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# 7α,25-dihydroxycholesterol suppresses hepatocellular steatosis through GPR183/EBI2 in mouse and human hepatocytes

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**Running title**: Oxysterol protects hepatic steatosis via GPR183

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**Abbreviations**: FAS, fatty acid synthase; LXR, liver X receptor; SREBP-1c, sterol regulatory element-binding protein 1c; PPAR-α, peroxisome proliferator-activated receptor α

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***Abstract***

Non-alcoholic fatty liver disease is a chronic inflammatory liver disease. It is associated with obesity and type 2 diabetes. Oxycholesterols are metabolites of cholesterol and several of them can act on the G protein-coupled receptor, GPR183/EBI2. We found expression of GPR183 in human hepatoma cell lines and *in vivo* induction of GPR183 expression in mouse livers after high-fat diet feeding. Therefore, the role of oxycholesterols and GPR183 in hepatocytes was studied using a model of hepatic steatosis induced by liver X receptor (LXR) activation. LXR activation by T0901317 resulted in fat accumulation in Hep3B human hepatoma cells. This lipid accumulation was inhibited by 7α,25-dihydroxycholesterol, the most potent agonist of GPR183. The protective effects of 7α,25-dihydroxycholesterol were suppressed by a specific GPR183 antagonist, NIBR189. T0901317 treatment induced expression of the major transcription factor for lipogenesis, SREBP-1c. 7α,25-dihydroxycholesterol inhibited the induction of SREBP-1c proteins in a GPR183-dependent manner. Using inhibitors specific for intracellular signaling molecules, 7α,25-dihydroxycholesterol-induced suppression of hepatocellular steatosis was shown to be mediated through $G_{i/o}$ proteins, p38 MAPKs, PI3K, and AMPK. In addition, the inhibitory effect of 7α,25-dihydroxycholesterol was validated in HepG2 cells and primary mouse hepatocytes. Therefore, the present report suggests that 7α,25-dihydroxycholesterol-GPR183 signaling may suppress hepatocellular steatosis in the liver.

**Significance statement**

Oxycholesterols, metabolites of cholesterol, act on the G protein-coupled receptor, GPR183/EBI2, which is expressed in human hepatoma cell lines and its expression is induced *in vivo* in mouse livers after high-fat diet feeding. Activation of GPR183 inhibits fat accumulation in primary mouse hepatocytes and HepG2 cells through $G_{i/o}$ proteins, p38 MAPKs, PI3K, and AMPK.

## Introduction

Oxycholesterols are 27-carbon molecules with a steroid backbone and a methylheptyl side chain. They were long considered as mere byproducts of cholesterol metabolism (Guillemot-Legris et al., 2016b; Mutemberezi et al., 2016a). However, they are now recognized as metabolic intermediates for bile acid production and as key mediators of a variety of physiological processes through specific receptor molecules (Guillemot-Legris et al., 2016b; Mutemberezi et al., 2016a). Among receptor molecules, the G protein-coupled receptor, GPR183 (a.k.a. EBI2), binds to oxycholesterols and plays important immunomodulatory roles (Birkenbach et al., 1993; Hannedouche et al., 2011; Liu et al., 2011). From extracts of sheep and pig livers (Hannedouche et al., 2011) and from rat spleen extracts (Liu et al., 2011), 7α,25-dihydroxycholesterol was identified as a naturally occurring endogenous ligand of GPR183. Among tested oxycholesterols, 7α,25-dihydroxycholesterol was the most potent endogenous agonist for GPR183 (Benned-Jensen et al., 2011; Hannedouche et al., 2011; Liu et al., 2011). In secondary lymphoid tissues, GPR183 is necessary for migration of B cells to intra- and extra-follicular sites (Hannedouche et al., 2011; Liu et al., 2013a). 7α,25-dihydroxycholesterol has promoted T cell migration through GPR183 activation (Chalmin et al., 2015). 7α, 25-dihydroxycholesterol is generated *in vivo* and regulates activation, migration, and functions of B cells, dendritic cells, monocytes/macrophages, T cells, and astrocytes (Sun and Liu, 2015). Dysregulation of oxycholesterol synthesis and

GPR183 activation is linked to inflammation and autoimmune diseases, as well as metabolic diseases such as obesity, dyslipidemia, and diabetes (Sun and Liu, 2015; Guillemot-Legris et al., 2016b). Levels of 7α,25-dihydroxycholesterol are estimated as 4.7 ng/g and 0.5 ng/ml in mouse liver and plasma, respectively (Crick et al., 2015; Mutemberezi et al., 2016b) and 0.4 ng/ml in human plasma (Iuliano et al., 2015). Although the liver is the organ responsible for oxycholesterol generation and oxycholesterol levels may change during metabolic syndrome (Guillemot-Legris et al., 2016b), the function of GPR183 has not been studied in the liver in relation to non-alcoholic fatty liver disease.

Non-alcoholic fatty liver disease is a condition of excessive fat accumulation in the liver and is associated strongly with insulin resistance, obesity, and type 2 diabetes (Postic and Girard, 2008a). *De novo* lipogenesis has been shown to contribute significantly to hepatic steatosis (Donnelly et al., 2005; Postic and Girard, 2008b). Liver X receptor α (LXRα), a member of nuclear receptor family, is an important regulator of hepatic lipogenesis, and its activation subsequently results in activation of sterol regulatory element binding protein 1c (SREBP-1c), a master transcription factor for hepatic lipid synthesis (Peet et al., 1998). In steatosis patients, increased expression of hepatic LXRα and SREBP-1c was observed (Higuchi et al., 2008). Moreover, in animals, treatment with an LXRα agonist enhances levels of hepatic triglycerides and increases expression of SREBP-1c (Schultz et al., 2000).

We found expression of GPR183 in human hepatoma cell lines and *in vivo* induction of GPR183 expression in mouse livers after high-fat diet feeding. Therefore, the aim of the present study was to assess the role of 7α,25-dihydroxycholesterol and GPR183 in hepatocellular steatosis by LXR activation in a human hepatoma cell line, Hep3B cells, and primary mouse hepatocytes. We used 7α,25-dihydroxycholesterol as an agonist of GPR183 and NIBR189 as an antagonist.

## Materials and methods

### *Materials*

Pertussis toxin (PTX), 7α,25-dihydroxycholesterol, and rat tail collagen were purchased from Gibco (Gran Island, NY, USA). NIBR189, SB202190, LY294002, SP600125, and PD98059 were obtained from Tocris Bioscience (Bristol, UK). T0901317 was obtained from Cayman Chemical (Ann Arbor, MI, USA) and compound C from Abcam (Cambridge, UK).

### *Cell culture and treatment*

Human Hep3B and HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Hep3B and HepG2 cells were cultured in Dulbecco's modified Eagle medium with high glucose (Welgene, Daegu, Korea) with 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum at 37ºC in a 5% $CO_2$-containing atmosphere. Hep3B and HepG2 cells were used between passage numbers 3 and 15. Cells ($2\times10^5$ cells) were seeded onto 6-well culture plates and cultured overnight (18 h) for cell adhesion. Serum-free media with 0.1% bovine serum albumin (BSA) was then used. The cells were first treated with 7α,25-dihydroxycholesterol at concentrations indicated, and after 1 h, T0901317 was treated at a concentration of 1 μM. After 48 h, samples for proteins, triglycerides, and Oil red O staining were made. Each experiment was carried out on

at least three independent occasions.

***Isolation of primary mouse hepatocytes***

Eight-week old female Balb/c mice were used to isolate primary hepatocytes by a collagenase perfusion (Chu et al., 2013). Livers were perfused with Hanks' Balanced Salt Solution (HBSS) without magnesium and calcium for 3 min and then with 0.1% collagenase for 5 min. The liver was excised and gently minced in PBS. After filtering with a 70 μM filter and centrifugation for 1 min at 50 *g*, the cells were washed with PBS three times. The pellet of cells was suspended with DMEM containing 50 μg/mL streptomycin, 100 units/mL penicillin, and 10% FBS. Isolated cells were seeded at a density of $3.6\times10^5$ cells/mL in collagen-coated 8-well chambers (SPL, Pocheon, Korea).

***Oil red O staining***

We performed Oil red O staining in Hep3B, HepG2, and primary mouse hepatocytes (Park et al., 2014). Cells fixed with formalin were stained with freshly prepared Oil red O working solution. After rinsing with tap water, nuclei were lightly stained with hematoxylin. After rinsing with tap water, the slides were mounted with aqueous mountant. Six different fields were photograp hed for each slide, and ImageJ software was used to analyze red-stained areas and intensity in each photo (NIH, Bethesda, MD, USA).

***Measurement of cellular triglyceride content***

We extracted cellular lipids with methanol/chloroform (1:2; v/v) in Hep3B cells. Hep3B cells ($4\times10^5$ cells) were collected, mixed with 750 μL methanol/chloroform solvent, and vortexed well. After standing for 30 min at room temperature, we evaporated solvent in 60°C and suspended lipids in 20 μL deionized water. By using a kit from Asan Pharm (Chungcheong, South Korea), we determined the levels of triglycerides.

***Transfection for GPR183 silencing***

A double-stranded interfering RNA (siRNA) specific for human GPR183 were obtained along with a scrambled control siRNA from Bioneer Inc (Daejeon, Korea). We transfected Hep3B cells ($0.5\times10^5$ cells/well) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) (Liu et al., 2013b). After 48 h incubation, reverse transcription PCR detected the suppressed expression of GPR183.

***Reverse Transcription-PCR***

We isolated total RNA from Hep3B cells by using TRIzol reagent (Invitrogen, USA), and determined concentrations of RNA by a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Transcription was performed with one microgram of RNA in the Reverse Transcription System of Promega ImProm-II (Madison, WI, USA). Primers for each gene and synthesized cDNA products were used for PCR. Specific primers were used to amplify gene fragments (detailed information in supplemental table 1). Aliquots of PCR products were

electrophoresed in agarose gels (1.2%) and stained with Nucleic acid gel stain (Real Biotech Corp., Taiwan) (Lee et al., 2017).

***Western blot***

Harvested Hep3B cells were suspended in lysis buffer. Concentrations of proteins were determined by using a BCA protein assay kit (Thermo scientific, Rockford, IL, USA). After separation by SDS-PAGE (8%) and transferring to nitrocellulose paper electrophoretically, cell lysates (30 μg protein) were incubated with primary antibodies for GPR183 (cat no. 12377-1-AP, Proteintech, Rosemont, USA), SREBP-1c (cat no. sc-366, Santa Cruz Biotechnology, CA, USA), FAS (cat no. 3180, Cell Signaling Technology, Danvers, MA, USA) or β-actin at 4°C overnight. After incubating with HRP-conjugated secondary antibody, blots were developed with ECL detection reagents (Huang et al., 2018). By using a ChemiDoc Touch Imaging System (Bio-Rad), luminescence was detected and analyzed with the ImageLab software (Bio-Rad).

***Animals and diets***

From Daehan Biolink (DBL, Seoul, Korea) we obtained male C57BL/6 mice. Mice had *ad libitum* access to water and food in the laboratory animal facility at PNU. Eight-week-old mice were divided randomly into 2 groups. Control C57BL/6 mice were fed with a normal chow diet for 4 weeks (n = 7) and high-fat diet (HFD) C57BL/6 mice with a synthetic diet supplemented with 60% (w/w) fat (HFD, Efeed, Korea) for 4 weeks (n = 7). The animal protocol used in this study was

reviewed and approved by the PNU Institutional Animal Care Committee with respect to the ethics of the procedures and animal care (PNU-2017-1445).

***Statistical analysis***

Statistical significances of differences were determined by analysis of variance (ANOVA) and Tukey's multiple comparison test. All results were expressed as mean ± SD. A $p$ value < 0.05 was considered statistically significant.

## Results

### *Expression of GPR183 in hepatocytes and induction of GPR183 expression in liver after HFD feeding.*

Because the liver is the responsible organ for oxycholesterol generation and oxycholesterol levels change during metabolic syndrome (Guillemot-Legris et al., 2016b), we tested the possibility that GPR183 has an oxycholesterol-sensing function in hepatocytes. First, we determined the expression of GPR183 in Hep3B cells, HepG2 human hepatoma cells, and primary mouse hepatocytes. In Hep3B cells mRNA of GPR183 was detected, and in both hepatoma cells, and primary mouse hepatocytes proteins of GPR183 were detected as shown in Fig 1-A and B. Second, effect of HFD feeding on GPR183 expression was tested. HFD feeding for 4 weeks successfully induced liver steatosis in C57BL/6 mice as proved by increased TG levels and Oil red O staining in livers from mice fed HFD comparing to those from mice fed normal chow diet (data not shown). We found that the protein level of GPR183 was significantly higher in livers of mice fed HFD than those of mice fed normal chow diet (Fig. 1-C and D).

### *7α,25-dihydroxycholesterol suppress fat accumulation by LXR activation through GPR183 in Hep3B cells.*

Next, 7α,25-dihydroxycholesterol, the most potent endogenous agonist for GPR183 (Benned-Jensen et al., 2011; Hannedouche et al., 2011; Liu et al., 2011), was employed to test the function

of GPR183 in fat accumulation. LXR activation by T0901317 treatment strongly increased number of lipid droplets in Hep3B cells (Fig 2-A). 7α,25-dihydroxycholesterol pretreatment inhibited the fat accumulation in a dose-dependent manner (Fig 2-A and B).

To further verify the effect of 7α,25-dihydroxycholesterol, cells were treated with palmitic acid to induce fat accumulation in Hep3B cells. The number of lipid droplet was strongly increased by treatment of 0.1 mM palmitic acid comparing with non-treated controls in the cells (Fig 2-C). Pretreatment of 7α,25-dihydroxycholesterol suppressed the effect of palmitic acid (Fig 2-C).

Furthermore, triglycerides levels were measured to validate the fat accumulation detected by Oil red O staining. As shown in Fig 2-D, T0901317 increased triglyceride content and 7α,25-dihydroxycholesterol reversed this increase in Hep3B cells.

We used NIBR189 (a competitive GPR183 antagonist) to verify that the suppressive effect of 7α,25-dihydroxycholesterol on fat accumulation is mediated by GPR183 (Gessier et al., 2014). NIBR189 blunted the 7α,25-dihydroxycholesterol-induced inhibition of lipid accumulation in a dose-dependent manner (Fig 3-A and B).

***Involvement of GPR183 in 7α,25-dihydroxycholesterol suppression of SREBP-1c in Hep3B cells.***

SREBP-1c is a transcription factor for lipogenic genes in hepatic steatosis (Shimano et al., 1999;

Yahagi et al., 1999) and fat accumulation by LXR activation has been shown to be mediated through SREBP-1c induction (Repa et al., 2000). Thus, the effects of T0901317 and 7α,25-dihydroxycholesterol on SREBP-1c and fatty acid synthase (FAS) expression were determined. Treatment of T0901317 increased the expression of SREBP-1c and FAS proteins (Fig 4). However, the induction of SREBP-1c and FAS by LXR was inhibited markedly by 7α,25-dihydroxycholesterol treatment (Fig 4). Chemical inhibition by NIBR189 and gene silencing methods was utilized to confirm if GPR183 is involved in the 7α,25-dihydroxycholesterol-induced suppression of SREBP-1c expression. Treatment with NIBR189 significantly blunted the expression suppression of SREBP-1c and FAS induced by 7α,25-dihydroxycholesterol in the cells (Fig 4). Application of double stranded RNA interference silenced the GPR183 expression in Hep3B cells. Knockdown of GPR183 by siRNA transfection was confirmed at mRNA and protein levels (Fig 5-A and B). Knockdown of GPR183 blunted the 7α,25-dihydroxycholesterol-induced suppression of SREBP-1c expression (Fig 5-C and D). Transfection with a scrambled siRNA had no effect (Fig 5-C and D).

Furthermore, we investigated PPARα (peroxisome proliferator-activated receptor α) expression. PPARα is a key regulatory factor for lipolysis such as β-oxidation. PPARα showed no significant change with T0901317 (data not shown)

Therefore, these data show that activation of GPR183 by 7α,25-dihydroxycholesterol suppresses

expression of SREBP-1c and FAS by LXR in hepatocytes, leading to the suppression of fat accumulation.

***Cellular signaling in the 7α,25-dihydroxycholesterol-induced effect in Hep3B cells.***

To elucidate the cellular signaling of the 7α,25-dihydroxycholesterol-GPR183 response, we used inhibitors specific for $G_{i/o}$ proteins, ERKs, JNK, or p38 MAPKs. As GPR183 is commonly reported to be a $G_i$-coupled receptor (Rosenkilde et al., 2006; Benned-Jensen et al., 2011; Hannedouche et al., 2011; Liu et al., 2011), a specific inhibitor for $G_{i/o}$–type G proteins, PTX was used to confirm the $G_{i/o}$ proteins involvement. The inhibition of lipid accumulation by 7α,25-dihydroxycholesterol was suppressed by pretreatment with PTX (100 ng/mL, 24 h) (Fig 6). This means the involvement of $G_{i/o}$ proteins in the GPR183 signaling by 7α,25-dihydroxycholesterol. This is the same with the known G protein coupling of GPR183 (Rosenkilde et al., 2006; Benned-Jensen et al., 2011; Benned-Jensen et al., 2013; Eibinger et al., 2013).

GPR183 has been reported to induce cellular migration through activation of ERK1/2 and p38 MAPK (Benned-Jensen et al., 2011; Benned-Jensen et al., 2013; Rutkowska et al., 2015). Therefore, the total lipid content in Hep3B cells was measured in the presence of PD98059 (10 μM), an ERK1/2 inhibitor (Benned-Jensen et al., 2011); SP600125 (10 μM), a JNK inhibitor; or SB202190 (10 μM), a p38 MAPK inhibitor. As shown in Fig 7-A, SB202190 inhibited 7α,25-

dihydroxycholesterol-induced suppression of lipid accumulation in Hep3B cells. Neither PD98059 nor SP600125 inhibited depletion of LXR-activation-induced lipid accumulation (Fig 7-B). These results suggest $G_{i/o}$ proteins and p38 MAPK as signaling components of the 7α,25-dihydroxycholesterol-induced suppression of fat accumulation in Hep3B hepatocytes.

Furthermore, involvement of PI3K and AMPK was assessed using their specific inhibitors, LY294002 and compound C, respectively. As shown in Fig 7-A, both inhibitors totally blunted the 7α,25-dihydroxycholesterol-induced suppression of fat accumulation in Hep3B cells. This implies that PI3K and AMPK are signaling components of GPR183-mediated suppression of fat accumulation in Hep3B hepatocytes.

***7α,25-dihydroxycholesterol suppress fat accumulation induced by LXR activation in HepG2 cells and primary mouse hepatocytes***

To further establish the suppressive effects of 7α,25-dihydroxycholesterol on lipogenesis, fat accumulation was investigated by measuring cellular fat in another human hepatoma cell line (HepG2 cells) and primary mouse hepatocytes. LXR activation-induced fat accumulation was decreased markedly in a dose-dependent manner in the presence of 7α,25-dihydroxycholesterol in HepG2 cells and primary hepatocytes (Fig 8 A and B), which is agreeing with the results from Hep3B cells. NIBR189 pretreatment blocked the effect of 7α,25-dihydroxycholesterol in HepG2

cells and primary mouse hepatocytes (Fig 8 A and B). These results imply that 7α,25-dihydroxycholesterol has also suppressive effects on fat accumulation in HepG2 cells and primary mouse hepatocytes.

## Discussion

This study shows for the first time negative regulation of 7α,25-dihydroxycholesterol on liver fat accumulation. Key findings are following four things. First, GPR183 is expressed in human hepatocytes and its expression is induced *in vivo* by high-fat diet feeding in mice. Second, 7α,25-dihydroxycholesterol reduces fat accumulation mediated by LXR in human Hep3B cells, HepG2 cells, and primary mouse hepatocytes. Third, 7α,25-dihydroxycholesterol inhibits LXR-induced expression of lipid-synthesizing SREBP-1c. Forth, 7α,25-dihydroxycholesterol signaling is mediated via GPR183, $G_{i/o}$ protein, AMPK, p38 MAPK, and PI3K (Fig 9).

GPR183 is mainly coupling to PTX-sensitive $G_{i/o}$ proteins, thereby resulting in adenylyl cyclase inhibition and activation of p38 MAPK and ERK signaling pathways (Rosenkilde et al., 2006; Benned-Jensen et al., 2011; Benned-Jensen et al., 2013; Eibinger et al., 2013; Rutkowska et al., 2015). In the present study, blockage of p38 MAPK or PI3K resulted in 7α,25-dihydroxycholesterol dysfunction, suggesting that the cellular components of p38 MAPK and PI3K are signaling pathways in regulating fat metabolism in Hep3B cells. AMPK was reported as an energy sensor that maintains cellular energy homeostasis. And activation of hepatic AMPK could suppress SREBP-1c-dependent lipogenesis and protect against hepatic steato sis (Li et al., 2016). p38 MAPK and PI3K, therefore, are suggested to be the kinases upstream of AMPK, because AMPK has been shown to be a key downstream signaling component in

hepatic steatosis (Oh et al., 2011; Zheng et al., 2015; Huang et al., 2017; Woods et al., 2017; Yun et al., 2017).

Simple calculation of 4.7 ng/g mouse liver would be 11 nM. That is ten times lower than what we used in the experiment (100 nM). However, 7α,25-dihydroxycholesterol, the most potent agonist of GPR183, is not the only ligand. There are other mono-hydroxycholesterols, such as 7α-, 7β-, and 25-hydroxycholesterols and dihydroxycholesterols such as, 7α,25-, 7β,25-, 7α,27-, and 7β,27-dihydroxycholesterols, which are also active ligands for GPR183 (Liu et al., 2011). Especially tissue concentration of 25-hydroxycholesterol increased from 5.8 ng/mg protein in liver tissue of normal chow diet-fed mice to 18.5 ng/mg of high-fat diet-fed mice (Wooten et al., 2014). And its potency on GPR183 is similar to that of 7α,25-dihydroxycholesterol in cAMP measurement (Liu et al., 2011). Levels of 7α-, 25-, and 27-hydroxycholesterols have been significantly increased during obesogenic diet in liver (Guillemot-Legris et al., 2016a). Therefore, the concentration of 7α,25-dihydroxycholesterol we used may not represent physiological conditions by itself. However, levels of other mono-hydroxycholesterols and dihydroxycholesterols, which are active ligands for GPR183, are significantly high enough in the liver. GPR183 may be activated by oxysterols in *in vivo* conditions.

It is interesting that oxysterols are natural ligands for both LXR and GPR183. Oxysterols activate LXR to up-regulate genes involved in cholesterol efflux and cholesterol clearance, such as

ABCA1, ABCG1, ABCG5, and apolipoprotein E (Edwards et al., 2002). LXR target genes, in addition, are critical for fatty acid synthesis and triglycerides metabolism, such as SREBP-1c, FAS, cholesterol ester transfer protein and lipoprotein lipase (Edwards et al., 2002; Zhao and Dahlman-Wright, 2010). LXR-activating oxysterols are 24(*S*),25-epoxycholesterol, 22(*R*)-, 24(*S*)-, 25-, 27-, and 20(*S*)-hydroxycholesterols (Forman et al., 1997; Lehmann et al., 1997; Janowski et al., 1999; Zhang et al., 2001). On the other, some oxysterols, such as 7α,25-, 7β,25-, 7α,27-, and 7β,27-dihydroxycholesterols, and 7α-, 7β-, and 25-hydroxycholesterols, are active ligands of GPR183 (Liu et al., 2011). And as shown above, activation of GPR183 may function to reduce liver steatosis, which is contrasting to LXR-mediated steatosis. The most abundant oxysterol in the liver is 4β-hydroxycholesterol, which is followed by 5β,6β-epoxycholesterol, 5α,6α-epoxycholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 7-ketocholesterol, 27-hydroxycholesterol, and 25-hydroxycholesterol (Wooten et al., 2014; Guillemot-Legris et al., 2016a). Among them, 7α-, 7β-, or 25-hydroxycholesterol could activate GPR183 and 25- or 27-hydroxycholesterol could be an activator of LXR. In obesity conditions induced by high-fat diet or genetic models (db/db and ob/ob), levels of 7α-hydroxy-4-cholesten-3-one and 25-, 27-hydroxycholesterol are increased (Wooten et al., 2014; Guillemot-Legris et al., 2016a). Therefore, based on analyzed data, under normal diet conditions, 7α-, 7β-, and 25-hydroxycholesterols may activate GPR183 to protect against steatosis. Under high-fat diet conditions, 25- and 27-

hydroxycholesterols may start to activate LXR to regulate excess cholesterol (Musso et al., 2013). By feeding an atherogenic diet containing cholesterol, cholate, and lard for 2-3 weeks, increases of 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, and 25-hydroxycholesterol in liver homogenates were observed (Zhang et al., 2001). Those LXR activators may detoxify excess cholesterol. LXR not only induces fatty acid synthesis through SREBP-1c, but also stimulates cholesterol efflux, transport, conversion to bile acids, and excretion, when excess cholesterols are loaded. It would be an interesting topic to study effects of GPR183 on cholesterol homeostasis and detoxification in near future (Edwards et al., 2002; Zhao and Dahlman-Wright, 2010).

In this study, *in vivo* HFD feeding significantly induced GPR183 expression in the liver. Although precise mechanism needs to be elucidated, under steatosis conditions GPR183 may be increased and function to reduce hepatic steatosis. The immune regulatory function of GPR183 has been studied in B cells, dendritic cells, T cells, and macrophages/osteoclasts (Hannedouche et al., 2011; Liu et al., 2011; Liu et al., 2013a; Chalmin et al., 2015; Nevius et al., 2015; Sun and Liu, 2015). Recently, GPR183 was found to be expressed in astrocytes and 7α,25-dihydroxycholesterol was found to regulate cellular signaling and induce cellular migration (Rutkowska et al., 2015). The present data indicate protective functions of 7α,25-dihydroxycholesterol and GPR183 in liver steatosis and thus, identify potential therapeutic targets. In conclusion, our results show that 7α,25-dihydroxycholesterol can protect against fat accumulation promoted by activation of LXR

in human and mouse hepatocytes. These findings identify the 7α,25-dihydroxycholesterol-GPR183 signaling as a therapeutic target for nonalcoholic fatty liver diseases.

**Authorship contributions**:

Designed the experiments: Huang and Im.

Performed the experiments and analyzed the data: Huang, Lee, Kang, and Choi.

Wrote the manuscript: Huang and Im.

**Conflict of interest**: The authors declare that there is no conflict of interest.

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## Footnotes

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## Figure Legends

**Figure 1. Expression of GPR183 in hepatocytes and induction of GPR183 expression in mouse liver after HFD feeding.**

(A) GPR183 RT-PCR result in Hep3B cells. (B) Western blotting of GPR183 in Hep3B cells, HepG2 cells, and primary hepatocytes. (C and D) C57BL/6 mice were fed with HFD or normal chow diet for 4 weeks. Then, livers were collected after mice were killed. (C) Western blotting result of GPR183 expression in those mice. (D) Quantified results of Western blotting analysis of GPR183 in those mice. Data are from seven mice for each group. *** $p < 0.001$, comparing with the normal chow-diet group.

**Figure 2. 7α,25-dihydroxycholesterol inhibits LXR- or palmitic acid-mediated lipid accumulation in Hep3B cells.**

(A) Hep3B cells were treated with different concentrations of 7α,25-dihydroxycholesterol. After 1 h, cells were treated with T0901317 (1 μM) for 48 h. Cells were fixed with formalin and the slides were stained with Oil red O working solution. The red staining shows lipid droplets, bar = 20 μm. Representative images of three independent experiments. B) Histogram of lipid accumulation. Six different fields were photographed for each slide, and ImageJ software was used to analyze red-stained areas and intensity in each photo (NIH, Bethesda, MD, USA). (C) Hep3B cells were treated with 7α,25-dihydroxycholesterol. After 1 h, cells were treated with palmitic acid (PA, 0.1 mM) for 48 h. After Oil red O staining, ImageJ software was used to analyze red-stained areas and intensity. (D) Hep3B cells were treated with 7α,25-dihydroxycholesterol. After 1 h cells were treated with 1 μM T0901317 for 48 h. Then, triglyceride contents are analyzed and shown as histograms. The results shown are representative of three independent experiments. Results are presented as the means ± SDs of three separate experiments. *** $p < 0.001$ *vs.* the non-treated group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, *vs.* the T0901317-treated group or the PA-treated group.

**Figure 3. NIBR198 antagonizes 7α,25-dihydroxycholesterol inhibition of fat accumulation by LXR activation**

(A) Hep3B cells were treated with NIBR189 for 30 min. Then, Hep3B cells were treated with 100

nM 7α,25-dihydroxycholesterol for 1 h, and with T0901317 (1 μM) for 48 h. With formalin cells were fixed and Oil red O working solution was used to stain the slides. The red staining shows lipid droplets. Representative images from three independent experiments. (B) Histogram of lipid accumulation. Six different fields were photographed for each slide, and ImageJ software was used to analyze red-stained areas and intensity in each photo (NIH, Bethesda, MD, USA). Results from three individual experiments are expressed as mean ± SD. *** $p < 0.001$, compared with the non-treated group; ### $p < 0.001$, comparing with T0901317-treated group; $$$ $p < 0.001$, comparing with T0901317 plus 7α,25-dihydroxycholesterol-treated group.

**Figure 4. 7α,25-dihydroxycholesterol reduces SREBP-1c and FAS protein expression.**

(A) Hep3B cells were pretreated with vehicle or NIBR189 (10 μM) for 1 h, 100 nM 7α,25-dihydroxycholesterol for another 1 h, and then treated with T0901317 (1 μM) for 48 h. Western blot analysis was conducted for SREBP-1c and FAS protein expression in Hep3B cells. (B) Quantified results of Western blotting analysis of SREBP-1c (pre-form and mature form) and FAS in Hep3B cells. Results from three individual experiments are expressed as mean ± SD. *** $p < 0.001$, compared with the non-treated group; # $p < 0.05$, ## $p < 0.01$, comparing with T0901317-treated group; $ $p < 0.05$, comparing with T0901317 plus 7α,25-dihydroxycholesterol-treated group.

**Figure 5. 7α,25-dihydroxycholesterol reduces expression of SREBP-1c protein through GPR183.**

(A) RT-PCR result of GPR183 mRNA expression in siRNA-transfected Hep3B cells. (B) Western blotting analysis of GPR183 protein expression in siRNA-transfected Hep3B cells. (C) Hep3B cells were transfected with scrambled siRNA or GPR183 siRNA and incubated for 24 h. Then, Hep3B cells were treated with 7α,25-dihydroxycholesterol (100 nM) for 1 h, followed by T0901317 (1 μM) for 48 h. SREBP-1c expression was analyzed by Western blotting in the GPR183-knockdown Hep3B cells. (D) Quantified results of Western blotting analysis of SREBP-1c in the GPR183-knockdown Hep3B cells. Results are expressed as mean ± SD from three individual experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, comparing with the non-treated group; # $p < 0.05$, comparing with T0901317-treated group.

**Figure 6. Effect of pertussis toxin on 7α,25-dihydroxycholesterol inhibition on fat accumulation in Hep3B cells.**

(A) Hep3B cells were treated with PTX (100 ng/mL) for 24. Then, Hep3B cells were treated with 7α,25-dihydroxycholesterol (1 μM) for 1 h and with T0901317 (1 μM) for 48 h. With formalin cells were fixed and Oil red O working solution was used to stain the slides. Six different fields

were photographed for each slide, and ImageJ software was used to analyze red-stained areas and intensity in each photo (NIH, Bethesda, MD, USA). Results are expressed as mean ± SD from three independent experiments. *** $p < 0.001$, comparing with the control group.

**Figure 7. Signaling pathways of 7α,25-dihydroxycholesterol inhibition on fat accumulation in Hep3B cells.**

(A) Hep3B cells were treated with the p38 MAPK inhibitor, AMPK inhibitor, compound C (10 μM); SB202190 (10 μM); or PI3K inhibitor, LY294002 (5 μM) for 30 min. Then, after treatment of 7α,25-dihydroxycholesterol (100 nM) for 1 h and treatment with T0901317 (1 μM) for 48 h, Hep3B cells were fixed for Oil red O staining analysis. (B) Hep3B cells were treated with the ERK1/2 inhibitor PD98059 (10 μM) or JNK inhibitor, SP600125 (10 μM) 30 min. Then, after treatment of 7α,25-dihydroxycholesterol (100 nM) for 1 h and treatment with T0901317 (1 μM) for 48 h, Hep3B cells were fixed for Oil red O staining analysis. Results are expressed as mean ± SD from three independent experiments. *** $p < 0.001$, comparing with the control group; ## $p < 0.01$, ### $p < 0.001$, comparing with the T0901317-treated group.

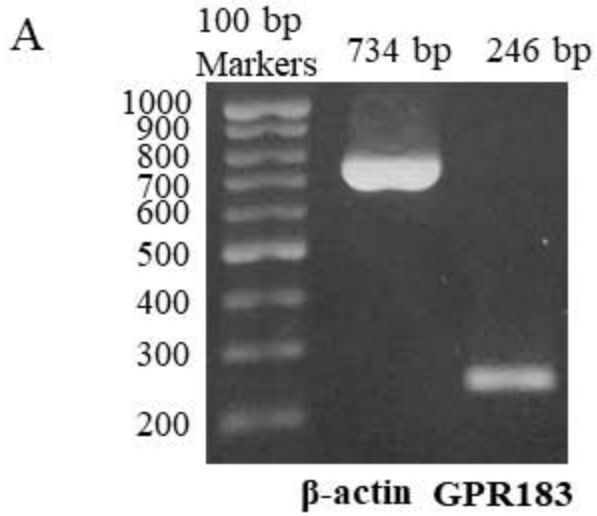
**Figure 8. 7α,25-dihydroxycholesterol inhibits fat accumulation by LXR activation in HepG2 cells and primary mouse hepatocytes.**

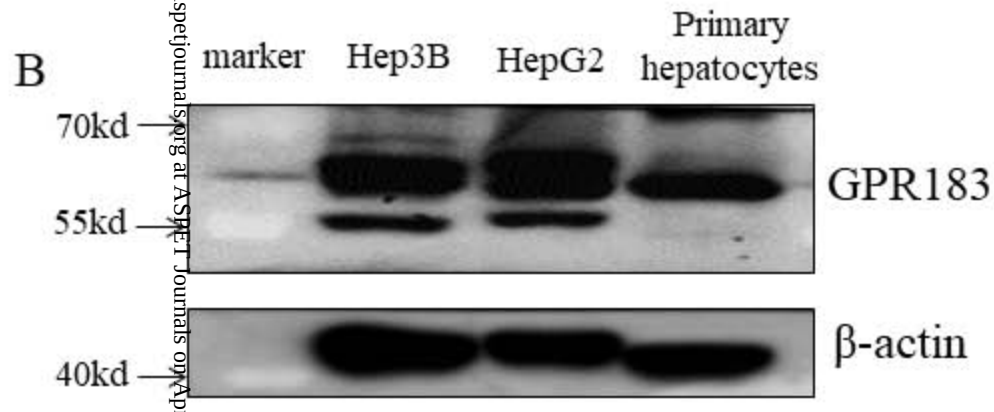
HepG2 cells (A) or mouse primary hepatocytes (B) were pretreated with vehicle or 10 μM NIBR189 for 30 min. Then, after treatment with different concentrations of 7α,25-dihydroxycholesterol for 1 h, and with T0901317 (1 μM) for 48 h, Oil red O staining was conducted and quantitatively analyzed using ImageJ software. Results are expressed as mean ± SD from three independent experiments. *** $p < 0.001$, comparing with the non-treated group; ## $p < 0.01$, ### $p < 0.001$, comparing with T0901317-treated group; $ $p < 0.05$, comparing with T0901317 plus 7α,25-dihydroxycholesterol-treated group.

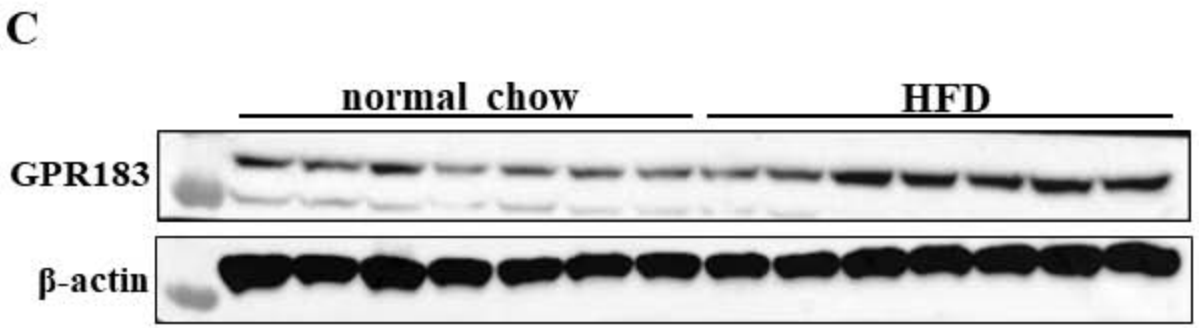
**Figure 9. Illustrated mechanism of 7α,25-dihydroxycholesterol inhibition on fat accumulation in hepatocytes.**

Fig 1





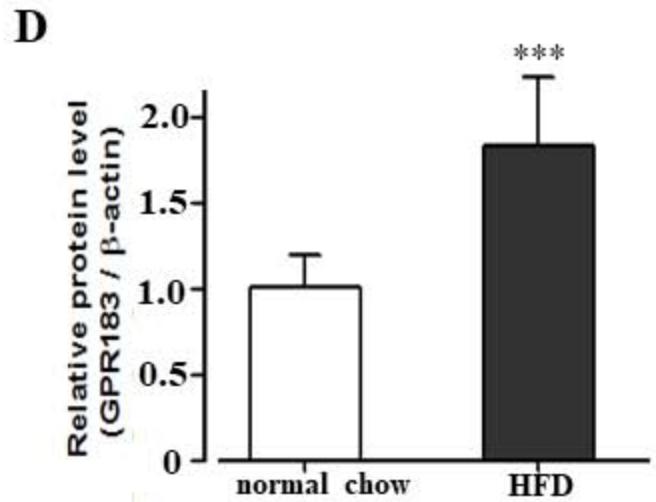
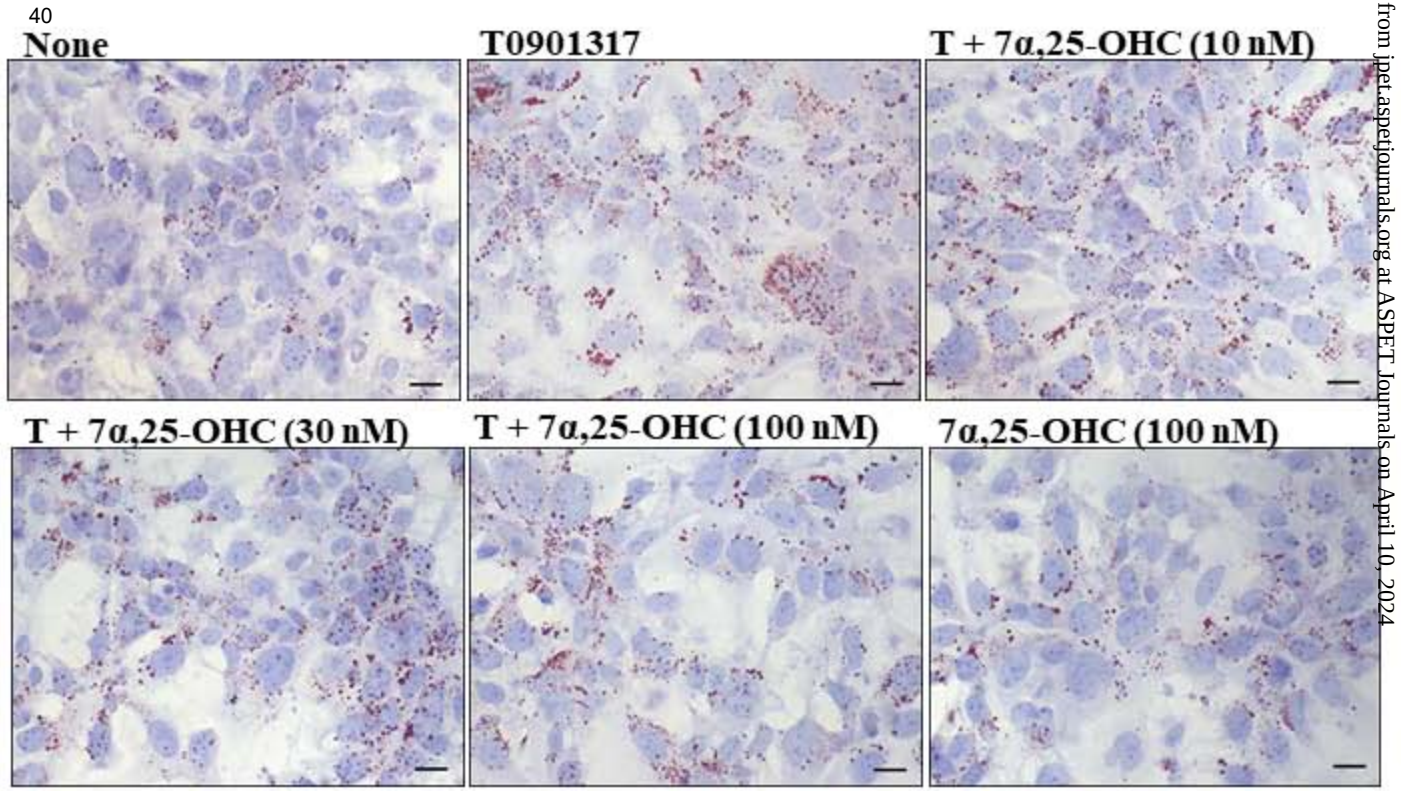



Fig 2 A

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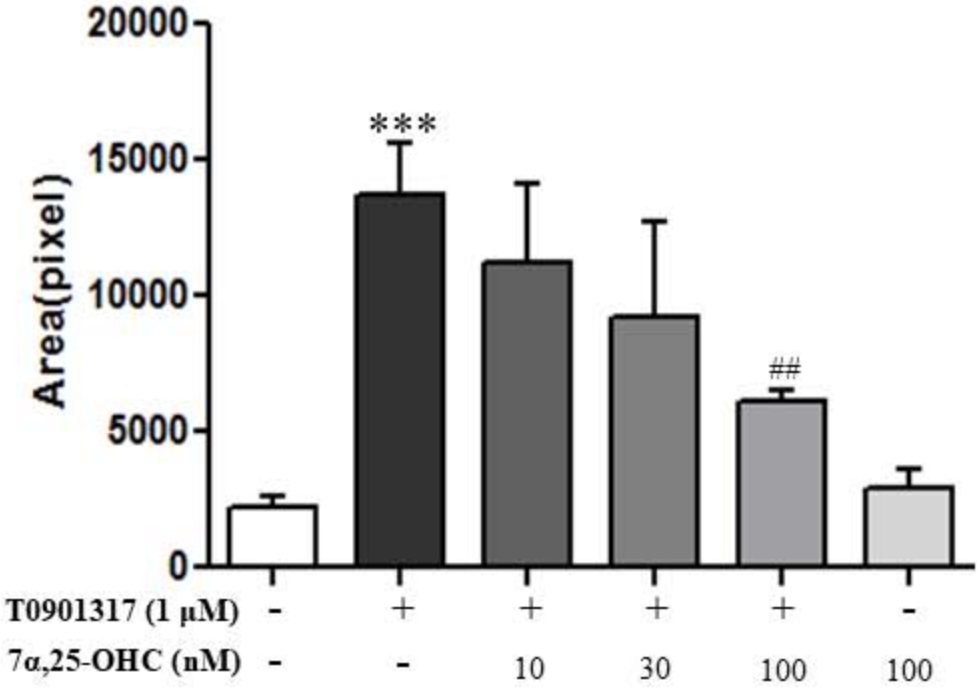


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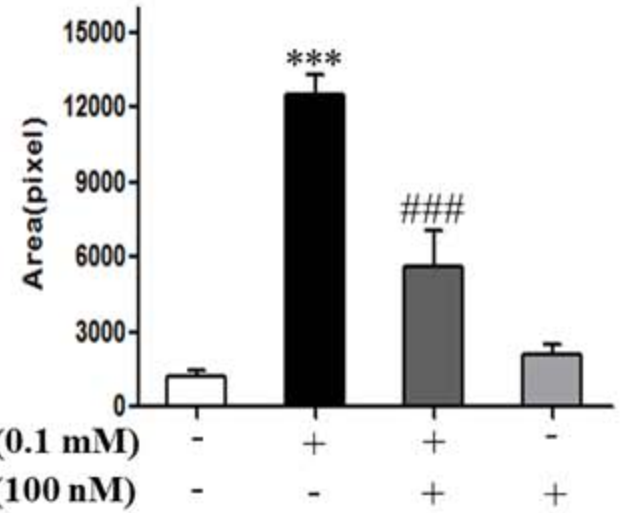


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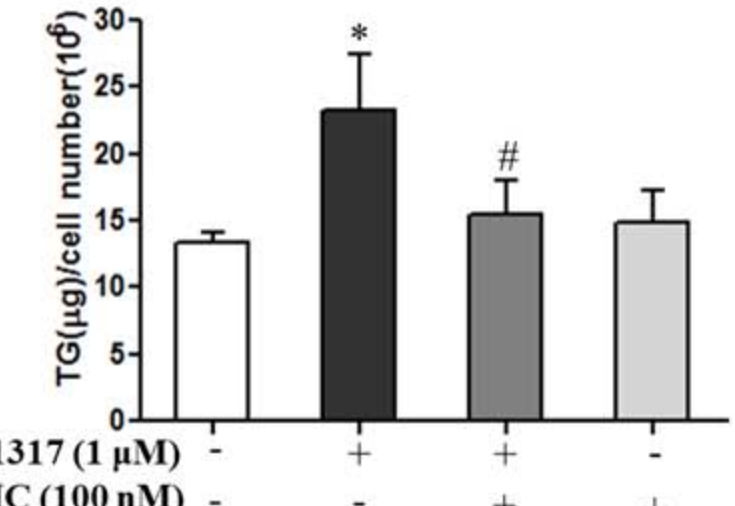
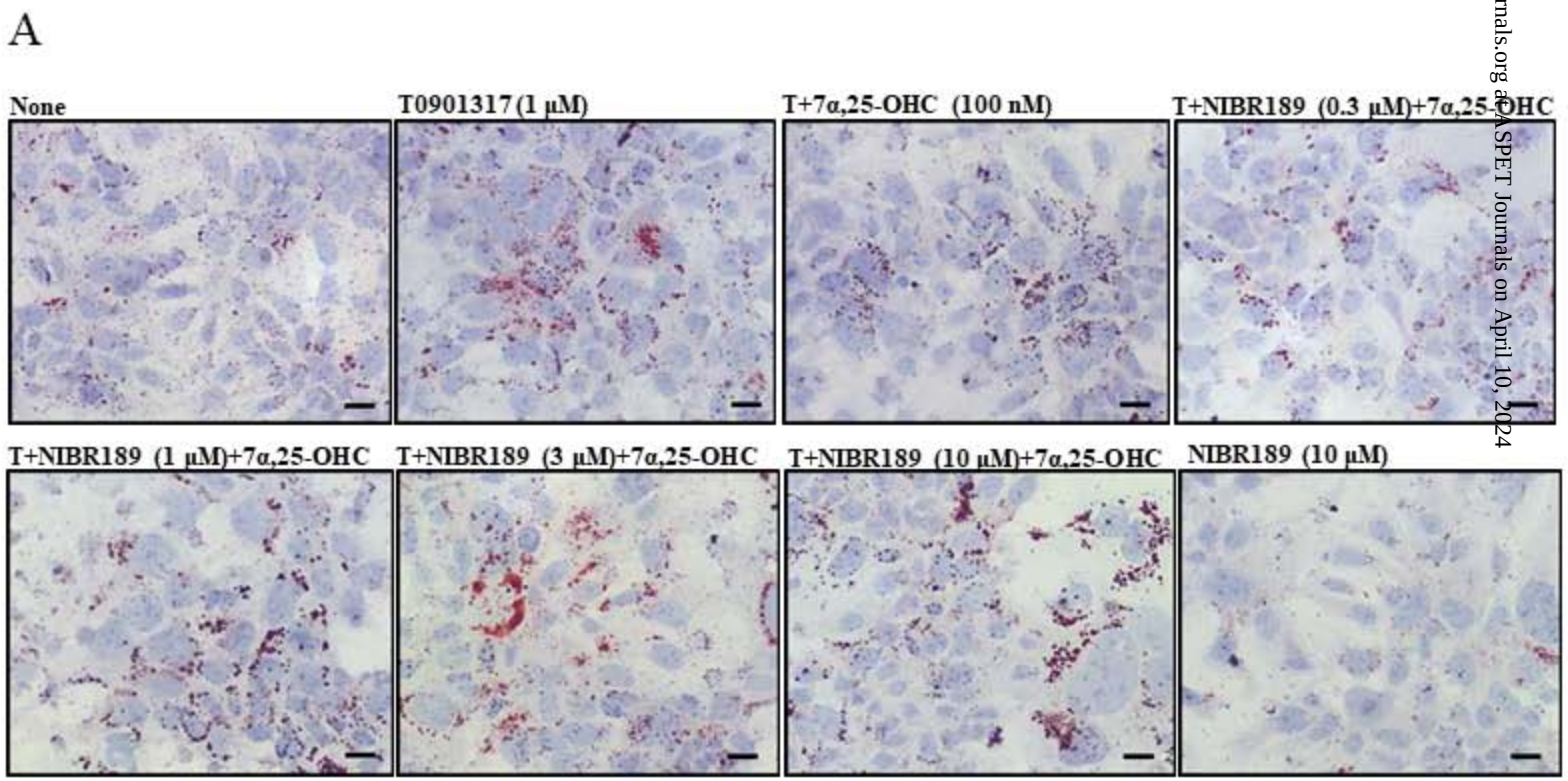


Fig 3







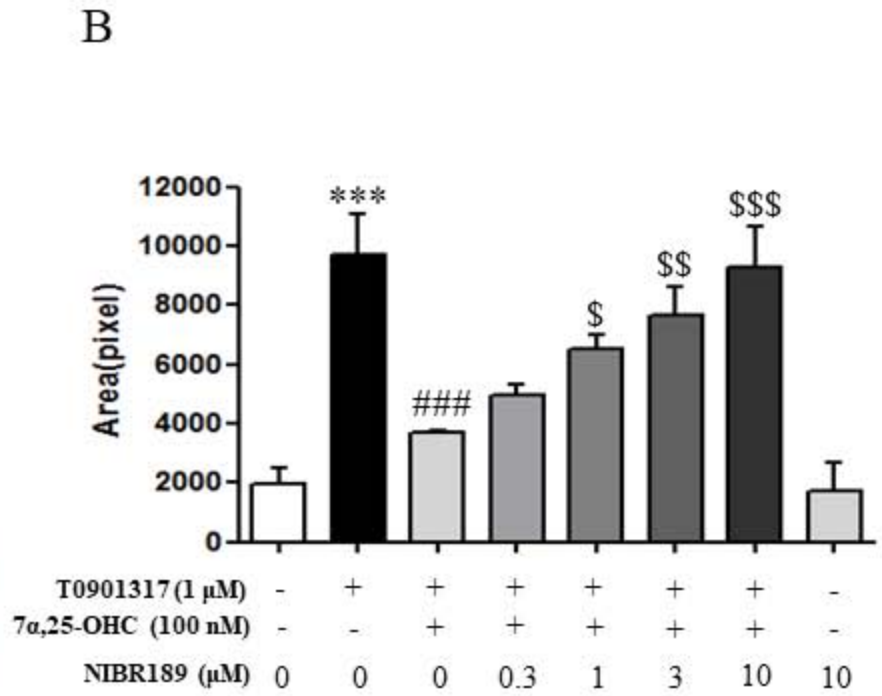



Fig 4



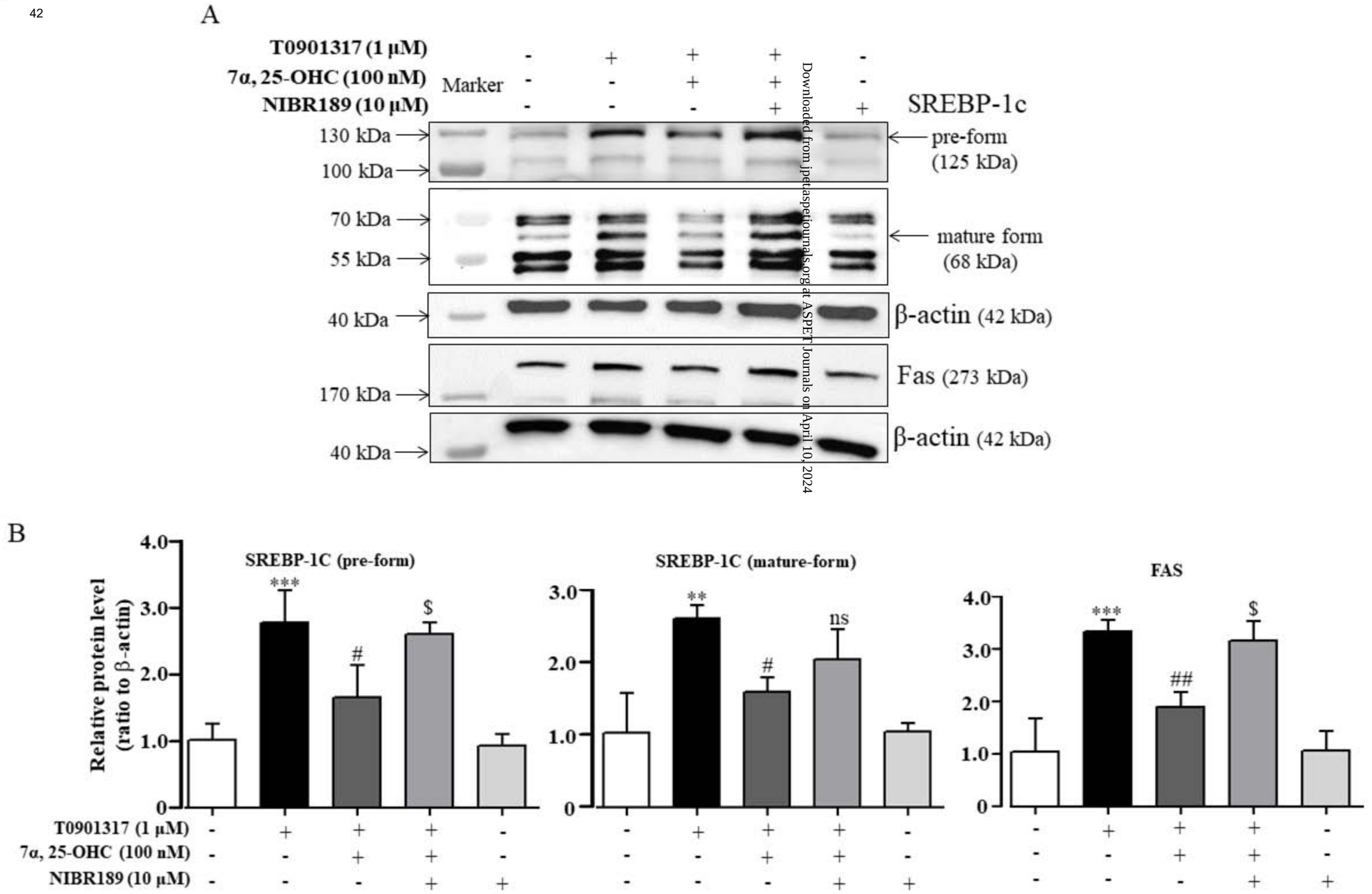


Fig 5



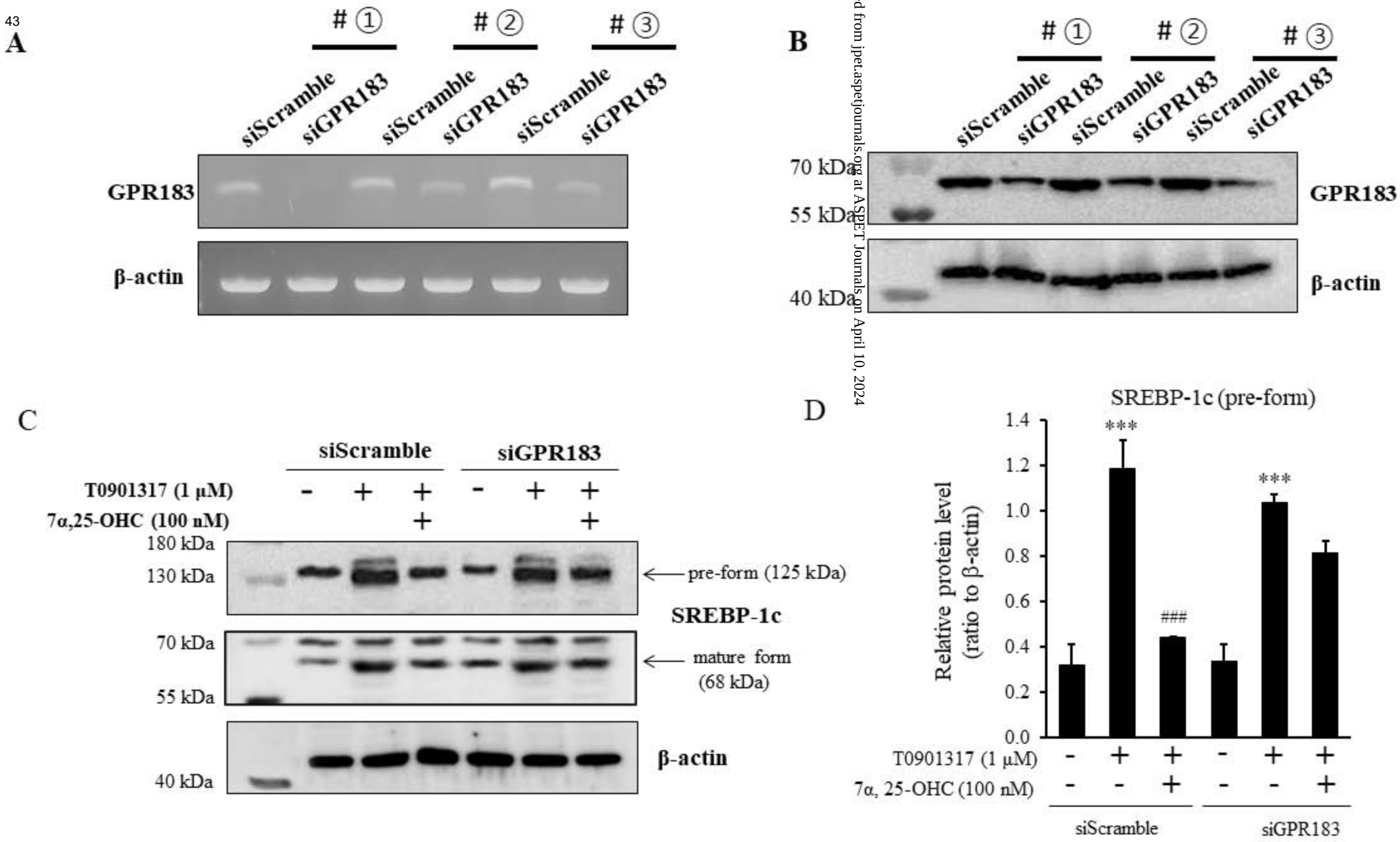
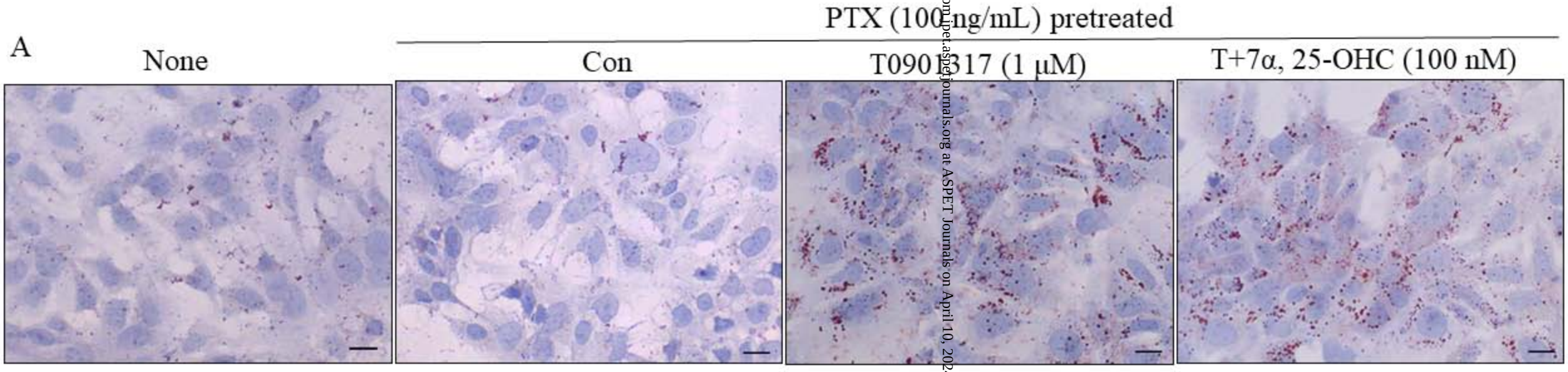


Fig 6







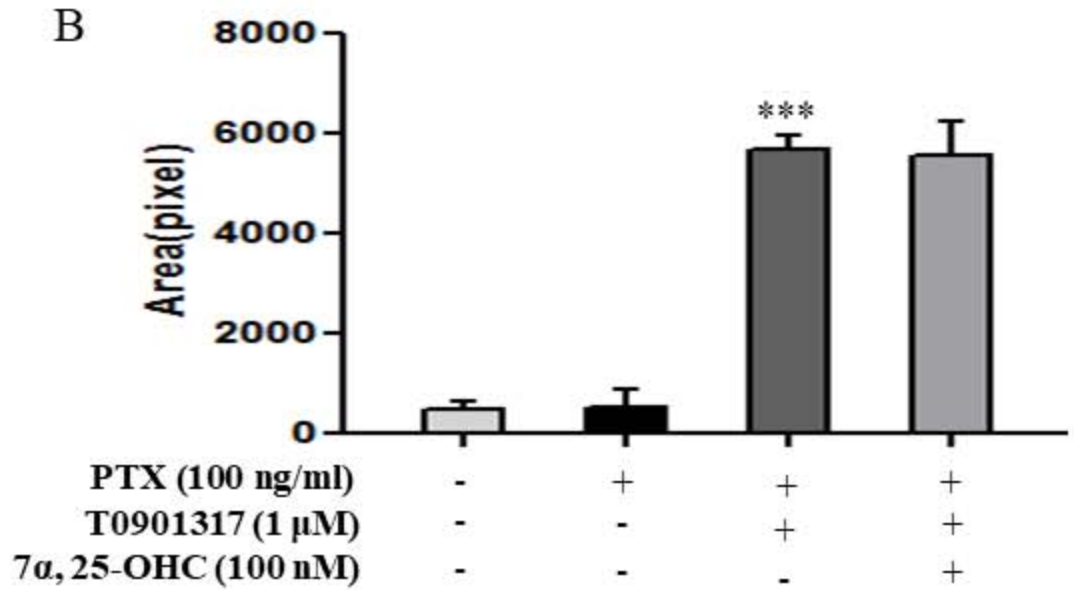



Fig 7



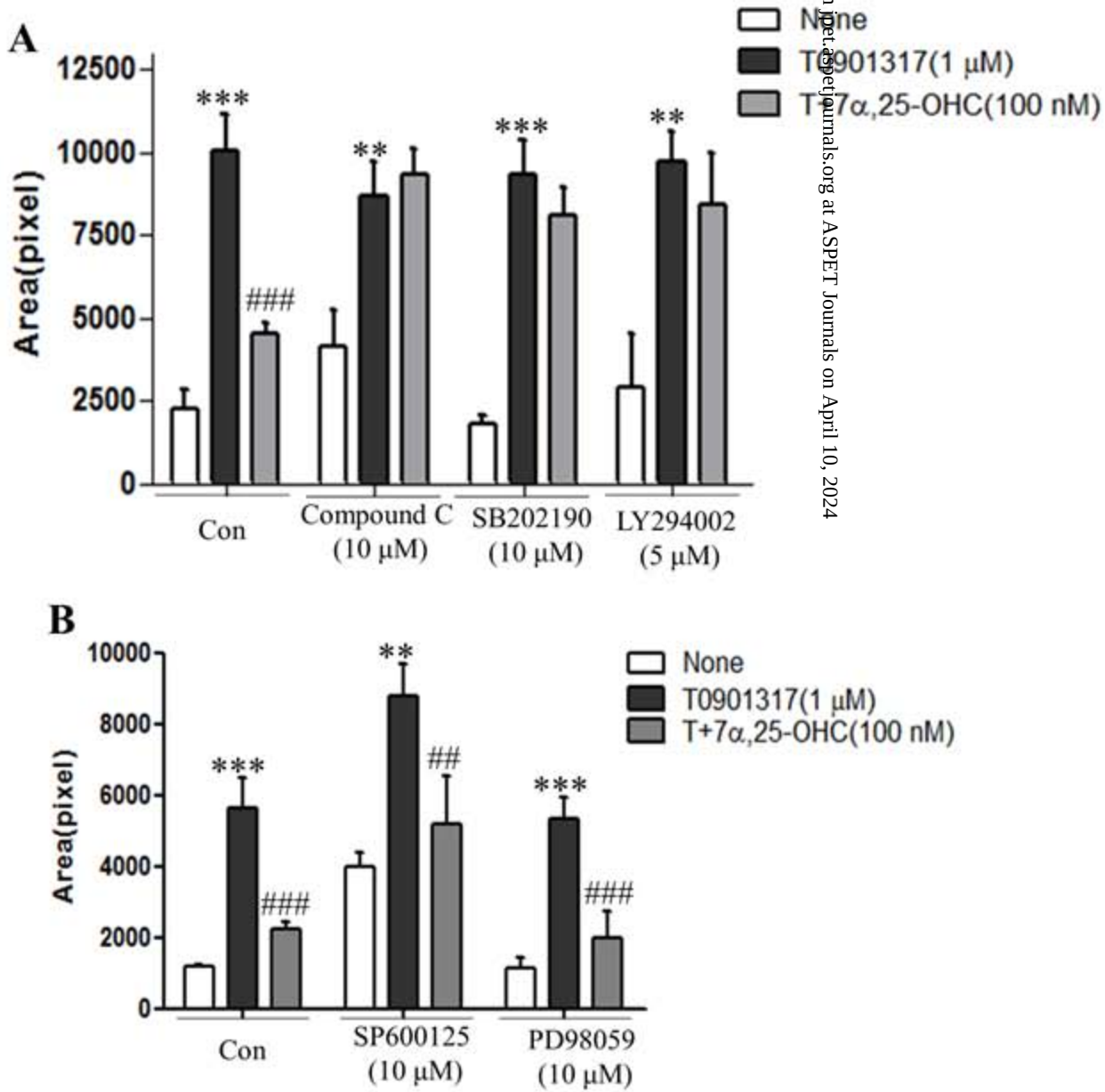


Fig 8



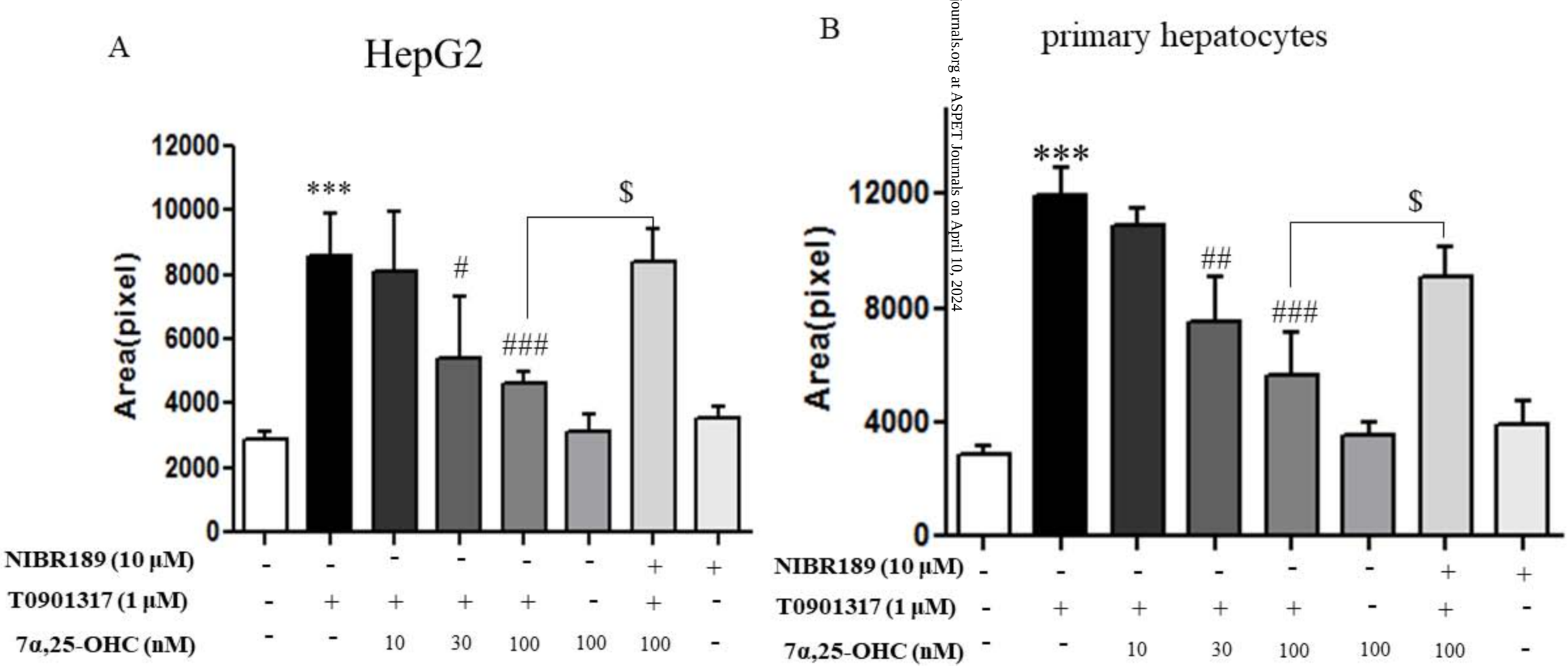


Fig 9