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Hepatitis B virus X protein mutant HBxΔ127 promotes proliferation of hepatoma cells through up-regulating miR-215 targeting PTPRT

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ABSTRACT

The mutant of virus is a frequent event. Hepatitis B virus X protein (HBx) plays a vital role in the development of hepatocellular carcinoma (HCC). Therefore, the identification of potent mutant of HBx in hepatocarcinogenesis is significant. Previously, we identified a natural mutant of the HBx gene (termed HBxΔ127). Relative to wild type HBx, HBxΔ127 strongly enhanced cell proliferation and migration in HCC. In this study, we aim to explore the mechanism of HBxΔ127 in promotion of proliferation of hepatoma cells. Our data showed that both wild type HBx and HBxΔ127 could increase the expression of miR-215 in hepatoma HepG2 and H7402 cells. However, HBxΔ127 was able to significantly increase miR-215 expression relative to wild type HBx in the cells. We identified that protein tyrosine phosphatase, receptor type T (PTPRT) was one of the target genes of miR-215 through targeting 3′UTR of PTPRT mRNA. In function, miR-215 was able to promote the proliferation of hepatoma cells. Meanwhile anti-miR-215 could partially abolish the enhancement of cell proliferation mediated by HBxΔ127 *in vitro*. Knockdown of PTPRT by siRNA could distinctly suppress the decrease of cell proliferation mediated by anti-miR-215 in HepG2-XΔ127/H7402-XΔ127 cells. Moreover, we found that anti-miR-215 remarkably inhibited the tumor growth of hepatoma cells in nude mice. Collectively, relative to wild type HBx, HBxΔ127 strongly enhances proliferation of hepatoma cells through up-regulating miR-215 targeting PTPRT. Our finding provides new insights into the mechanism of HBx mutant HBxΔ127 in promotion of proliferation of hepatoma cells.

1. Introduction

The mutant of virus is a frequent event. Hepatitis B virus (HBV) is a major etiological factor of hepatocellular carcinoma (HCC), in which the oncoprotein HBV X protein (HBx) plays a vital role in the pathogenesis of HCC [1–4]. A growing number of studies have shown that HBx has multiple biological functions, including regulating cell proliferation, apoptosis, migration and cell cycle progress [5–8]. HBx deletion, especially the COOH-terminal deletion of HBx, occurs frequently in HBV-associated HCC tissues [9]. It has been reported that the COOH-terminal–truncated HBx rather than the wild type HBx could effectively transform immortalized liver cell line MIHA, which suggests that the COOH-terminal–truncated HBx plays an important role in hepatocarcinogenesis [10]. Therefore, the identification of potent mutant of HBx in hepatocarcinogenesis is significant. We previously identified a natural mutant of the HBx gene with COOH-terminal-deletion from 382 to 401 bp (termed HBxΔ127) [11]. Relative to wild type HBx, HBxΔ127 could remarkably increase the proliferation and migration of hepatoma cells through up-regulating NF-κB, survivin, human telomerase reverse transcriptase (hTERT), c-Myc, proliferating cell nuclear antigen (PCNA), 5-lipoxygenases (5-LOX), fatty acid synthase (FAS) and osteopontin [12–14]. Therefore, HBxΔ127 plays a curial role in hepatocarcinogenesis. However, the underlying mechanism of HBxΔ127 in hepatocarcinogenesis needs further elucidation.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that are implicated in post-transcriptional regulation through binding to the 3′ untranslated regions (3′UTRs) of the target mRNAs [15].

Abbreviations: HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; PCNA, proliferating cell nuclear antigen; 5-LOX, 5-lipoxygenases; FAS, fatty acid synthase; RCC, renal cell carcinoma; GC, gastric carcinomas; NS, non-neoplastic stomach; ALCAM, activated leukocyte cell adhesion molecule; PTP, protein tyrosine phosphatase; FBS, fetal bovine serum; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, small interfering RNA; miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; UTR, untranslated regions; wt, wild type; NC, negative control.

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Growing evidence suggests that miRNAs may function as oncogenes or tumor suppressors involving in development, cell proliferation, migration and metabolism [15–18]. It has been reported that miR-215 is implicated in renal cell carcinoma (RCC) and colon cancer by inhibiting cell proliferation and triggering cell cycle arrest at G2 phase as a tumor suppressor [19,20]. However, the expression of miR-215 is higher in gastric carcinomas (GC) and HCC than their noncancerous counterparts [21,22]. These studies suggest a context-dependent pattern for miR-215 in tumorigenicity. Protein tyrosine phosphatases (PTPs) are known to be signaling molecules that regulate a variety of cellular processes including differentiation, cell proliferation, mitotic cycle and oncogenic transformation [23]. Emerging evidence has revealed that mutations in PTPs play vital roles in tumorigenesis [23]. PTPRT, also known as PTPρ, is a member of the PTPs family. Recently, it has been reported that PTPRT is the most frequently mutated PTP gene in colorectal cancers (CRCs) [24]. PTPRT normally acts as a tumor suppressor and over-expression of PTPRT inhibits CRC cell growth [24].

In this study, we demonstrate that relative to wild type HBx, HBxΔ127 strongly enhances proliferation of hepatoma cells through up-regulating miR-215, which directly targets PTPRT mRNA. Our finding provides new insights into the mechanism of hepatocarcinogenesis mediated by HBxΔ127.

2. Materials and methods

2.1. Cell culture and tissue specimen

The human hepatocellular carcinoma H7402 and H7402-XΔ127 (an HBxΔ127 stably transfected H7402 cell line) cell lines [13,14] were cultured in RPMI Medium 1640 (Gibco, Grand Island, NY, USA). HepG2 and HepG2-XΔ127 (an HBxΔ127 stably transfected HepG2 cell line) were cultured in Dulbecco's modified Eagle's medium (Gibco). The media were supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin.

2.2. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

QRT-PCR was explored to detect the expression of miR-215 and PTPRT using Absolute Blue qRT-PCR SYBR green mix according to the manufacturer's instructions. Double-stranded DNA specific expression was examined by the comparative Ct method using $2^{-\Delta\Delta Ct}$. Primers were listed in Supplementary Table 1.

2.3. Western blot analysis

Western blot analysis assay protocols were described previously [25,26]. Primary antibodies were rabbit anti-PTPRT (Santa cruz biotechnology, Santa Cruz, CA, USA), and mouse anti-β-actin (Sigma–Aldrich, St. Louis, St. Louis, MO, USA).

2.4. Treatment of cultured cells

Cells were cultured in 6-well or 24-well plates for 12 h and then were transfected with plasmid or siRNA using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). pSilencer-X produced small interfering RNAs (siRNAs) targeting HBxΔ127 (pSi-XΔ127) and pSilencer-control (pSi-NC) was used as negative control [27]. The plasmids were transfected into cells for qRT-PCR, Western blot analysis or luciferase reporter gene assays. siRNA duplexes and miR-215 mimic were synthesized and purified by RiboBio (Guang Zhou, China). The siRNA duplexes sequences were listed in Supplementary Table 1.

2.5. Construction of the 3′UTR of PTPRT

The fragment containing the target site of miR-215 in 3′UTR of PTPRT was inserted into the FseI/XbaI site, the downstream of the luciferase gene in the pGL3-Control vector (Promega, Madison, WI, USA). The resulting vector was sequenced and named pGL3-PTPRT 3′UTR wt. Site-directed mutants of the miR-215 target sites in pGL3-PTPRT 3′UTR was named pGL3-PTPRT 3′UTR mut. All primers were listed in Supplementary Table 1.

2.6. Luciferase reporter gene assays

Cells were transferred at 3×10^4 cells per well into 24-well dishes. After 12 h, pGL3-PTPRT 3′UTR wt/mut and miR-215/anti-miR-215 were transiently co-transfected with the pRL-TK plasmid (Promega) into cells. After 48 h, cells were collected and lysed in 1× passive lysis buffer. The luciferase activity was determined using the Dual-Luciferase Reporter® Assay System (Promega) according to the manufacturer's instructions.

2.7. Cell proliferation analysis

For quantitative proliferation assays, HepG2/HepG2-XΔ127 (or H7402/H7402-XΔ127) cells were seeded into 96 well plates (1000 cells/well) for 12 h before transfection with indicated reagents and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assays were explored to evaluate cell proliferation. With following 3 days, the absorbance value (OD) at 490 nm of each well was measured every day. Five-ethynyl-2′-deoxyuridine (EdU) incorporation assays were carried out using the Cell-Light TM EdU imaging detecting kit according to the manufacturer's instructions (RiboBio).

2.8. Colony formation analysis

For clonogenicity analysis, 24 h after transfection with indicated reagents, 1000 viable transfected cells were placed in 6-well plates and maintained in complete medium for 2 weeks. Colonies were fixed with methanol and stained with methylene blue.

2.9. Animal transplantation

Nude mice were housed and treated according to guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For tumor transplantation in nude mice, HepG2-XΔ127 cells were pretreated with anti-NC or anti-miR-215 for 24 h, and then were harvested and re-suspended at 2×10^7 per ml with sterile normal saline. Groups of 4-week-old female BALB/c athymic nude mice (Experiment Animal Center of Peking, China) (each group, $n = 5$) were subcutaneously injected at the shoulder with 0.2 ml of the cell suspensions. Tumor growth was measured after 10 days from injection and then every 5 days. Tumor volume (V) was monitored by measuring the length (L) and width (W) with calipers and calculated with the formula $(L \times W^2) \times 0.5$. After 30 days, tumor-bearing mice and controls were sacrificed, and the tumors were excised and measured.

2.10. Statistical analysis

Each experiment was repeated at least three times. Statistical significance was assessed by comparing mean values (± SD) using a Student's t-test for independent groups and was assumed for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

3. Results

3.1. HBxΔ127 strongly up-regulates miR-215 compared to wild type HBx in hepatoma cells

Previous studies showed that the expression levels of miR-215 were up-regulated in GC and HCC relative to their noncancerous counterparts [21,22] and HBx played a critical role in the development of HCC [1–4]. Thus, to investigate the effect of HBx on miR-215 expression, we performed over-expression of HBx using pCMV-HBx plasmid in HCC cells. Our data showed that HBx was able to up-regulate miR-215 in the cells. As a natural mutant of the HBx, HBxΔ127 remarkably enhanced cell proliferation and migration in HCC relative to wild type HBx [12–14]. More importantly, we found that HBxΔ127 could significantly increase the expression of miR-215 relative to HBx (Fig. 1A and B). Conversely, down-regulation of HBxΔ127 could abolish the up-regulation of miR-215 in HepG2-XΔ127 and H7402-XΔ127 cells (Fig. 1C and D). Therefore, these data suggest that HBxΔ127 can significantly up-regulate the expression of miR-215 compared to HBx, which may play an important role in hepatocarcinogenesis.

3.2. MiR-215 directly targets PTPRT mRNA

To search for the miR-215 target genes, we identified one complementary sequence to its seed region in the 3′UTR of tumor suppressor PTPRT using TargetScan (http://www.targetscan.org/) (Fig. 2A). Then, we cloned the 3′UTR of PTPRT and its mutants, respectively. The luciferase reporter gene assays showed that miR-215 could decrease the luciferase activity through targeting 3′UTR of PTPRT, but it failed to work when the target site was mutated (Fig. 2B). Meanwhile, the anti-miR-215 increased the luciferase activity in HepG2-XΔ127 cells and the effect could be abolished when the target site was mutated (Fig. 2C). In addition, miR-215 mimics was able to up-regulate the expression of PTPRT in HepG2 cells (Fig. 2D and F) and anti-miR-215 resulted in the down-regulation of PTPRT expression in HepG2-XΔ127 cells (Fig. 2E and F). Thus, our data support the notion that PTPRT is one of the targets of miR-215.

3.3. HBxΔ127 enhances proliferation of hepatoma cells via up-regulating miR-215 in vitro

It has been reported that HBxΔ127, miR-215 and PTPRT all are implicated in tumor growth [12–14,21,22,24]. Therefore, we are

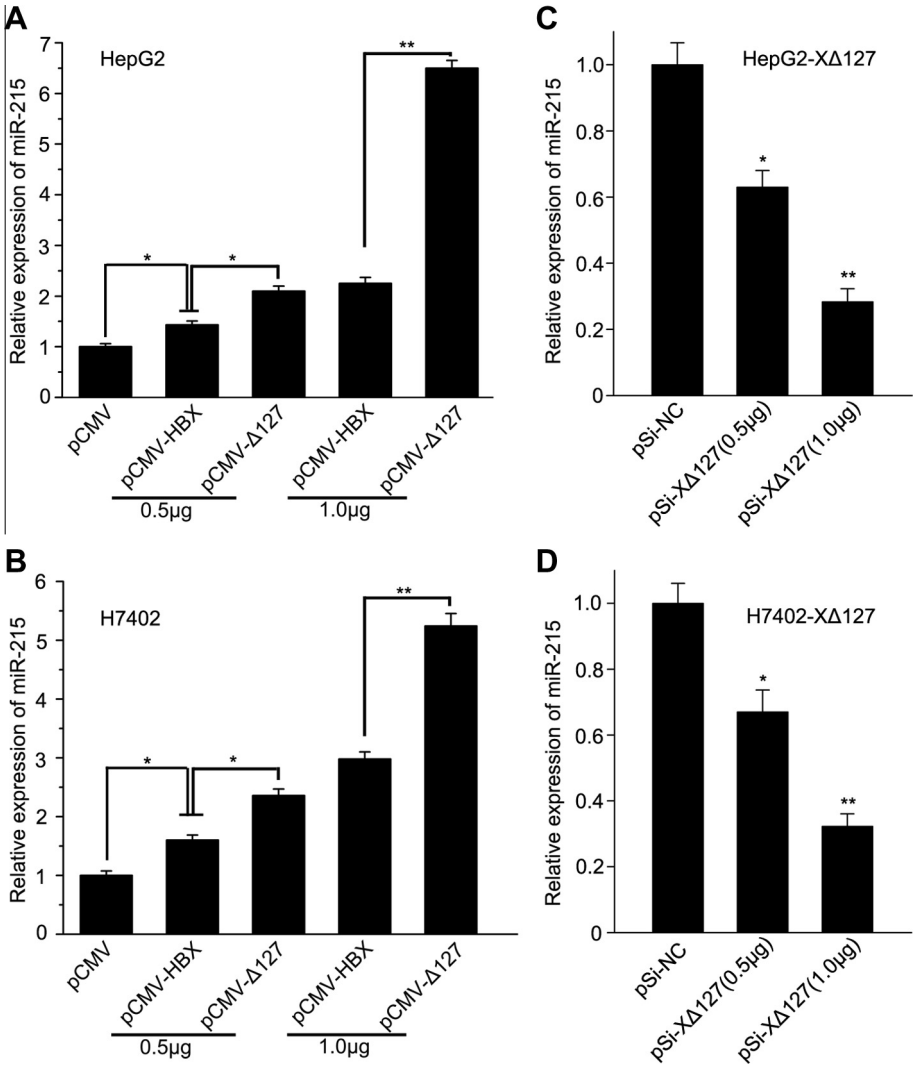

Fig. 1. HBxΔ127 strongly up-regulates miR-215 compared to wild type HBx in hepatoma cells. (A and B) The expression of miR-215 in HepG2/H7402 cells transfected with HBxΔ127 (or pCMV, HBx) was detected by qRT-PCR. (C and D) The expression of miR-215 in HepG2-XΔ127/H7402-XΔ127 cells transfected with pSi-XΔ127 (or pSi-NC) was examined by qRT-PCR. Statistical significant differences are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

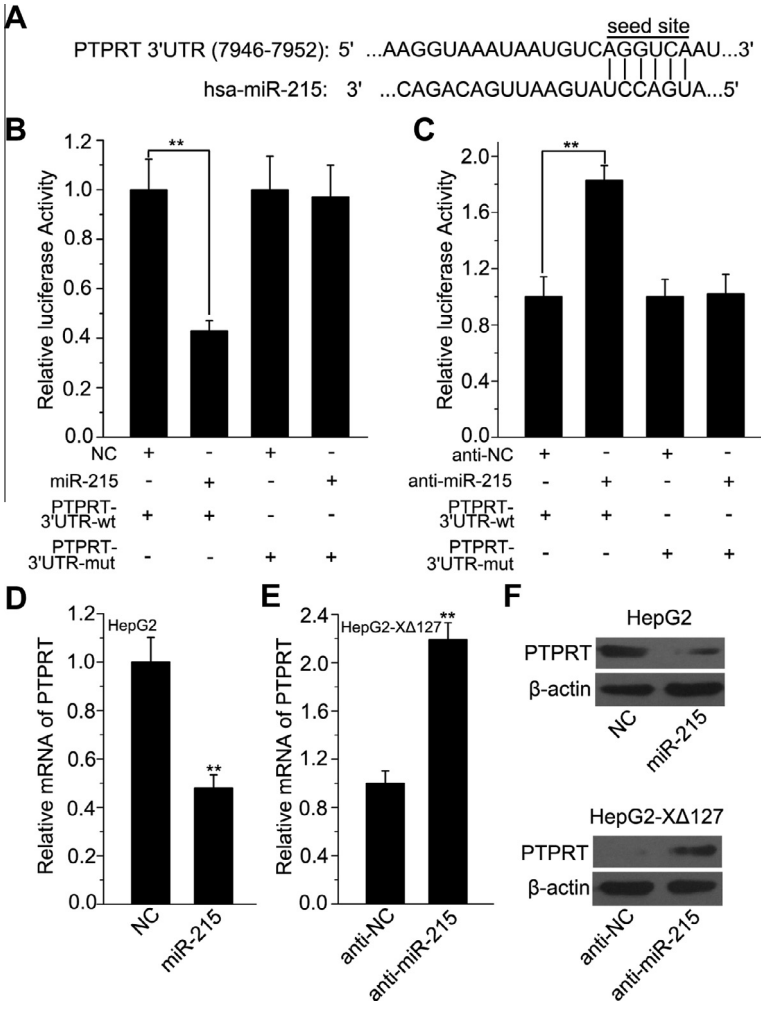

Fig. 2. MiR-215 directly targets PTPRT mRNA. (A) Sequence alignment between miR-215 and 3′UTR of PTPRT mRNA was shown. (B) The effect of miR-215 on promoter activity of PTPRT 3′UTR was detected by luciferase reporter gene assays. Co-transfection was performed using PTPRT-3′UTR-wt/PTPRT-3′UTR-mut with scrambled/miR-215 in HepG2 cells. (C) HepG2-XΔ127 cells were co-transfected with PTPRT-3′UTR-wt/PTPRT-3′UTR-mut and anti-miR-NC/anti-miR-215. The promoter activities were measured by luciferase reporter gene assays. (D–F) The expression of PTPRT in scrambled or miR-215 stimulated-HepG2 cells (or anti-miR-NC, anti-miR-215 stimulated-HepG2-XΔ127 cells) was detected by qRT-PCR and Western blot, respectively. Statistical significant differences are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

interested in whether HBxΔ127 enhances proliferation of hepatoma cells through up-regulating miR-215. As indicated in Fig. 3A and B, the proliferation of hepatoma cells transfected with miR-215 was increased sharply relative to controls. However, anti-miR-215 suppressed the proliferation of HepG2-XΔ127 and H7402-XΔ127 cells (Fig. 3A and B). To determine the effect of PTPRT on hepatoma cell proliferation mediated by HBxΔ127/miR-215 signaling, the HepG2-XΔ127 and H7402-XΔ127 cells transfected with anti-miR-215 were treated with NC or si-PTPRT, respectively. MTT, EdU and colony formation assays showed that depletion of PTPRT remarkably suppressed the down-regulation of cell proliferation mediated by anti-miR-215 in HepG2-XΔ127 and H7402-XΔ127 cells (Fig. 3C–F). Thus, we conclude that HBxΔ127 promotes proliferation through up-regulating miR-215 targeting PTPRT in hepatoma cells *in vitro*.

3.4. Anti-miR-215 suppresses growth of hepatoma cells mediated by HBxΔ127 in vivo

To better understand the contribution of HBxΔ127 to growth of hepatoma cells through up-regulating miR-215, we performed tumor xenograft analysis in which miR-215 was inhibited by anti-miR-215. Animal transplantation analysis showed that anti-miR-215 was able to decrease the tumor weight and volume derived from HepG2-XΔ127 cells relative to controls (Fig. 4A–C). Moreover, the expression of PTPRT was increased in tumors treated with anti-miR-215, which further validated that PTPRT was one of the targets of miR-215 (Fig. 4D). Thus, we conclude that anti-miR-215 is responsible for suppressing growth of hepatoma cells mediated by HBxΔ127 through up-regulating PTPRT expression *in vivo*.

4. Discussion

HBV infection plays crucial roles in the development of hepatocarcinogenesis [3]. A high rate of viral turnover, combined with an error-prone polymerase, leads to a very high frequency of mutational events during HBV replication [28]. Emerging evidences have shown that COOH-terminal truncated HBx is implicated in proliferation, apoptosis, migration and transformation of hepatoma cells [10]. However, the underlying mechanism needs further elucidated. In the study, we report that HBxΔ127 enhances cell proliferation through up-regulating miR-215, which directly targets tumor suppressor PTPRT.

Recent studies have revealed that miRNAs participate in multiple cellular processes including proliferation, migration, and differentiation. In addition, many of them act as tumor suppressors or

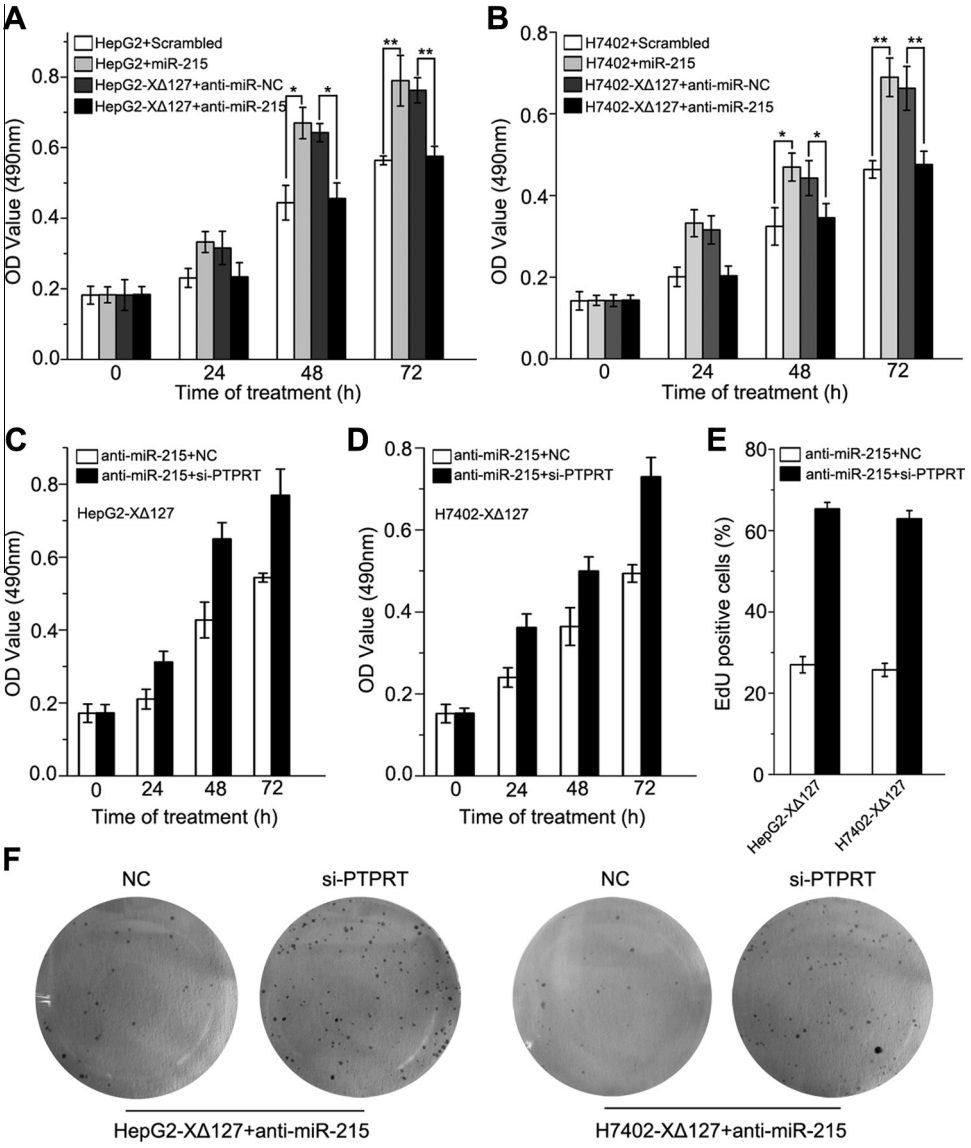

Fig. 3. HBxΔ127 enhances proliferation of hepatoma cells *via* up-regulating miR-215 *in vitro*. (A and B) The effect of miR-215 on HBxΔ127-enhanced cell proliferation was determined by MTT assays. HepG2/H7402 (or HepG2-XΔ127/H7402-XΔ127) cells were transfected with scrambled or miR-215 (or anti-miR-NC, anti-miR-215), respectively. (C–F) The effect of PTPRT on cell proliferation mediated by HBxΔ127 and miR-215 was determined by MTT, EdU and colony formation assays. Anti-miR-215 pre-treated HepG2-XΔ127/H7402-XΔ127 cells were transfected with si-NC or si-PTPRT. Statistical significant differences are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

oncogenes implicated in the development of HCC mediated by HBx [18,26]. MiR-215 acts a tumor suppressor or oncogene in tumorigenicity in a context-dependent pattern [19–22]. However, in this study, we found that miR-215 was up-regulated by HBx in hepatoma cells. As a natural mutant of HBx, HBxΔ127 possessed much more tumorigenesis capability to regulate genes expression, which was involved in the control of cell proliferation and migration [13,14]. Consistent with the notion, we found that HBxΔ127 could significantly increase the expression of miR-215 relative to HBx in hepatoma cells. It has been reported that wild type HBx contains two function domains: oncogenic domain (the NH2-terminal through middle peptide) and proapoptotic domain (the COOH-terminal peptide). There is a balance between these two functions in HBV-infected hepatocytes. Once the proapoptotic domain is deleted by an unknown mechanism during the viral integration, the balance is broken and the oncogenic function becomes dominant, resulting in the subsequent development of HCC [10]. In this study, we revealed that the stability of HBxΔ127 was not changed compared to wild type HBx protein (Supplementary Fig. 1). Therefore, our data are consistent with the above reported finding, because of HBxΔ127 is deleted with 27 amino acids in the COOH-terminal. It has been reported that frequently up-regulated miR-215 in gastric cancer may influence cell proliferation by targeting RB1 [29]. Thus, our data suggest that miR-215 may play an important role in the development of HCC.

Next, we predicted the target genes of miR-215 by bioinformatics analysis and found that the 3′UTR of PTPRT contains binding site of miR-215. PTPRT is the most frequently mutated PTPs in human cancers. Previous study reported that signal transducer and activator of transcription 3 (STAT3) was a substrate of PTPRT [30]. Over-expression of PTPRT in colorectal cancer cells could reduce the expression of target genes in STAT3 pathway. In this study, our results showed that miR-215 was able to inhibit the expression of PTPRT by directly targeting its 3′UTR. It has been reported that STAT3 pathway plays a vital role in tumorigenesis. Moreover, STAT3 was involved in the HBx enhanced proliferation of hepatoma cells [31]. Thus, miR-215 and its target PTPRT might be also implicated in the proliferation of hepatoma cells. As antic-

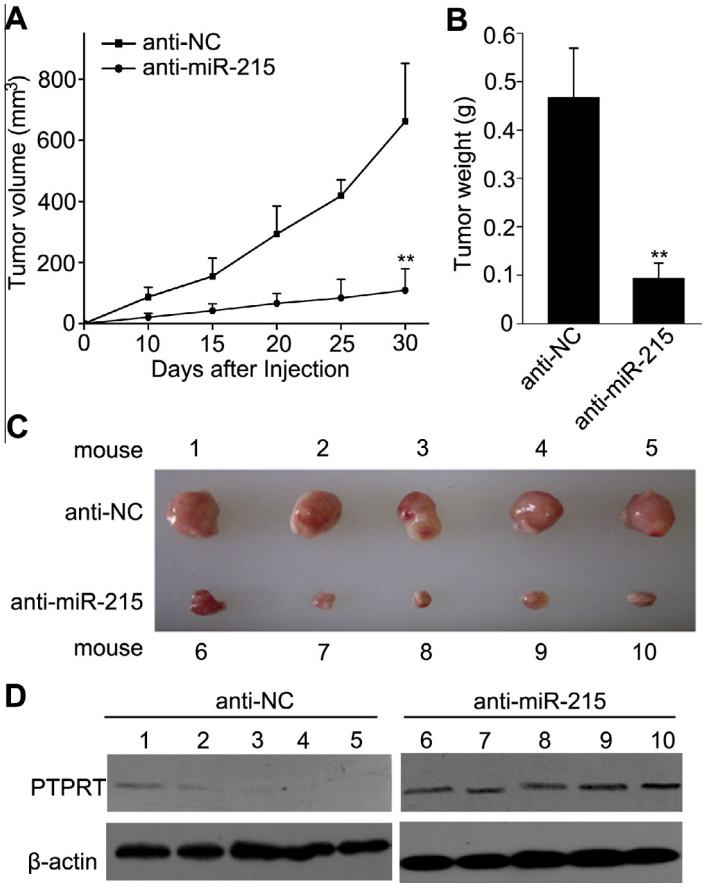

Fig. 4. Anti-miR-215 suppresses growth of hepatoma cells mediated by HBxΔ127 *in vivo*. (A) The growth curves of tumors derived from HepG2-XΔ127 cells transfected with anti-miR-NC or anti-miR-215. (B) The average weight of tumors. (C) The image of dissected tumors from nude mice ($n = 5$). (D) The relative protein expression of PTPRT in the tumor tissues from mice was determined by Western blot analysis. Statistical significant differences are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$, Student's t-test.

ipate, cell proliferation and animal transplant analysis showed that HBxΔ127 promoted proliferation of hepatoma cells through up-regulating miR-215 which inhibited PTPRT. Our previous studies showed that HBxΔ127 could remarkably up-regulate NF-κB, survivin, human telomerase reverse transcriptase (hTERT), c-Myc, PCNA, 5-LOX, FAS and osteopontin, respectively, relative to wild type HBx in hepatoma cells [12–14]. In this study, we found that HBxΔ127 suppressed PTPRT through up-regulating miR-215 expression. Bioinformatics analysis showed that those genes were not targets of miR-215. Interestingly, it has been reported that PTPRT is able to inhibit STAT3 signaling which can up-regulate survivin, hTERT, c-Myc and Rac1 [32–35]. Rac 1 is involved in the regulation of 5-LOX [36]. *5-LOX* is able to promote NF-κB activity [37]. In addition, osteopontin can activate JAK2/STAT3 signaling to promote tumor growth [38]. Therefore, we conclude that HBxΔ127 enhances proliferation of hepatoma cells in a network manner involving NF-κB, survivin, hTERT, c-Myc, PCNA, 5-LOX, FAS, osteopontin, miR-215 and PTPRT.

In summary, the central finding of this work is that HBxΔ127 promotes proliferation of hepatoma cells through up-regulating miR-215 targeting PTPRT. Thus, our finding provides new insights into the mechanism of promotion of cell growth mediated by HBx mutants. Therapeutically, HBx/HBxΔ127 may serve as a target of HBV-associated liver cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.004.

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