Targeting protein palmitoylation decreases palmitate-induced sphere formation of human liver cancer cells

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Abstract. Although non-alcoholic fatty liver disease (NAFLD) is considered a benign disorder, hepatic steatosis has been proposed to be involved in the tumorigenesis of liver cancer. However, the underlying mechanism for carcinogenesis in fatty liver diseases remains unclear. Cancer stem cells (CSCs) have been hypothesized to serve a key role in tumorigenesis. Tumor formation begins with a subset of heterogeneous cells that share properties with stem cells, such as self-renewal and undifferentiated properties. Our previous study reported that the saturated fatty acid palmitate (PA) significantly enhanced the CSC properties of the HepG2 human liver cancer cell line; however, its underlying mechanisms are unknown. In the present study, a proteomic approach was used to investigate the palmitoylation of proteins in HepG2 CSCs. CSC behavior was induced in HepG2 cells via 200 μM PA. Proteomic analysis was performed to identify post-transcriptional modifications of proteins in HepG2 CSCs in response to PA treatment. The present study identified proteins modified by palmitoylation in HepG2 CSC spheres formed following PA treatment. It was therefore hypothesized that palmitoylation may be crucial for CSC sphere formation. Furthermore, the present study demonstrated that two palmitoylation inhibitors, tunicamycin (5, 10 and 25 μg/ml) and 2-bromohexadeca-noic acid (25, 50 and 150 μM), significantly decreased CSC sphere formation without affecting cell viability. An association was identified between sphere formation capacity and tumor-initiating capacity of CSCs. The results of the present study demonstrated that protein palmitoylation may influence the PA-induced CSC tumor-initiating capacity, and that the inhibition of palmitoylation may be a suitable chemopreventive strategy for treating patients with NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disease that is a global public health concern. From 1989 to 2015, the incidence rate of NAFLD was 28.01-52.34 per 1,000 people for Asia and Israel (1). In 2018, the prevalence of NAFLD was ~25% of the world population (2). The clinical process of NAFLD includes simple steatosis, steatohepatitis, hepatic fibrosis/cirrhosis and hepatocellular carcinoma (HCC) (3). The two-hit hypothesis of non-alcoholic steato-hepatitis (NASH) suggests that hepatic steatosis ('first hit') is a prerequisite for subsequent events ('second hits') that lead to hepatic injury (4). Free fatty acids (FFAs) are the primary mediators of excessive lipid accumulation in the liver. Notably, circulating FFA levels are significantly increased in patients with NAFLD, and evidence indicates that plasma FFAs are associated with disease severity (5). Excess FFA-induced hepatic injury results from the limited capacity of hepatocytes to transform FFA into triglycerides in lipid droplets (5). Excess lipid droplets in cells can result in mitochondrial dysfunction and oxidative stress, potentially contributing to hepatic inflammation and fibrosis in NASH (5).

The initiation of cancer may be the result of mutations in stem cells that interfere with differentiation. Certain tumor cells share properties with stem cells, which indicates that cancer stem cells (CSCs) may be responsible for cancer initiation and progression. These distinctive properties of CSCs are the capacities for self-renewal and cell proliferation (spherogenesis), which are primary causes of cancer recurrence and metastasis (6). The sonic hedgehog (Shh) signaling pathway and stemness-connected transcription factors (such as Sox2 and Oct4) are primarily responsible for CSC proliferation (7,8). CSCs have been hypothesized to be responsible for the carcinogenesis of HCC. An increasing number of studies have demonstrated that growth factors, such as epidermal growth factor (EGF) or fibroblast growth factor 2 (FGF2), can stimulate cell proliferation

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of CSCs and sphere formation in a three-dimensional culture system. Sphere formation is a practical approach for enriching certain CSC subpopulations with self-renewal properties and CSC marker expression levels (9,10).

Research has demonstrated that liver CSC subpopulations can be isolated by certain cell surface markers, specifically CD133 (11,12), CD90 (13-15), CD44 (16), the epithelial cell adhesion molecule (17) and CD13 (18). A previous study demonstrated that undifferentiated multipotent neural stem cells could be expanded via suspension using sphere assay (19). Sphere assays have been used to study adult stem cells in numerous organs and tissues, including the liver, nerves, prostate and mammary (9,20-22). Furthermore, sphere formation assay is widely recognized for its ability to enrich potential CSC subpopulations via stimulation of EGF or FGF2 (9,23-25).

Our previous study reported that exposure of hepatocytes to palmitate (PA) induced reactive oxygen species (ROS) production, NFκB activation and inflammatory cytokine expression levels in primary rat hepatocytes (PRHs) (26) and HepG2 cells (27). This increase in proinflammatory cytokines induced by PA could serve a key role in hepatic stellate cell activation and the development of hepatic fibrosis. Furthermore, it was demonstrated that PA induced CSC sphere formation in HepG2 cells but not in PRHs. Conversely, inhibition of NFκB activity significantly limited HepG2 CSC sphere formation (27). In the present study, it was hypothesized that PA would induce CSC properties via palmitoylation, as palmitoylation has been demonstrated to regulate CSC markers, estrogen receptors and epidermal growth factor (EGF) receptors (28). Liquid chromatography-tandem mass spectrometry (LC/MS/MS) was used to identify the palmitoylation of proteins in PA-treated HepG2 CSC spheres. The present study demonstrated that numerous proteins were palmitoylated in CSC spheres formed following PA treatment. After treating the HepG2 cells with two palmitoylation inhibitors, tunicamycin and 2-bromohexadecanoic acid, the PA-treated CSC spheres were notably inhibited. Taken together, the results of the present study verified the hypothesis that PA-induced palmitoylation is key for HepG2 CSC sphere formation. Thus, the inhibition of palmitoylation may be a chemopreventive strategy for use in treating patients with NAFLD.

Materials and methods

Chemicals. PA (cat. no. 102553) and BSA were acquired from Sigma-Aldrich (Merck KGaA). PA stock solution was prepared as previously described (27). In brief, 400 mM PA stock solution was prepared in DMSO. The 3% (w/v) FFA-free BSA solution was prepared in DMEM and maintained at 55˚C. A 5 mM FFA/BSA solution was prepared by dissolving the 128.2 mg of PA in 1 ml (500 mM) ethanol and heating at 70˚C for 10 min. 10% BSA solution was made in DMEM medium, filtered and incubated at 37˚C for 10 min. Subsequently, 10 ul of dissolved PA was added to 990 μl of 10% BSA, and the solution was vortexed and incubated at 55˚C for 30 min. The final PA concentration in BSA solution was 5 mM. The FFA/BSA solution was diluted to the concentration used in the experiments using DMEM. All solutions were freshly prepared before use. The 1 mg/ml tunicamycin and 1 mM 2-bromohexadecanoic acid (cat. nos. T7765 and 21604, respectively; both

Sigma-Aldrich; Merck KGaA) stock solutions were prepared in ethanol and stored at -20˚C.

Cell culture. The human liver cancer cell line HepG2 was obtained from the Bioresource Collection and Research Center. Authentication of the HepG2 cell line used in the present study was performed via the short tandem repeat-PCR method. Cells were maintained as a monolayer in culture medium (Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified 5% CO_2 incubator. During sphere assays, cells were harvested and washed with PBS to remove serum. Cells were then suspended in ultralow-attachment 6-well plates (Corning, Inc.) at a density of 5,000 cells/well. Serum-free DMEM/F12 culture medium was supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml human recombinant EGF, 10 ng/ml human recombinant FGF2, 2% B27 supplement and 1% N_2 supplement (Thermo Fisher Scientific, Inc.). Mycoplasma testing was performed for the cell lines used. The maintenance and culture of HepG2 cells were performed as previously described (27). Cell morphology was observed and recorded using a light microscope (magnification, x40) and the most representative image was selected.

Oil Red O staining. After HepG2 cells were exposed to 200 μM PA (Sigma-Aldrich; Merck KGaA) for 12 h at 37˚C, cells were stained with Oil Red O to investigate the amount of fat accumulation in cells. Briefly, cells were washed with PBS and fixed in 10% neutral formalin at room temperature (RT) for 30 min. After two washes with propylene glycol, 10% Oil Red O was added and cells were stained for 7 min at RT before being washed with 85% propylene glycol. Cells were rinsed in distilled water and counterstained with 10% hematoxylin solution at RT for 8 min. A total of three images were captured per well using a light microscope (magnification, x40) and the most representative image was selected.

CSC sphere formation assay. After cells were cultured under serum-free medium supplemented with EGF and FGF2 for sphere formation, spheres were collected via centrifugation at 700 x g at RT for 3 min. Subsequently, cells were dissociated using trypsin-EDTA and mechanically disrupted with a pipette to obtain single cells. The sphere-derived single cells were then centrifuged at 700 x g at RT for 3 min to remove the trypsin-EDTA and resuspended in serum-free medium for cell counting using trypan-blue exclusion assay (Thermo Fisher Scientific, Inc.) using a light microscope (magnification, x100) and the replication of sphere assays. The spheres were passaged every 7 days until they reached a diameter of 100 μm.

Mass spectrometry. PA-treated HepG2 CSCs were collected to extract protein lysates using lysis buffer [1 ml ice-cold buffer (20 mM Tris-Hcl, pH 7.4; 150 mM NaCl; 1% (v/v) Triton X-100; 0.5% (v/v) Nonidet P40; 1 mM EDTA; 1 mM PMSF; 2 μg/ml Antipain C; 50 μg/ml tosyl phenylalanyl chloromethyl ketone; 10 μg/ml Leupeptin; 1 mM NaF; 1 mM $NaVO_3$; 5 mM $Na_4P_2O_7$)]. A total of 10 μg of protein lysates were further separated using 10% SDS-PAGE and stained with 0.25% Coomassie brilliant blue R-250 solution (Bio-Rad
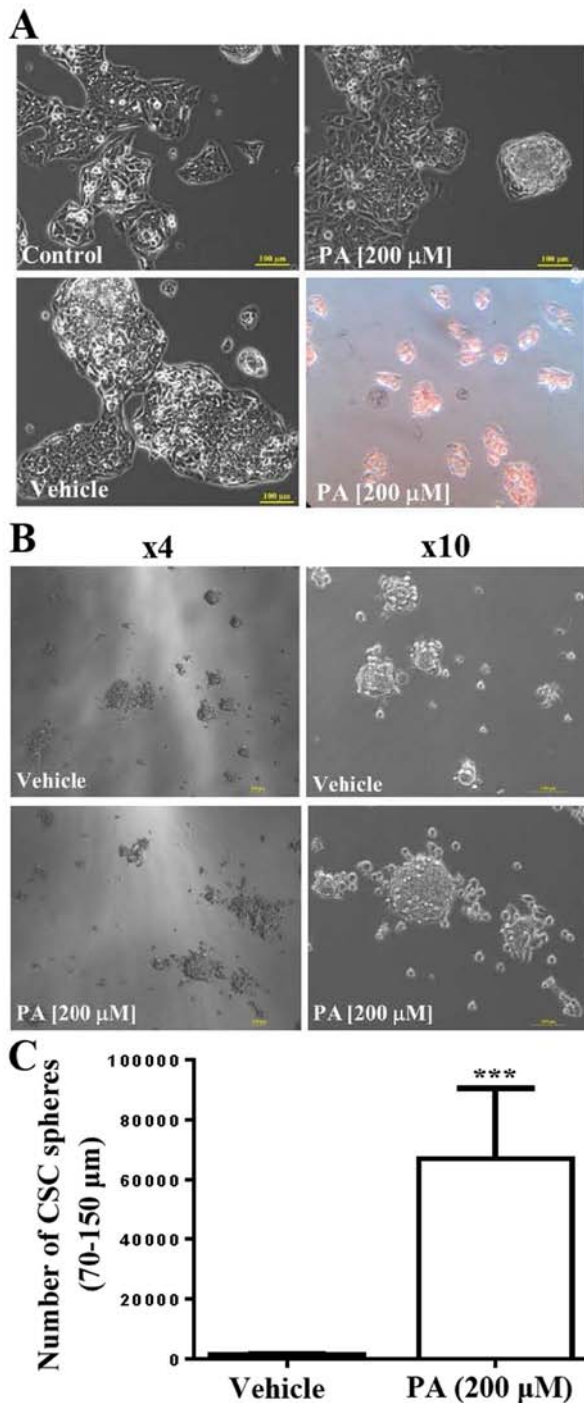
Figure 1. Effects of PA on human HepG2 cells. (A) PA did not affect cell growth but induced lipid accumulation in HepG2 cells under 10% FBS culture medium. Scale bar, 100 μm. The 3% (w/v) FFA-free BSA solution was prepared in DMEM (vehicle) and was used to prepare PA solution. (B) Effects of PA on human HepG2 CSCs under sphere formation culture conditions. Scale bar, 100 μm. (C) PA significantly increased the number of human HepG2 CSC spheres. Data are presented as the mean ± standard error of the mean; n=3. ***P<0.001. PA, palmitate; CSC, cancer stem cell.

Laboratories, Inc.) at RT for 1 h. All subsequent procedures for in-gel digestion and mass spectrometry were performed as previously described (29).

Statistical analysis. Data are presented as the mean ± standard error of the mean. One-way analysis of variance was performed
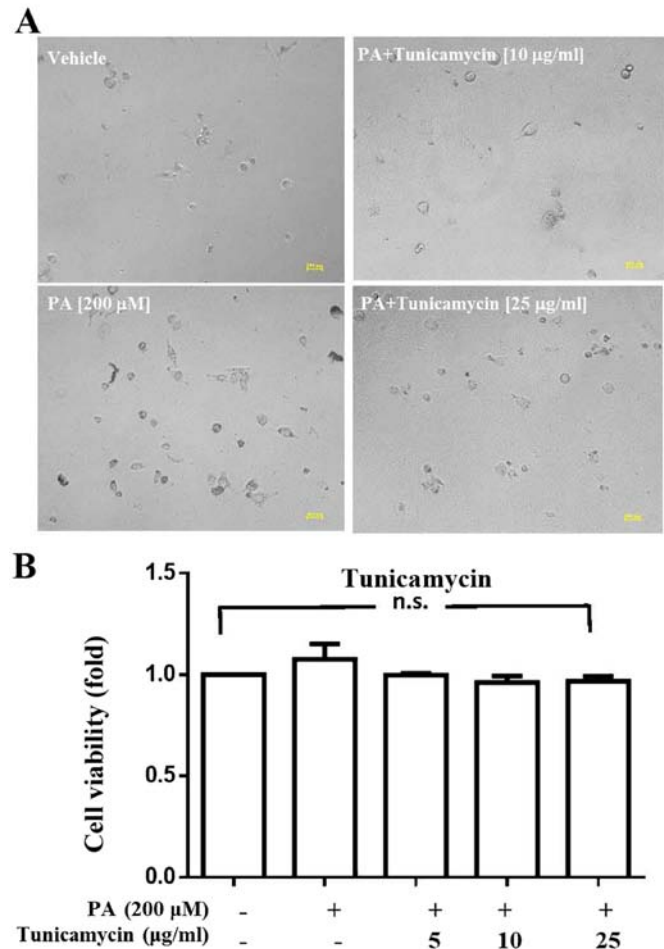

Figure 2. Effects of tunicamycin on viability of human HepG2 cells in 10% FBS culture medium. (A) Morphology of tunicamycin-treated HepG2 cells. Scale bar, 100 μm. Tunicamycin did not alter the morphology of HepG2 under 10% serum culture condition. (B) Tunicamycin (5, 10 and 25 μg/ml) did not affect viability of PA-treated human HepG2 cells in 10% FBS culture medium. Data are presented as the mean ± standard error of the mean; n=3. PA, palmitate; n.s., not significant.

to compare the statistical differences in biochemical and molecular parameters. The post hoc test performed was Tukey's multiple comparisons test. PRISM software (version 6.0; GraphPad Software, Inc.) was used for this purpose. A total of three independent experiments were performed. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of PA on HepG2 cells. The present study investigated the underlying mechanisms of the effects of PA on human HepG2 cells. The results indicated that, at a concentration of 200 μM, PA did not affect the growth and survival rate of HepG2 cells but did induce morphological changes in HepG2 cells in the 10% FBS culture medium (Fig. 1A). The results of Oil Red O staining indicated that lipid droplets accumulated inside HepG2 cells following PA treatment (Fig. 1A). Furthermore, the present study investigated the effects of PA on the cancer sphere formation ability of HepG2 cells; it was demonstrated that PA treatment significantly increased the cancer sphere formation ability of HepG2 cells (n=3; P<0.001). Representative images are presented in Fig. 1B and C.

Table I. Identification of novel types of palmitoylation in palmitate-induced HepG2-CSC spheres using mass spectrometry.

Protein name	Protein ID	Modified peptides[a]	Position[a]	Calc. m/z	Obs. m/z	dM, calc.-Obs.[b]	dM, ppm[c]
α-enolase	P06733	(-)IEEELGS#K	413-420	381.568	381.576	-0.008	-20.959
C-myc promoter-binding protein 1	E2DRY6	(-)IEEELGS#K	317-324	381.568	381.576	-0.008	-20.959
Epididymis luminal protein 114	V9HWK2	(-)NLGPGMTK#MAK	163-173	693.416	693.405	0.011	16.103
Epithelial protein lost in neoplasm β variant (Fragment)	Q53GG0	(-)RS#NTENLSQHFR	54-65	432.495	432.501	-0.005	-12.570
		(-)KGWSM*SEQSEES#VGGR	614-629	502.758	502.770	-0.012	-24.776
LIM domain and actin-binding protein 1	Q9UHB6	(-)RS#NTENLSQHFR	54-65	432.495	432.501	-0.005	-12.570
		(-)KGWSM*SEQSEES#VGGR	614-629	502.758	502.770	-0.012	-24.776
AHNAK	Q09666	(-)M*KM*PTFS#TPGAK	631-642	392.225	392.224	0.001	2.163
		(-)IS#M*PDFDLNLK	3,671-3,681	387.476	387.479	-0.003	-6.553
RNA-binding motif protein, X chromosome	P38159	(-)PSFES#GRRGPPPPPR	87-101	624.700	624.707	-0.007	-11.174
		(-)DYGHSS#SRDDYPSR	245-258	470.735	470.746	-0.012	-24.794
		(-)DYSDHPSGGSYRDS#YESYGNSR	271-292	685.065	685.071	-0.006	-9.169
Plectin	Q15149	(-)S#EFERLECLQR	523-533	584.995	584.995	0.000	-0.554
		(-)AS#FEKAAAGKAELELELGR	2,069-2,087	557.828	557.816	0.012	20.636
Spectrin α chain, non-erythrocytic 1	Q13813	(-)SCK#KFM*LFR	1,090-1,098	380.480	380.477	0.003	6.954
SFPQ protein (Fragment)	Q9BSV4	(-)DMRMGGGGAMNM*GDPYGS#GGQK	536-557	607.785	607.785	0.000	-0.257
Thyroid hormone receptor-associated protein 3	Q9Y2W1	(-)SSSDRS#RR	149-156	396.906	396.904	0.003	7.305
		(-)SSSNHSRVESS#K	162-173	514.954	514.955	-0.001	-1.561
		(-)S#GKWEGLVYAPPGK	468-481	432.509	432.501	0.008	18.812
Vinculin	P18206	(-)NLGPGMTK#MAK	163-173	693.416	693.405	0.011	16.103

Modified peptide sites are marked with a hash (#). [a]Amino acid positions of the first and the last residues in accession number; [b]dM (Obs.-calc.), mass accuracy; [c]modified peptide mass accuracy (ppm).
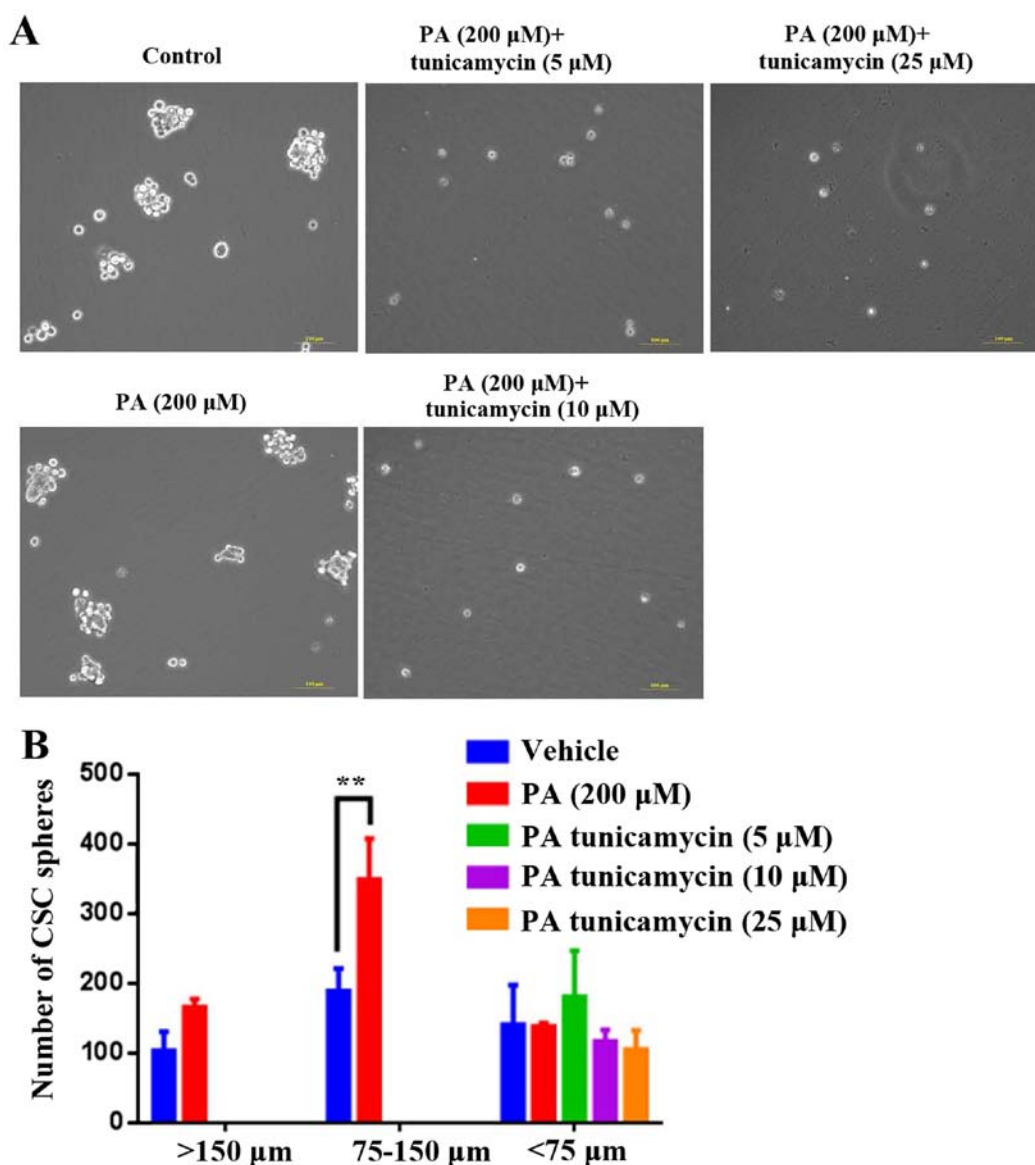
Figure 3. Effects of tunicamycin on CSC sphere formation of human HepG2 cells under CSC sphere formation culture condition. (A) CSC sphere morphology of tunicamycin-treated HepG2 cells. Scale bar, 100 μm. (B) Tunicamycin at concentrations of 5, 10 and 25 μg/ml notably decreased the formation of HepG2-CSC spheres (75-150 μm and >150 μm). Data are presented as the mean ± standard error of the mean; n=3. **P<0.01. CSC, cancer stem cell; PA, palmitate.

LC/MS/MS analysis to identify sites of PA-induced HepG2-CSC sphere palmitoylation on cell proteins. PA treatment has previously been shown to significantly increase sphere formation of HepG2 cells (27). In the present study, it was demonstrated that 200 μM PA significantly increased HepG2 CSC sphere formation, particularly at the sphere size 75-150 μm (Fig. 1). Subsequently, PA-treated HepG2 CSC spheres were collected and total proteins were isolated for LC/MS/MS analysis to investigate the effects of PA treatment on the palmitoylation of HepG2-CSC spheres. It was noted that certain proteins were palmitoylated, namely α-enolase, c-Myc promoter-binding protein 1, epididymis luminal protein 114, epithelial protein lost in neoplasm β variant (fragment), LIM-domain and actin-binding protein 1, AHNAK, RNA-binding motif protein, X chromosomes, plectin, spectrin α chain, non-erythrocytic 1 SFPQ protein (fragment), thyroid hormone receptor-associated protein 3 and vinculin (Table I).

Analysis of the effects of tunicamycin on viability and CSC sphere formation ability of HepG2 cells. After demonstrating that numerous proteins were palmitoylated, the present study investigated whether inhibition of palmitoylation alters cell viability and CSC sphere formation. The results indicated that tunicamycin, a specific inhibitor of palmitoylation, did not affect the viability of PA-treated HepG2 cells at concentrations of 5, 10 or 25 μg/ml (Fig. 2); however, at these concentrations, tunicamycin notably decreased PA-induced HepG2-CSC sphere formation (>150 μm and 75-150 μm; Fig. 3).

Analysis of the effects of 2-bromohexadecanoic acid on viability and CSC sphere formation ability of HepG2 cells. The present study demonstrated that 2-bromohexadecanoic acid, another inhibitor of palmitoylation, did not affect viability of PA-treated HepG2 cells at concentrations of 25, 50 and 150 μM (Fig. 4); however, it significantly decreased
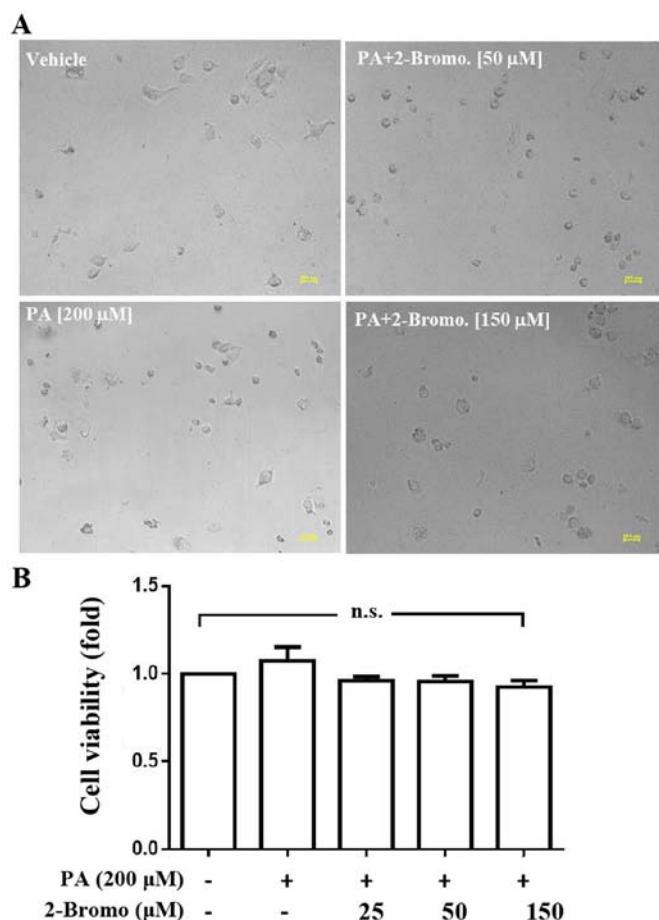
A

B

Figure 4. Effects of 2-bromo on viability of human HepG2 cells in 10% FBS culture medium. (A) Cell morphology of 2-bromo-treated HepG2 cells. Scale bar, 100 μm. (B) 2-Bromo (25, 50 and 150 μM) did not affect viability of PA-treated human HepG2 cells in the 10% FBS culture medium. Data are presented as the mean ± standard error of the mean; n=3. 2-Bromo, 2-bromodecahexanoic acid; PA, palmitate; n.s., not significant.

PA-induced HepG2-CSC sphere formation (75-150 μm) at these concentrations (Fig. 5).

Discussion

NAFLD is the most common type of liver disease worldwide and is expected to become a primary cause of HCC in the near future. Previous research has investigated the effects and underlying mechanisms of saturated FFAs, such as PA, on liver cells. Our previous study demonstrated that PA increased intracellular hydrogen peroxide levels in PRHs and the human liver cancer cell line HepG2, and induced the expression levels of proinflammatory cytokines, such as TNF-α, IL6 and intercellular adhesion molecule 1. Furthermore, PA activated hepatic stellate cells and contributed to steatosis-associated hepatic fibrogenesis (30). In addition, PA has been reported to significantly increase CSC sphere formation in HepG2 cells but not in PRH (27). Furthermore, PA (50, 100 and 200 μM) activated the expression levels of pluripotent genes, including Sox2, Oct4 and Shh in a dose-dependent manner, and 200 μM PA significantly increased the sphere formation capacity of HepG2 cells (27). In the present study, 200 μM PA significantly increased the sphere formation capacity of HepG2 cells.

Notably, in a previous study, PA (10, 50, 100 and 200 μM) dose-dependently induced production of ROS in primary hepatocytes and HepG2 cells but did not affect the viability of primary hepatocytes and HepG2 cells (30). Furthermore, PA not only induced proinflammatory responses in hepatocytes and HepG2 cells (30) but also activated the CSC-like properties of HepG2 cells (27) to increase sphere formation. However, long-term PA treatment may cause cells to become senescent, as certain types of cell are prone to senescence rather than apoptosis following high exogenous stress (31).

The primary saturated fatty acid in HepG2 cells is PA, which undergoes post-translation palmitoylation. Research indicates that palmitoylation modulates protein function at all stages of protein processing (26). In addition, other saturated fatty acids, such as myristic and stearic acid, and unsaturated fatty acids, such as oleic and arachidonic acid, also undergo post-translational modification. The present study used LC/MS/MS to identify palmitoylation-modified proteins in PA-treated HepG2 CSC spheres (Fig. 1); numerous proteins were demonstrated to be palmitoylated.

Tunicamycin is a nucleoside antibiotic that can inhibit protein palmitoylation. Tunicamycin has been reported to inhibit palmitoylation of the endothelial isoform of nitric oxide synthase, promote nitric oxide synthesis in aortic endothelial cells and increase the influx of Ca^{2+} across the plasma membrane (32). In the present study, tunicamycin did not affect HepG2 cell viability in 10% FBS culture medium but decreased PA-induced formation of HepG2 CSC spheres in the sphere formation culture medium. Although 2-bromohexadecanoic acid (2-bromopalmitate) is used as a non-selective inhibitor of lipid metabolism, it has been demonstrated to be a general inhibitor of protein S-palmitoylation. It has been hypothesized that 2-bromopalmitate is converted to 2-bromopalmitoyl-CoA in cells. Notably, both 2-bromopalmitate and 2-bromopalmitoyl-CoA interact with DHHC palmitoyl acyl transferases, which catalyze protein S-palmitoylation. The present study demonstrated that 2-bromohexadecanoic acid did not affect HepG2 cell viability in the 10% FBS culture medium, but decreased PA-induced formation of HepG2 CSC spheres in the sphere formation culture medium. Neither 2-bromopalmitate nor tunicamycin affected viability of HepG2 cells under serum-containing culture conditions. However, they both significantly decreased the number of HepG2 spheres formed under serum-free culture conditions. One explanation for this finding is that serum supplementation may activate cell survival to eliminate ER stress when cells are treated with 2-bromopalmitate or tunicamycin. The present study investigated the effects of PA and two inhibitors, 2-bromopalmitate and tunicamycin, on palmitoylation. Although 2-bromopalmitate is traditionally used as an inhibitor of PA, its effects extend beyond protein palmitoylation. Tunicamycin can also function as an inhibitor of protein N-glycosylation and induce cellular ER stress. Further research is necessary to determine other mechanisms of 2-bromopalmitate and tunicamycin and to investigate their ability to decrease PA-induced protein palmitoylation.

The effects of tunicamycin at concentrations of 5, 10 and 25 μg/ml, and 2-bromopalmitate at a concentration of 150 μM observed in the present study are consistent with those reported in the literature (6,7). Sobocińska *et al* (33) used 2-bromopalmitate (125 and 250 μM) to inhibit lipopolysaccharide-induced
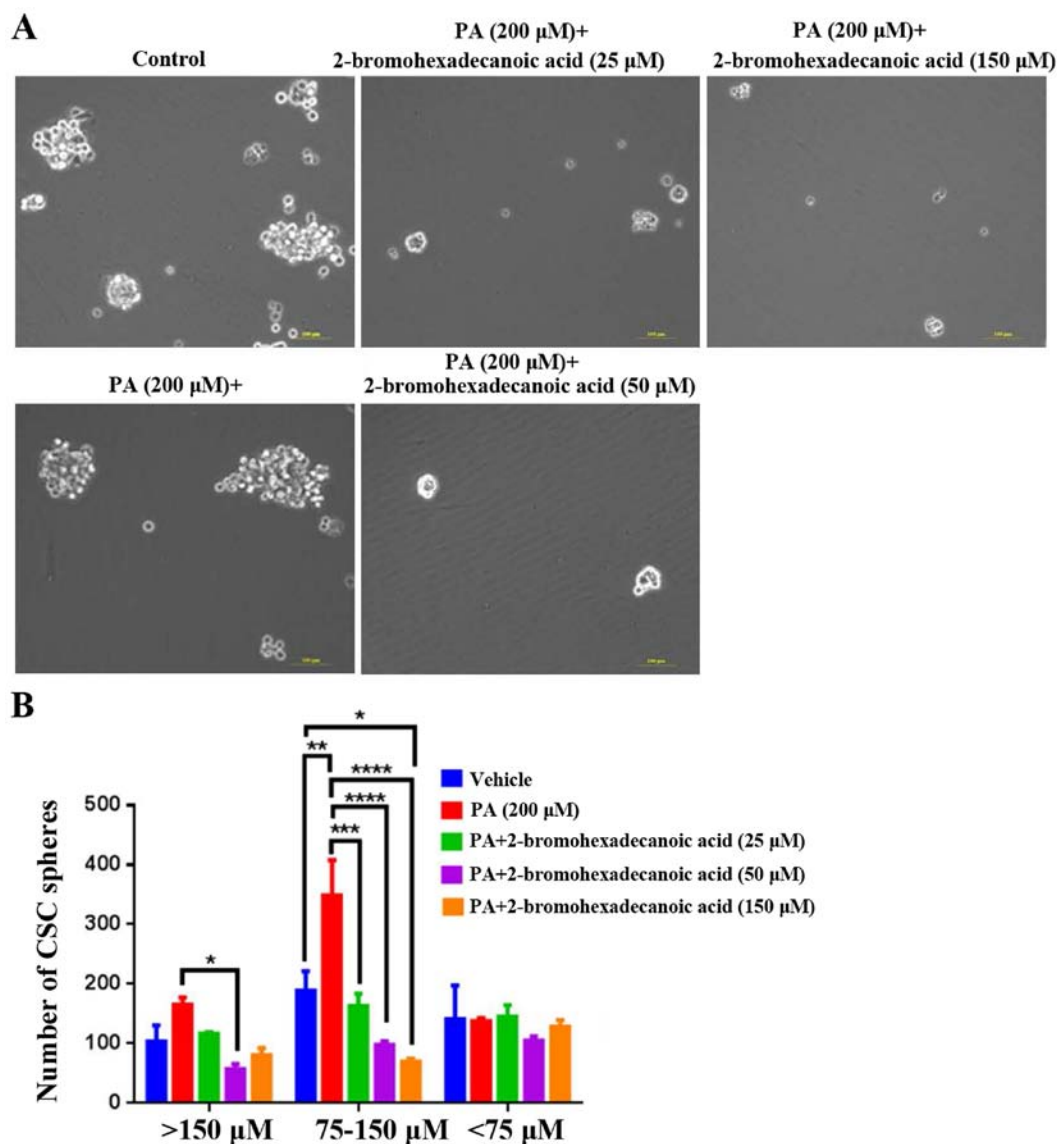
Figure 5. Effects of 2-bromohexadecanoic acid on CSC sphere formation of human HepG2 cells under CSC sphere formation culture condition. (A) Sphere morphology of tunicamycin-treated HepG2 cells. Scale bar, 100 μm. (B) 2-Bromohexadecanoic acid (25, 50 and 150 μM) significantly decreased the number of HepG2 CSC spheres (75-150 μm and >150 μm). Data are presented as the mean ± standard error of the mean; n=3. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CSC, cancer stem cell.

S-palmitoylation and activation of PI4KIIβ; this activation generated phosphatidylinositol-4-phosphate, which is involved in signaling pathways controlling the production of proinflammatory cytokines. Patterson and Skene (34) demonstrated that tunicamycin (10 μg/ml) significantly inhibited growth cone protein palmitoylation in intact neuronal cells. On the other hand, the results of the present study demonstrated that PA may induce protein palmitoylation and CSC properties via inducing expression levels of carnitine O-palmitoyltransferase 1 and the CSC marker CD44. Additionally, tunicamycin and 2-bromopalmitate treatment notably suppressed the expression levels of carnitine O-palmitoyltransferase 1 and CD44, respectively (Data S1), indicating a novel mechanism of tunicamycin and 2-bromopalmitate in the inhibition of palmitoylation.

In breast cancer, palmitoylation has been hypothesized to control the function of commonly dysregulated genes, including estrogen and EGF receptors, and CSC marker proteins. Palmitoylation also regulates the formation of complexes,

such as integrins, at the plasma membrane (26). However, it is unclear whether aberrant palmitoylation can contribute to tumor initiation and growth in the liver. The results of the present study indicated that PA-mediated palmitoylation may be a key factor for inducing CSC activation in the fatty liver of patients with NAFLD. In conclusion, the development of specific inhibitors for the suppression of PA-mediated palmitoylation may present a promising chemopreventive strategy to treat patients with NAFLD.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LWC, YCH and CCL were involved in the conceptualization, supervision and funding acquisition of the present study. LWC, YCH was involved in project administration. LWC, CLT, KCY, CCL and YCH were involved in the investigation, methodology and analysis of the present study, and in the writing of the manuscript. The final manuscript was read and approved by LWC, CLT, KCY, CCL and YCH.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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