PSMD1 and PSMD2 regulate HepG2 cell proliferation and apoptosis via modulating cellular lipid droplet metabolism

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Abstract

Background: Obesity and nonalcoholic steatohepatitis (NASH) are well-known risk factors of hepatocellular carcinoma (HCC). The lipid-rich environment enhances the proliferation and metastasis abilities of tumor cells. Previous studies showed the effect of the ubiquitin–proteasome system (UPS) on tumor cell proliferation. However, the underlying mechanism of UPS in regulating the proliferation of lipid-rich tumor cells is not totally clear.

Results: Here, we identify two proteasome 26S subunits, non-ATPase 1 and 2 (PSMD1 and PSMD2), which regulate HepG2 cells proliferation via modulating cellular lipid metabolism. Briefly, the knockdown of PSMD1 and/or PSMD2 decreases the formation of cellular lipid droplets, the provider of the energy and membrane components for tumor cell proliferation. Mechanically, PSMD1 and PSMD2 regulate the expression of genes related to de novo lipid synthesis via p38-JNK and AKT signaling. Moreover, the high expression of PSMD1 and PSMD2 is significantly correlated with poor prognosis of HCC.

Conclusion: We demonstrate that PSMD1 and PSMD2 promote the proliferation of HepG2 cells via facilitating cellular lipid droplet accumulation. This study provides a potential therapeutic strategy for the treatment of lipid-rich tumors.

Keywords: PSMD1, PSMD2, Lipid droplet, Cell proliferation

Background

Hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related deaths worldwide [1]. The chronic liver damage and inflammation caused by chronic hepatitis B virus or hepatitis C virus infections are common reason for HCC development. However, the incidence of nonviral HCC is rapidly increasing, especially in developed countries. Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), which are usually enhanced by obesity, are well-known risk factors of HCC [2, 3]. Patients with obesity and NAFLD/NASH show an increased intake of dietary fatty acids (FAs), and meanwhile, insulin resistance enhances lipolysis of adipose tissue, which causes an increased exogenous FA supply and results in the development of a “lipid-rich” environment for hepatocytes. The lipid-rich environment is considered to promote the proliferation and metastasis of tumor cells [4–6]. Compared to normal cells, the tumor cells usually uptake larger amount of lipids, accompanied by enhanced lipogenesis and cholesterol (CH) production and increased fatty acid (FA) β-oxidation [7, 8]. Furthermore, FA synthetases such as FASN and ACS play an important role in tumor cell proliferation and tumorigenicity [9, 10]. Lipids are required for the proliferation process. For example, the ovarian tumor cells can obtain lipids from the adipocytes grown in coculture experiments [11]. Besides enhanced lipid uptake, tumor cells often accumulate a larger amount of cellular lipid droplets (LDs) compared to normal cells [12], which might promote the proliferation process, thereby favoring the development of a more
aggressive phenotype [13]. Moreover, evidence indicates that a high level of LDs in cancer cells is associated with higher tumor aggressiveness [14, 15]. Recently, LDs showed a powerful effect on cellular lipid metabolism, which regulates lipid synthesis, lipolysis, and lipid turnover [16, 17]. The proteins decorated in the monolayer of phospholipids of LDs control the activities of LDs [18–20]. Therefore, the LD-related proteins are involved in the regulation of cellular lipid metabolism.

The ubiquitin–proteasome system (UPS) is a huge and complex system that regulates the degradation of up to 80% of cellular proteins, consisting of ubiquitin (Ub), Ub-activating enzymes (E1s, two classes, nearly 10 members), Ub-conjugating enzymes (E2s, approximately 40 members), and deubiquitinating enzymes (DUBs, roughly 90 members), and the heart of the system, the 26S proteasome complex [21–25]. Previous studies showed that UPS is involved in a wide range of biological processes, including cell growth, the cell cycle, DNA metabolism, and signal transduction [26, 27]. The deregulation of UPS or its components results in severe pathological disorders and changes in the expression levels of many tumor promoters and suppressors [21, 28–31]. The 26S proteasome is a multi-enzyme complex that responds to the degradation of intracellular proteins in eukaryotic cells [32–34]. PSMD1 and PSMD2 are two subunits of the 19S regulatory complex of the 26S proteasome [35–38]. Recent studies indicate that knockdown of PSMD1 and/or PSMD2 is able to suppress tumor cell proliferation [39–41]. Many studies about the proteomes of LDs have found that PSMD1 and PSMD2 are LD-related proteins in several species such as humans, mice, and C. elegans [42–48]. However, the regulatory roles of PSMD1 and PSMD2 in cellular lipid metabolism are unclear.

In the present study, we chose a hepatocellular carcinoma cell line, HepG2, to investigate the roles of PSMD1 and PSMD2 in cell proliferation and cellular lipid metabolism. HepG2 cells were derived from 15-year-old white liver cancer tissue and were utilized in the study about hepatocyte metabolism. Firstly, we showed that the expression levels of PSMD1 and PSMD2 affected cell proliferation and apoptosis. The knockdown of PSMD1 and PSMD2 inhibited cell proliferation and promoted cell apoptosis, and overexpression of PSMD1 and PSMD2 showed the opposite effects. Furthermore, the expression of PSMD1 and PSMD2 affected several proliferation and apoptosis related molecules. Because cellular lipid content is associated with cell proliferation and apoptosis, we further investigated the effects of PSMD1 and PSMD2 expression on cellular lipid metabolism. The knockdown of PSMD1 and PSMD2 decreased the content of cellular lipids. On the contrary, the overexpression of PSMD1 and PSMD2 promoted lipid formation and increased the cellular lipid content. Mechanically, PSMD1 and PSMD2 inhibition suppressed fatty acid and lipid synthesis by downregulating SREBF1 and PPARy via ASK-p38-JNK and AKT signaling. Moreover, through bioinformatic analysis, high expression levels of PSMD1 and PSMD2 were significantly correlated with poor prognosis of liver hepatocellular carcinoma. Therefore, the high expression levels of PSMD1 and PSMD2 probably enhanced hepatocellular carcinoma tumor cell proliferation. PSMD1 and PSMD2 could be potential therapeutic targets for this type of tumor.

Materials and methods

Cell culture and transfection

HepG2 and Huh7 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Wuhan, China). Cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, AusGeneX, Molendinar, Australia) at 37 °C in a humidified atmosphere of 5% CO2. Cells were transfected with Lipofectamine™ Transfection Reagent (#C0533, Beyotime, Nanjing, China). HepG2 cells were seeded on the cell slide in a 6-well plate and transfected with the plasmid vector in accordance with the transfection reagent instructions.

Oleic acid treatment

For oleic acid treatment, a 20 mM oleic acid (LPS free)-phosphate buffer saline (PBS) mixture and 20% FA-free bovine serum albumin (BSA) medium were prepared, and both media were heated in a 70 °C water bath for 30 min. Finally, the media were mixed. The 10 mM oleic acid–BSA mixture was added to the cell cultural medium at 1:49 (v:v). To identify the best treatment time, a time course was performed. The cells were treated with 200 μM oleic acid (OA) for 0, 1, 2, 3, 4, 5, and 6 h respectively, and then were stained by BODIPY to indicate the cellular LDs. The images showed that cellular LDs were able to be observed at 2 h after OA treatment. Many LDs formed “grape-like” structures at 5 h after OA treatment, and additionally, the image from the 6 h treatment showed little difference from the image from the 5 h treatment (Additional file 1: Fig. S1A). Therefore, the 6 h treatment was considered to be the best choice for the experiment. The cells that were seeded on the slides or plates were washed 3 times using PBS. One millilitre of
oleic acid medium was added to the well (200 μM), and the cells were cultured for 6 h.

**MTT assay**

HepG2 cell proliferation was detected using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Nanjing, China). Briefly, cells were seeded at a density of 4 × 10^4/well in 96-well plates 24 h after transfection. At different time points, 10 μL MTT (5 mg/mL) was added to each well and cells were cultured for another 4 h. Furthermore, 100 μL Formazan solution was added into each well and the optical density (OD) at 570 nm was measured on a microplate reader (PerkinElmer, Germany).

**CCK8 assay**

HepG2 and Huh7 cells activity after oleic acid treatment was detected by Cell Counting Kit-8 (CCK8) kit (#CK04, DOJINDO LABORATORIESE, Shanghai, China). Briefly, cells were seeded at a density of 4 × 10^5/well in 96-well plates. The cells were treated with 200 μM oleic acid for 0 h, 6 h, 12 h and 24 h. Then 10 μL CCK8 was added to each well and cells were cultured for another 4 h. The optical density (OD) at 450 nm was measured on a microplate reader (PerkinElmer, Germany).

**Flow cytometric analysis of the cell cycle**

Cells were collected and fixed in 70% ice-cold ethanol at 4 °C overnight. Then, the cells were centrifuged and washed with PBS. After incubation with 100 μL RNase A (0.1 mg/mL) for 30 min at 37 °C, 2 μL propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) was added to the cells and incubated for another 30 min at room temperature in the dark. Finally, cell cycle profiling was performed using a FACSCalibur Flow Cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed using ModFit software (Verity Software House).

**Cell apoptosis assay**

At 48 h after transfection, the percentage of apoptotic cells was measured using an Annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, USA) and analyzed by flow cytometry (BD Biosciences, USA) in accordance with the manufacturer’s protocol.

**5-Ethynyl-2'-deoxyuridine (EdU) assay**

Cells seeded in 12-well plates were cultured to 50% confluence and then transfected. Forty-eight hours after transfection with siRNAs or overexpression plasmid, the cells were fixed and stained with a BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, China) in accordance with the instructions. A fluorescence microscope (Olympus, Japan) was used to capture three randomly selected fields to visualize the number of EdU-positive cells.

**RNA oligonucleotides and plasmid construction**

siRNA oligos against PSMD1 and PSMD2 were obtained from Ribobio (Guangzhou, China). The sequence of the siRNA against the PSMD1 coding region was 5’-GAG AAAGACAGUGACUCGAGGAAA-3’, and that of the siRNA against the PSMD2 coding region was 5’-CCCAAGGUGCCUGAUGCAUCUACA-3’ [49]. The expression vector used in this study was constructed according to the instructions of a Seamless Cloning Kit (Vazyme Biotech Co., Ltd.). The primers used for plasmid vector construction are listed in Additional file 2: Table S1.

**RNA interference and overexpression assay**

The siRNAs targeting PSMD1 and PSMD2 were obtained from Ribobio (Guangzhou, China). Cells were seeded onto 6-well plates in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂. Cells at 70–80% confluence were transfected with 10 μL of siRNA using Lipo8000™ Transfection Reagent (#C0533, Beyotime, Nanjing, China). For the overexpression assay, 4 μg of the constructed plasmid was transfected with 10 μL of Lipo8000™ Transfection Reagent (#C0533, Beyotime, Nanjing, China). All transfections were performed in triplicate, and the data are presented as the mean ± standard deviation (SD).

**RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and real-time quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted using TRIzol (Life Technologies Inc., Carlsbad, CA, USA). RNA concentrations were determined using spectrophotometry with a NanoDrop 2000 (Thermo Fisher Scientific). A total of 1 μg of RNA was then subjected to reverse transcription to cDNA using a Reverse Transcription Kit (#R232-01; HiScript II Q Select RT SuperMix for qPCR; Vazyme, Nanjing, China). qRT-PCR was performed with SYBR Green I Real-Time PCR Master Mix (#Q131-02; AceQ qPCR SYBR Green Master Mix; Vazyme; Nanjing, China) on a LightCycler® 480 Real-Time System (Roche) and QuantStudio 6 Flex Real-Time PCR System (ABI, Thermo Fisher, Shanghai, China). The primer sequences used for qRT-PCR are listed in Additional file 2: Table S2. GAPDH and ACTB, the most frequently used housekeeping genes, were chosen as candidates. Then, the stability of the candidate reference genes was analyzed through standard curve and geNorm methods (Additional file 2: Tables S3, S4). Consequently, GAPDH was chosen as the reference
standard for normalization. The related method was based on that used in Costantini et al’s study [50]. The relative gene expression was calculated by the 2−ΔΔCt method, and the Student’s t test was used for statistical analysis.

**Western blotting**

Western blotting was performed as reported previously [51]. The following antibodies were used: anti-PSMD1 (#A16420; rabbit polyclonal antibody; ABclonal; 1:1000 dilution), anti-PSMD2 (#14748-1-AP; rabbit polyclonal antibody; Proteintech; 1:1000 dilution), anti-ASK1 (#A6274; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-AS1 (#A11027; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-p-ASK1 (#AP0394; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-p-AKT (#A11119; rabbit polyclonal antibody; Proteintech; 1:1000 dilution), anti-ASK1 (AP0394; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-p-AKT (#AP0140; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-p-p38 (#A01832; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-p-p38 (#AP0297; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-JNK1/2 (#A11119; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-p-JNK1/2 (#AP0631; rabbit polyclonal antibody; ABclonal; 1:2000 dilution), anti-GAPDH (Servicebio, Wuhan, China; 1:2000 dilution), anti-Tubulin (Servicebio, Wuhan, China; 1:2000 dilution), HRP-labeled Goat Anti-Rabbit IgG (H+L) (#GB23301-1, Servicebio, Wuhan, China), and HRP-labeled Goat Anti-Mouse IgG (H+L) (#GB23301, Servicebio, Wuhan, China).

**Lipid droplet marking and observation**

Cells were seeded on slides on a 24-well plate and were cultured for 24 h. The slides were fixed in 4% paraformaldehyde (Servicebio, Wuhan, China) for 15 min at room temperature. The slides were stained with BODIPY 493/503 (#D3922, Invitrogen, Carlsbad, CA, USA) for 45 min at 37 °C and were then stained with DAPI (#G1012, Servicebio) for 10 min at 37 °C. After washing three times with PBS for 10 min each, the slides were sealed with an anti-fluorescent quenching solution (#P36961, ProLong™ Diamond Antifade Mountant, Invitrogen™, Carlsbad, CA, USA) for use in a confocal laser scanning microscope (Zeiss LSM 800, Carl Zeiss, German) observation.

**Co-localization of PSMD1/PSMD2 and LDs**

The fluorescence labelling PSMD1, PSMD2, and PLIN2 expression vectors (PSMD1-mCherry, PSMD2-mCherry and PLIN2-EGFP) were co-transfected into HepG2 cells. After 48 h, the cells were treated with 200 μM OA for another 6 h. Then, the cells were fixed and then sealed with an anti-fluorescent quenching solution for confocal laser scanning microscope observation. The fluorescence plot analysis was performed by ImageJ software according to the instructions (https://imagej.en.softonic.com/). For the co-localization of PSMD1/PSMD2 and newly formed LDs, Livedrop was introduced to this experiment. The Livedrop [52] has been reported to be a useful fluorescence marker to label the newly formed LDs from the ER. The newly formed LDs were detected by Live-drop-EGFP within 30 min. Then, the same experiment was performed. In our study, we detected that the newly formed LDs were 45 min old.

**Survival analysis and normal/cancer gene expression comparison analysis**

Survival predication was performed using the GEPIA database (http://gepia.cancer-pku.cn/). The prognosis analysis and gene expression analysis were performed according to the instructions of the creator of this database [53].

**Statistical analysis**

All experiments were repeated at least three times. All results are presented as the mean ± SD. Statistical significance was assessed using Student’s t-test. A p value <0.05 was deemed to indicate statistical significance.

**Results**

**Both PSMD1 and PSMD2 knockdown suppresses HepG2 cell proliferation but promotes apoptosis**

The idea that PSMD1 and PSMD2 play important roles in the proliferation process of tumor cells, such as breast cancer cells and lung cancer cells, is not novel. However, few studies have reported their roles in regulating the proliferation and apoptosis process in HepG2 cells. Thus, we first investigated the functions of PSMD1 and PSMD2 in HepG2 cells. The specific RNAi fragments were prepared to block the expression of PSMD1 and PSMD2—individually and together. The cell proliferation capacity was detected by the MTT method, indicating that the cell proliferation process was significantly inhibited by the downregulation of PSMD1 and PSMD2 (p < 0.05, Fig. 1a). We then performed a cell cycle analysis to illustrate the cells in different phases. The results indicated that more cells were blocked in the S-stage by PSMD1 and PSMD2 knockdown (Fig. 1b, c). Then, the apoptosis analysis was performed through the flow cytometry method. As expected, more cells underwent apoptosis with low expression levels of PSMD1 and PSMD2 (Fig. 1d, e). To investigate the potential regulatory mechanism, we detected the expression levels of proliferation-related (Fig. 1f) and apoptosis-related genes (Fig. 1g). The expression levels of CCND1, CCND2, CCNB1, CCNE1,
and ki67 were downregulated significantly \((p < 0.05)\) in the siPSMD1, siPSMD2, and siPSMD1 + siPSMD2 group cells, while CASP3, CASP8, CASP9, and FAS were upregulated significantly \((p < 0.05)\). To validate this result, the EdU assay was performed to detect cell proliferation. The number of proliferating cells was approximately
60% of the total cells in the control group, whereas the percentages were about 40%, 39%, and 38% in the siPSMD1, siPSMD2, and siPSMD1 + siPSMD2 groups, respectively (Fig. 1h, i). Taken together, *PSMD1* and *PSMD2* played important roles in the cell proliferation and apoptosis processes.
**PSMD2 and PSMD2 overexpression promotes HepG2 cell proliferation but suppresses apoptosis**

To further investigate the functions of *PSMD1* and *PSMD2* in cell proliferation, *PSMD1* and *PSMD2* were overexpressed individually or together. The MTT assay indicated that *PSMD1* and *PSMD2* overexpression increased the cell proliferation capacity slightly but not significantly (Fig. 2a). We then performed the cell cycle analysis. There were no significant changes among different groups of cells (Fig. 2b, c). Subsequently, the apoptosis analysis was performed, and interestingly, the number of cells undergoing apoptosis increased only in groups with both *PSMD1* and *PSMD2* overexpression (Fig. 2d, e). Therefore, we detected the expression levels of genes related to proliferation (Fig. 2f) and apoptosis (Fig. 2g). Interestingly, the expression levels of *CCND1*, *CCND2*, and *ki67* increased slightly but significantly (p < 0.05, Fig. 2f), while the expression levels of *CASP3* and *FAS* decreased in the *PSMD1* OE + *PSMD2* OE group (Fig. 2g). To validate the result, the EdU assay was performed to detect the number of proliferating cells. There were no significant differences observed among the different treatment groups (Fig. 2h, i).

**PSMD1 and PSMD2 knockdown inhibits the formation of cellular lipid droplets**

Cellular lipid metabolism is important for cell proliferation and apoptosis. We investigated the effects of *PSMD1* and *PSMD2* on the formation of lipid droplets (LDs). Usually, cells form the fatty acid and lipid droplets de novo. The cellular LDs were marked by BODIPY493/503, a specific neutral lipid targeting dye, and the numbers and sizes of LDs in different cell groups were analyzed based on the images captured by the confocal laser scanning microscope (Fig. 3a). The LD number in the siPSMD1, siPSMD2, and siPSMD1 + siPSMD2 cell groups was significantly less than the number of control group cells (p < 0.05, Fig. 3b). Furthermore, we detected the capacity of neutral lipid synthesis of different group cells by treating them with 200 μM oleic acid (OA) for 12 h. Before OA treatment experiment, we detected the toxicity of OA on both HepG2 cells and Huh7 cells by utilizing CCK8 method. The results showed that OA treatment showed less toxicity on these two cell lines with the treatment time of 6 h, 12 h and 24 h (Additional file 1: Fig. S1C, D). Subsequently, we found that siPSMD1, siPSMD2, and both interfered cell groups contained fewer LDs than the control cells (p < 0.05, Fig. 3c). Then, we calculated the LD size and found that *PSMD1* and *PSMD2* interference did not change the LD size in the absence of OA, whereas the suppression of *PSMD1* and *PSMD2* increased the diameters of LDs in FA-rich medium (Fig. 3d, e). To further validate the regulatory roles of *PSMD1* and *PSMD2* in lipid metabolism, we performed the same experiments using another human hepatocellular carcinoma cell line, Huh7. We found that the knockdown of *PSMD1* and/or *PSMD2* decreased the cellular LD number significantly (p < 0.05, Additional file 3: Fig. S2A–C).

**PSMD1 and PSMD2 overexpression promotes the formation of lipid droplets**

To further investigate the effects of *PSMD1* and *PSMD2* on cellular LD formation and growth, we overexpressed these two genes in HepG2 cells and detected the LD number and size by fluorescence labeling of LDs. In both the absence and presence of OA, *PSMD1* and *PSMD2* overexpression significantly increased the number of cellular LDs compared with that of control cells (p < 0.05, Fig. 4a–c). Interestingly, larger LDs were observed in cells with *PSMD1* and/or *PSMD2* overexpression compared with control cells (Fig. 4a, d), whereas the difference in LD size between the overexpression and control group cells was decreased and almost disappeared in the presence of OA (Fig. 4e), although the overexpression group cells contained sporadic larger LDs with diameters between 1 and 2 μm (Fig. 4a, e). For further validation, the Huh7 cells were utilized to detect whether overexpression of *PSMD1* and/or *PSMD2* affected the cellular LD number. We found that the overexpression of *PSMD1* and/or *PSMD2* increased the LD diameter in Huh7 cells (Additional file 3: Fig. S2D–F). The phenotypes seen in the overexpression experiment contrasted with the results seen in the interference experiment, and therefore, *PSMD1* and *PSMD2* were potential positive regulators in the cellular lipid formation process.
Figure 8

(a) Immunofluorescence images of the subcellular localization of PLIN2 in HepG2 cells treated with different treatments. 

(b) Bar graphs showing the number of lipid droplets (LDs) per cell without OA. 

(c) Bar graphs showing the number of LDs per cell with 200 μM OA. 

(d) Bar graphs showing the percentage of LDs with different diameters without OA. 

(e) Bar graphs showing the percentage of LDs with different diameters with 200 μM OA. 

* indicates a significant difference compared to the control group.
**PSMD1 and PSMD2 regulate the expression level of FA and lipid synthesis-related genes**

The changes in LD number and size prompted us to investigate the effects of PSMD1 and PSMD2 on the expression levels of lipid metabolic-related genes including the FA-related genes (SREBF1, FASN, SCD1, and ACSL3) and neutral lipid synthesis-related genes (PLINs, LIPINs, FITMs, DGATs, PPARy, SEIPIN, and FSP27). We first investigated the effect of the suppression of PSMD1 and PSMD2 on the expression on these genes. The efficiency of interference was reflected by the mRNA levels of PSMD1 and PSMD2 detected by qPCR (Fig. 5a), and the expression levels of PSMD1 and PSMD2 decreased by approximately 70%. Then, the expression levels of FA and lipid synthesis-related genes were detected. As expected, the expression levels of SREBF1, FASN, SCD1, and ACSL3 were significantly decreased in the interference group cells compared with the control cells (p < 0.05, Fig. 5b), which suggests that the de novo FA synthesis capacity was decreased in the interference group cells. Moreover, the expression of neutral lipid synthesis-related genes, such as DGAT1, DGAT2 and PPARy, was decreased by the suppression of PSMD1 and/or PSMD2 (p < 0.05, Fig. 5c). Interestingly, we found that PSMD1 and PSMD2 showed different effects on the expression of PLIN2, a well-known LD marker gene. PSMD1 interference promoted the expression of PLIN2, whereas the PSMD2 interference decreased PLIN2 expression. Additionally, PSMD1 interference increased the expression of SEIPIN, also known as BSCL2, which plays an important role in cellular LD formation and budding off, whereas PSMD2 interference did not change the expression of SEIPIN. Subsequently, we also investigated the effects of PSMD1 and/or PSMD2 overexpression on the expression of these genes. PSMD1 and PSMD2 were highly overexpressed (Fig. 5d). The expression of SREBF1, FASN, SCD1, and ACSL3 was significantly increased in the PSMD1 OE + PSMD2 OE cell groups (p < 0.05, Fig. 5e). Furthermore, the expression levels of DGAT1, DGAT2, and PPARy were significantly upregulated by PSMD1 and PSMD2 overexpression (p < 0.05, Fig. 5f). It was interesting that the expression level of SEIPIN was downregulated by PSMD1 + PSMD2 OE (p < 0.05), which was in contrast to the results of the interference experiment. This result suggests that PSMD1 and PSMD2 may regulate SEIPIN expression.

To investigate the molecular mechanism by which PSMD1 and PSMD2 regulate FA and lipid synthesis-related genes, we detected two classical pathways, the ASK1-p38-JNK and AKT signaling pathways. We detected the phosphorylated ASK1, p38, and JNK1/2 levels and found that the p-ASK1, p-p38, and p-JNK1/2 levels were decreased in interference group cells compared with control cells (Fig. 5g, h). Additionally, the p-AKT level was also decreased in the interference group cells compared with the control cells (Fig. 5g, h). Subsequently, we investigated the effects of PSMD1 and PSMD2 overexpression on these two signaling pathways. As expected, the p-AKT and p-ASK1 levels increased significantly but mildly (p < 0.05, Fig. 5i, j). Additionally, the p-p38 level was not significantly different (Fig. 5i, j). Although the effects of PSMD1 and PSM2 overexpression on ASK1 and AKT signaling were not obvious, we also considered that PSMD1 and PSMD2 regulated these two signaling pathways, thereby regulating FA and neutral lipid synthesis.

**PSMD1 and PSMD2 regulate cellular lipid metabolism**

To validate that PSMD1 and PSMD2 regulate cell proliferation through modulating cellular FA and lipid content, we performed a rescue experiment that overexpressed SREBF1 or PPARγ in the PSMD1 and PSMD2 knockout cells. We showed that PSMD1 and PSMD2 knockout inhibited cell proliferation. Subsequently, we tried to increase the FA level by overexpressing SREBF1 in PSMD1 and PSMD2 knockout cells, because SREBF1 could promote the transcription of FA-synthesis-related enzymes. Then, the capacity of cell proliferation was detected by EdU assay. The results showed that SREBF1 overexpression increased the number of proliferating cells in the siPSMD1, siPSMD2, and siPSMD1 + siPSMD2 groups compared with the corresponding control groups (p < 0.05, Fig. 6a, b). Moreover, the expression levels of proliferation-related genes were also detected by qPCR. Although SREBF1 overexpression did not recover the
a

![Cell culture images with DAPI/BODIPY 493/503 staining](image)

b

**n=20 without OA**

- NC
- PSMD1 OE
- PSMD2 OE
- PSMD1+PSMD2 OE

![Box plots for number of LDs per cell without OA](image)

c

**n=20 with 200 µM OA**

- NC
- PSMD1 OE
- PSMD2 OE
- PSMD1+PSMD2 OE

![Box plots for number of LDs per cell with 200 µM OA](image)

d

**without OA**

- n=10

![Bar charts for percentage of LDs with different diameters](image)

e

**with 200 µM OA**

- n=10

![Bar charts for percentage of LDs with different diameters](image)
proliferation impaired by PSMD1 and/or PSMD2 interference totally, the proliferation capacity did increase compared with that of the control (Fig. 6b, c). Furthermore, PPARγ overexpression increased the proliferation capacity of PSMD1 and/or PSMD2 knockdown cells (Fig. 6d, e). Additionally, the expression levels of proliferation-related genes, including CCNs and ki67, increased compared with that of the corresponding control group (p < 0.05, Fig. 6f). These two rescue experiments indicated that increasing the lipid content
in cells could partly recover the PSMD1 and PSMD2 interference-induced inhibition of proliferation. The results showed that PSMD1 and PSMD2 can affect cell proliferation by regulating cellular lipid metabolism.

**PSMD1 and PSMD2 localize to the surface of lipid droplets**

We detected the subcellular localization of PSMD1 and PSMD2 and the co-localization of PSMD and LDs. PSMD1 and PSMD2 are the components of the 19S subunit of proteasome; therefore, they are mainly distributed in the cytoplasm. We labelled PSMD1 and PSMD2 by the mCherry-tag in their C-terminals, and their distribution was captured by a confocal laser scanning microscope (Fig. 7a). As expected, PSMD1 and PSMD2 were localized in the cytoplasm (Fig. 7a). Then, we labelled PSMD1 by an EGFP-tag in its C-terminal to determine the co-localization of PSMD1 and PSMD2. The results showed that PSMD1 and PSMD2 share the same localization (Fig. 7b). Furthermore, we investigated the co-localization of PSMD1/PSMD2 and cellular LDs. The signals of PSMD1-mCherry/PSMD2-mCherry and BODIPY493/503 were captured and the images of Fig. 7c, d showed that PSMD1/PSMD2 were localized to the outside of the LDs. To validate the PSMD1/PSMD2 localization accurately, the PLIN2-EGFP was co-transfected with the PSMD1-mCherry or PSMD2-mCherry expression vector, because PLIN2 is a well-known LD marker localized to the surface of LDs. By fluorescence distribution analysis, the signals of PSMD1/PSMD2 were almost overlapped with the signal of PLIN2 (Fig. 7e, f). It should be noted that PSMD1/PSMD2 are not strict peri-LD proteins, because we did not observe the “round-ring” signal like we did for PLIN2. According to these results, we conclude that PSMD1 and PSMD2 are able to localize to cellular LDs, which could be the potential method by which PSMD1 and PSMD2 regulate cellular LD formation and growth.

It is well known that LDs are generated from the endoplasmic reticulum (ER). The LDs can carry proteins from the ER and recruit proteins through the ARF-COPI system. Therefore, we further investigated the source of LD localized PSMD1/PSMD2 proteins. We detected the signals of Livedrop-EGFP and PSMD1-mCherry/PSMD2-mCherry at 45 min after OA treatment. The fluorescence analysis showed that two signals shared a high level of overlap, which indicated that PSMD1 and PSMD2 had localized to the newly formed LDs (Fig. 7g, h). This result suggests that LDs might carry PSMD1 and PSMD2 from the ER during their generation.

**High expression of PSMD1 and PSMD2 is associated with the poor prognosis of liver hepatocellular carcinomas**

To investigate the effect of high expression of PSMD1 or PSMD2 on the prognosis of liver hepatocellular carcinoma (LIHC), we analyzed the expression levels of PSMD1 and PSMD2 in LIHC via the GEPIA (gene expression profiling interactive analysis) database (http://geopia.cancer-pku.cn/). The data showed that PSMD1 and PSMD2 are highly expressed in LIHC (Fig. 8a, b). Furthermore, the results showed that LIHC with high expression of PSMD1 and PSMD2 is associated with a poor prognosis (Fig. 8c, d).

**Discussion**

In the present study, we identified a novel way in which PSMD1 and PSMD2 promote tumor cell proliferation—namely, via increasing cellular FA and lipid synthesis. It is not novel that the proteasome can regulate the proliferation and apoptosis of tumor cells [54, 55], because proteasomes are associated with the degradation of the majority of cellular proteins. Therefore, as the important subunits of the 26S proteasome, PSMD1 and PSMD2 also regulate tumor cell growth. Many previous studies also have reported their function in cell proliferation. For example, PSMD1 could regulate breast cancer cell growth [41], and PSMD2 can regulate tumor cell proliferation in lung cancer and breast cancer [39, 56]. Furthermore, PSMD2 reduces proliferation and induces apoptosis in lung cancer cells, and the overexpression of PSMD2 could stimulate cell growth in SMMC-7721 and NIH-3T3 cells [40, 57]. However, the way in which PSMD1 and PSMD2 regulate tumor cell proliferation is not totally clear. Previous studies demonstrated that PSMD1 or PSMD2 regulate the expression levels of p53, p21, and p27 [39–41], which are cell cycle and cell growth regulators. Our findings provide new insight into the regulatory roles of PSMD1 and PSMD2 in tumor cell proliferation via regulating lipid metabolism.
Recently, lipid metabolism was found to be important for the proliferation and growth of tumor cells. Cells need many kinds of lipids to support their duplication and growth, and they also require enough FAs for necessary biological processes. Cellular FAs and lipids are usually stored in LDs, novel organelles that have been known about for in decades, which play important roles in many biological processes such as lipid formation and degradation, cell proliferation and apoptosis, anti-stimulation, and cellular immunity [19, 58–63]. Recently, researchers found the LDs play an important role in tumor carcinogenesis and metastasis. Aboumrad et al. identified that a high number of lipid vesicles in the cytoplasm is a characteristic of mammary carcinoma [64]. Additionally, Ramos et al. reported that high lipid-containing mammary carcinoma has more aggressive behavior [6]. Lipid-rich characterization has become a clinically distinctive form of carcinoma [6, 65]. A high level of LDs is associated with higher tumor aggressiveness [15] and chemotherapy resistance [14], and additionally, tumorigenesis-related proteins can be recruited and stored in LDs [66–68], such as PI3K, ERK1, and ERK2. Tumor cells can regulate the LD content via the EGFR–PI3K–mTOR and FOXO/SIRT6 pathways, and LDs also regulate the proliferation and growth of tumor cells via membrane lipid and energy formation [69–74], which establishes a tight association between LDs and tumor cells.

It is very interesting that PSMD1 and PSMD2 can regulate the cellular LD content. As far as we know, no study has reported this regulatory function of PSMD1 and PSMD2 in tumor cells. It should be noted that the idea that the proteasome pathway regulates LD formation, stabilization, and degradation is not novel, and there are many studies about it. For example, the proteasome inhibitor MG132 can suppress the degradation of ADRP (also known as PLIN2) [75], and additionally, a proteasome-dependent pathway can regulate the level of ADRP to modulate the cellular TG content [76]. Moreover, Doa10, an ER-associated degradation ubiquitin ligase, is able to regulate the levels of some LD proteins [77], and additionally, the ubiquitin ligases and Spartin/SPG20 can regulate the number and size of LDs [78]. Furthermore, SIRT7 controls hepatic lipid formation and accumulation via regulating the ubiquitin–proteasome pathway, and SIRT7 knockout mice have shown resistance to high-fat-diet induced fatty liver [79]. Moreover, a previous functional genomic screen study in Drosophila S2 cells suggested that proteasomes are involved in the regulation of cellular lipids [80]. All of these studies demonstrated that UPS is essential for LD biology. However, ubiquitin–proteasome is a huge system, which consists of numerous components. The functions and regulatory mechanisms of these components are poorly understood. In this study, we focused on the two important subunits, PSMD1 and PSMD2, which could affect the cellular lipid content. We detected the FA and lipid synthesis enzyme expression levels during PSMD1/PSMD2 knockdown or cell overexpression, and the results indicated that PSMD1 and PSMD2 indeed affect the expression levels of FASN, SCD1, SREBF1, DGAT, and PPARγ (Fig. 5). Although some changes in expression were mild, significant changes in the LD number can be observed in Figs. 3a and 4a (p < 0.05), indicating that the capacity of cellular FA and de novo lipid synthesis indeed changed. Subsequently, we also investigated the pathways impacted by PSMD1 and PSMD2. Previous studies showed that ASK1-p38-JNK signaling regulates the expression level of SREBF1 and the activation of SREBP1c, which could regulate the de novo cellular FA synthesis process [81–85]. Additionally, AKT signaling is important for cellular metabolism as it regulates cell proliferation and differentiation [86–93]. We found that PSMD1/PSMD2 knockdown impaired ASK1 and AKT signaling, which indicates that cellular lipid formation and energy metabolism were suppressed. This genotype corresponds to the phenotype where cell proliferation was inhibited and apoptosis was promoted (Fig. 1). On the other hand, PSMD1/PSMD2 overexpression enhanced ASK1 and AKT signaling, although the changes were mild (Fig. 5i, j), which corresponds

(See figure on next page.)

**Fig. 7** PSMD1 and PSMD2 localize to the surface of lipid droplets. Fluorescence expression vectors were constructed, including PSMD1-mCherry, PSMD2-mCherry, PSMD1-EGFP, PLIN2-EGFP, and Livedrop-EGFP. **a** PSMD1-mCherry or PSMD2-mCherry was transfected into cells for 48 h. Then, the cells were fixed and stained by DAPI. The images were captured by laser scanning confocal microscope (bar = 10 μm). **b** PSMD1-EGFP and PSMD2-mCherry were co-transfected into cells for 48 h. Then, the cells were fixed and stained by DAPI. The images were captured by a laser scanning confocal microscope (bar = 10 μm). The fluorescence plot was analyzed by ImageJ software. **c, d** The cells were transfected with PSMD1-mCherry or PSMD2-mCherry for 48 h, and then the cells were treated with 200 μM oleic acid (OA) for another 6 h. Then, the cells were fixed and stained by BODIPY493/503 and DAPI. The images were captured by a laser scanning confocal microscope (bar = 10 μm). **e–h** The cells were co-transfected with PSMD1-mCherry or PSMD2-mCherry and PLIN2-EGFP vectors for 48 h, and then the cells were treated with 200 μM OA for another 6 h. Then, the cells were fixed and stained by BODIPY493/503 and DAPI. The images were captured by a laser scanning confocal microscope (bar = 10 μm).
to the phenotype where the cell-proliferation-related genes were upregulated and cell apoptosis was decreased (Fig. 2). For this phenotype, we considered that PSMD1 and PSMD2 are medium to highly expressed genes, so overexpression might not lead to obvious effects on cellular proliferation, and furthermore, previous studies have reported that the overexpression of PSMD2 could stimulate cell growth [40, 57]. Therefore, there is no doubt that PSMD1 and PSMD2 could enhance the cell proliferation process. We then tried to validate the idea that PSMD1/PSMD2 regulate cell proliferation via regulating lipid metabolism. Since PSMD1/PSMD2 knockdown downregulated the expression of FA and lipid synthesis genes, we recovered their expression by transfection with SREBF1 or PPARγ expression vectors. These two genes are upstream transcriptional factors of FA and lipid synthesis enzymes. The results indicated that cell proliferation was recovered partly when the cells were transfected with SREBF1 or PPARγ (Fig. 6), which suggests that this treatment was able to compensate for the impairment of PSMD1/PSMD2 knockdown on cell proliferation. We then investigated the co-localization of PSMD1/PSMD2

![Fig. 8 PSMD1 and PSMD2 show higher expression in liver hepatocellular carcinoma (LIHC) and are associated with a poor prognosis. Gene expression analysis and survival analysis were performed by the GEPIA (gene expression profiling interactive analysis) database (http://gepia.cancer-pku.cn/). a, b Box plot of expression of PSMD1 and PSMD2 in LIHC. c, d The survival analysis of PSMD1 and PSMD2 in LIHC.](image)
and LDs. Both the mature LDs (labelled by PLIN2-EGFP) and newly formed LDs (labelled by Livedrop-EGFP) were found to be related to the localization of PSMD1 and PSMD2 (Fig. 7f–h), which indicated that PSMD1/PSMD2 were localized to LDs during the generation progression. Moreover, the localization of PSMD1/PSMD2 on the LD surface suggests that proteasomes might degrade the proteins recruited on LD surfaces. However, the significance of the localization of PSMD1 and PSMD2 on LD surfaces still requires further study. A prognosis analysis was performed to illustrate the association between the PSMD1 and PSMD2 expression levels and survival rates. The results indicated that high expression levels of PSMD1 and PSMD2 decrease the survival rate of LIHC (Fig. 8c, d), which corresponds to our study where PSMD1 and PSMD2 increased the LD content and promoted tumor cell proliferation.

**Conclusion**

In the present study, we identified that PSMD1 and PSMD2 could regulate the cellular lipid content, thereby affecting cell proliferation progression. Mechanistically, PSMD1 and PSMD2 regulate FA and lipid synthesis-related gene expression through ASK1-p38-JNK and AKT signaling. The molecular mechanism of PSMD1/PSMD2 regulating cell proliferation is illustrated in Fig. 9. This study provides new insights into the PSMD1/PSMD2 regulatory mechanism in HCC cell proliferation and provides a potential novel therapeutic strategy for lipid-rich tumor.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12867-019-0141-z.

**Additional file 1:** Fig. S1. Time course experiment of oleic acid medium treatment. A The cells were treated with 200 μM oleic acid. The cellular lipid droplets were imaged at 1 h, 2 h, 3 h, 4 h, 5 h and 6 h after oleic acid treatment. B, C The cell activity of Huh7 cells and HepG2 cells was detected by CCK8 method after 200 mM oleic acid medium treatment for 6 h, 12 h and 24 h.

**Additional file 2:** Additional tables.

**Additional file 3:** Fig. S2. PSMD1 and PSMD2 expression level regulates the number and size of cellular lipid droplets in Huh7 cells. The Huh7 cells were seeded on the slide in a 24-well plate. Then, the cells were transfected with PSMD1/PSMD2 or NC siRNAs for 48 h for knockdown (A–C), or transfected with PSMD1/PSMD2 expression vector or NC vector for 48 h for overexpression (D–F). Subsequently, the cells were treated with 200 μM oleic acid for another 6 h. Then, the cells were fixed and stained by BODIPY493/503 and DAPI for observation by microscope. (B, E) The number of cellular LDs from different groups of cells. ImageJ software was used for the analysis. The statistical significance of differences between means was assessed using an unpaired Student’s t-test (n = 20; *p < 0.05) vs. NC. (C, F) The size of cellular LDs of different groups of cells. ImageJ software was used for the analysis. The statistical significance of differences between means was assessed using an unpaired Student’s t-test (n = 10; *p < 0.05) vs. NC.

**Abbreviations**

PSMD1: proteasome 26S subunit, non-ATPase 1; PSMD2: proteasome 26S subunit, non-ATPase 2; UPS: ubiquitin proteasome system; E1: Ub-activating enzyme; E2: Ub-conjugating enzyme; E3: Ub-protein ligase; DUB: deubiquitinating enzyme; PLIN1: perilipin1; PLIN2: perilipin2; PLIN3: perilipin3; PLIN4:
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