of abnormal ERK activation in this phenomenon. We observed enhanced ERK activation after STAT3 blockade in HBV-related HCC cells but not in non-HBV-related HCC cells. Combined ERK and STAT3 inhibition resulted in a greater inhibition of cell proliferation and eliminated the discrepancy between these two cell types.

Our data prompted us to speculate why ERK activation elevates in HBV-related cells after STAT3 inhibition. STAT3 is the primary mediator of interleukin 6 (IL-6) signaling. It has been shown that IL-6 moderately suppresses HBV transcription and protein levels [27], although conflicting results have also been reported [28]. We therefore assessed HBV expression after inhibition of STAT3 signaling. Our results indicated that HBV expression was reduced by STAT3 inhibition and, as such, the observed enhanced ERK activation could not be explained by the change of HBV expression. The exact mechanism for this phenomenon remains unclear.

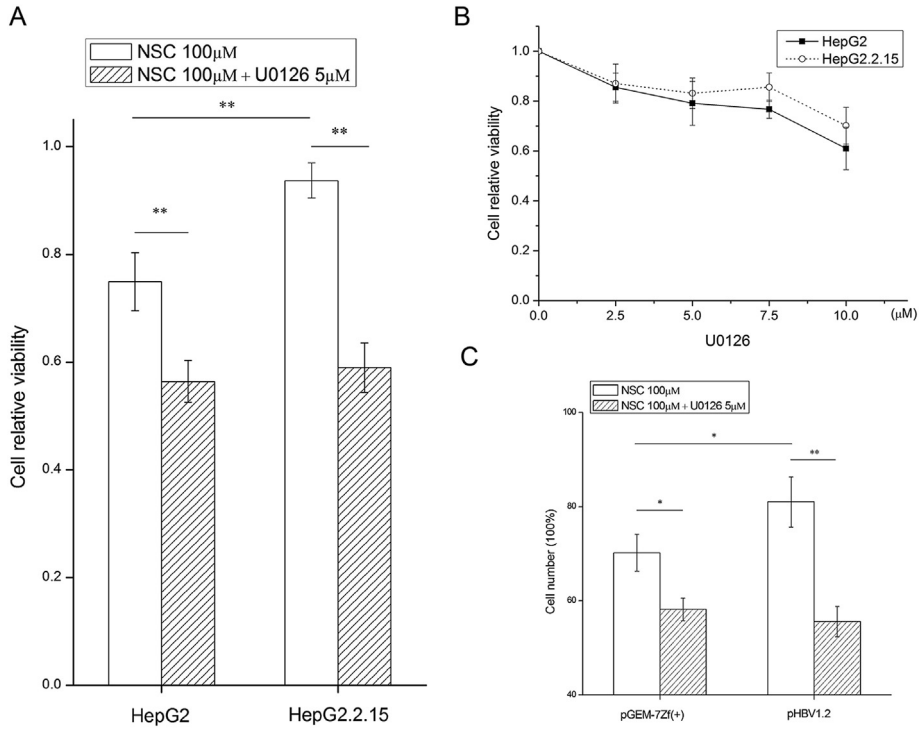

Fig. 3. Additional ERK inactivation eliminated the discrepancy in antiproliferation induced by STAT3 inhibition between non-HBV-related and HBV-related HCC cells. (A) HepG2 and HepG2.2.15 cells were treated with NSC 74859 alone, or NSC 74859 and U0126 in combination for 24 h. Relative cell viabilities of treated cells were determined by comparing with vehicle controls. (B) Cell viability was measured 24 h after treatment with the indicated concentration of U0126. (C) Huh7 cells were transiently transfected with pGEM-7Zf(+) or pHBV1.2 plasmid for 24 h, followed by treatment with NSC 74859 alone or in combination with U0126. Cell numbers were then assessed. $^{*}P < 0.05$; $^{**}P < 0.01$.

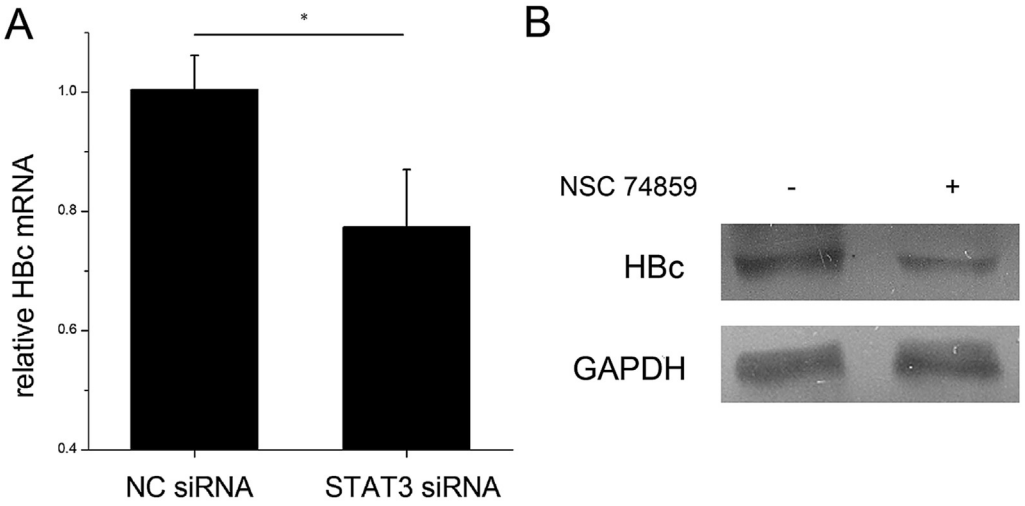

Fig. 4. STAT3 inhibition reduced HBV expression. (A) The intracellular HBc mRNA level was moderately depressed following treatment of HepG2.2.15 cells with STAT3 siRNA. (B) Western blot analysis showed reduction of HBc protein expression in HepG2.2.15 cells transfected with STAT3 siRNA. $^{*}P < 0.05$.

Interestingly, an earlier report described diminished STAT3 activation and increased ERK activation in IL-6 receptor alpha knockout mice compared with wild-type mice, also with unclear underlying mechanisms [29].

In conclusion, our study is the first to reveal the differential antiproliferative effect of STAT3 inhibition in non-HBV-related and HBV-related HCC cells. We also found that the abnormal ERK activation after STAT3 inhibition in HBV-related HCC cells was responsible for their resistance. Although our findings are preliminary, they underscore the complexity of antiproliferative effect of STAT3 inactivation in HBV-related HCC cells and they imply that we should beware of the background of HBV infection in STAT3 targeted therapy for HCC. However, further experiments and clinical trials are needed to confirm our inference.

Conflict of interest

None.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.058.

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