Akt1 is required to maintain lipid droplets for release of HCV infectious virions

Wei-Ping Lee
Taipei Veterans General Hospital
Keng-Li Lan
Taipei Veterans General Hospital
Shi-Xian Liao
Taipei Veterans General Hospital
Yi-Hsiang Huang
Taipei Veterans General Hospital
Ming-Chih Hou
Taipei Veterans General Hospital
Keng-Hsin Lan (khlan@vghtpe.gov.tw)
Taipei Veterans General Hospital

Research Article

Keywords: Akt1, HCV, life cycle, sterol regulatory element binding protein-1, lipid droplet

Posted Date: May 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-546614/v1

Abstract

Hepatitis C virus (HCV) relies on the interplay of viral and host factors to complete its life cycle. It has evolved to benefit from Akt activation at some point in the life cycle through a variety of mechanisms. Our preliminary result showed that Akt-specific inhibitor reduced cell culture-derived HCV (HCVcc) infectivity in a dose-dependent manner. To dissect the mechanism, we adopted a two-part cell culture-derived HCV infection protocol with Akt1 small interfering RNAs (siRNAs) to determine the role of Akt in the HCV life cycle. The result showed that Akt1 was a crucial host factor involved in the late stage of HCV life cycle. Akt1 depletion reduced viral particles released from Huh-7.5.1 cells to culture medium with consequent reduction of viral reinfection in cell culture system, which was restored by ectopic Akt1 expression. To further study the mechanism, we found that Akt1 enhanced lipogenic pathway through transcriptional activation of fatty acid synthase (FAS) by sterol regulatory element binding protein-1 (SREBP1) to maintain sufficient lipid droplet that is an essential organelle for assembly and release of HCV infectious virions.

Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It encodes a polyprotein which is processed by both host and viral proteases to yield the structural proteins, core, and envelope glycoproteins E1, E2, as well as the non-structural proteins, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B[1]. The HCV life cycle is initiated by binding of virus particles to cellular receptors, endocytosis, fusion of HCV glycoproteins with endosomal membranes, and release of the viral genome[2]. Internal ribosome entry site [3]-mediated translation of incoming viral RNA enables viral gene expression and processing, and replication occurs in the “membranous webs”[4]. Following replication, genomic RNAs in complex with NS5A protein transit to lipid droplets (LDs), where core protein localizes and virion assembly occurs[5]. After acquiring apolipoproteins B and E (apoB and apoE), components of beta-lipoproteins (very low-density lipoproteins [VLDL] and LDL), HCV infectious particles egress in a manner that parallels the VLDL secretory pathway[6, 7].

Akt, a serine/threonine kinase, is a key regulator in the phosphatidylinositol 3-kinase (PI3K) signaling pathway and plays an important role in an array of diverse cellular functions, such as cell growth, proliferation, survival, metabolism, and mobility. In mammals, there are three Akt isoforms, Akt1, 2, and 3. While Akt1 is the predominantly expressed isoform in most tissues, Akt2 is strongly expressed in insulin-responsive/dependent tissue such as brown fat, skeletal muscle, and liver. The expression of Akt3 is localized to the brain and testes [8]. Akt1 knockout mice have been reported to display growth retardation and increased apoptosis, indicating that Akt1 plays a critical role in cell survival [9]. Akt2 knockout mice have been shown to develop a type 2 diabetes-like phenotype, suggesting that Akt2 may play an important role in the maintenance of glucose homeostasis [10]. In rat hepatocytes, insulin activates only Akt1 and Akt2 but not Akt3, with the activity of Akt1 in response to insulin being four-fold higher than that of Akt2 [11]. These findings suggest that different cellular processes may be controlled by different Akt isoforms.

Mimicking a normal insulin response, ectopic expression of constitutively active Akt has been shown to stimulate lipogenesis and lipid droplets accumulation in 3T3-L1 adipocytes [12]. In addition, the expression of wild-type or kinase-dead (dominant negative) Akt in 3T3-L1 adipocytes increases or decreases the promoter activity of fatty acid synthase (FAS), a critical enzyme in *de novo* lipogenesis, respectively, indicating that Akt is involved in the transcriptional regulation of lipogenic genes [13]. The effect of Akt on *de novo* lipogenesis may be mediated by sterol regulatory element-binding protein 1 (SREBP1), as Akt activation reportedly induces SREBP1 mRNA accumulation in primary hepatocytes [14]. Among the lipogenic enzymes downstream of SREBP1, constitutively activation of Akt increased FAS and stearoyl-CoA desaturase 1 (SCD1) mRNA, whereas amounts of acetyl-CoA carboxylase (ACC) mRNAs did not change significantly [15]. Akt1 has been shown to be required for adipocyte differentiation in cultured mouse embryo fibroblasts [16]. Akt1 depletion has been shown to markedly attenuated peroxisome proliferator-activated receptor (PPARγ) transcriptional activity, which is implicated in modulating adipocyte differentiation [17]. Collectively, Akt1 is essential for lipid metabolism and therefore may play a critical role in the late stage of the HCV life cycle.

Previous reports have suggested that Akt may be important for HCV infection, including entry, replication, and translation. By applying siRNA coupled with an HCVcc system, we identified that Akt1 is involved in the late stage of the HCV life cycle, that is, Akt1 maintains sufficient lipid droplet that is required for assembly and release of infectious virions. Our data also answer to previous controversial results showing Akt inactivation promoted HCV replication but suppressed HCV translation [18, 19](refer to the section of Discussion).

Materials And Methods

Cell lines

Huh-7.5.1/SGR-JFH1 stable cells were established by transfection of pSGR-JFH1 RNA into Huh-7.5.1 cells [20], followed by selection with G418 (500 μg/ml) for 3 weeks. Huh-7.5.1/shLacZ, Huh-7.5.1/shluc, and Huh-7.5.1/shAkt1 stable cell lines were constructed by infection of Huh-7.5.1 with lentivirus harboring corresponding short hairpin RNA (shRNA) obtained from National RNAi Core Facility, Academia Sinica, Taiwan, followed by selection with 2 μg/ml of puromycin for 3 weeks.

Small interfering RNA transfections

ON-TARGETplus SMARTpools containing small interfering RNA (siRNA) targeting CD81, apoE, Akt1 and nontargeting siRNA were provided by Dharmacon (Lafayette, CO). Transfections using Oligofectamine (Invitrogen) were performed following the manufacturer's instruction.

Antibodies and reagents

The following antibodies were obtained commercially: Akt1 (clone C73H10; Cell Signaling Technology), mouse [8G-2] anti-HCV NS3 (ab65407; Abcam), rabbit anti-HCV NS5B (ab65410; Abcam), anti-β-actin (Sigma), mouse [C7-50] anti-HCV core (MA1-080; Thermo Scientific), rabbit [K-10] anti-SREBP1 (sc-367;

Santa Cruz Biotechnology, Dallas, TX), Alexa Fluor 488 goat anti-mouse IgG (A11001; Invitrogen). The anti-HCV core monoclonal antibody was produced from the anti-core 6G7 hybridoma cells provided by Drs. Harry Greenberg and Xiaosong He (Stanford University, Palo Alto, CA). Monoclonal antibody 9E10 (anti-NS5A) was a gift from Charles Rice (The Rockefeller University, New York, NY). MTT (3-(4,5-dimethylthiozol-2-ly)-2,5-diphenyltetrazolium bromide), Hoechst 33342 (Invitrogen), BODIPY 493/503 (D-3922; Invitrogen), Akt inhibitor VIII and Akt-I-1 inhibitor were purchased from Calbiochem and Symansis (Timaru, New Zealand), respectively.

In vitro **transcription and infectious HCV production**

HCV Con1 replicons (pFK-I389luc-NS3-3'/5.1)[21] and JFH1 DNA constructs pSGR-Luc-JFH1[22], J6/JFH1[23], and J6/JFH (p7-Rluc2A)[24] were linearized by digestion with *ScaI* and *XbaI*, respectively. *In vitro* RNA transcripts were generated from linearized plasmids using MEGAscript T7 kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction and transfected into Huh-7.5.1 cells for infectious HCV production as described previously[25].

Drug inhibition and cell viability study

Akt inhibitor VIII and Akt-I-1 inhibitor were re-suspended at 10 mM in dimethyl sulfoxide (DMSO). Akt inhibitor VIII is a cell-permeable and reversible quinoxaline compound that potently and selectively inhibits Akt1/Akt2 activity (IC_{50} = 58 nM, 210 nM, and 2.12 μM for Akt1, Akt2, and Akt3, respectively, in in vitro kinase assays). Akt-I-1 is an Akt1 selective inhibitor, with IC_{50} = 4.6 μM, > 250 μM, and > 250 μM for Akt1, Akt2, and Akt3, respectively. Huh-7.5.1 cells seeded in a 96-well plate were treated with 50 μL of various concentrations of Akt inhibitors (Akt inhibitor VIII or Akt-I-1 inhibitor) or equivalent volume of DMSO, diluted in complete growth medium followed by inoculation with an equal volume of J6/JFH(p7-Rluc2A). After 48 h, the cells were subjected to luciferase activity and MTT assay as described previously[25].

TaqMan Real-Time PCR Analysis

Huh-7.5.1 cells were treated with indicated concentrations of Akt inhibitor VIII one hour before infection with J6/JFH1 viral particles followed by incubation for additional 72 h. To determine the amount of HCV RNA, extracellular and intracellular RNA were extracted with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from 140 μL culture medium and with RNeasy Mini Kit (Qiagen, Hilden, Germany) from whole-cell lysate, respectively. Copy numbers of HCV RNA were determined by quantitative RT-PCR reactions with the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Primers and probe were selected from a highly conserved region of the 5' UTR [26]. PCR parameters consisted of 1 cycle of 50°C for 5 min, then 95°C for 20 sec, followed by 40 cycles of PCR at 95°C for 3 sec, and 60°C for 30 sec.

Plasmid and DNA transfection

Single-round infectious HCV-like particles (HCV-LP) were generated by co-transfection of pHH/SGR-luc, a bicistronic subgenomic reporter replicon with a Pol I promoter/terminator, with a core-NS2 expressing pCAG/C-NS2 plasmid [27]. The pcDNA3.1-Flag-SREBP-1c plasmid was provided by Dr. Timothy F.

Osborne (University of California, Irvine, CA)[27]. pFASwt-luc, a plasmid bearing the luciferase gene directed by a 178-bp promoter fragment of the human fatty acid synthase (FAS) gene, was provided by Johannes V. Swinnen (University of Leuven, Leuven, Belgium)[28]. DNA transfection was performed using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions.

HCV replicon assay

Huh-7.5.1 cells were transfected with the indicated siRNAs in 6 cm dishes for 48 h and were then transferred into a 96-well plate. After 24 h, cells were transiently transfected with *in vitro* transcribed RNA from pFK I389 luc/NS3-3'/5.1 (Con1) or pSGR-Fluc-JFH1 subgenomic replicon. After 48 h, cell were harvested for luciferase assay.

Immunoblotting

Huh-7.5.1 cells seeded in 6 cm dishes were transfected with siRNAs against Akt1 at 50 nM using Oligofectamine (Invitrogen). After 72 h, the cells were harvested and the lysates (100 μg) were subjected to SDS/PAGE, transferred to a polyvinylidene difluoride membrane, and detected using Akt1 (clone C73H10; Cell Signaling Technology). Huh-7.5.1/SGR-JFH1 cells were treated with the indicated concentrations of Akt-I-1 inhibitor or an equivalent volume of DMSO for 48 h. The cell lysates were subjected to immunoblotting using antibodies directed against the proteins of interest: HCV NS3 (clone 8G2; Abcam), HCV NS5A (clone 9E10), HCV NS5B (ab65410; Abcam), and β-actin (Sigma). The relative band intensity was quantified by densitometric analysis, and the relative expression levels of NS3, NS5A, or NS5B were normalized to their respective β-actin protein levels.

siRNA transfection and core staining

ON-TARGETplus SMARTpools containing small interfering RNA (siRNA) targeting CD81, apoE, Akt1, and nontargeting #2 siRNA were purchased from Dharmacon (Lafayette, CO). Huh-7.5.1 cells were transfected with siRNAs at a concentration of 50 nM using a reverse transfection protocol with Oligofectamine (Invitrogen) in a 96-well plate [29]. After 72 h, the cells were infected with the J6/JFH1 virus for an additional 48 h (part-one infection). The supernatant was replica plated onto a plate with Huh 7.5.1 cells (part-two infection). After 48 h, both part-one and part-two infected cells were stained with anti-HCV core 6G7 monoclonal antibody followed by Alexa Fluor 488 antibody (Invitrogen). Nuclear DNA was stained with Hoechst 33342 (Invitrogen). Images were captured with an automated Image Express Micro (IXM) microscope (Molecular Devices) and analyzed using Metamorph Cell Scoring software (Molecular Devices Inc.) to determine the total cells per field and the percentage of core positive cells in each field (percent infected).

Intracellular and extracellular HCV infection assay

Huh-7.5.1 cells were seeded on a 12-well plate and then transfected with control NT#2 or Akt1-specific siRNAs as described above. After 72 h of siRNA transfection, cells were transfected with 1 μg/well of J6/JFH(p7-Rluc2A) RNA using DMRIE-C Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After additional 48 h, the culture medium was collected, filtered through a

0.45-μm-pore-size filter and then used as the “extracellular” viral infection source. To prepare “intracellular” viral infection source, we followed the protocol described by Tellinghuisen *et al.* [30] with some modifications. Briefly, the above HCV RNA-transfected cells were washed twice with PBS and trypsinized. After centrifugation at 5,000× g for 5 minutes, the cells were resuspended in 1 ml of DMEM/10% FBS and lysed by four rounds of freezing and thawing. The lysate supernatant was collected after centrifugation at 5,000× g for 5 minutes and then filtered through a 0.45-μm-pore-size filter. The resulting solution was used as the intracellular viral infection source. The collected intracellular and extracellular J6/JFH(p7-Rluc2A) particles were used to infect naïve Huh-7.5.1 cells. After 48 h, the intracellular and extracellular infectivity was determined by luciferase assay. The mean luciferase value of the NT#2 siRNA-transfected cells was defined as 100%, and the relative infectivity was then calculated as a percentage of NT#2 siRNA-transfected cells.

HCV pseudotype particle production

The luciferase-based HCV pseudotyped retroviral particle (HCVpp) was produced by transfection of plasmid DNA encoding HCV (genotypes 1a, 1b or 2a) or vesicular stomatitis virus (VSV) glycoproteins with the Env-deficient HIV vector carrying a luciferase reporter gene (pNL4-3-Luc-R-E-) into the 293T producer cells. Supernatants containing the pseudo-typed particles were collected 72 h post-transfection, filtered through a 0.45-μm pore-size filter (Millipore, Cork, Ireland), aliquoted and frozen for later use. Huh-7.5.1 cells were inoculated with an equal amount of viral particles for 48 h and then subjected to luciferase assay.

Flow cytometry and quantification of lipid droplet contents

Huh-7.5.1, Huh-7.5.1/shluc, and Huh-7.5.1/shAkt1 cells seeded in 6-well plates at a density of 5 x 10^5 cells/well were trypsinized and washed twice with PBS. Cellular lipid droplets were stained with 5 μM BODIPY 493/503 (D-3922; Invitrogen) on ice for 20 minutes. The cells were then filtered on a cell strainer capped tube (#352235; BD) and analyzed on a BD FACSCalibur (BD Biosciences).

Statistical analysis

The results were presented as mean ± standard deviation (SD). The two-tailed unpaired Student’s *t* test was used for statistical analysis. The graphs were prepared using GraphPad Prism 5.0 software (GraphPad Software, Inc, San Diego, CA).

Results

Akt inactivation inhibited HCV infection

To investigate the effect of Akt on HCV infectivity, Huh-7.5.1 cells were treated with Akt inhibitor VIII at doses of 0, 2, 4, and 8 μM first and then infected with J6/JFH (p7-Rluc2A) reporter virus. The infected cells were incubated for 72 h at standard culture condition, during which time the virus re-infected the cells in view of HCV doubling time of approximately 22 h in culture [20]. Suppression of Akt activity by

Akt inhibitor VIII was shown in Fig. 1A with decreased phosphorylation at Ser473 and Thr308. As shown in Fig. 1B, relative to the DMSO-treated control, Akt inhibitor VIII inhibited HCVcc infectivity in a dose-dependent manner. The dose of Akt inhibitor VIII did not affect cell viability up to 8 μM (Fig. 1C).

Akt1 is involved in the late stage of HCV life cycle

To further dissect the target stage of Akt inhibitor VIII in HCV life cycle, we measured HCV RNA levels within the cells (Fig. 2A) and in the culture supernatant (Fig. 2B) in HCV-infected Huh-7.5.1 cells treated with Akt inhibitor VIII. The inhibitor did not affect intracellular HCV RNA level (Fig. 2A) but caused progressive reduction of viral RNA with drug doses elevated in the culture supernatants (Fig. 2B), indicating that Akt may modulate viral release from cells in late stage of HCV life cycle. To further determine whether Akt really affected viral production in late stage of HCV life cycle, we adopted a two-part HCV infection protocol as described previously with some modifications [29]. We used siRNA to knock down the expressions of Akt1, CD81, or ApoE. Then cells were infected with J6/JFH1 for 48 h (part-one infection), and the supernatant was used to inoculate naïve Huh-7.5.1 cells (part-two infection). CD81 is a cell membrane receptor for HCV entry and can represent early stage i.e. part-one infection. ApoE is an apo-lipoprotein required for maturation of infectious HCV particles and can represent late stage i.e. part-two infection. As shown in Fig. 2C, non-targeting siRNA (siNT #2) transfection gave basal fluorescence values of HCV core-staining (100%) for part-one and part-two infections. While Akt1 knockdown had no significant influence in part-one infection (Fig. 2D), it resulted in significant decrease in part-two infection (Fig. 2E), similar to the core-staining pattern of ApoE knockdown but in sharp contrast to CD81 knockdown that suppressed part-one infection with consequent suppression of part-two infection. The similar core-staining patterns between Akt1 silencing and ApoE silencing further suggest that Akt1 is involved in late stage of the HCV life cycle.

Akt1 silencing did not affect HCV entry and replication

As shown in Fig. 2, Akt1 silencing did not affect part-one infection of HCV. We next made an attempt to determine the effects of Akt1 silencing on HCV entry and RNA replication. Huh-7.5.1 cell line stably expressing shLacZ or shAkt1 was established by lentiviral infection and puromycin selection. To evaluate the effect of Akt1 on HCV entry, we infected Huh-7.5.1/shLacZ and Huh-7.5.1/shAkt1 cells with luciferase-carrying HCV pseudo-particles (HCVpp) of different genotypes or with vesicle stomatitis virus pseudo-typed virus (VSVpp) as a control. As shown in Fig. 3A, Akt1 silencing did not cause significant change in luciferase activity either in HCVpp infection, regardless of genotype, or in VSVpp infection, indicating that Akt1 does not affect HCV entry.

To evaluate the effect of Akt1 silencing on HCV replication, Huh-7.5.1 cells were transfected first with control siRNA(NT#2) or Akt1-specific siRNA and then transfected with the HCV subgenomic replicons [31], pFK I_{389}luc/NS3-3'/5.1 (genotype 1b) or pSGR-Fluc-JFH1 (genotype 2a). HCV replication of the two replicons was represented by luciferase activity as shown in Fig. 3B (Con1) and Fig. 3C (JFH1), i.e. Akt1 silencing did not affect HCV replication. To investigate whether Akt1 suppression affected expression of

HCV non-structural proteins, Huh-7.5.1/SGR-JFH1 cells were treated with the Akt1-specific inhibitor Akt-I-1 and then subjected to immunoblotting. The expression levels of NS3, NS5A, and NS5B did not change under Akt1 suppression (Fig. 3D). Taken together, our results indicate that Akt1 is not involved in early stage of HCV life cycle including entry and viral RNA replication.

Akt1 silencing inhibited HCV release

As shown in Fig. 2. silencing of Akt1 did not affect core protein expression in part-one infection but caused profound inhibition in part-two infection (Fig. 2C and 2E), suggesting that Akt1 depletion may impede HCV release, leading to accumulation of intracellular HCV particles. We therefore adopted a single cycle infection system [27] to gain a more mechanistic insight into the function of Akt1 in HCV particle production. Naïve Huh-7.5.1 cells were infected with culture supernatants in which single-round infectious HCV-like particles were produced from control (Huh-7.5.1/shLacZ) or Akt1-silenced (Huh-7.5.1/shAkt1) cells that had been transfected with pHH/SGR-luc and pCAG/C-NS2. The entry amounts of infectious HCV-like particles within Huh-7.5.1 cells were determined by luciferase activity at 48 and 72 h after infection. Figure 4A showed that the release of infectious HCV-like particles from the Huh-7.5.1/shAkt1 cells was significantly reduced over time (*black bars*; $P = 0.004$ and $P = 0.002$ at 48 h and 72h, respectively) compared to that from the control Huh-7.5.1/shLacZ cells (*white bars*).

To further examine whether such an Akt1 silencing-mediated inhibition in extracellular HCV infectivity was attributable to defective virion assembly or impaired virion release, we did intracellular and extracellular viral infections as described in Materials and Methods [6]. Akt1 silencing significantly dampened the amount of extracellular infectious virions (Fig. 4B; $P < 0.001$) but did not affect intracellular infectious virions in comparison with those in control NT#2 siRNA-transfected cells, indicating that Akt1 is crucial for the release of infectious virions.

To further clarify the importance of Akt1 on viral egress, we re-expressed Akt1 in Huh-7.5.1 cells whose Akt1 had been knocked down by siRNA to determine whether ectopic Akt1 could restore HCV production in cells with Akt1 depletion. Huh-7.5.1 cells with Akt1 silencing by siRNA were transfected with Akt1 cDNA and then infected with J6/JFH(p7-Rluc2A) viral particles. The culture supernatants were harvested to inoculate naïve Huh-7.5.1 cells with which to determine the amounts of released infectious virus in medium by luciferase assay. As shown in Fig. 4C, Akt1 silencing significantly inhibited infectious J6/JFH(p7-Rluc2A) virion production compared to the control NT#2 siRNA transfected, and ectopic Akt1 expression rescued the release of infectious HCV particles in Akt1-depleted cells compared to control vector transfection. The direct mechanistic link between Akt1 and HCV release was reconfirmed by infection of Huh-7.5.1/shAkt1 cells, in which Akt1 was re-expressed, with J6/JFH(p7-Rluc2A) viral particles. The culture supernatants were harvested to inoculate naïve Huh-7.5.1 cells with which to determine HCV RNA in culture medium. As shown in Fig. 4D, ectopic expression of Akt1 restored HCV production in Huh-7.5.1/shAkt1 cells.

Akt1 silencing inhibited lipid droplet formation

Lipid droplets are required for HCV to produce infectious viral particles [5]. On the other hand, cytoplasmic activated protein kinase Akt regulates lipid-droplet accumulation in Drosophila nurse cells [32], and expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis [33]. To determine whether Akt1 participates in lipid droplet formation in Huh-7.5.1 cells, we quantified cellular lipid droplets in Huh-7.5.1/shluc and Huh-7.5.1/shAkt1 cells using BODIPY 493/503 staining, followed by flow cytometry (Fig. 5A). As shown in Fig. 5B (histogram from Fig. 5A), shAkt1-lentivirus-infected Huh-7.5.1 cell showed significantly decreased lipid droplet formation (43% reduction relative to mock infection, $p = 0.03$). The lipid droplet formation did not change in cells with shluc-lentiviral infection ($p = 0.18$). The inhibitory effect of Akt1 silencing on lipid droplets formation was further confirmed by immunofluorescence (Fig. 5C). Huh-7.5.1 cells were infected first with shAkt1- or shluc-carrying lentivirus and then with J6/JFH1 viral particles, followed by lipid droplet staining with BODIPY 493/503 (green fluorescence)and HCV core immunostaining with anti-HCV core antibody (red fluorescence) (Fig. 5C). HCV core and lipid droplets overlapped to show abundant yellow fluorescence in Huh-7.5.1/shluc cells (Fig. 5C), but the amount of lipid droplet decreased in Huh-7.5.1/shAkt1 cells with weakened yellow fluorescence in merge. These results indicate that Akt1 upregulates lipid droplet formation and thereby helps HCV particles release from cells.

Akt1 silencing inhibited lipogenesis

To further elucidate the mechanistic link between Akt1-mediated lipid metabolism and HCV infectious virion production, we focused on the pivotal lipogenic transcriptional factor SREBP1 (sterol regulatory element-binding protein 1) and its downstream FAS (fatty acid synthase). As shown in Fig. 6A, the expression levels of both precursor and mature forms of SREBP1 were downregulated by 25% and 35%, respectively, in Akt1-depleted cells compared to those in control shLacZ cells. FAS also decreased in Akt1-depleted cells. To further confirm the link between Akt1 and lipid metabolism, we transfected the pFASwt-luc plasmid into Huh-7.5.1/shAkt1 and Huh-7.5.1/shLacZ cells (Fig. 6B). Compared to the control shLacZ, Akt1 silencing downregulated the transcription activation of FAS because SREBP1 was suppressed (Fig. 6B). Exogenous expression of SREBP1c had no effect on FAS transcription in the control shLacZ cells, but it enhanced 2.3-fold transcription activation of FAS in Akt1-depleted cells (Fig. 6B). In addition, the FAS transcription was upregulated by ectopic expression of Akt1 in a dose-dependent manner (Fig. 6C). These results indicate that Akt1 maintains sufficient lipid droplets that HCV utilizes to produce infectious viral particles.

Discussion

Targeting a host pathway on which HCV relies, rather than a viral protein, may offer a novel treatment strategy for more recalcitrant genotypes and resistant strains in the development of anti-HCV agents [34]. In HCV-replicating cells, PI3K/Akt signaling and its downstream target mTOR are activated [18]. HCV infection transiently activates Akt during the early stage of viral infection to enhance HCV entry into host cells [35]. These results highlight the important role of PI3K/Akt signaling pathway in the HCV life cycle. Since Akt is ubiquitously present in all human cells with a steady expression and temporary activation in

response to stimulation, “how important is Akt for HCV propagation?” remains an unsolved issue in the understanding of the HCV life cycle. In this report, we applied Akt1-specific silencing approach in the HCVcc system to prove that Akt1 is a host factor involved in the late stage of the HCV life cycle. Akt1 silencing resulted in significant decrease in HCV production comparable to the extent exerted by silencing of apoE, a well-known host factor essential for the assembly and release steps of the HCV life cycle.

Accumulated evidence has established the critical role of lipid metabolism in the HCV life cycle [5, 36, 37]. Lipid droplet is actually an essential organelle for the assembly of infectious HCV virions [38]. Liver lipogenesis is triggered by insulin that activates Akt signaling pathway to up-regulate the expressions of lipogenic transcription factors, among which SREBP1c is the dominant isoform in the liver responsible for inducing lipogenic gene expression and promoting fatty acid synthesis [39]. Akt is required for ER-to-Golgi proteolytic maturation of SREBP1c [40]. Mouse embryo fibroblasts with Akt1 silencing has also been shown to have decreased lipid droplet stained by Oil Red O [16]. Reintroduction of Akt1 into mouse embryo fibroblasts devoid of Akt1 has been reported to restore lipid accumulation [41]. In addition, activated Akt has also been shown to cause lipid-droplet accumulation in Drosophila nurse cells [32] and in mammary gland of mice [33]. So far, very few, if any, reports give clear immunofluorescence image showing that Akt1-silenced Huh-7 cells permissive for HCV infection have diminished merge of core protein and lipid droplet. Our results provide evidence that Akt1 is required for Huh-7.5.1 cells to maintain lipid droplets for HCV assembly and ensuing release (Fig. 5).

Akt is activated transiently only at 4–8 h after HCV infection in permissive Huh-7 cells, the activation of which may be associated with viral entry [35]. However, reinfection of new viruses released from cells is quiescent without disturbing cells leading to Akt activation. The importance of this short-time activation of Akt in HCV entry step remains to be clarified. According to our experimental results, Akt1 affects little if any in the step of viral entry (Figs. 2 and 4). On the other hand, Akt1 is also involved in HCV RNA replication [18]. In that report, Mannova *et al.* showed that Akt inactivation by the PI3K inhibitor Ly294002 results in enhanced HCV RNA replication. The reason can be explained that viral RNA is replicated but accumulated within cells because Akt is required for release of viral particles but inactivated. Furthermore, Shi *et al.* reported that Akt1 silencing down-regulated HCV RNA translation by transfection of *in vitro* transcribed monocistronic HCV RNA into Huh-7.5 cells. They concluded that the PI3K-Akt signaling pathway positively regulates HCV translation through SREBPs [19]. As described above, Akt is activated transiently only at 4–8 h after HCV infection in permissive Huh-7 cells, and thereafter Akt is not on purpose to be activated in steady state when HCV RNA is replicated and translated. However, when Akt1 is silenced, lipid droplet downsizes, but HCV RNA is still being replicated, leading to excess viral proteins and immature viral particles accumulating within cells and therefore impeding further HCV RNA translation. Our results of intracellular and extracellular infections (Fig. 4B) answer to the *in vitro* phenomenon reported by Shi *et al.*

In this report, we have focused our study in Akt1 that is required to maintain sufficient lipid droplets for HCV assembly and release. The role of Akt2 is scanty in that core staining feature relative to those shown in Fig. 2C did not support that Akt2 silencing caused reduction of either part-one or part-two infection

(Supplementary Fig. 1B). In addition, Akt2-silenced Huh-7.5.1 cells did not show defect in viral release from cells to medium (Supplementary Fig. 1C). Akt3 was not detected in our Huh7.5.1 HCVcc system (Supplementary Fig. 1A). In conclusion, our data provide a hitherto unrecognized function of Akt1 as a host factor critical for HCV infectious virions release. We have demonstrated that Akt1 regulates SREBP1-mediated lipogenesis pathway and thereby maintains lipid droplets for HCV assembly and release. It is conceivable that a host factor essential for the HCV life cycle, such as Akt1, may be a novel target for antiviral drug design to overcome the potential viral resistance of current direct-acting antiviral agents.

Declarations

Acknowledgments

The authors are grateful to Dr. Ralf Bartenschlager, Dr. Francis V. Chisari, Dr. Takaji Wakita, Dr. Jose C. Rodriguez-Rey, Dr. Charles. M. Rice, Dr. Johannes V. Swinnen, Dr. Timothy F. Osborne, Dr. Tetsuro Suziki, Drs. Harry Greenberg and Xiaosong He for the generous provision of reagents.

Funding

This study was supported by grants from the Ministry of Science and Technology, Taiwan (MOST-106-2314-B-075-055 to Keng-Hsin Lan; MOST-107-2314-B-075-049 -MY2 to Wei-Ping Lee), and Taipei Veterans General Hospital (V107C-137 to Keng-Hsin Lan; V108C-206 to Wei-Ping Lee), Taipei, Taiwan.

Competing interests

All authors declare no competing interests.

References

[1] M. Hijikata, N. Kato, Y. Ootsuyama, M. Nakagawa, K. Shimotohno, Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis, Proceedings of the National Academy of Sciences of the United States of America, 88 (1991) 5547-5551.

[2] M.E. Burlone, A. Budkowska, Hepatitis C virus cell entry: role of lipoproteins and cellular receptors, The Journal of general virology, 90 (2009) 1055-1070.

[3] J.O. Link, J.G. Taylor, L. Xu, M. Mitchell, H. Guo, H. Liu, D. Kato, T. Kirschberg, J. Sun, N. Squires, J. Parrish, T. Keller, Z.Y. Yang, C. Yang, M. Matles, Y. Wang, K. Wang, G. Cheng, Y. Tian, E. Mogalian, E. Mondou, M. Cornpropst, J. Perry, M.C. Desai, Discovery of ledipasvir (GS-5885): a potent, once-daily oral NS5A inhibitor for the treatment of hepatitis C virus infection, Journal of medicinal chemistry, 57 (2014) 2033-2046.

[4] D. Moradpour, F. Penin, C.M. Rice, Replication of hepatitis C virus, Nat Rev Microbiol, 5 (2007) 453-463.

[5] Y. Miyanari, K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, K. Shimotohno, The lipid droplet is an important organelle for hepatitis C virus production, Nat Cell Biol, 9 (2007) 1089-1097.

[6] P. Gastaminza, G. Cheng, S. Wieland, J. Zhong, W. Liao, F.V. Chisari, Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion, J Virol, 82 (2008) 2120-2129.

[7] H. Huang, F. Sun, D.M. Owen, W. Li, Y. Chen, M. Gale, Jr., J. Ye, Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins, Proceedings of the National Academy of Sciences of the United States of America, 104 (2007) 5848-5853.

[8] A. Bellacosa, J.R. Testa, R. Moore, L. Larue, A portrait of AKT kinases: human cancer and animal models depict a family with strong individualities, Cancer Biol Ther, 3 (2004) 268-275.

[9] W.S. Chen, P.Z. Xu, K. Gottlob, M.L. Chen, K. Sokol, T. Shiyanova, I. Roninson, W. Weng, R. Suzuki, K. Tobe, T. Kadowaki, N. Hay, Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene, Genes Dev, 15 (2001) 2203-2208.

[10] H. Cho, J. Mu, J.K. Kim, J.L. Thorvaldsen, Q. Chu, E.B. Crenshaw, 3rd, K.H. Kaestner, M.S. Bartolomei, G.I. Shulman, M.J. Birnbaum, Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta), Science, 292 (2001) 1728-1731.

[11] K.S. Walker, M. Deak, A. Paterson, K. Hudson, P. Cohen, D.R. Alessi, Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha, Biochem J, 331 (Pt 1) (1998) 299-308.

[12] A.D. Kohn, S.A. Summers, M.J. Birnbaum, R.A. Roth, Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation, J Biol Chem, 271 (1996) 31372-31378.

[13] D. Wang, H.S. Sul, Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway. Involvement of protein kinase B/Akt, J Biol Chem, 273 (1998) 25420-25426.

[14] M. Fleischmann, P.B. Iynedjian, Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin and protein kinase B/cAkt, Biochem J, 349 (2000) 13-17.

[15] H. Ono, H. Shimano, H. Katagiri, N. Yahagi, H. Sakoda, Y. Onishi, M. Anai, T. Ogihara, M. Fujishiro, A.Y. Viana, Y. Fukushima, M. Abe, N. Shojima, M. Kikuchi, N. Yamada, Y. Oka, T. Asano, Hepatic Akt activation induces marked hypoglycemia, hepatomegaly, and hypertriglyceridemia with sterol regulatory element binding protein involvement, Diabetes, 52 (2003) 2905-2913.

[16] X.D. Peng, P.Z. Xu, M.L. Chen, A. Hahn-Windgassen, J. Skeen, J. Jacobs, D. Sundararajan, W.S. Chen, S.E. Crawford, K.G. Coleman, N. Hay, Dwarfism, impaired skin development, skeletal muscle atrophy,

delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2, Genes Dev, 17 (2003) 1352-1365.

[17] S.P. Kim, J.M. Ha, S.J. Yun, E.K. Kim, S.W. Chung, K.W. Hong, C.D. Kim, S.S. Bae, Transcriptional activation of peroxisome proliferator-activated receptor-gamma requires activation of both protein kinase A and Akt during adipocyte differentiation, Biochem Biophys Res Commun, 399 (2010) 55-59.

[18] P. Mannova, L. Beretta, Activation of the N-Ras-PI3K-Akt-mTOR pathway by hepatitis C virus: control of cell survival and viral replication, J Virol, 79 (2005) 8742-8749.

[19] Q. Shi, B. Hoffman, Q. Liu, PI3K-Akt signaling pathway upregulates hepatitis C virus RNA translation through the activation of SREBPs, Virology, 490 (2016) 99-108.

[20] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.R. Burton, S.F. Wieland, S.L. Uprichard, T. Wakita, F.V. Chisari, Robust hepatitis C virus infection in vitro, Proceedings of the National Academy of Sciences of the United States of America, 102 (2005) 9294-9299.

[21] N. Krieger, V. Lohmann, R. Bartenschlager, Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations, J Virol, 75 (2001) 4614-4624.

[22] P. Targett-Adams, J. McLauchlan, Development and characterization of a transient-replication assay for the genotype 2a hepatitis C virus subgenomic replicon, J Gen Virol, 86 (2005) 3075-3080.

[23] B.D. Lindenbach, M.J. Evans, A.J. Syder, B. Wolk, T.L. Tellinghuisen, C.C. Liu, T. Maruyama, R.O. Hynes, D.R. Burton, J.A. McKeating, C.M. Rice, Complete replication of hepatitis C virus in cell culture, Science, 309 (2005) 623-626.

[24] C.T. Jones, C.L. Murray, D.K. Eastman, J. Tassello, C.M. Rice, Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus, J Virol, 81 (2007) 8374-8383.

[25] K.H. Lan, Y.W. Wang, W.P. Lee, K.L. Lan, S.H. Tseng, L.R. Hung, S.H. Yen, H.C. Lin, S.D. Lee, Multiple effects of Honokiol on the life cycle of hepatitis C virus, Liver Int, 32 (2012) 989-997.

[26] J. Bukh, R.H. Purcell, R.H. Miller, Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay, Proc Natl Acad Sci U S A, 89 (1992) 187-191.

[27] T. Masaki, R. Suzuki, M. Saeed, K. Mori, M. Matsuda, H. Aizaki, K. Ishii, N. Maki, T. Miyamura, Y. Matsuura, T. Wakita, T. Suzuki, Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription, J Virol, 84 (2010) 5824-5835.

[28] J.V. Swinnen, H. Heemers, L. Deboel, F. Foufelle, W. Heyns, G. Verhoeven, Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway, Oncogene, 19 (2000) 5173-5181.

[29] M. Flint, D.M. Tscherne, Cellular receptors and HCV entry, Methods Mol Biol, 510 (2009) 265-277.

[30] T.L. Tellinghuisen, K.L. Foss, J. Treadaway, Regulation of hepatitis C virion production via phosphorylation of the NS5A protein, PLoS pathogens, 4 (2008) e1000032.

[31] S. Arora, S. Kalishman, K. Thornton, D. Dion, G. Murata, P. Deming, B. Parish, J. Brown, M. Komaromy, K. Colleran, A. Bankhurst, J. Katzman, M. Harkins, L. Curet, E. Cosgrove, W. Pak, Expanding access to hepatitis C virus treatment-Extension for Community Healthcare Outcomes (ECHO) project: Disruptive innovation in specialty care, Hepatology, (2010).

[32] N. Vereshchagina, C. Wilson, Cytoplasmic activated protein kinase Akt regulates lipid-droplet accumulation in Drosophila nurse cells, Development, 133 (2006) 4731-4735.

[33] K.L. Schwertfeger, J.L. McManaman, C.A. Palmer, M.C. Neville, S.M. Anderson, Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation, J Lipid Res, 44 (2003) 1100-1112.

[34] M.P. Manns, G.R. Foster, J.K. Rockstroh, S. Zeuzem, F. Zoulim, M. Houghton, The way forward in HCV treatment--finding the right path, Nat Rev Drug Discov, 6 (2007) 991-1000.

[35] Z. Liu, Y. Tian, K. Machida, M.M. Lai, G. Luo, S.K. Foung, J.H. Ou, Transient activation of the PI3K-AKT pathway by hepatitis C virus to enhance viral entry, The Journal of biological chemistry, 287 (2012) 41922-41930.

[36] P. Targett-Adams, Steeve Boulant , Mark W. Douglas and John McLauchlan, Lipid Metabolism and HCV Infection, Viruses, 2 (2010) 1195-1217.

[37] M.F. Bassendine, D.A. Sheridan, S.H. Bridge, D.J. Felmlee, R.D. Neely, Lipids and HCV, Seminars in immunopathology, 35 (2013) 87-100.

[38] A. Filipe, J. McLauchlan, Hepatitis C virus and lipid droplets: finding a niche, Trends Mol Med, 21 (2015) 34-42.

[39] J.D. Horton, J.L. Goldstein, M.S. Brown, SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver, J Clin Invest, 109 (2002) 1125-1131.

[40] X. Du, I. Kristiana, J. Wong, A.J. Brown, Involvement of Akt in ER-to-Golgi transport of SCAP/SREBP: a link between a key cell proliferative pathway and membrane synthesis, Mol Biol Cell, 17 (2006) 2735-2745.

[41] A. Baudry, Z.Z. Yang, B.A. Hemmings, PKBalpha is required for adipose differentiation of mouse embryonic fibroblasts, Journal of cell science, 119 (2006) 889-897.

Figures

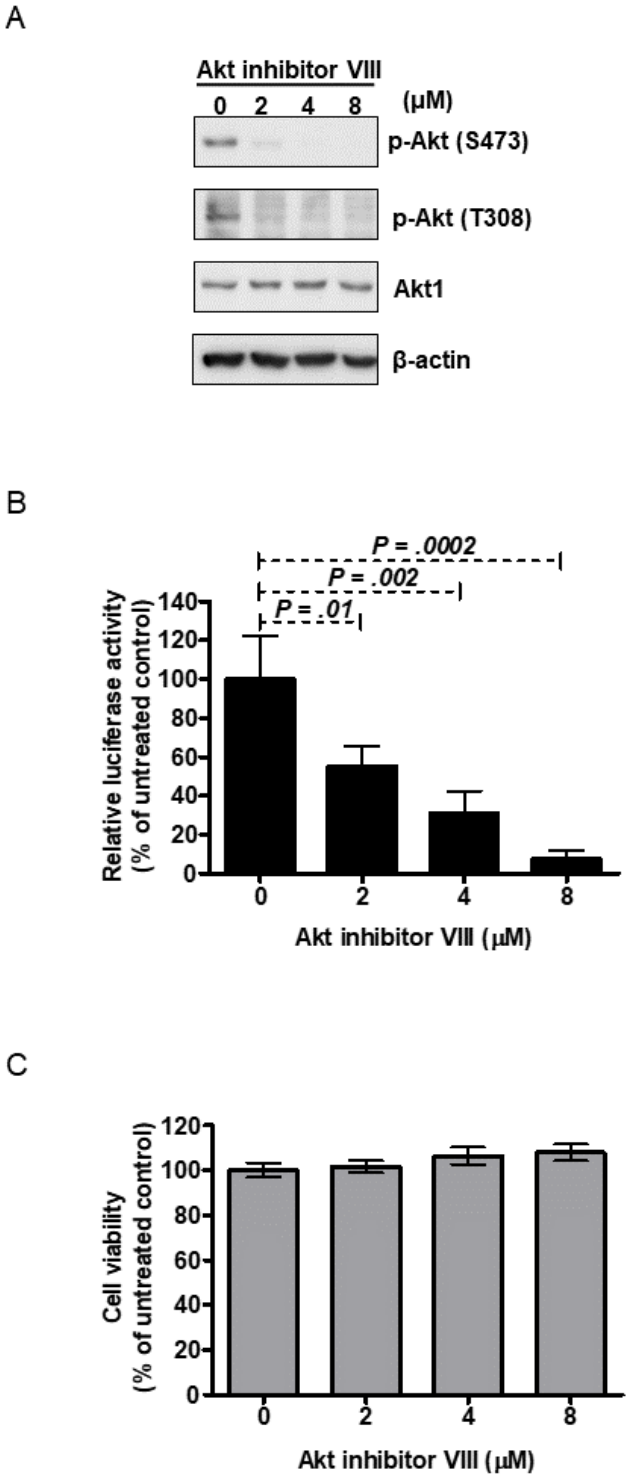

Figure 1

Akt inactivation by Akt inhibitor VIII inhibited HCV infectivity. (A) Huh-7.5.1 cells were treated with indicated concentrations of Akt inhibitor VIII for 48 h followed by Western blot to determine Akt activation. Akt phosphorylation on Thr308 and Ser473 was inhibited by Akt inhibitor VIII treatment. (B) Huh-7.5.1 cells were treated with indicated concentrations of Akt inhibitor VIII one hour before infection with J6/JFH(p7-Rluc2A) viral particles. After incubation for 72 h, cell lysates were subjected to firefly

luciferase assay. Akt repression by Akt inhibitor VIII suppressed J6/JFH(p7-Rluc2A) infection in a dose-dependent manner. (C) Akt inhibitor VIII was non-cytotoxic at the indicated concentrations. P values were determined by Student's t test.

Figure 2

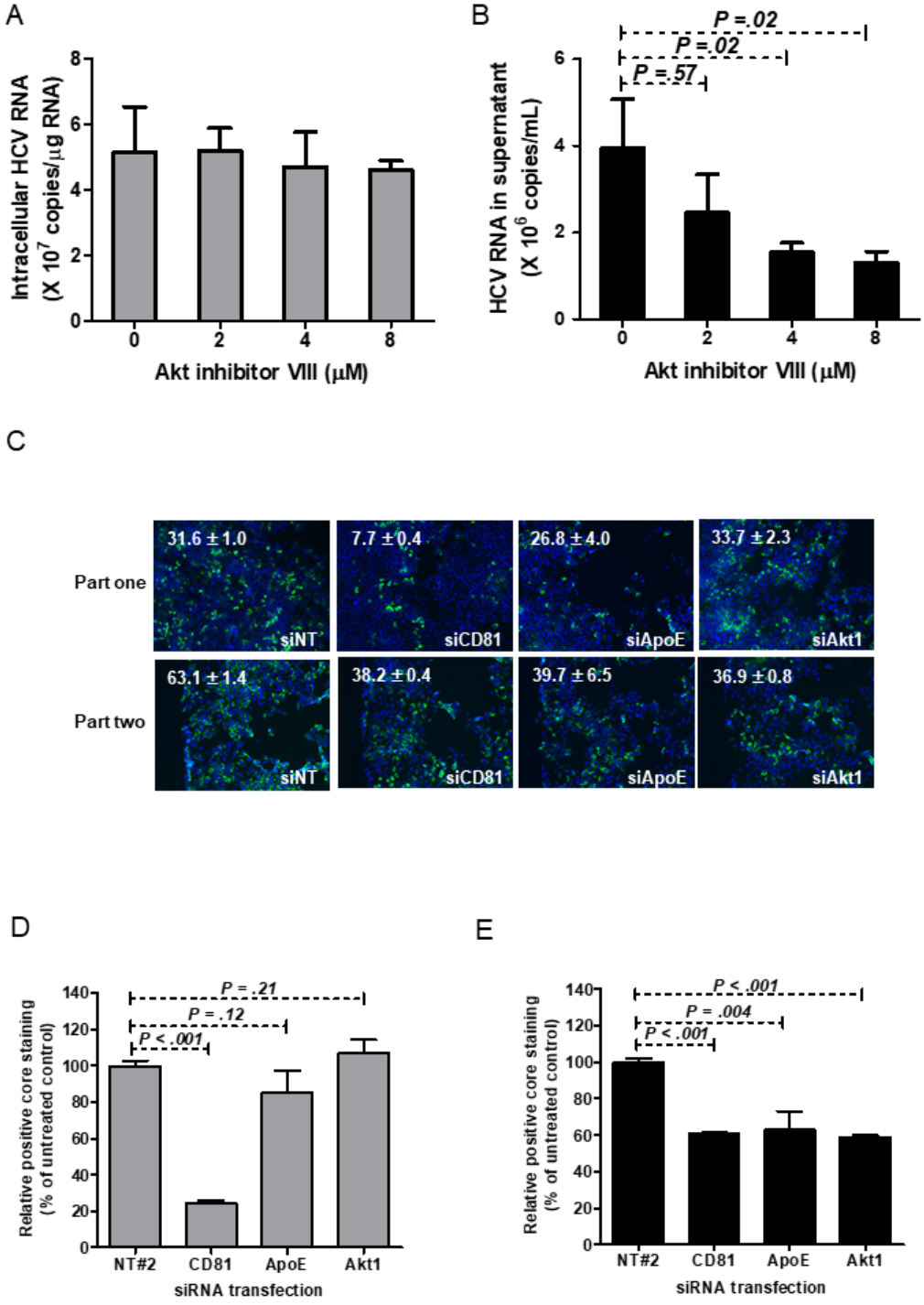

Figure 2

Akt1 was involved in late stage of HCV life cycle. Huh-7.5.1 cells were treated with indicated concentrations of Akt inhibitor VIII one hour before infection with J6/JFH1 viral particles. After incubation

for 72 h, the extracellular and intracellular RNA were extracted from culture medium and whole cell lysates, respectively. Copy numbers of HCV RNA were determined by quantitative RT-PCR reactions. (A) Akt inhibitor VIII had no effect on intracellular HCV RNA, as determined by TaqMan real-time PCR for J6/JFH1 RNA extracted from cultured cells. (B) Akt inhibitor VIII reduced extracellular HCV RNA, vs. untreated control, as determined by TaqMan real-time PCR for J6/JFH1 RNA extracted from culture medium. P values were determined by Student's t test. (C) Huh-7.5.1 cells transfected with the indicated siRNAs were infected with J6/JFH1 for 48 h (part-one infection), and the supernatant was used to inoculate naïve Huh-7.5.1 cells (part-two infection). Both part-one- and part-two-infected cells were stained with anti-HCV core antibody and Hoechst 33342 to detect HCV-infected cells and nuclear DNA, respectively (green: HCV core; blue: nuclear DNA). Magnification, 20X. NT#2, non-targeting siRNA #2. The percentages of HCV-infected cells in each field were shown in the figure. (D) Histogram of part-one infection in cells with indicated siRNA transfections was drawn with bars of percentages relative to siNT#2 transfection. (E) Histogram of part-two infection was drawn as described in panel D. The values were represented as means ± SD (n = 3).

Figure 3

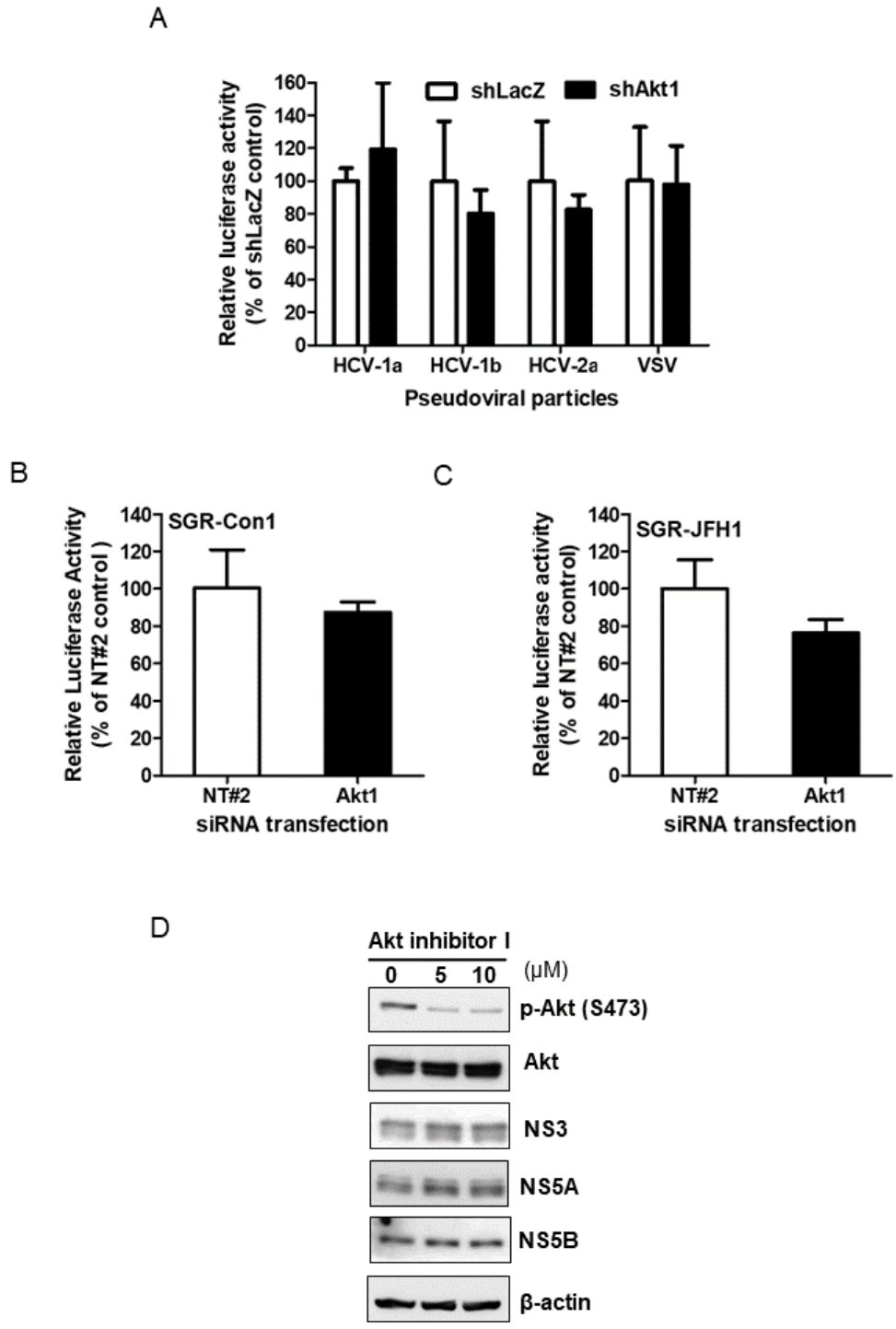

Figure 3

Akt1 silencing did not affect HCV entry and replication. (A) Huh-7.5.1 cells expressing shLacZ or shAkt1 were infected with the indicated genotypes of HCVpp or VSVpp for 48 h and then subjected to luciferase assay. The values were shown as a percentage of the control Huh-7.5.1/shLacZ infected with respective HCVpp or VSVpp. (B-C) Huh-7.5.1 cells transfected with the indicated siRNAs were transfected with in vitro transcribed RNA from the reporter subgenomic replicons of Con1 (B) or JFH1 (C) strain. Values were

shown as a percentage of the control NT#2 siRNA. (D) Huh-7.5.1/SGR-JFH1 cells treated with the indicated concentrations of Akt-I-1 inhibitor for 48 h were harvested for immunoblotting with the indicated antibodies.

Figure 4

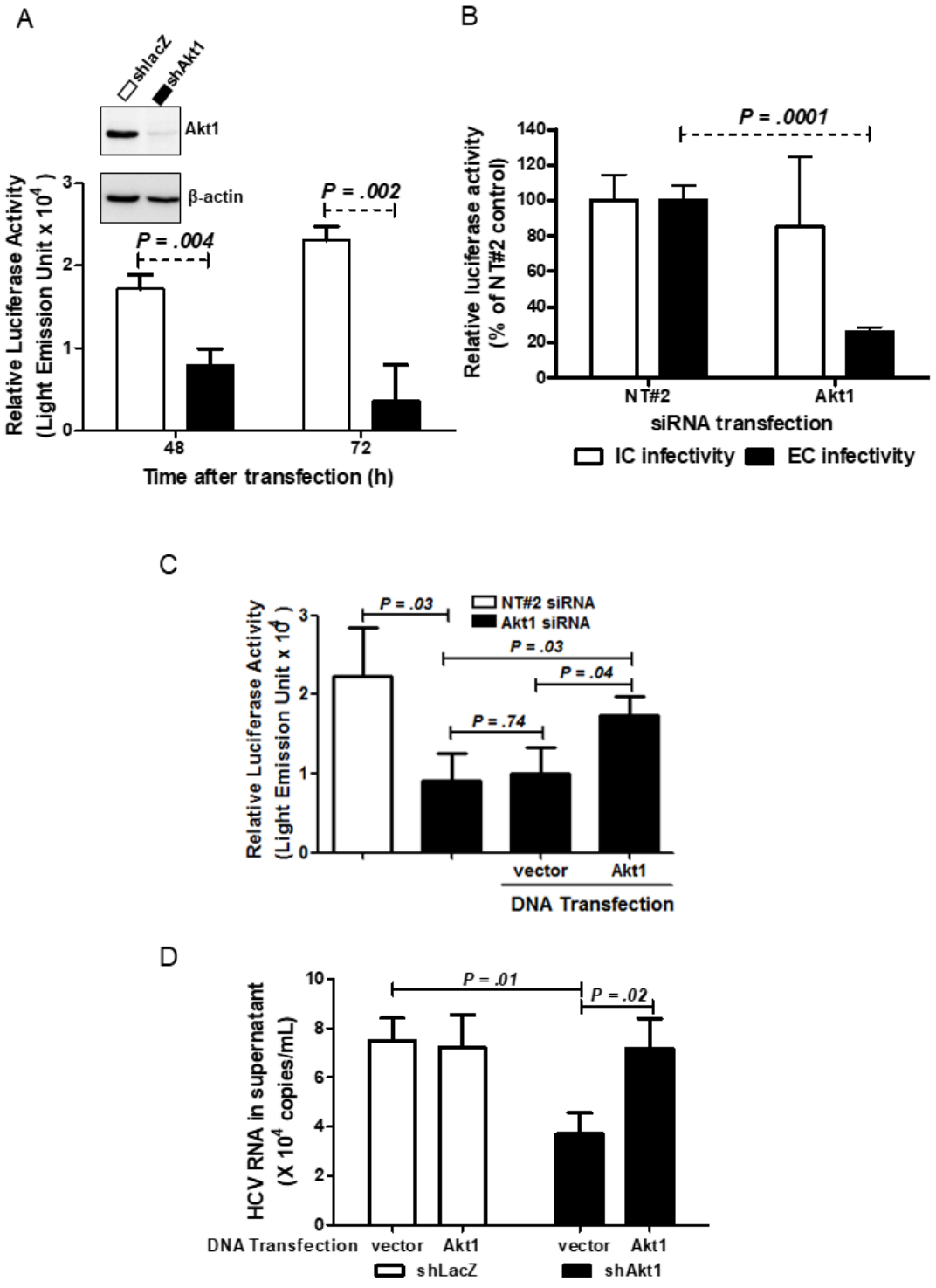

Figure 4

Akt1 silencing inhibited infectious HCV release. (A) Akt1 silencing decreased the release of single-round infectious HCV-like particle (HCV-LP) following co-transfection of pHH/SGR-luc and pCAG/C-NS2 over

time. (B) Akt1 silencing decreased infectious virion secretion into medium (extracellular) but not intracellular viral particles in Huh-7.5.1 cells transfected with the indicated siRNAs and J6/JFH(p7-Rluc2A) RNA. Data shown are luciferase values expressed as a percentage of NT#2 siRNA ($P < 0.001$). (C) Ectopic Akt1 expression restored HCV particle release in the supernatant of Huh-7.5.1 cells with Akt1 silencing by siRNA. Huh-7.5.1 cells with Akt1 silencing by siRNA were transfected with Akt1 cDNA and then infected with J6/JFH(p7-Rluc2A) viral particles. The culture supernatants were harvested to inoculate naïve Huh-7.5.1 cells with which to determine the amounts of released infectious virus in medium by luciferase assay. (D) Ectopic Akt1 expression restored HCV RNA levels in the supernatant of Huh-7.5.1 cells with Akt1 silencing by shAkt1. Huh-7.5.1/shAkt1 cells were transfected with Akt1 cDNA and then infected with J6/JFH1 viral particles. The culture supernatants were harvested to inoculate naïve Huh-7.5.1 cells with which to determine HCV RNA in culture medium.

Figure 5

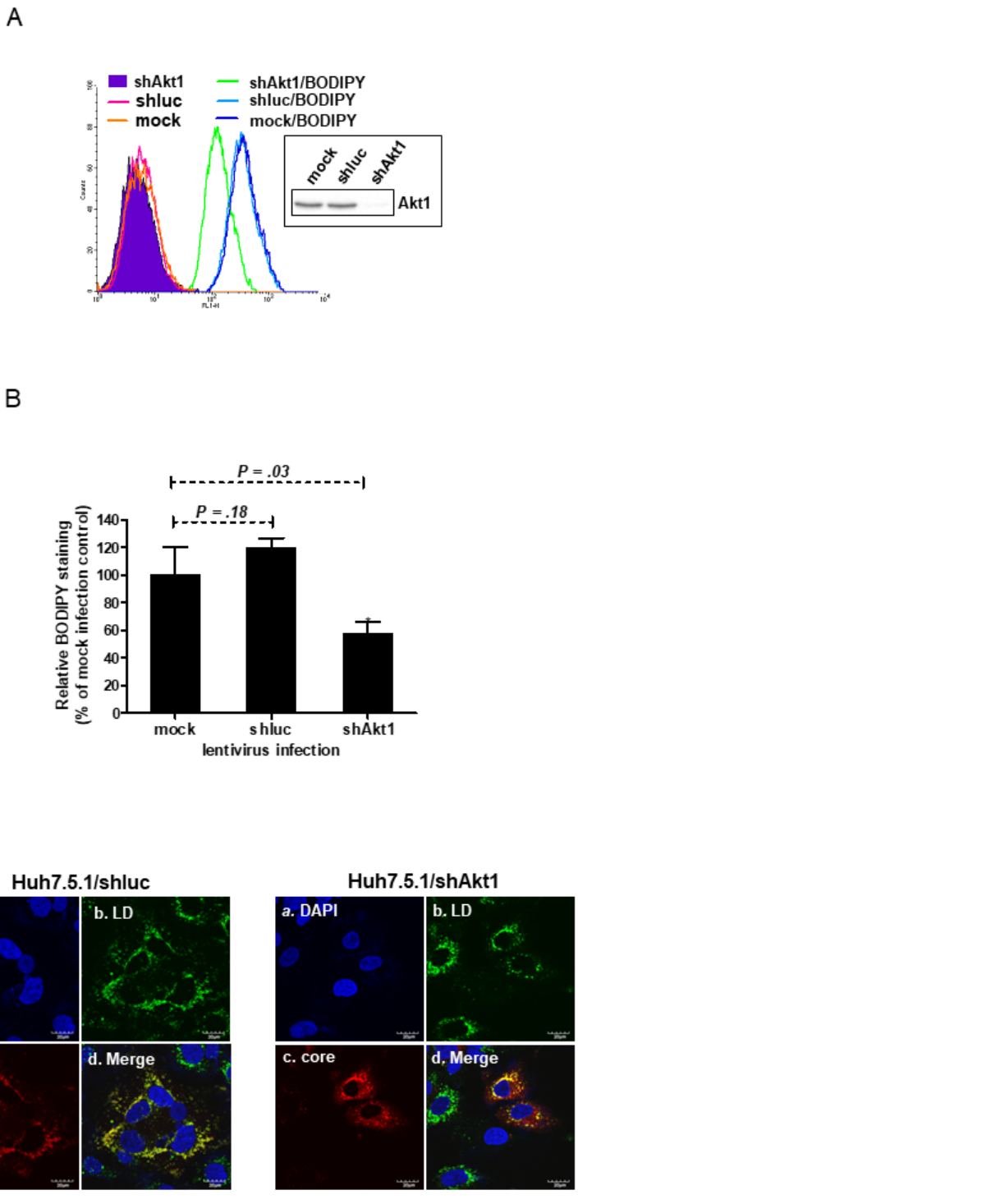

Figure 5

Akt1 silencing inhibited lipid droplet formation. (A) Quantification of lipid droplet formation by BODIPY 493/503 staining, followed by flow cytometry in Huh-7.5.1 cells with mock, shluc, or shAkt1 lentiviral infection. Akt1 silencing by shAkt1 was examined by immunoblotting (inset). (B) Values in panel A were shown in histogram with bars relative to the control mock infection. (C) Akt1 silencing decreased lipid droplet formation with co-localization of HCV core and lipid droplet (LD). Huh-7.5.1 cells were infected

first with shAkt1- or shluc-carrying lentivirus and then with J6/JFH1 viral particles, followed by immunostaining for lipid droplet (LD, green fluorescence) and HCV core antigen (red fluorescence)

Figure 6

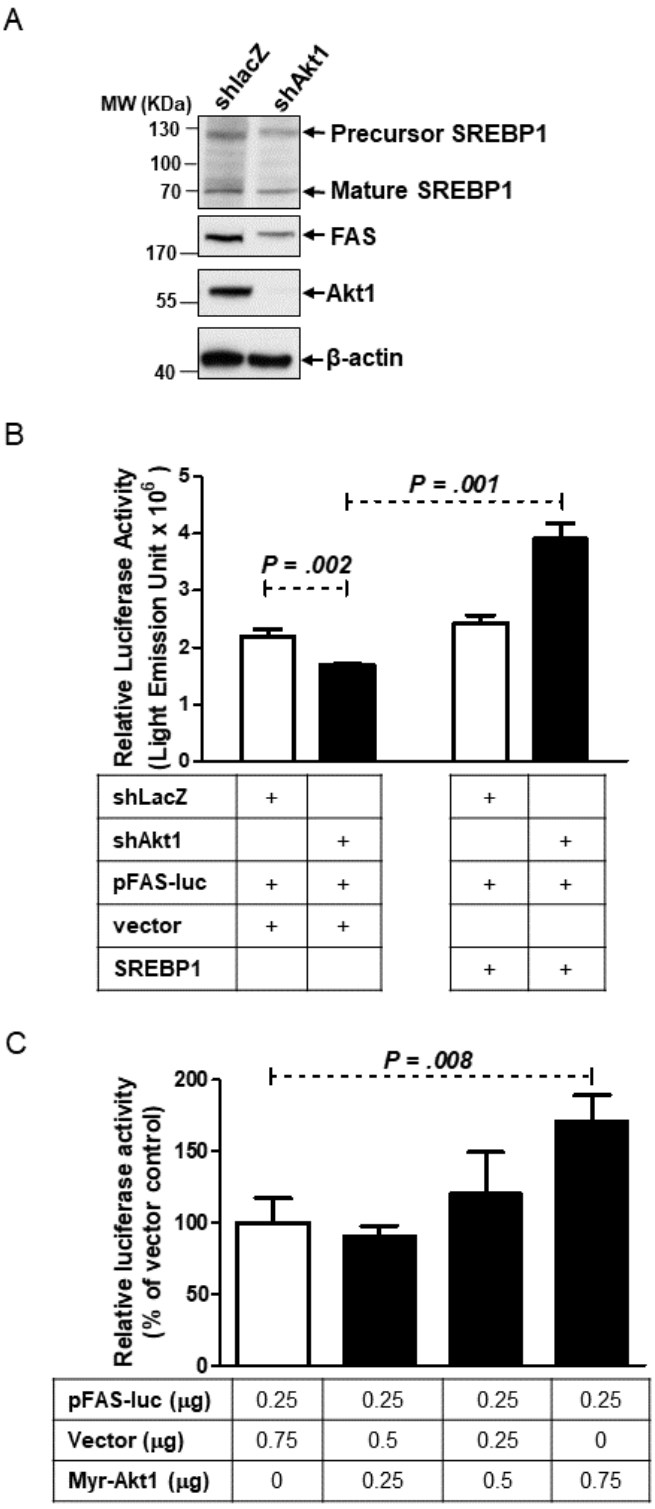

Figure 6

Akt1 silencing inhibited lipogenesis. (A) Western blot showing Akt1 silencing decreased the expression levels of precursor SREBP1, mature SREBP1, and fatty acid synthase (FAS). (B) SREBP-1c cDNA transfection restored the transcriptional activity of FAS which was inhibited by Akt1 silencing. Huh-7.5.1

cells were infected with lentiviral shLacZ or shAkt1 and then transfected with pFASwt-luc plus pcDNA3.1 or pFASwt-luc plus pcDNA3.1-Flag-SREBP-1c. After 48 h, cells were subjected to luciferase assay. (C) Ectopic Akt1 cDNA transfection induced FAS transcriptional activation in a dose-dependent manner. Huh7.5.1 cells were transfected with pFASwt-luc and the indicated doses of pCMV-myr-Akt1. After 48 h, cells were subjected to luciferase assay.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.pptx