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Phospholipase D1 decreases type I collagen levels in hepatic stellate cells via induction of autophagy

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ABSTRACT

Hepatic stellate cells (HSCs) are major players in liver fibrogenesis. Accumulating evidence shows that suppression of autophagy plays an important role in the development and progression of liver disease. Phospholipase D1 (PLD1), which catalyzes the hydrolysis of phosphatidylcholine to yield phosphatidic acid (PA) and choline, was recently shown to modulate autophagy. However, little is known about the effects of PLD1 on the production of type I collagen that characterizes liver fibrosis. Here, we examined whether PLD1 regulates type I collagen levels in HSCs through induction of autophagy. Adenovirus-mediated overexpression of PLD-1 (Ad-PLD1) reduced type I collagen levels in the activated human HSC lines, hTERT and LX2. Overexpression of PLD1 in HSCs led to induction of autophagy as demonstrated by increased LC3-II conversion and formation of LC3 puncta, and decreased p62 abundance. Moreover, inhibiting the induction of autophagy by treating cells with bafilomycin or a small interfering (si)RNA for ATG7 rescued Ad-PLD1-induced suppression of type I collagen accumulation in HSCs. The effects of PLD on type I collagen levels were not related to TGF-β/Smad signaling. Furthermore, treatment of cells with PA induced autophagy and inhibited type I collagen accumulation. The present study indicates that PLD1 plays a role in regulating type I collagen accumulation through induction of autophagy.

1. Introduction

During liver fibrogenesis, hepatic stellate cells (HSCs) are activated to form myofibroblast-like cells, a process that is controlled by many cytokines and growth factors [1]. The mechanisms underlying HSC activation are complex and involve the conversion of a resting, vitamin A-storing cell into a proliferating HSC without vitamin A droplets, which is then capable of secreting large quantities extracellular matrix (ECM) components such as type I collagen [2,3]. Accumulated ECM alters the hepatic architecture by forming a fibrous scar, and leads to the subsequent development of nodules of regenerating hepatocytes that define cirrhosis [4,5].

Autophagy is a self-degradative process that is important for cellular homeostasis and defense [6,7]. The process of autophagy involves formation of double membrane vesicles called autophagosomes, which envelop and sequester cytoplasmic components, including protein aggregates and damaged intracellular organelles, for bulk degradation through a lysosomal pathway [7]. Autophagy is increasingly appreciated as a protective mechanism against the progression of human diseases. A growing body of evidence suggests the importance of autophagy in the pathogenesis of liver diseases, including ischemia reperfusion injury [8], alpha-1 antitrypsin deficiency[9], alcoholic hepatitis [10], nonalcoholic fatty liver disease [11], and hepatocellular carcinoma [6,7,12].

Phospholipase D1 (PLD1) hydrolyzes the phosphodiester bond of phosphatidylcholine, resulting in the production of phosphatidic acid (PA) and choline. PLD1 activity facilitates budding from the Golgi complex, as well as exocytosis, endocytosis, and phagocytosis [13,14]. Intracellular signaling mediated by PLD1 has been examined in studies of cell proliferation, migration, inflammation, and

Abbreviations: HSCs, hepatic stellate cells; PA, phosphatidic acid; PLD, phospholipase D; ECM, extracellular matrix; TGF-β, transforming growth factor-β; BFM, bafilomycin A1.

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tumor development [15,16]. A recent study demonstrated that PLD1 induced autophagy in mice upon nutrient starvation, and that PLD1 knockout mice exhibited reduced hepatic autophagy compared with wild-type mice after starvation [17]. Inhibition of PLD1 in neuronal cells results in impaired autophagic flux and accumulation of α-synuclein; these changes are rescued by overexpression of PLD1 [18]. However, little is known about the effects of PLD1 on type I collagen production in liver fibrosis. Here, we examined whether PLD1 induces autophagy in HSCs and, thereby, decreases type I collagen accumulation.

2. Materials and methods

2.1. Chemicals

Recombinant human transforming growth factor-beta (TGF-β) was purchased from R&D systems (Minneapolis, MN) and bafilomycin A1 (BFM) was purchased from Sigma (St. Louis, MO).

2.2. Cell culture

The hTERT-HSC human HSC line was a kind gift from Dr. Pak (University of Sungkyunkwan, Suwon, Korea) and the LX2 human HSC line was a kind gift from Dr. Jeong (Korea Advanced Institute of Science and Technology, Daejeon, Korea). The hTERT-HSC and LX2 cells were cultured in 5% CO_2/95% air at 37 °C in DMEM (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics. Cells in serum free medium were infected with adenoviruses expressing PLD1 (Ad-PLD1) for 2 h and then treated as indicated below.

2.3. Generation of recombinant adenovirus

The cDNA encoding PLD1 was inserted into the pAd-Track-CMV shuttle vector. To produce the recombinant adenoviral plasmid, the resultant shuttle vector was electroporated into BJ5138 cells containing the AdEasy adenoviral vector. The recombinant adenoviral plasmids were then transfected into HEK-293 cells to amplify Ad-PLD1 and the viruses were purified using CsCl density centrifugation (Sigma, St. Louis, MO). The viruses were then collected and desalted, and the titer determined using an Adeno-X Rapid titer kit (BD Bioscience, San Jose, CA). Control adenoviruses (Ad-GFP) were prepared using the same method. The efficiency of adenoviral infection was assessed using a recombinant adenovirus encoding PLD1 fused to green fluorescent protein (GFP; data not shown).

2.4. Western blot analysis

The hTERT-HSC and LX2 cell were harvested in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.25% Na-deoxycholate] containing protease inhibitors and dithiothreitol. The proteins were resolved by SDS–PAGE and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated in a blocking buffer, incubated with a primary antibody followed by the appropriate horseradish peroxidase-conjugated secondary antibody, and then developed using the Clarity™ Western ECL substrate kit (Bio-Rad, Richmond, CA). The antibodies used in this study were anti-PLD1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-type I collagen (Abcam, Cambridge, MA), anti-p62 (Abcam, Cambridge, MA), anti-LC3 (Cell Signaling Technology, Beverly, MA), and anti-ATG7 (Cell Signaling Technology, Boston, MA). The membrane was re-blotted with anti-actin to verify equal loading of protein in each lane. Densitometry using the UN-SCAN-IT software was used to quantify the results (Skik Scientific Corp., Orem, UT).

2.5. Immunofluorescence analysis

Cells were fixed in 4% formaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After incubation with anti-LC3 and a Cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA), the cells were imaged using a confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.6. siRNA interference of ATG7

A pre-designed siRNA specific for ATG7 and a control siRNA were purchased from Santa Cruz Biotechnology. Cells were transfected with 100 nM siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The effect of the ATG7-siRNA on the expression of endogenous ATG7 was measured by Western blot analysis.

2.7. Statistical analysis

Data are expressed as the mean ± SEM. ANOVA and Duncan's new multiple range test were used to determine significant differences. Significant differences are indicated in the figure legends. All experiments were performed at least three times.

3. Results

3.1. Adenovirus-mediated overexpression of PLD1 reduces type I collagen levels in activated HSCs

Consistent with previous reports [19] showing that cultured hTERT-HSC and LX2 cells behave as activated HSCs, we found that the activated HSC marker alpha-SMA is highly expressed in these cells. To investigate whether PLD1 inhibited type I collagen levels in activated HSCs, we measured the effect of adenovirus-mediated overexpression of PLD1 (Ad-PLD1) on type I collagen levels by Western blot analysis. Ad-PLD1 decreased type I collagen abundance in hTERT-HSC and LX2 cells in a concentration-dependent manner (Fig. 1A and B, respectively). Because the TGF-β and Smad3 signaling pathways play key roles in HSC activation [2,5], we next investigated whether PLD1 inhibited TGF-β-induced fibrotic gene expression in HSCs. Ad-PLD1 decreased TGF-β-stimulated type I collagen accumulation in LX2 cells (Fig. 1C), but did not change Smad3 phosphorylation (Fig. 1D), suggesting that the PLD-1 induced reduction in type I collagen levels is independent of the Smad pathway.

3.2. Adenovirus-mediated overexpression of PLD1 leads to induction of autophagy in activated HSCs

Next, we examined whether PLD1 inhibits type I collagen production by inducting autophagy. To determine whether PLD-1 induces autophagy in HSCs, we monitored two biochemical markers of autophagy: the expression of p62 protein and the conversion of type I LC3 (LC3-I) to type II (LC3-II). Ad-PLD1 reduced the p62 level and increased LC3 II conversion in hTERT-HSC cells (Fig. 2A) and LX2 cells (Fig. 2B). Induction of autophagy by Ad-PLD1 was further demonstrated by formation of LC3 puncta (Fig. 2C).

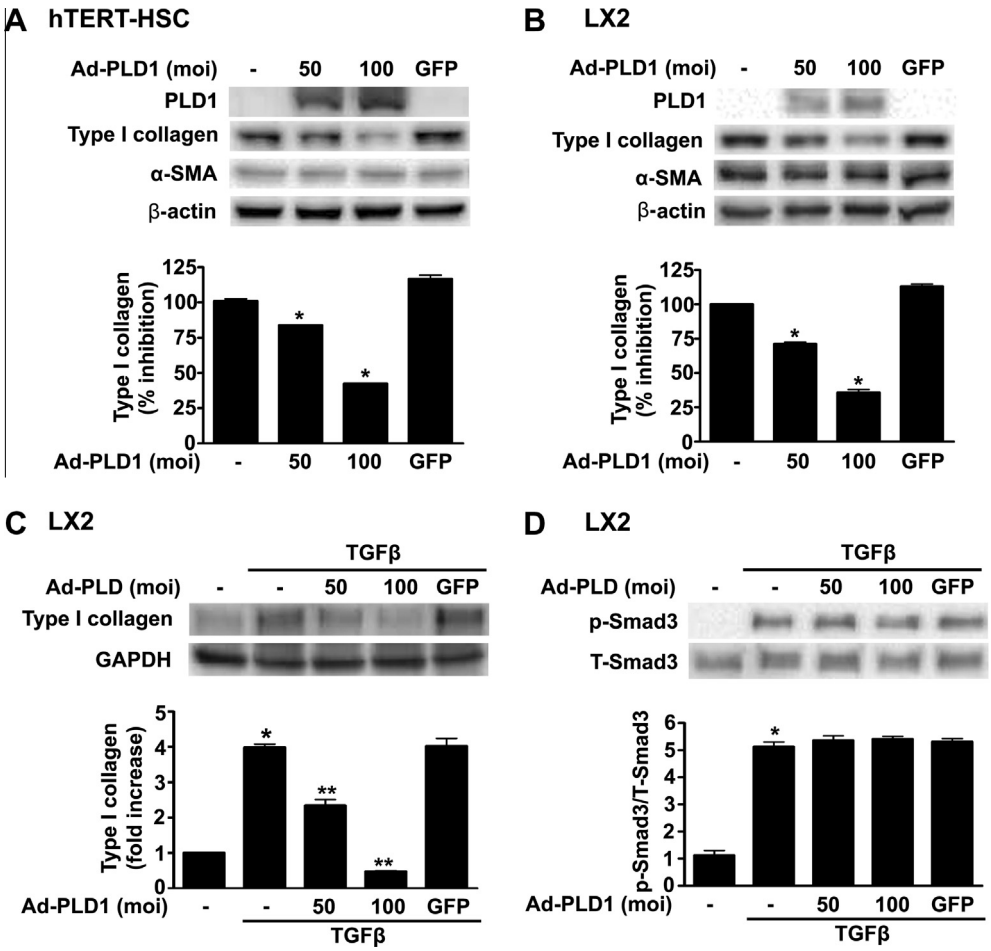

Fig. 1. Adenovirus-mediated overexpression of PLD1 in HSCs reduces the accumulation of type I collagen. (A and B) Representative Western blot showing the effect PLD1 overexpression (Ad-PLD1) on type I collagen levels in hTERT-HSC (A) and LX2 (B) cells. The bar graph shows the relative expression (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control. (C) Representative Western blot showing the effect PLD1 overexpression on TGF-β-stimulated type I collagen levels. LX2 cells were infected with the indicated concentrations [multiplicity of infection (moi)] of Ad-PLD1 for 24 h and then treated with TGF-β for 24 h. The bar graph shows the relative expression (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control, $^{**}P < 0.01$ compared with TGF-β alone. (D) Representative Western blot showing the effect of PLD1 overexpression on TGF-β-stimulated Smad3 phosphorylation. LX2 cells were infected with the indicated concentrations (moi) of Ad-PLD1 for 24 h and then treated with TGF-β for 1 h. The bar graph shows the relative level of Smad3 phosphorylation (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control.

3.3. Inhibition of autophagy increases type I collagen levels in activated HSCs

To determine whether autophagy regulates type I collagen levels in HSCs, we examined autophagic flux using the autophagy inhibitor BFM. Treatment with 100 nM BFM induced a marked increase in the levels of p62 and LC3-II (Fig. 3A and B). Moreover, BFM increased type I collagen accumulation, suggesting that inhibition of autophagy increases type I collagen accumulation (Fig. 3A and B). Consistent with the results of chemical inhibition of autophagy, inhibition of autophagy by siRNA-mediated down-regulation of ATG7 increased the type I collagen level in hTERT-HSC cells (Fig. 3C).

3.4. Induction of autophagy is required for the PLD1-induced reduction in type I collagen levels in activated HSCs

To determine the relationship between Ad-PLD1, autophagy, and type I collagen accumulation, we examined the effect of Ad-PLD on type I collagen and the induction of autophagy in presence of BFM. Treatment with BFM increased LC3-II levels; however, treatment with both BFM and Ad-PLD1 further increased LC3-II levels, indicating that autophagic flux is higher in Ad-PLD1 infected cells. Moreover, the Ad-PLD1-induced reduction in type I collagen levels was restored by treatment with BFM (Fig. 4A and B). Additionally, knockdown of ATG7 rescued the inhibitory effect of PLD1 on type I collagen accumulation (Fig. 4C). These results suggest that PLD1 inhibits hepatic fibrosis by inducing autophagy. Finally, because PA is produced by PLD1 [17], we examined whether PA treatment inhibits fibrosis and induces autophagy. PA-treated LX2 cells exhibited a decrease in type I collagen levels and an increase in LC3-II conversion (Fig. 4D).

4. Discussion

Here, we showed that Ad-PLD1 and its product, PA, reduce type I collagen levels in activated HSCs through the induction of autophagy. The effect of PLD1 on type I collagen levels was not associated with TGF-β/Smad signaling.

Some studies report that PLD activity is correlated with tissue fibrosis, and that inhibition of PLD activity decreases TGF-β induced type I collagen synthesis in human primary dermal fibroblasts [20]. Another study showed that both the levels and activity of PLD2 protein increase in the scar tissue formed as a result of heart failure, but PLD1 is not detected, suggesting that differential changes in PLD isozymes may be correlated with the processes of scar tissue remodeling after myocardial infarction [21]. In the pres-

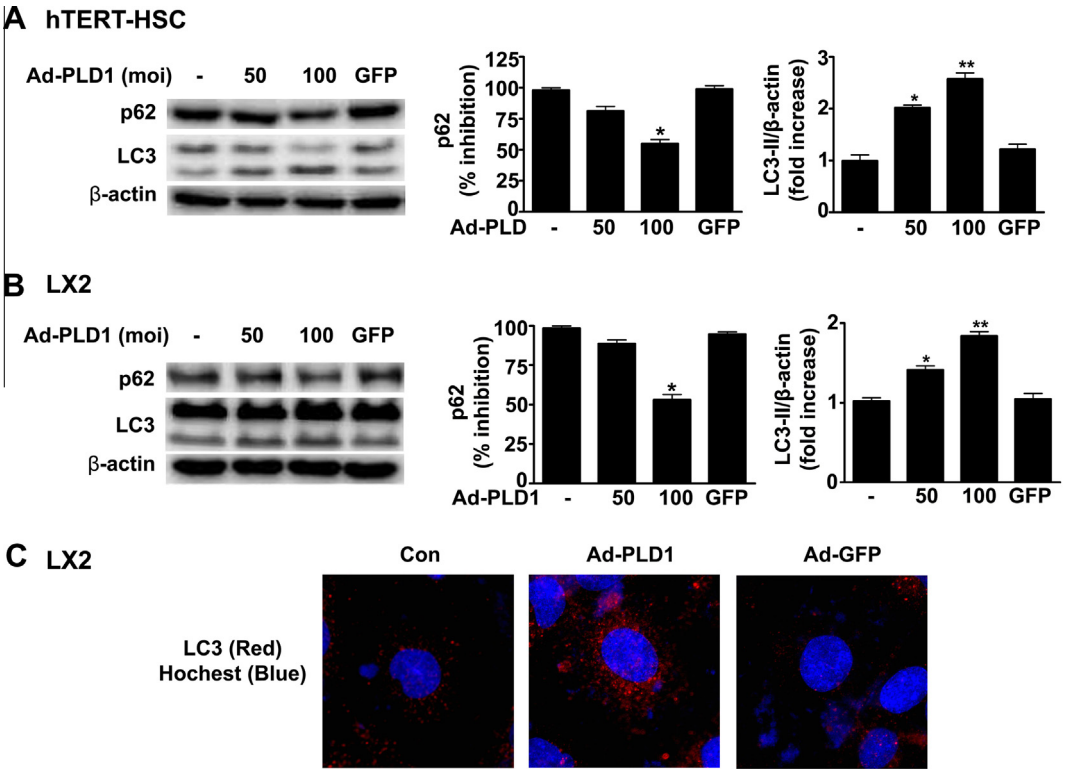

Fig. 2. Adenovirus-mediated overexpression of PLD1 in HCSs leads to induction of autophagy. (A and B) Representative Western blot showing the effect of PLD1 overexpression on p62 and LC3 protein levels in hTERT-HSC (A) and LX2 (B) cells. The bar graph shows the relative p62 and LC3 levels (mean ± SEM; $n = 3$ three independent measurements). $^{*}P < 0.01$ and $^{**}P < 0.05$ compared with control. (C) LX2 cells were infected with Ad-PLD1 for 24 h and LC3 puncta formation was analyzed by immunofluorescence. Original magnification, 800×.

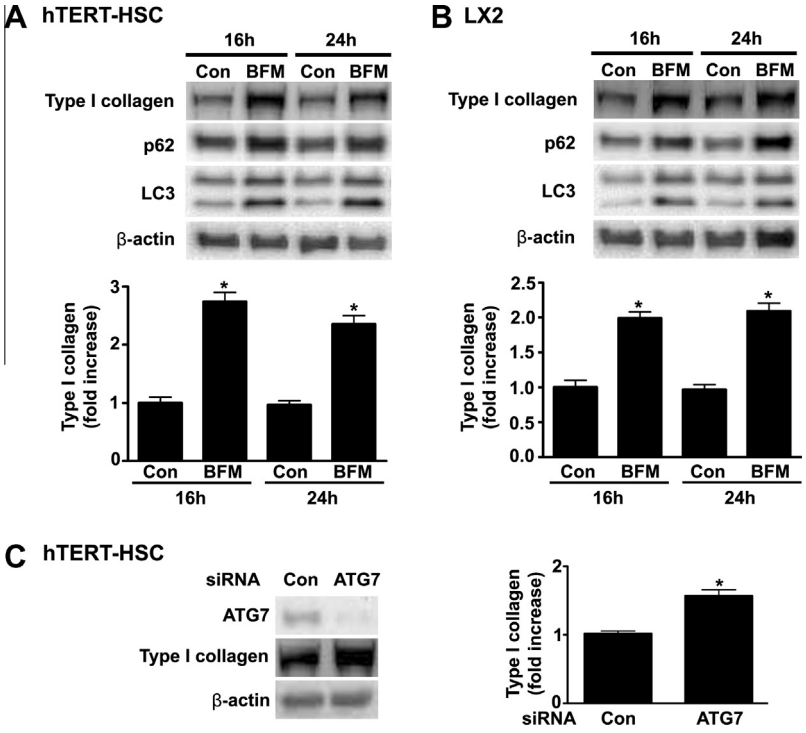

Fig. 3. Inhibition of autophagy increases the levels of type I collagen in HSCs. (A and B) Representative Western blot showing the effect of bafilomycin A1 (BFM) on type I collagen levels in hTERT-HSC (A) and LX2 (B) cells. The bar graph shows the relative protein levels (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control. (C) hTERT-HSC cells were transfected with 100 nM ATG7-siRNA or a control siRNA (Con). The bar graph shows the relative collagen levels (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control siRNA.

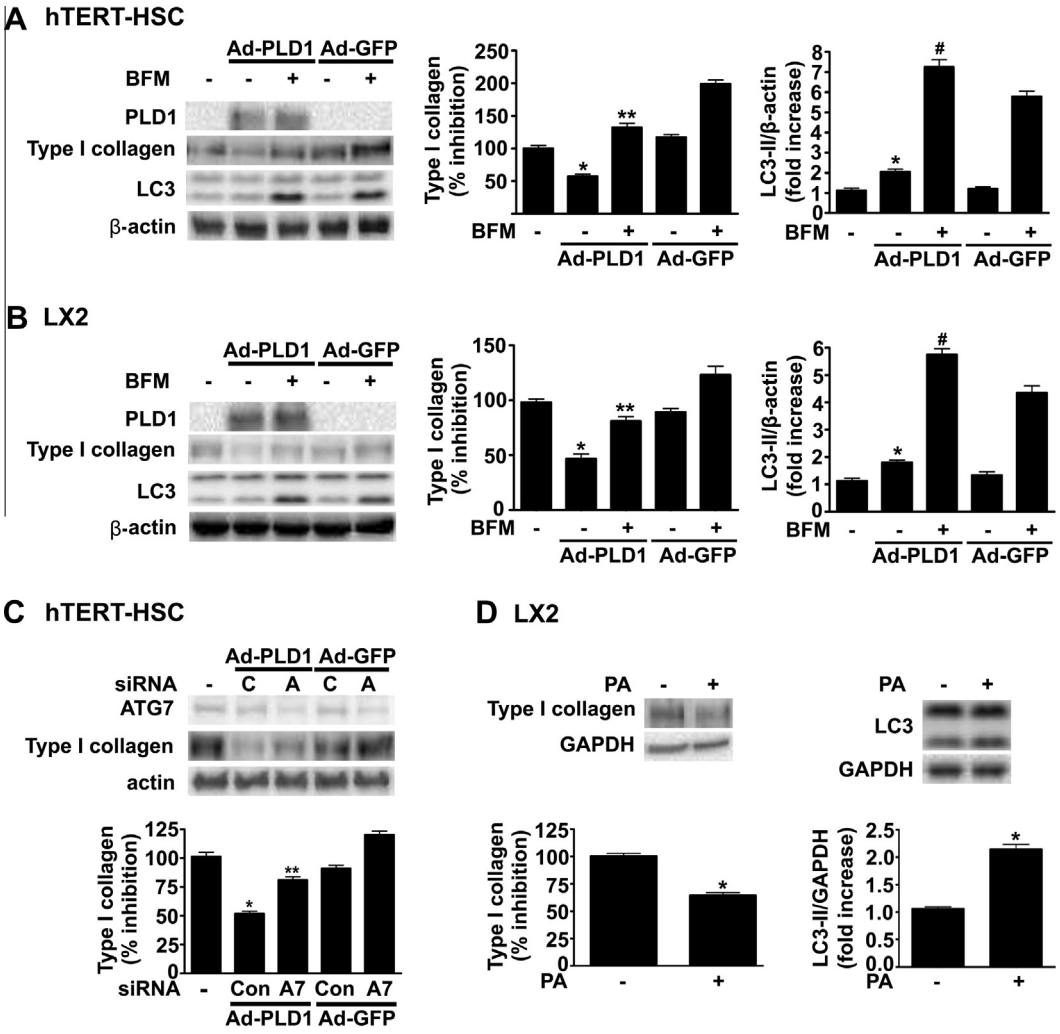

Fig. 4. Induction of autophagy is required for PLD1-induced reduction of type I collagen in HSCs. (A and B) Representative Western blot showing the effect of overexpression of PLD1 and of bafilomycin A1 (BFM, 100 nM) on type I collagen and LC3 protein levels in hTERT-HSC (A) and LX2 (B) cells. The bar graph shows the relative protein levels (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control, $^{**}P < 0.01$ compared with Ad-PLD1 only, $^{\#}P < 0.05$ compared with Ad-GFP + BFM. (C) hTERT-HSC cells were transfected with 100 nM ATG7-siRNA or a control siRNA (Con) and then infected with Ad-PLD1. The bar graph shows the relative decrease in collagen levels (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control, $^{**}P < 0.01$ compared with Ad-PLD1 + siRNA Con. (D) Representative Western blot showing the effect of phosphatidic acid (PA, 100 μM) on type I collagen, and LC3 levels in LX2 cells. The bar graph shows the relative protein levels (mean ± SEM; $n = 3$ independent measurements). $^{*}P < 0.01$ compared with control.

ent study, we found that PLD1 overexpression in activated HSCs reduced the levels of type I collagen, suggesting that PLD1 plays a protective role against hepatic disease by blocking fibrogenesis. During the phase of HSC activation in which fibrogenesis occurs, TGF-β is the most potent fibrogenic stimulus [5]; the effects of TGF-β are classically mediated by intracellular signaling via Smad proteins [22]. However, in the present study, Ad-PLD1 inhibited TGF-β-stimulated type I collagen accumulation, but there was no change in Smad3 phosphorylation. These results suggest that the effect of Ad-PLD1 on liver fibrosis is not related to inhibition of the TGF-β/Smad3 pathway.

The present study also showed that inhibition of autophagy by treatment of activated HSCs with BFM or an ATG7-siRNA increased type I collagen levels, suggesting that autophagy induction in activated HSCs helps to reduce excess type I collagen accumulation. Contrary to our results, previous studies show that autophagic flux increased during HSC activation and promoted hepatic fibrogenesis through changes in lipid metabolism during activation of stellate cells [23,24]. However, a growing body of evidence supports the hypothesis that autophagy has a protective effect against fibrosis in various tissues [25,26]. Autophagy in the kidney plays a cytoprotective role by promoting intracellular degradation of type 1 collagen in response to TGF-β without altering the type I collagen mRNA level [27], and provides protection against tubular cell apoptosis and tubulointerstitial fibrosis after unilateral urethral obstruction [28]. However, Hernadez-Gea et al. reported that autophagy induced fibrogenesis in mouse mesangial cell as well as in HSCs [24]. These distinct roles of autophagy in the kidney may be the result of the different strategies used to inhibit autophagy and the different experimental conditions, such as basal autophagy versus induced autophagy [29] . Although the reasons for the discrepancies between the results of the present study and those of previous studies [23,24] are unclear, one reason may be the use of HSCs in different states of activation. When quiescent HSCs are activated by liver injury, autophagy increases and promotes hepatic fibrosis through changes in lipid metabolism in HSCs [23,24]; however, if autophagy is induced after activation of HSCs, as in the present study, autophagy could inhibit fibrosis through degradation of type I collagen proteins.

Collectively, the present study shows that PLD1 reduces type I collagen levels in activated HSCs through induction of autophagy, suggesting that PLD1 plays an anti-fibrogenic role in hepatic fibro-

sis. This study raises the possibility that PLD1 may be a suitable target for preventing hepatic fibrosis.

Acknowledgments

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