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NS5ATP9 Promotes Beclin 1-Dependent Starvation-Induced Autophagy of Hepatoblastoma Cells

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ABSTRACT

NS5ATP9, a gene up-regulated by NS5A, plays a crucial oncogenic role in several types of human tumours. However, the underlying mechanisms remain unclear. Autophagy, an evolutionarily conserved catabolic process, maintains cellular homeostasis under stress conditions, such as starvation, and plays a crucial role in tumour initiation and progression. Here, we report that NS5ATP9 mRNA and protein expression was up-regulated in starved HepG2 cells and that the up-regulated NS5ATP9 played a functional role in starvation-induced autophagy. Overexpression or silencing of this gene showed contrasting effects on Beclin 1 and on starvation-induced autophagy. Furthermore, NS5ATP9-mediated autophagy is required for promotion of tumour cell growth, and this effect could be inhibited with 3-methyladenine, chloroquine or by Beclin 1-silencing. Thus, the mechanism for NS5ATP9-promoted autophagy is Beclin 1-dependent in the condition of starvation, and for hepatoblastoma cell growth is also Beclin 1-dependent. J. Cell. Biochem. 116: 1574–1582, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: AUTOPHAGY; NS5ATP9; STARVATION; BECLIN 1; PROLIFERATION

NS5ATP9 (GenBank accession No. AF529370), also is known as KIAA0101, p15PAF, L5 or OEACT-1. According to the National Center for Biotechnology Information (NCBI) database, the NS5ATP9 gene is located at 15q22.1 and it consists of 336 bp that encode a 111-amino acid residue protein. The gene product of NS5ATP9 was firstly identified as a proliferating cell nuclear antigen (PCNA)-binding protein by yeast two-hybrid assay [Yu et al., 2001], and as a target gene transactivied by HCV NS5A protein by suppression substraction hybridisation (SSH) technique by this group [Shi et al., 2008]. Recent studies have shown that NS5ATP9 expression is significantly elevated in some types of tumour tissues [Mizutani et al., 2005; Yuan et al., 2007; Jain et al., 2011; Kato et al., 2012; Liu et al., 2012]. There were studies that NS5ATP9 is involved in the regulation of diverse processes such as DNA repair, apoptosis, cellular signalling pathway, cell cycle control and cell growth [Turchi et al., 2009; Emanuele et al., 2011; Jain et al., 2011]. Our previous studies had shown that NS5ATP9 is up-regulated by NS5A protein of HCV and that NF-κB could bind to the NS5ATP9 gene promoter [Shi et al., 2008]. The recent study has also showed that variant 1 of NS5ATP9 was overexpressed in hepatocellular carcinoma and could prevent doxorubicin-induced apoptosis by inhibiting p53 activation [Liu et al., 2012]. It is not known whether NS5ATP9 is involved in carcinogenesis.

Autophagy, is an intracellular process that allows for the degradation of proteins and organelles [Ohsumi, 2001; Shintani and Klionsky, 2004; Lum et al., 2005]. Morphologically, autophagy is characterized by the formation of a double-membrane structure termed the autophagosome. In yeast, the process of autophagy can be stimulated by the withdrawal of various nutrients, many components are involved in the autophagosome formation and autophagy-related genes (ATG) especially play an essential role in this process [Lee et al., 2008]. Autophagy plays dual roles in tumours [Choi, 2012]. Some data

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support the idea that autophagy can be classified as an anti-oncogenic mechanism [Czyzyk-Krzeska et al., 2012]; however, accumulating evidence strongly suggests that autophagy enhances tumourigenesis and protects tumour cells from death [Ogier-Denis and Codogno, 2003]. Autophagy, as a compensatory response under various stresses, ensures recovery of energy in tumour cells, which is then used for survival and the reprogramming of the metabolism of these cells to accommodate rapid cell growth and proliferation [Lin et al., 2009]. Many tumour suppressors, such as TP53 [Yamaki et al., 2001], PTEN [Arico et al., 2001], DAPK1 [Inbal et al., 2002] and TSC1/TSC2 [Kvam et al., 2005] are involved in the regulation of autophagy [Song et al., 2013]. Our aim was to determine whether NS5ATP9 also affects autophagy under starvation conditions and, if so, to define the underlying mechanism(s).

The process of autophagosome formation is regulated by several autophagy-related genes, such as ATG5, ATG6 (also known as Beclin 1), and ATG8 (the microtubule-associated protein 1 light chain 3 gene, LC3). Beclin 1, which acts upstream of autophagosome formation, governs the autophagy process by regulating the activity of the PIK3C3/VPS34 [Tassa et al., 2003] and the subsequent recruitment of additional ATG proteins for initiating autophagosome formation [Juhasz et al., 2008]. Expression of NS5ATP9 is related to several types of tumours, and NS5ATP9 is involved in the regulation of cell autophagy that is evoked by the HCV NS5A protein. However, little is known about whether NS5ATP9 plays a role in tumour cell autophagy under conditions of starvation. In a previous study, we have shown that NS5A- and NS5ATP9-enhanced autophagy is Beclin 1-dependent [Quan et al., 2014]. We then hypothesized that NS5ATP9 may interfere in the formation of the autophagosome by targeting autophagy-associated genes under starvation conditions. The results presented in this paper indicated that the expression of NS5ATP9 was up-regulated by starvation and that Beclin 1 was involved in autophagy induced in hepatoblastoma cells by starvation.

MATERIALS AND METHODS

CELL CULTURE

Hepatoblastoma cell line HepG2 and hepatic cell line L02 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, Life Technology, Grand Island, NY), 100 U/ml of penicillin G, and 100 μg/ml of streptomycin (SW30010, BD, Franklin Lakes, NJ) at 37°C in a 5% CO_2 atmosphere. Autophagy was induced by amino acid deprivation in Earle's Balanced Salt Solution (EBSS, Sigma–Aldrich, St. Louis, MO, E2888-500ML) for different time points. 3-methyladenine (3-MA, Sigma–Aldrich, M9281) and chloroquine (CQ, InvivoGen, Shatin, Hong Kong, tlrl-chq) was used to block autophagy.

CONSTRUCTION OF PLASMIDS

pcDNA3.1/myc-His(−)-NS5ATP9, pEGFP-C1-NS5ATP9, pGL4.10-Beclin 1-promoter, all of these plasmids as described in our previous study [Quan et al., 2014].

LUCIFERASE REPORTER ASSAY

HepG2 cells were seeded in a 48-well culture plate. Transfection assays were performed using jetPRIME™ (Polyplus-transfection, Inc., Illkirch, France) in 48-well culture dishes with 250 ng of Beclin 1 promoter reporter plasmids (pGL4.10-Beclin 1-P) and 25 ng of Renilla luciferase vector (pRL-TK) DNA 24 h after seeding. In some experiments, HepG2 cells were cotransfected with 250 ng of the indicated expression vector (pcDNA3.1/myc-His(−)-NS5ATP9) DNA, whereas the pcDNA 3.1/myc-His(−) empty vector DNA was used to normalize the amount of total DNA. At 4 h post-transfection, HepG2 cells were left for 24 h in complete induction medium. After 24 h of transfection, the HepG2 cells were lysed in the Passive Lysis Buffer (E1941, Promega, Madison, WI). Beclin 1 promoter activity was measured in a microplate luminometer according to the technical manual for the Dual-Luciferase Reporter Assay System (E1910, Promega).

RNA ISOLATION, REVERSE TRANSCRIPTION AND QUANTATIVE POLYMERASE CHAIN REACTION (qPCR)

Total RNA from various transfected HepG2 cells was prepared using Total RNA Kit (R6834, Omega, Norcross, GA) according to the manufacturer's instructions. Total RNA was reverse transcribed into single strand cDNA by PrimeScript® RT reagent Kit (DRR037A, TaKaRa, Shanghai, China). A one-fourth aliquot of the cDNA was subjected to qPCR (4367659, ABI, Grand Island, NY) amplification using gene-specific primers. The relative amounts of Beclin 1 mRNA were calculated with the comparative Ct method (ΔΔCt) and normalized to the endogenous levels of β-actin. The Beclin 1 sense and anti-sense primers were 5′-GAGGGATGGAAGGGTCTAAG-3′ and 5′-GCCTGGGCTGTGGTAAGT-3′, respectively, and the β-actin sense and anti-sense primers were 5′-CATCCGCAAAGACCTG-TACGC-3′ and 5′-AGTACTTGCGCTCAGGAGGAG-3′, respectively (Sangon, Shanghai, China).

AUTOPHAGY ASSAYS

For starvation, the HepG2 cells were incubated in amino acid deprivation in EBSS (Sigma–Aldrich, E2888-500 ML) for indicated number of hours. Autophagy was visualized in HepG2 cells by cotransfection with pCMV6-AN-RFP-LC3 (pRFP-LC3) (RC100053, Origene, Rockville, MD) and pEGFP-C1-NS5ATP9, the control HepG2 cells were cotransfected with RFP-LC3 and pEGFP-C1. The nuclei were stained with 4′, 6-diamidino-2-pheny-lindole (DAPI) (D9542, Sigma), followed by analysis with confocal microscopy (LSM510Meta, Zeiss, German) observation. For most experiments, the number of pRFP-LC3 punctate was assessed from at least six random high-power fields. A single random Z-section was used for each field, and a minimum of 30 cells per sample were counted with triplicate samples per condition per experiment. A LC3 punctate was considered to be a totally isolated RFP-positive structure greater than ≈1 μm in diameter.

WESTERN BLOTTING

HepG2 cells were lysed in lysis buffer containing a protease inhibitor cocktail (5872 s, CST, USA). The protein concentration was determined by the Pierce BCA assay (23225, Thermo Scientific, Rockford, IL). Equal amounts of protein were separated by 12% Bis–Tris Gel/MOPS (NP0034, Invitrogen, Grand Island, NY) and transferred to a PVDF membrane (ISEQ00010, Milipore, Billerica, MA) by electro-blotting. After blocking with 5% nonfat dry milk (2321000, BD), the

membranes were reacted with primary antibodies such as anti-LC3B antibody (3868, CST), anti-Beclin 1 antibody (3495, CST), anti-NS5ATP9 (ab56773, Abcam, HongKong), anti-PI3KIII (4362, CST), anti-ATG-4B (5299, CST), anti-ATG-5 (8540, CST), anti-GAPDH (5174, CST), in Tris-buffered saline containing 0.1% Tween-20 supplemented with 5% nonfat dry milk. The bands were detected using an enhanced chemiluminescence system (32209, Thermo Scientific). All the Western blot data were quantified by using Bio1D software (VILBER, S:11.640150, France).

CELL PROLIFERATION ASSAY

HepG2 cells were seeded at a density of 5,000 cells per well to overnight at 37°C, subjected to siRNA interference or plasmid transfection. OD value was measured at 450 nm using Cell Counting Kit-8 (Dojindo, Shanghai, China, CK04) at various time points according to the guidance of the manufacturer. Cell viability was calculated according to the formula: experimental OD value/control OD value.

siRNA OLIGONUCLEOTIDES

siRNA duplexes against human NS5ATP9 and a negative control siRNA that does not target any mammalian genes were synthesized and purchased from Invitrogen as described previously [Quan et al., 2014]. The siRNA were Beclin 1 siRNA (sc-29797) and the negative control siRNA (sc-37007) were purchased from Santa Cruz.

STATISTICAL ANALYSIS

Triplicate experiments represent data pooled from three independent experiments. Statistical evaluation for data analysis was determined by using the paired Student's *t*-test. All data were showed as the mean ± SEM. A statistical difference of $P < 0.05$ was considered significant.

RESULTS

NS5ATP9 EXPRESSION WAS UP-REGULATED IN STARVED HepG2 CELLS

Autophagy was induced in the hepatoblastoma cell line, HepG2, by starvation in EBSS for 2–48 h. NS5ATP9 mRNA levels were detected in post-starvation HepG2 cells using qPCR, as shown in Figure 1A. NS5ATP9 mRNA levels were up-regulated according to the duration of starvation. After 24 h of starvation, NS5ATP9 mRNA levels had increased by 2.8-fold. The mRNA levels of autophagy-related genes also increased in starved HepG2 cells (Fig. 1B). Proteins encoded by autophagy-related genes as well as the NS5ATP9 protein were also detected by western blot in cells after 0, 1, 2, 4 and 6 h of starvation (Fig. 1C). These results showed that the NS5ATP9 protein level was up-regulated post starvation, consistent with the conversion of LC3-I to LC3-II, an important marker of autophagy [Mizushima et al., 2010] (Fig. 1C). According to the results of autophagy induced by starvation in HepG2 cells (Fig. 1C), the conversion of LC3-I to LC3-II peaked at 6 h post-starvation; thus, in the subsequent experiment, autophagy was induced by 6 h of starvation. Furthermore, NS5ATP9 and autophagy-related gene mRNA levels were up-regulated at 6 h post-starvation, and RFP-LC3 punctate formation increased by this time, in contrast to the control (Fig. 1D and E). All these results showed that starvation-induced autophagy is accompanied by an increase in NS5ATP9 mRNA and protein.

EXOGENOUS OVEREXPRESSION OR KNOCKDOWN OF NS5ATP9 SHOWED CONTRASTING EFFECTS ON BECLIN 1 REGULATION AND STARVATION-INDUCED AUTOPHAGY

Our previous study had demonstrated that the NS5ATP9 protein could up-regulate Beclin 1 promoter activity in HepG2 cell line [Quan et al., 2014]. In this study, we demonstrated that the promoter activity of Beclin 1 was also up-regulated in starved cells, however, down-regulated in NS5ATP9-silenced cells (Fig. 2A). Furthermore, the Beclin 1 promoter activity could not be up-regulated in NS5ATP9-silenced cells even though starvation for 6 h. Consistent with these results, the mRNA level of Beclin 1 could be up-regulated in starved cells, however, attenuated in NS5ATP9-silenced cells (Fig. 2B). The western blot results showed that the Beclin 1 expression and the conversion of LC3-I to LC3-II were increased in NS5ATP9-overexpressing HepG2 cells (Fig. 2D), and down-regulated in NS5ATP9-silenced cells that had been starved for 6 h (Fig. 2C). These results were consistent with the RFP-LC3 punctate formation detected by confocal microscopy (Fig. 2E and F). All of these results suggested that NS5ATP9 plays an important role in starvation induced autophagy, the knockdown of NS5ATP9 attenuated the autophagy induced by starvation (Fig. 2C).

NS5ATP9-PROMOTED AUTOPHAGY IS BECLIN 1-DEPENDENT IN THE CONDITION OF STARVATION

Beclin 1 siRNA was used to knockdown Beclin 1 expression in HepG2 cells, and autophagy levels were determined by western blotting and confocal microscopy. Beclin 1 siRNA significantly reduced the number of RFP-LC3 punctate formation under starvation conditions (Fig. 3A and B) and blocked the conversion of endogenous LC3-I to LC3-II both in the presence and in the absence of starvation (Fig. 3C). NS5ATP9 did not enhance accumulation of punctate RFP-LC3 (Fig. 3D and E) or the conversion of endogenous LC3-I to LC3-II in the Beclin 1-silenced HepG2 cells after 6 h of starvation (Fig. 3F).

STARVATION-MEDIATED AUTOPHAGY COULD BE INHIBITED BY 3-MA AND CQ

The hypothesis that NS5ATP9 is involved in cell proliferation is not totally new. In order to observing NS5ATP9-mediated cell growth have relationship with autophagy, 3-MA and CQ were used to inhibit autophagy, respectively. The HepG2 cells were starved (cultured in EBSS) for 6 h, with or without 3-MA or CQ, two important agents for blocking autophagy. Western blot results showed that the conversion of endogenous LC3-I to LC3-II was inhibited, in 3-MA-treated HepG2 cells (Fig. 4A). 3-MA is found to promote autophagy flux when treated under nutrient-rich conditions with a prolonged period of treatment, whereas it is still capable of suppressing starvation-induced autophagy [Wu et al., 2010]. We also detected several autophagy related genes mRNA level treated with 3-MA in the condition of starvation for 6 h, which also down-regulated (Fig. 4B). CQ is a well-known inhibitor of autophagic protein degradation, the LC3-II expression increased at 6 h after CQ treatment as western blot results showed (Fig. 4A). These results were confirmed by quantification of punctate RFP-LC3 formation. Consistent with

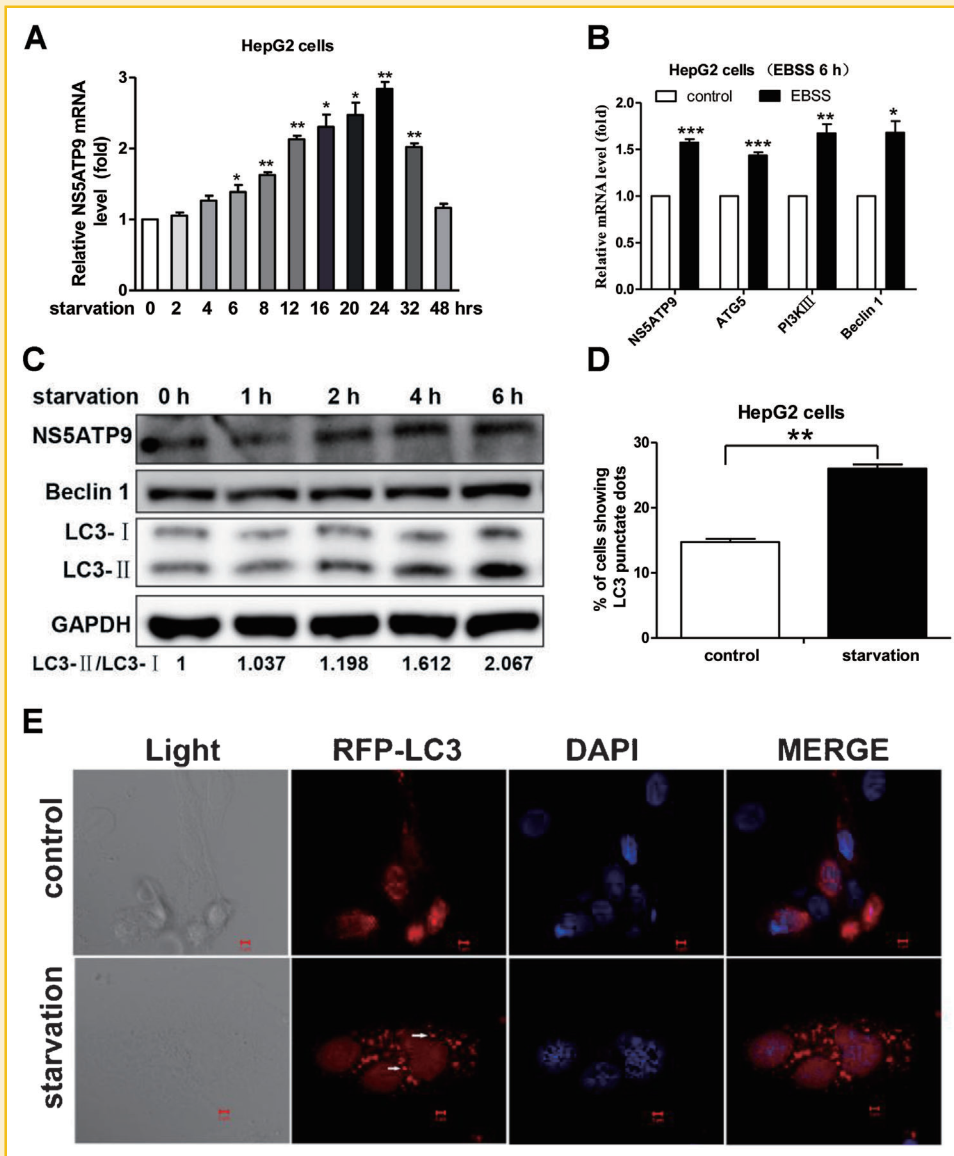

Fig. 1. NS5ATP9 expression is up-regulated in starved HepG2 cells. (A) HepG2 cells were starved for different time periods (0–48 h) in EBSS medium; NS5ATP9 mRNA expression was examined by qPCR. The results shown are the mean ± SEM values ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$). (B) HepG2 cells were starved for 6 h, and the cells without starvation as control; mRNA expression of autophagy-related genes and NS5ATP9 was then examined by qPCR ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (C) HepG2 cells were starved for different time periods (0–6 h); NS5ATP9 and autophagy-related genes proteins expresed levels were then detected by western blotting. The band intensities of LC3-II, LC3-I and GAPDH were quantified, and the relative LC3-II/LC3-I/GAPDH ratios are shown below the blots, and presented as LC3-II/LC3-I. (D–E) HepG2 cells were transfected with pRFP-LC3 for 42 h and then starved for 6 h. Confocal microscopy images showing subcellular localization of LC3 are presented (red). The nuclei were stained with DAPI (blue) at 48 h after transfection. The arrows indicated the punctuate LC3 dots. Punctate LC3 dots were then counted and results are presented in a bar graph. Scale bars, 5 μm ($n = 3$, $^{**}P < 0.01$).

western blot results, formation of punctate RFP-LC3 decreased in 3-MA-treated cells and increased in CQ-treated cells after 6 h of starvation (Fig. 4C and D).

NS5ATP9-MEDIATED AUTOPHAGY IS REQUIRED FOR PROMOTION OF TUMOUR CELL GROWTH

We next investigated the contribution of NS5ATP9-mediated autophagy to tumour cell growth. As shown in Figure 5A, NS5ATP9-overexpressing cells, with high levels of autophagy, grew significantly faster than did control cells after 6 h of starvation. However, the blockage of autophagy by 3-MA or CQ two autophagy-blocking agent (Fig. 4A and B), weaken HepG2 cell viability (Fig. 5A and B), Furthermore, NS5ATP9-silenced cells with lower autophagy levels grew significantly less than control cells (Fig. 5C and D), suggesting that attenuated autophagy is required for inhibition of tumour cell survival. To confirm these data, Beclin 1-siRNA treatment was used to abrogate autophagy, as shown in Figure 3A and C. Silencing Beclin 1 caused attenuation of autophagy in the HepG2 cells, and the cell viability could not be enhanced by overexpression of NS5ATP9 in HepG2 cells (Fig. 5E). Correspondingly, the cell viability,

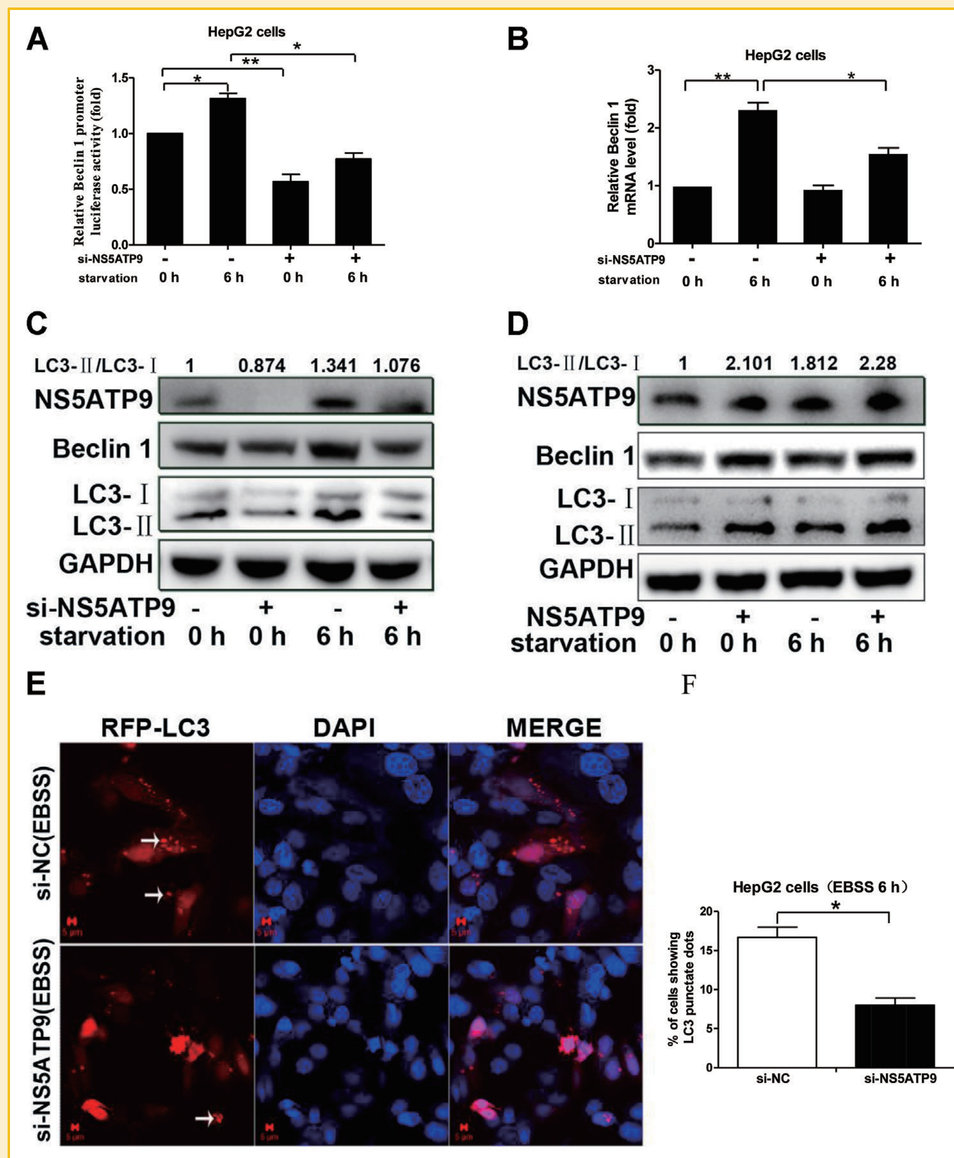

Fig. 2. Knockdown of NS5ATP9 attenuates up-regulation of Beclin 1 and the induction of autophagy by starvation. (A) HepG2 cells were cotransfected with NS5ATP9-siRNA and the Beclin 1-p construct or were cotransfected with control siRNA and the Beclin 1-p construct for 24 h, with or without starvation for 6 h. Beclin 1 promoter activity was then assayed ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). (B) HepG2 cells were transfected with NS5ATP9-siRNA and control siRNA. Total RNA was isolated at 24 h after transfection. Beclin 1 mRNA expression was quantified by qPCR, with the actin gene used as an internal control ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). (C and D) HepG2 cells were transfected with pNS5ATP9 or NS5ATP9-siRNA, and their control for 48 h, respectively, with or without starvation for 6 h. Cell lysates were then analysed for Beclin 1, and LC3 expression by western blot. The band intensities of LC3-II, LC3-I and GAPDH were quantified, and the relative LC3-II/LC3-I/GAPDH ratios are shown below the blots, and presented as LC3-II/LC3-I. (E) HepG2 cells were transfected with NS5ATP9-siRNA or a control siRNA for 24 h, and were then transfected with pRFP-LC3 for 48 h with starvation for 6 h. The arrows indicated the punctuate LC3 dots. (F) Punctate LC3 dots were counted and the results are presented in a bar graph. Scale bars: 5 μm ($n = 3$, $^{*}P < 0.05$).

after treatment with Beclin 1-siRNA was similar to that of cells treated with 3-MA and CQ. Collectively, these data indicated that NS5ATP9-mediated autophagy is required for HepG2 cell proliferation.

DISCUSSION

Accumulating evidence has demonstrated that tumour oncogenes are involved in the regulation of autophagy. NS5ATP9 has also been shown to have oncogenic activity and may be a promising target for the development of novel anticancer therapies [Hosokawa et al., 2007]. Earlier studies have shown that NS5ATP9 expression was significantly elevated in some types of tumours, including pancreatic cancer [Hosokawa et al., 2007], colorectal adenoma and adenocarcinoma [Notterman et al., 2001], non-small-cell lung cancer [Petroziello et al., 2004] and anaplastic thyroid carcinoma [Mizutani et al., 2005]. Liu et al. [2012] reported that both mRNA and protein levels of NS5ATP9 variant 1 were higher in human hepatocellular carcinoma (HCC) tissues than in normal tissues. Our previous studies showed that NS5ATP9 is up-regulated by NS5A of HCV by binding to the NS5ATP9 promoter [Shi et al., 2008]; moreover, we have

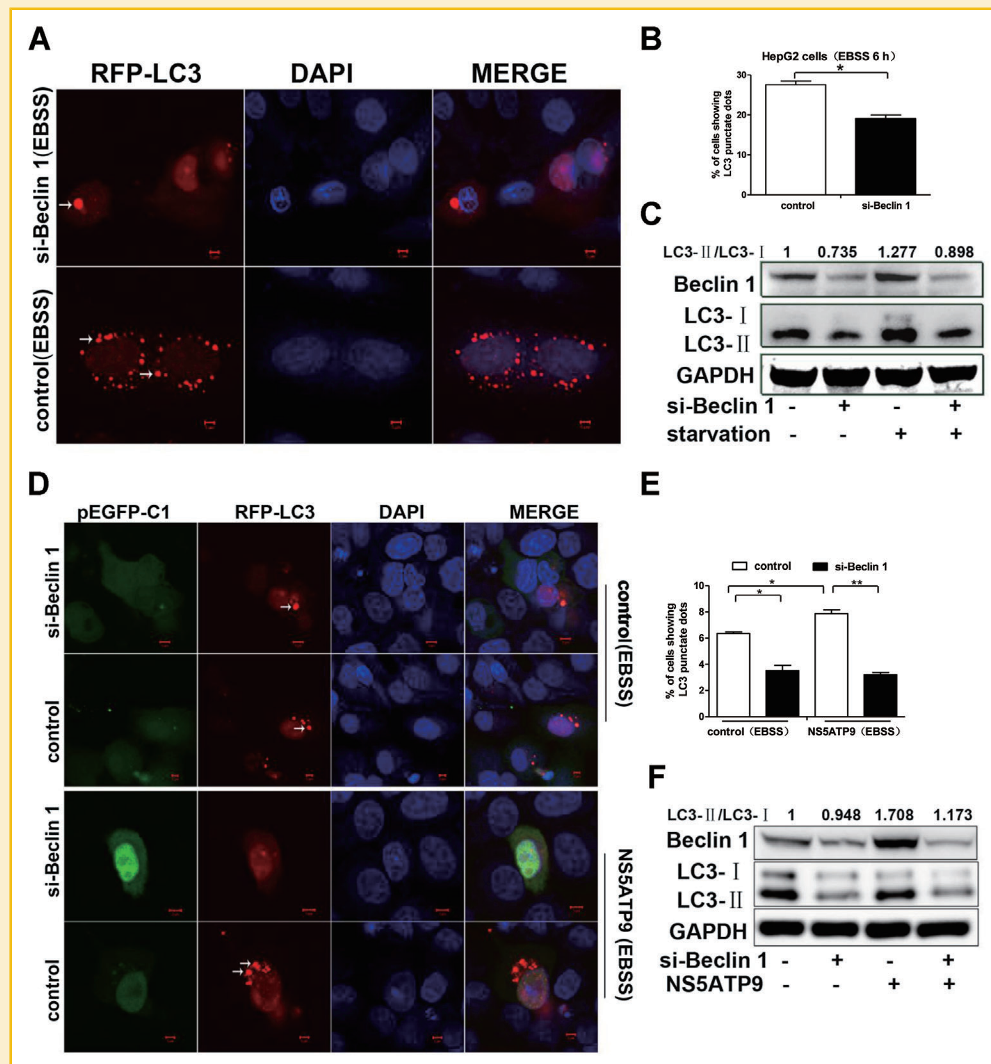

Fig. 3. NS5ATP9-mediated autophagy is Beclin 1-dependent under conditions of starvation. (A and B) HepG2 cells were transfected with Beclin 1-siRNA or control siRNA for 24 h, and were then transfected with pRFP-LC3 for 48 h, followed by starvation for 6 h. (C) HepG2 cells were transfected with Beclin 1-siRNA or control siRNA, with or without starvation for 6 h, cell lysates were then analysed for Beclin 1 and LC3 expression by western blot. (D and E) HepG2 cells were transfected with Beclin 1-siRNA or control siRNA for 24 h, and were then cotransfected with pRFP-LC3 and pEGFP-NS5ATP9 for 48 h, followed by starvation for 6 h. The arrows indicated the punctuate LC3 dots. (F) HepG2 cells were transfected with Beclin 1-siRNA or control siRNA for 24 h, and then transfected with pNS5ATP9 for 48 h, followed by starvation for 6 h; cell lysates were then analysed for Beclin 1 and LC3 expression by western blot. The band intensities of LC3-II, LC3-I and GAPDH were quantified, and the relative LC3-II/LC3-I/GAPDH ratios are shown below the blots, and presented as LC3-II/LC3-I. In (B and E) punctate LC3 dots were counted and the results are presented in a bar graph. Scale bars: 5 μm (n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$).

demonstrated that NS5ATP9 plays a role in NS5A-mediated autophagy [Quan et al., 2014]. Interestingly, NS5ATP9 mRNA and protein levels were up-regulated under starvation conditions, this provides a clue to the role of NS5ATP9 in the autophagy that is induced by starvation. Here, we demonstrated that NS5ATP9 up-regulates endogenous Beclin 1 mRNA and protein levels in a hepatoblastoma cell line in the presence or absence of starvation. We performed some experiments to gain insights into the mechanism by which Beclin 1 is regulated by NS5ATP9 and found that Beclin 1 promoter activity could be transactivated by NS5ATP9 in two cell lines (HepG2 and L02), indicating that NS5ATP9 up-regulates Beclin 1 expression at the transcription level. In our current study, NS5ATP9-induced autophagy was dependent on Beclin 1 up-regulation under conditions of starvation. No elevation in Beclin 1 promoter activity was observed in NS5ATP9-silenced cells under conditions of starvation.

The hypothesis that NS5ATP9 is involved in cell proliferation is not totally new. Consistent with our results, Mizutani et al. [2005] showed that NS5ATP9 knockdown markedly inhibited the growth of anaplastic thyroid cancer cells. Overexpression of NS5ATP9 promotes cancer cell growth, whereas attenuation of its expression by siRNA leads to reduced cell proliferation [Hosokawa et al., 2007]. A study by Hosokawa et al. [2007] demonstrated the potential oncogenic role of NS5ATP9 in pancreatic cancer. They overexpressed NS5ATP9 exogenously in NIH3T3 murine fibroblast cells, which led to cancer cell growth in vivo, confirming the growth-

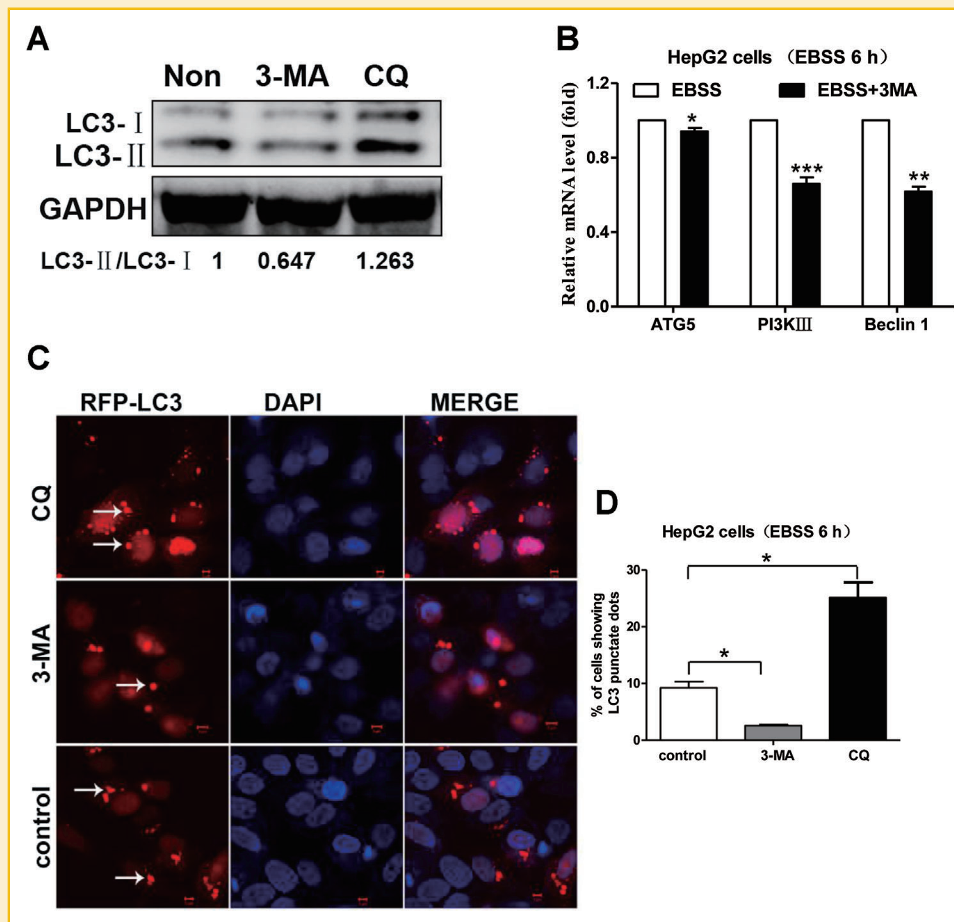

Fig. 4. Starvation-mediated autophagy could be inhibited by 3-MA and CQ. (A) HepG2 cells were starved for 6 h, with or without 3-MA (10 mM) and CQ (10 μM) for 6 h; cell lysates were then analysed for LC3 expression by western blot. Blots were re-probed with an antibody to GAPDH for comparison of protein load. The band intensities of LC3-II, LC3-I and GAPDH were quantified, and the relative LC3-II/LC3-I/GAPDH ratios are shown below the blots, and presented as LC3-II/LC3-I. (B) HepG2 cells were starved for 6 h with 3-MA (10 mM), and the control group without 3-MA; mRNA expression of autophagy-related genes was then examined by qPCR ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (C) HepG2 cells were transfected with pRFP-LC3 for 42 h and then starved for 6 h, in the presence or absence of 3-MA and CQ. Punctate localization of LC3 in autophagic vacuoles was detected by confocal microscopy. The arrows indicated the punctuate LC3 dots. Punctate LC3 dots were then counted and results are presented in a bar graph. Scale bars: 5 μm ($n = 3$, $^{*}P < 0.05$).

promoting and oncogenic nature of this protein. Similarly, in hepatocellular carcinoma (HCC), it has been shown that NS5ATP9 overexpression is associated with higher vascular invasion and contributes to poor prognosis [Yuan et al., 2007]. These results indicated that NS5ATP9 has a growth-promoting role; however, the molecular mechanisms underlying its effects have yet to be identified.

Autophagy plays a dual role in cancer, that is, it is a tumour suppressor [Qu et al., 2003; Mathew et al., 2009] and a tumour promoter [Fung et al., 2008; Yoo et al., 2009]. Although it is still a matter of debate whether autophagy is ultimately a protective response or a detrimental process, it seems clear that autophagy can lead to adverse effects in cells under certain pathological conditions [Ogier-Denis and Codogno, 2003]. Therefore, autophagy signalling and functions are necessary, but should be under tight negative regulation. Previous studies using complementary DNA (cDNA) microarray screens and northern blot analysis found that Beclin 1 was up-regulated in hepatocellular carcinoma tissues [Song et al., 2004]. Therefore, we hypothesized that NS5ATP9 overexpression in HCC is related to Beclin 1 expression and autophagy, for NS5ATP9 could up-regulate Beclin 1 with starvation or not (Fig. 3F). In our study, NS5ATP9 was up-regulated under starvation conditions and played an important role in regulated Beclin 1 and starvation-induced autophagy (Figs. 1 and 2). We investigated the contribution of NS5ATP9-mediated autophagy to tumour cell growth and found that NS5ATP9-mediated autophagy is required for promotion of tumour cell proliferation (Fig. 5). In this study, we observed that the combined treatment of NS5ATP9-overexpression and CQ or 3-MA led to dramatic tumour repression contrast with the control without CQ or 3-MA. These results indicate that inhibition of autophagy impairs NS5ATP9-mediated cell proliferation, In conclusion, we showed that inhibition of autophagy may be a efficicacy way to

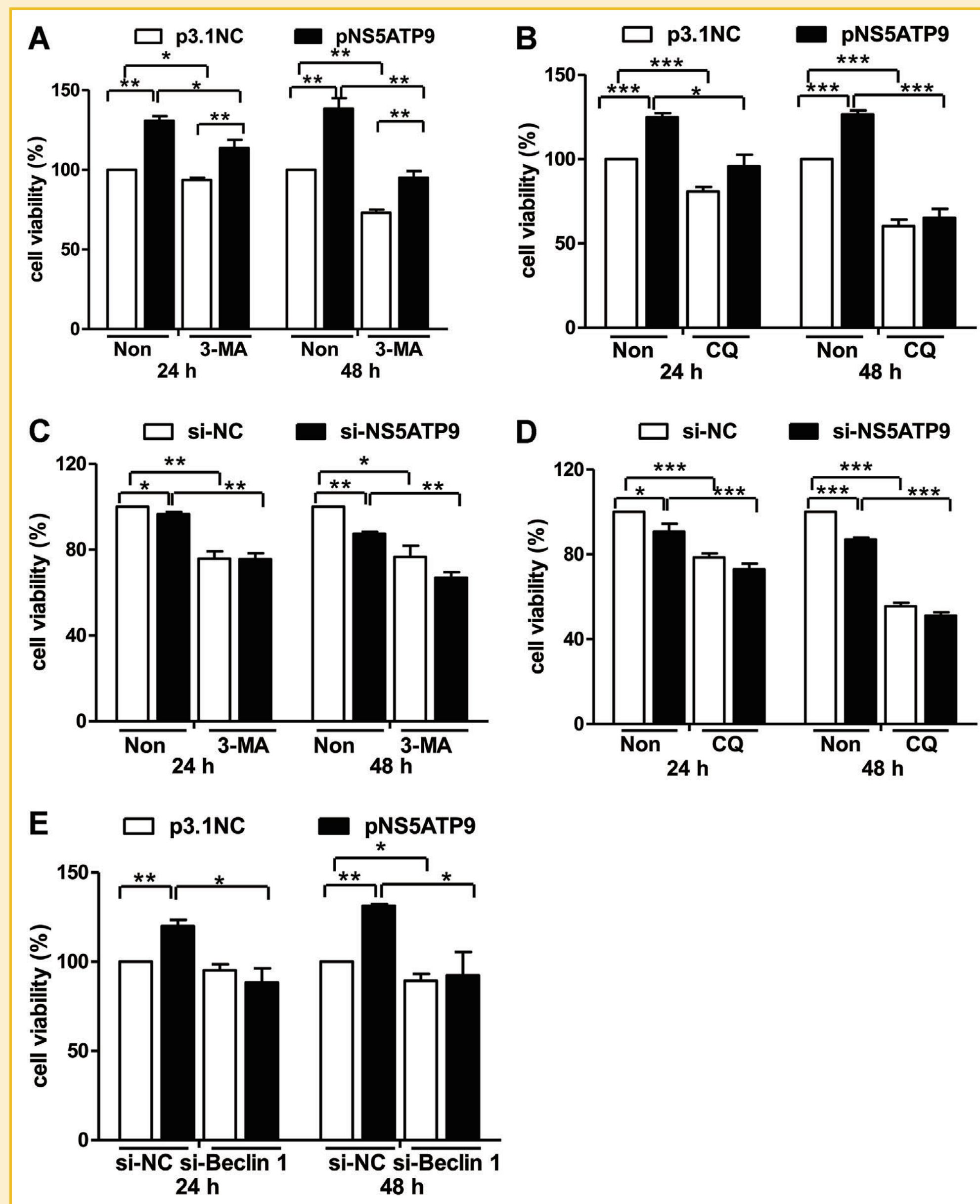

Fig. 5. NS5ATP9-mediated autophagy is required for promotion of tumour cell growth. (A–D) HepG2 cells were transfected with pcDNA3.1/myc-His(−) (p3.1NC) pcDNA3.1/myc-His(−)-NS5ATP9 (pNS5ATP9), or NS5ATP9-siRNA, siRNA control (si-NC) respectively, in the presence or absence of 3-MA (10 mM) or CQ (10 μM) to block autophagy for 24 h. Cell viability was measured, using a CCK-8 kit, at 24 and 48 h after transfection. (E) HepG2 cells were transfected with Beclin 1-siRNA or a control siRNA for 24 h and were then transfected with pNS5ATP9 or NS5ATP9-siRNA for 24 h. Cell viability was then measured using a CCK-8 kit at 24 and 48 h after transfection ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$).

inhibite the cell proliferation of tumours in which NS5ATP9 is overexpressed.

However, in another study of HCC, KIAA0101 was down-regulated and found to be a growth-inhibitory gene [Guo et al., 2006]. Specifically, depending on the tumour type and cell line studied, KIAA0101 has been found to have both growth inhibitory and stimulatory effects [Yu et al., 2001; Guo et al., 2006; Simpson et al., 2006; Hosokawa et al., 2007; Yuan et al., 2007; Turchi et al., 2009]. Such paradoxical effects on growth and cell cycle progression (growth promoting or inhibiting effects) via key cell cycle regulating factors, such as p21 and p27, have also been observed to depend on cell type and external cell stimuli or stress factors [Aguilar and Fajas, 2010].

In conclusion, our study indicated that NS5ATP9 may regulate the function of Beclin 1 and participate in the autophagy process in starved cells. The hepatoblastoma cell line also exploits the NS5ATP9-promoted autophagy pathway for promotion of tumour cell proliferation, and therefore, NS5ATP9 may also be a promising candidate target for the inhibition of tumour cell proliferation, and inhibition of autophagy may be a promising novel strategy with broad applications in cancer therapy.

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