



Original Article



O-GlcNAcylation Coordinates Glutaminolysis by Regulating the Stability and Membrane Trafficking of ASCT2 in Hepatic Stellate Cells

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Abstract

Background and Aims: Recognition of excessive activation of hepatic stellate cells (HSCs) in liver fibrosis prompted us to investigate the regulatory mechanisms of HSCs. We aimed to examine the role of O-GlcNAcylation modification of alanine, serine, cysteine transporter 2 (ASCT2) in HSCs and liver fibrosis. **Methods:** The expression of O-GlcNAcylation modification in fibrotic mice livers and activated HSCs was analyzed by western blotting. Immunoprecipitation was used to assess the interaction of ASCT2 and O-GlcNAc transferase (OGT). In addition, ASCT2 protein stability was assayed after cycloheximide (CHX) treatment. The O-GlcNAcylation site of ASCT2 was predicted and mutated by site-directed mutagenesis. Real-time PCR, immunofluorescence, kit determinations and Seahorse assays were used to clarify the effect of ASCT2 O-GlcNAcylation on HSC glutaminolysis and HSC activation. Western blotting, immunohistochemistry, and immunohistochemistry were used to analyze the effect of ASCT2 O-GlcNAcylation *in vivo*. **Results:** We observed significantly increased O-GlcNAcylation modification of ASCT2. ASCT2 was found to interact with OGT to regulate ASCT2 stability. We predicted and confirmed that O-GlcNAcylation of ASCT2 at Thr122 site resulted in HSC activation. We found Thr122 O-GlcNAcylation of ASCT2 mediated membrane trafficking of glutamine transport and attenuated HSC glutaminolysis. Finally, we validated the expression and function of ASCT2 O-GlcNAcylation after injection of AAV8-ASCT2 shRNA in CCl₄-induced liver fibrosis mice *in vivo*. **Conclusions:** Thr122 O-GlcNAcylation regulation of ASCT2 resulted in stability and membrane traf-

ficking-mediated glutaminolysis in HSCs and liver fibrosis. Further studies are required to assess its role as a putative therapeutic target.

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Introduction

During progressive liver fibrosis, there is excessive accumulation of myofibroblasts in the injured liver that accelerates liver fibrosis.¹ Quiescent hepatic stellate cells (Q-HSCs) are the source of fibrogenic myofibroblasts in chronic liver injury.² Quiescent HSCs store vitamin A and transform to myofibroblasts, which are predominant in the extracellular matrix (ECM).³ What is the underlying molecular mechanism of the transition of the phenotype of Q-HSCs? An appealing perspective is the investigation of metabolic demands in myofibroblastic HSCs. The bioenergetics and biosynthetic requirements of myofibroblastic HSCs are similar to those of highly proliferative cancer cells.⁴ A growing body of evidence suggests that glutaminolysis is a key component of metabolic reprogramming necessary to fuel cancer cell growth.⁵ In a study by Du *et al.*,⁶ inhibition of glutaminolysis was shown to block the accumulation of myofibroblasts and fibrogenesis in mice with liver fibrosis. Glutamine is the most abundant amino acid in mammalian plasma. Specifically, the glutaminolysis pathway converts glutamine to glutamate and glutamate to alpha-ketoglutarate (α-KG).⁷ Glutamine-derived α-KG is regenerated to enhance the activity of tricarboxylic acid cycle, increasing both ATP production and key metabolic intermediates for the biosynthesis of amino acids, nucleic acids, and lipids.⁸

There has been much interest in alanine, serine, cysteine transporter 2 (ASCT2; SLC1A5), which participates in glutamine transport and metabolism.⁹ ASCT2 is an amino acid exchanger, and has more recently been described as an amino acid “harmonizer” that ensures adequate intra-

Keywords: ASCT2; O-GlcNAcylation; Stability; Trafficking; Liver fibrosis.

Abbreviations: α-KG, alpha-ketoglutarate; α-SMA, alpha smooth muscle actin; ASCT2, alanine, serine, cysteine transporter 2; CCl₄, carbon tetrachloride; CHX, cycloheximide; Col1α1, alpha-1 type I collagen; ECM, extracellular matrix; HSC, hepatic stellate cell; OCR, oxygen consumption rate; OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase; PTMs, post-translational modifications.

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cellular levels of all its substrates.¹⁰ Therefore, ASCT2 is an attractive and putative therapeutic target because of its high expression in many cancers. One of the most intriguing aspects of cell biology is the regulation of proteins via interactions with other proteins and/or by post-translational modifications (PTMs).¹¹ Protein glycosylation is acknowledged as one of the major post-translational modifications, with significant effects on protein folding, conformation, distribution, stability, and activity.¹² It is thought that N-glycosylation of ASCT2 suggests the existence of the coordinated regulation of glucose and glutamine metabolism.¹³ Therefore, it is important to elucidate the biological significance of these PTMs. An important form of serine/threonine-linked (O-linked) glycosylation is also a major modification of many cell surface and secreted proteins.¹⁴ Here we examined whether there is O-GlcNAcylation modification of ASCT2 and investigated its role in HSCs activation and liver fibrosis. We also explored the effects of specific O-GlcNAcylation modification sites of ASCT2 on its expression and function, which could provide a putative therapeutic target.

Methods

Cell culture and transfection

LX2 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin in a sterile incubator at 37°C with 5% CO₂. ASCT2 siRNA (# sc-60210) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). ASCT2 plasmid, ASCT2^{WT} plasmid, and ASCT2^{T122A} mutation plasmid were constructed by KeyGEN BioTECH Co. Ltd. (Nanjing, China). The sequences for plasmid constructs are listed in Supplementary Tables 1 and 2. Cell transfection was performed as recommended by the manufacturer of the transfection reagent. Briefly, LX2 cells were inoculated in a 6-well plate and grown to 60% to 70% confluence. Lipofectamine 2000 transfection reagent (# 1668-019; Life Technologies, Waltham, USA) was used following the manufacturer's instructions. After transfection, the cells were cultured in a 5% CO₂ incubator at 37°C for the subsequent experiments.

Western blotting

LX2 cells were seeded in cell culture dishes and cultured as described above. The cells were scraped off the culture dishes with ice-cold lysis buffer, and the lysates were harvested after agitation for 30 m at 4°C. Livers were dissected on ice, and disrupted with an electric homogenizer in ice-cold lysis buffer after centrifugation in a microcentrifuge for 20 m at 4°C, the lysates were heated to 95°C for 15 m to denature the proteins after addition of the denatured lysis buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used for western blot assays as described elsewhere.¹⁵ The antibodies used to assay relative protein expression are listed in Supplementary Table 3.

Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen). cDNA was generated using Superscript II reverse transcriptase (Yeasen, Shanghai, China) following the manufacturer's instructions. Real-time PCR was performed with

SYBR Green PCR kits (Yeasen) following the manufacturer's instructions. Results were normalized to mouse GAPDH or human GAPDH based on the threshold cycle (C_t) and relative fold-change calculated by the 2^{-ΔΔC_t} method. The sequences of primers for real-time PCR are listed in Supplementary Table 4.

Immunoprecipitation

LX2 cells lysates were obtained as above. Immunoprecipitation was used for antigen detection and identifying physiologically relevant protein-protein interactions. Protein A/G Plus MaqPoly Beads (RM09008; ABclonal, Wuhan, China) were prepared and performed in an immunoprecipitation assay. The detailed procedure has been previously described.¹⁶ The antibodies used are listed in Supplementary Table 3.

Immunofluorescence assay

LX-2 cells were used for immunofluorescence assay as described elsewhere.¹⁷ 4',6-Diamidino-2-phenylindole (DAPI) (#D9542; Sigma-Aldrich, Darmstadt, Germany) was used for nuclear staining. Representative images were examined by fluorescence microscopy (Zeiss, Oberkochen, Germany).

Determination of protein half life

Cycloheximide (CHX) was used to inhibit protein synthesis. LX2 cells were treated with CHX for 0, 2, 4, 8, and 24 h, and then cells lysates were obtained as above and protein expression was assayed by western blotting. CHX treatment time was plotted on the abscissa, and the amount of protein was plotted on the ordinate. The time needed to degrade half the protein was the protein half-life.

Prediction of O-GlcNAcylation sites

The ASCT2 protein sequence was acquired from (https://www.ncbi.nlm.nih.gov/nuccore/NM_001145144.2). The O-linked β-N-acetylglucosamine (O-GlcNAc) site of ASCT2 was predicted as described in the instructions (<http://glycomine.erc.monash.edu/Lab/GlycoMine/>).¹⁸

Confocal microscope

Colocalization of proteins and organelles was observed with a laser confocal scanning microscope (TCS SP5; Leica, Wetzlar, Germany) and Dil dye (# C1036; Beyotime Biotechnology, Shanghai, China) a red fluorescent probe was used for the localization of cell membranes. Determination of protein was consistent with the above immunofluorescence assay.

Glutamine, glutamate, α-KG and GSH measurements

LX2 cells lysates were obtained as above. Intracellular glutamine, glutamate, α-KG, and GSH levels were determined using glutamine kits (# JL19428; Jianglai, Shanghai, China), glutamate kit (# A074-1-1; Jiancheng, Nanjing, China), α-KG kit (# JL48612; Jianglai), and glutathione kit (# BB-4711; BestBio, Nanjing, China), following the manufacturer's instructions.

Extracellular flux analysis (Seahorse assay)

The assays were performed with an Agilent Seahorse XF24 bioanalyzer (Agilent, Santa Clara, USA). Briefly, LX2 cells were isolated and spun onto XF24 Cell-Tak (# 354240 BD Bioscience, New Jersey, USA) coated plates in Seahorse XF RPMI 1640 media supplemented with glutamine, sodium pyruvate, and glucose. For immediate metabolic response, cells were treated with 1.0 μ M concentrations of oligomycin (ATP synthase inhibitor of complex V), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, electron transport chain uncoupler), and rotenone with antimycin A (which are electron transport chain complex I and III uncouplers, respectively) throughout the analysis. Mitochondrial oxidative phosphorylation was measured by the oxygen consumption rate (OCR). All assays were performed in triplicate, and repeats were performed for this experiment.

Animal experiments

Animal experiments were approved by the Nanjing University of Chinese Medicine (Nanjing, China) and the local animal protection and utilization committee (Ethics Committee approval number: 202009A043). Forty SPF male institute of Cancer Research mice, weighing 20–25 g, were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) Before performing the procedures, the animals were fed adaptively for 1 week and housed in a controlled environment at a room temperature of 20 \pm 2°C, relative humidity: 40 \pm 5%, and a 12 h light/dark cycle). AA8-ASCT2 short hairpin RNA were administered by tail vein injection for knockdown of ASCT2. Intraperitoneal injection of CCl₄ in olive oil mixture (1:9 (v/v), 5 mL/kg body weight), was administered to model liver fibrosis in mice for 8 weeks. After the last administration, mice were sacrificed and liver sections were cut and fixed in 4% buffered paraformaldehyde for histopathological examination. The remaining tissue was immediately stored in a freezer at -80°C for western blot assays.

Immunohistochemical staining for histological analysis

Fixed livers sections were used for immunohistochemical (IHC) staining of alpha smooth muscle actin (α -SMA), O-GlcNAcylation, OGT, and ASCT2, as previously described.¹⁹

Immunohistofluorescence

For triple immunohistofluorescence, 5 μ m sections of liver tissue were fixed in acetone for immunohistofluorescence, and stained with antibodies against α -SMA, O-GlcNAcylation, and ASCT2 and 4',6-diamidino-2-phenylindole (DAPI), and examined with a confocal laser scanning microscope (Leica).

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data were reported as means \pm standard error of the mean. Between-group differences were assessed with Student's *t*-tests; multigroup comparisons were performed with one-way analysis of variance and *post-hoc* Dunnett's test. *P*-values <0.05 were considered significant.

Results

Increased O-GlcNAcylation modification of ASCT2 regulated activation of HSCs

We first characterized O-GlcNAcylation modification, ASCT2 expression, and HSC activation in mice with CCl₄-induced liver fibrosis. Expression of ASCT2, O-GlcNAcylation, OGT, and the HSC activation marker α -SMA was increased in fibrotic mice livers. We found positive correlations between ASCT2 and α -SMA, and between ASCT2 and O-GlcNAcylation modification (Fig. 1A). These findings indicated a role of ASCT2 in O-GlcNAcylation and HSC activation. LX2 cells, which had a myofibroblastic phenotype indicating activated HSC were transfected by ASCT2 siRNA and ASCT2 plasmids to regulate the expression of ASCT2. ASCT2 siRNA strongly inhibited ASCT2 expression, and ASCT2 plasmid transfection increased the expression of ASCT2 in LX2 cells (Fig. 1B). Importantly, western blot assays revealed greatly increased O-GlcNAcylation level in LX2 cells overexpressing ASCT2, which were significantly lower than the expression in ASCT2-knockdown LX2 cells. O-GlcNAcylation is catalyzed by a highly conserved O-GlcNAc transferase (OGT) and O-GlcNAc hydrolase (OGA). We also examined the expressions of OGT and OGA in HSCs, and found positive expression of OGT with O-GlcNAcylation signal, whereas there was no change in OGA expression in LX2 cells (Fig. 1B). We further tested the binding of O-GlcNAcylation and ASCT2. Immunoprecipitation analysis revealed that ASCT2 interacted with O-GlcNAcylation and confirmed that ASCT2 was strongly O-GlcNAcylated (Fig. 1C). Next, we evaluated the effects of O-GlcNAcylated ASCT2 on HSCs activation. Results of RT-PCR, western blotting, and immunofluorescence analysis showed a significant positive correlation between ASCT2 expression and the activation marker α -SMA. Detection of another myofibroblastic marker alpha-1 type I collagen (Col1 α 1) also was consistent with that result (Fig. 1D, F). The findings support the association of HSC and activation by O-GlcNAcylation of ASCT2.

O-GlcNAcylation of ASCT2 interacted with OGT contributing to ASCT2 protein stability in activated HSCs

The above results demonstrated that OGT was positively expressed with O-GlcNAcylation modification of ASCT2. To better understand the connection between OGT and O-GlcNAcylation of ASCT2, we performed co-immunoprecipitation assay in LX2 cells. We found that ASCT2 interacted with OGT, which demonstrated that OGT as indeed involved in O-GlcNAcylation of ASCT2 in activated HSCs (Fig. 2A). We next investigated the mechanism by which OGT-mediated O-GlcNAcylation regulated ASCT2 expression. As showed in cycloheximide (CHX) chase experiments, we found that treatment with UDP-GlcNAc prolonged the half-life of ASCT2 protein, but the OGT inhibitor OSMI-1 reversed the effect (Fig. 2B–D). The results demonstrated that interaction with OGT-mediated O-GlcNAcylation is capable of enhancing ASCT2 stability in HSCs.

O-GlcNAcylation of ASCT2 at Thr122 is required for activation of HSCs

O-GlcNAcylation has been shown to involve serine/threonine residues.²⁰ Therefore, we used the GlycoMine website (<https://glycomine.erc.monash.edu/Lab/GlycoMine/>) to predict the sites of O-GlcNAcylation modification of ASCT2. The thr122 site was predicted as a potential O-GlcNAcylation

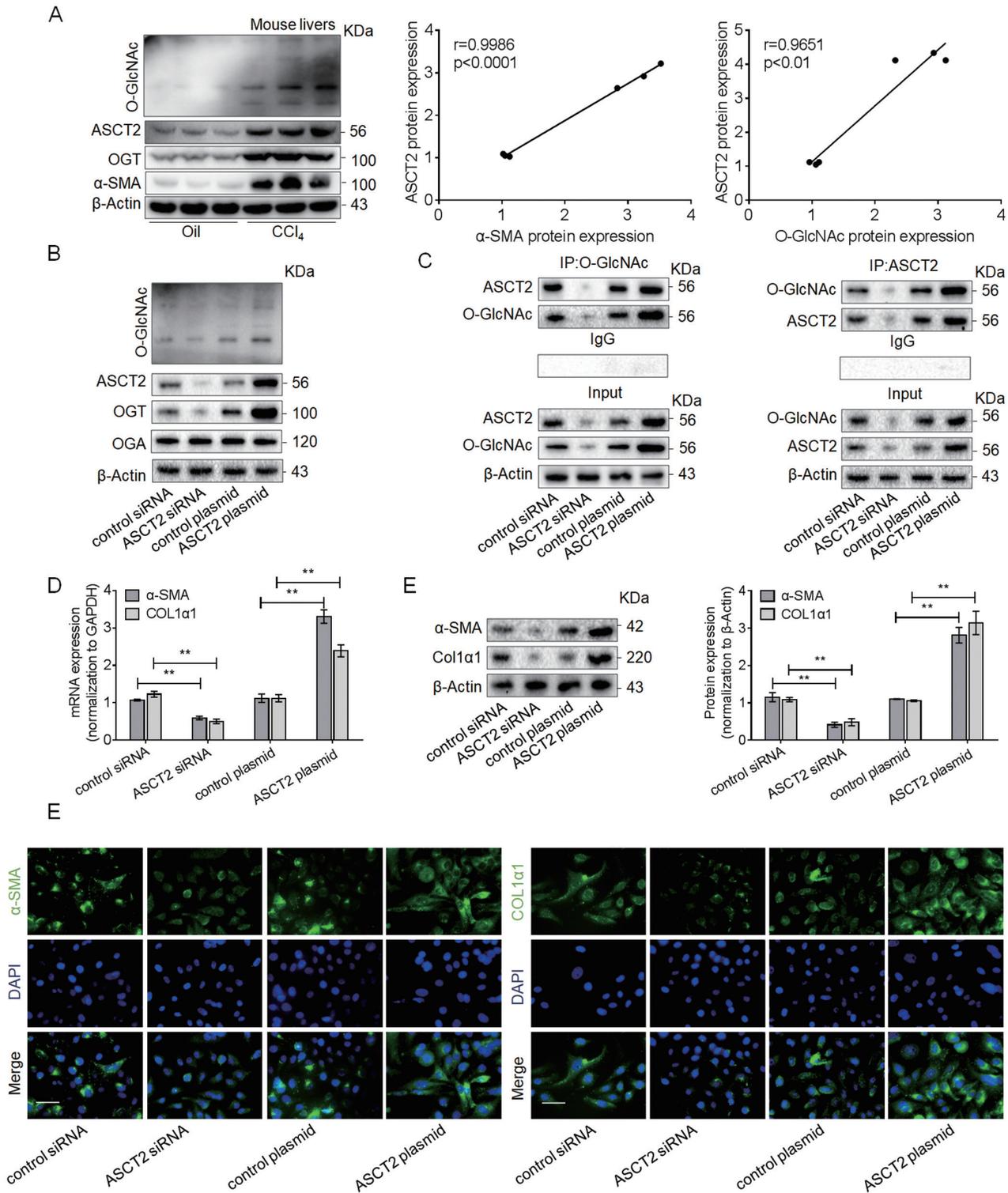


Fig. 1. Increased O-GlcNAcylation levels in ASCT2-regulated activation of hepatic stellate cells. Mice were given CCl₄ to induce liver fibrosis. *In vitro* studies were conducted in HSCs; LX2 cells were transfected with ASCT2 siRNA or ASCT2 plasmids. (A) Western blot results of protein expression and Spearman's *r* correlation of ASCT2, O-GlcNAcylation, and α-SMA in fibrotic mouse livers. (B) Western blot assay of indicated protein expression in LX2 cells. (C) ASCT2 or O-GlcNAcylation immunoprecipitated by an anti-ASCT2 antibody or anti-O-GlcNAcylation antibody. (D) RT-PCR analysis of α-SMA and Col1a1 expression normalized to those of GAPDH. Mean ± standard deviation of three independent assays. **p* <0.05, ***p* <0.01, and ****p* <0.001 vs. control siRNA or control plasmid. (E) Western blot analysis of the α-SMA and Col1a1 expression normalized to β-actin. Mean ± SD values of three independent assays. **p* <0.05, ***p* <0.01, and ****p* <0.001 vs. control siRNA or control plasmid. (F) Immunofluorescence for α-SMA (left green) and Col1a1 (right green) in LX2 cells. DAPI-stained nuclei (blue). Scale bar = 20 μm. α-SMA, alpha smooth muscle actin; ASCT2, alanine, serine, cysteine transporter 2; CCl₄, carbon tetrachloride; Col1a1, alpha-1 type I collagen.

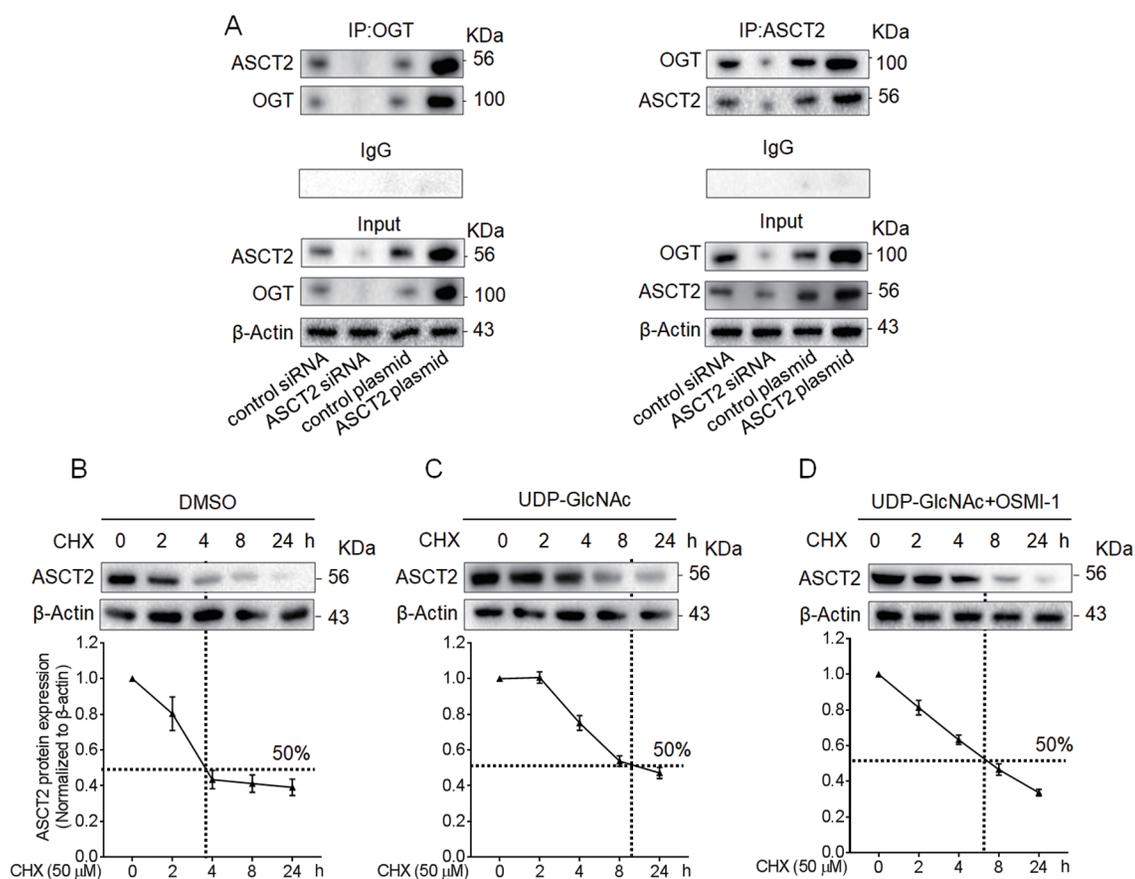


Fig. 2. O-GlcNAcylation of ASCT2 interacted with OGT contributing to ASCT2 protein stability in hepatic stellate cells. *In vitro* studies of HSCs, with LX2 cells transfected with ASCT2 siRNA or ASCT2 plasmids. (A) Co-immunoprecipitation of OGT and ASCT2, and OGT were observed by immunoprecipitation with anti-ASCT2 antibodies. ASCT2 was detected by immunoprecipitation with anti-OGT antibodies. (B–D) LX2 cells were harvested after addition of CHX (50 μM). Western blot assay of ASCT2 expression normalized to that of β-actin with 0 h as 100%. Representative images are shown, and mean ± standard deviation from three independent assay. α-SMA, alpha smooth muscle actin; ASCT2, alanine, serine, cysteine transporter 2; CHX, cycloheximide; Col1α1, alpha-1 type I collagen; OGT, O-GlcNAc transferase.

tion site of ASCT2 (Fig. 3A). We next used point mutation experiments to define the predicted sites. Western blotting showed that when Thr122 was mutated to alanine (ASCT2^{T122A} plasmid), O-GlcNAcylation of ASCT2 was significantly inhibited compared with ASCT2^{WT}. That suggested that Thr122 is the O-GlcNAc site of ASCT2. We also assayed the expression of ASCT2 and OGT. After Thr122 was mutated, the levels of ASCT2 and OGT were also significantly reduced (Fig. 3B). We also found that the expression of ASCT2 switched from nuclear to cytoplasmic in ASCT2^{WT} compared with ASCT2^{T122A} (Fig. 3C). That may have an important use and piqued our interest. RT-PCR, western blotting, and IHC found that the expression of α-SMA and Col1α1 were also down-regulated in ASCT2^{T122A} relative to ASCT2^{WT} (Fig. 3D, F). The findings demonstrated that O-GlcNAcylation of ASCT2 at Thr122 is required for the activation of HSCs.

Thr122 O-GlcNAcylation mediates membrane trafficking of ASCT2 for glutaminolysis in activated HSCs

To further uncover the effect of O-GlcNAcylation on the function of ASCT2, we analyzed the ASCT2 sequence (PDB: 6GCT) and found the T122 O-GlcNAcylation site was located in the transmembrane region domain of ASCT2 isoform. The N-glycosylation of ASCT2 has been investigated as the ca-

nonical way to indicate the definitive localization of a protein in the plasma membrane.⁹ Therefore, we performed confocal immunofluorescence staining with a Dil tracker for cell membrane tracking and staining with anti-ASCT2 antibody for detection of ASCT2. We found that ASCT2^{WT} significantly translocated ASCT2 to the cell membrane. However, the ASCT2^{T122A} mutant significantly increased the nuclear accumulation of ASCT2 (Fig. 4A, B). The findings suggest that the O-GlcNAcylation of ASCT2 influenced the definitive localization in the plasma membrane. Previous studies indicated that ASCT2 enhanced expression in highly proliferative cells to fulfill the increased glutamine demand. In addition, inhibition of glutaminolysis disrupted the transdifferentiation of HSCs. Next, we characterized the functional role of ASCT2 O-GlcNAcylation at T122 on HSCs activation. To address this question, we drew a schematic diagram of glutaminolysis in HSCs (Fig. 4G). We investigated glutaminolysis activity by glutamine, glutamate, α-KG, and GSH assays. In that step, LX2 cells were transfected with ASCT2^{WT} and mutant ASCT2^{T122A}. The results indicated that WT ASCT2 significantly increased the glutaminolysis activity of HSCs. On the contrary, the T122A mutant down-regulated glutaminolysis activity (Fig. 4C–F). We also showed that transfection of LX2 cells with ASCT2^{WT} induced changes in mitochondrial respiration (Fig. 4H). Compared with cells transfected with control plasmids, cells transfected with ASCT2^{WT} plasmids

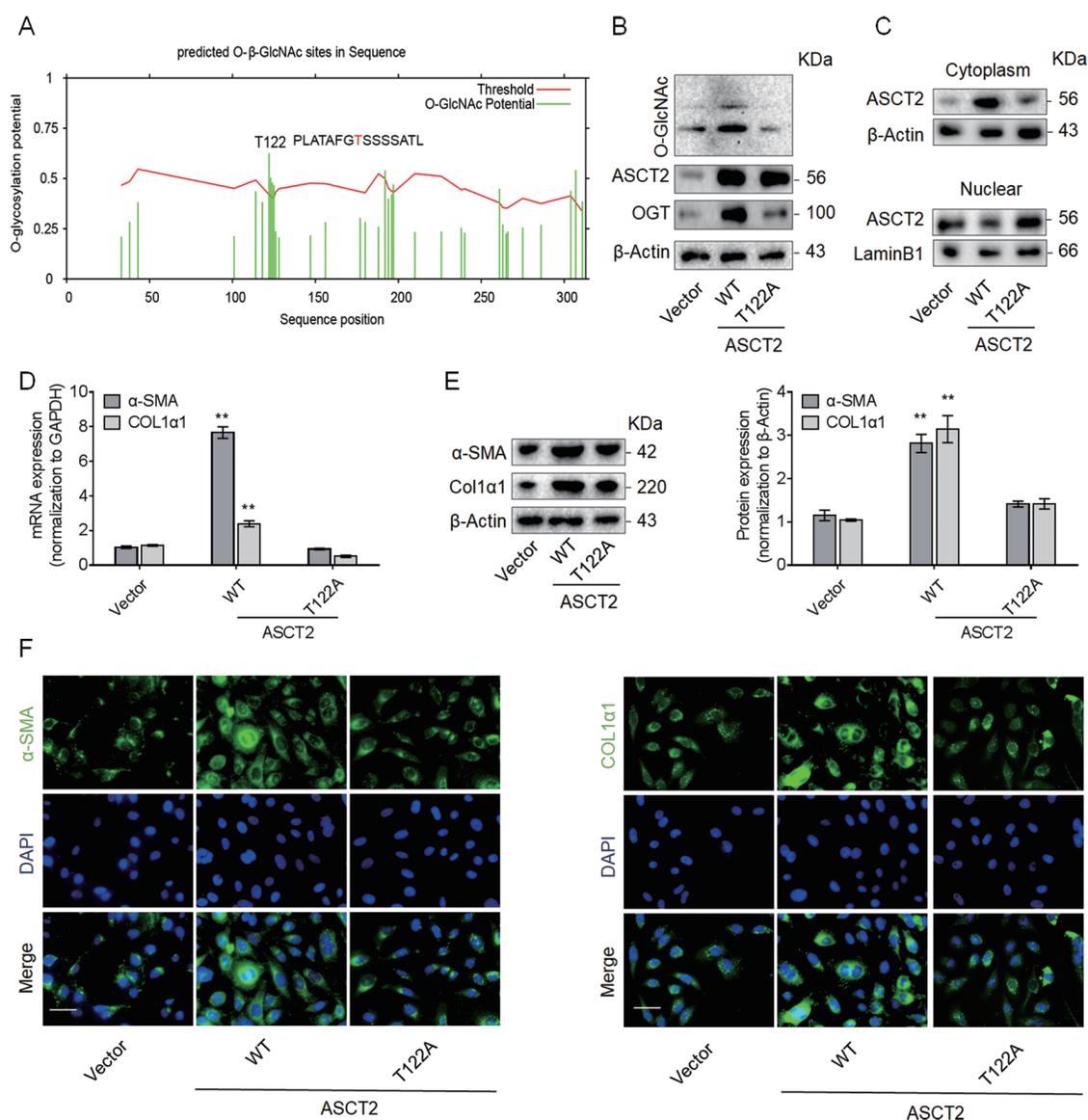


Fig. 3. O-GlcNAcylation of ASCT2 at Thr122 is required for the activation of hepatic stellate cells. (A) GlycoMine website was used to predict the site of O-GlcNAc modification on ASCT2. Representative images are shown; T122 was the predicted site. (B) *In vitro* studies were conducted in HSCs; ASCT2 WT or ASCT2 T122A was transfected into LX2 cells, and cells were treated as shown for 24 h before analysis. Results of western blot analysis showing the expression of the indicated proteins in LX2 cells. (C) Western blot analysis of ASCT2 expression in the nucleus and cytoplasm. (D) Real-time PCR analysis of the α -SMA and COL1 α 1 expression normalized to GAPDH. Mean \pm standard deviation of three independent assays. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. control siRNA or control plasmid. (E) Western blot analysis of α -SMA and COL1 α 1 expression normalized to β -actin. Mean \pm standard deviation of three independent assays. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. control siRNA or control plasmid. (F) Immunofluorescence for α -SMA (left green) and Col1 α 1 (right green) in LX2 cells. DAPI-stained nuclei (blue). Scale bar = 20 μ m. α -SMA, alpha smooth muscle actin; ASCT2, alanine, serine, cysteine transporter 2; Col1 α 1, alpha-1 type I collagen; DAPI, 4',6-Diamidino-2-phenylindole.

showed increased basal and maximal respiratory capacity-linked OCR, but the effect was removed by the ASCT2^{T122A} mutation. This indicated that ASCT2 affected mitochondrial respiration (Fig. 4I, J). The observations indicate that the O-GlcNAcylation status of ASCT2 in Thr122 is definitive localization in the membrane and glutaminolysis in HSCs.

O-GlcNAcylation of ASCT2 and OGT is increased in carbon tetrachloride-induced mice liver fibrosis *in vivo*

Given the significant role of ASCT2 O-GlcNAcylation at Thr122 in HSC activation, we examined its effects on liver

fibrogenesis *in vivo*. To determine whether ASCT2 plays an active role in this process, AAV-ASCT2 shRNA was used to delete ASCT2 *in vivo*. Hematoxylin and eosin staining, Sirius red staining, and IHC of α -SMA found typical fibrotic pathological changes in the model mouse livers compared with the control mice. However, the pathological fibrosis was improved by ASCT2 deletion (Fig. 5A). To better define the expression of ASCT2 O-GlcNAcylation, IHC found increased expression of O-GlcNAcylation, OGT, and ASCT2 protein in mice with liver fibrosis that was reversed by ASCT2 deletion (Fig. 5B). In addition, treatment with CCl₄ markedly induced HSC activation, O-GlcNAcylation, and ASCT2 both in mouse liver and mouse primary HSC cultures; however, the

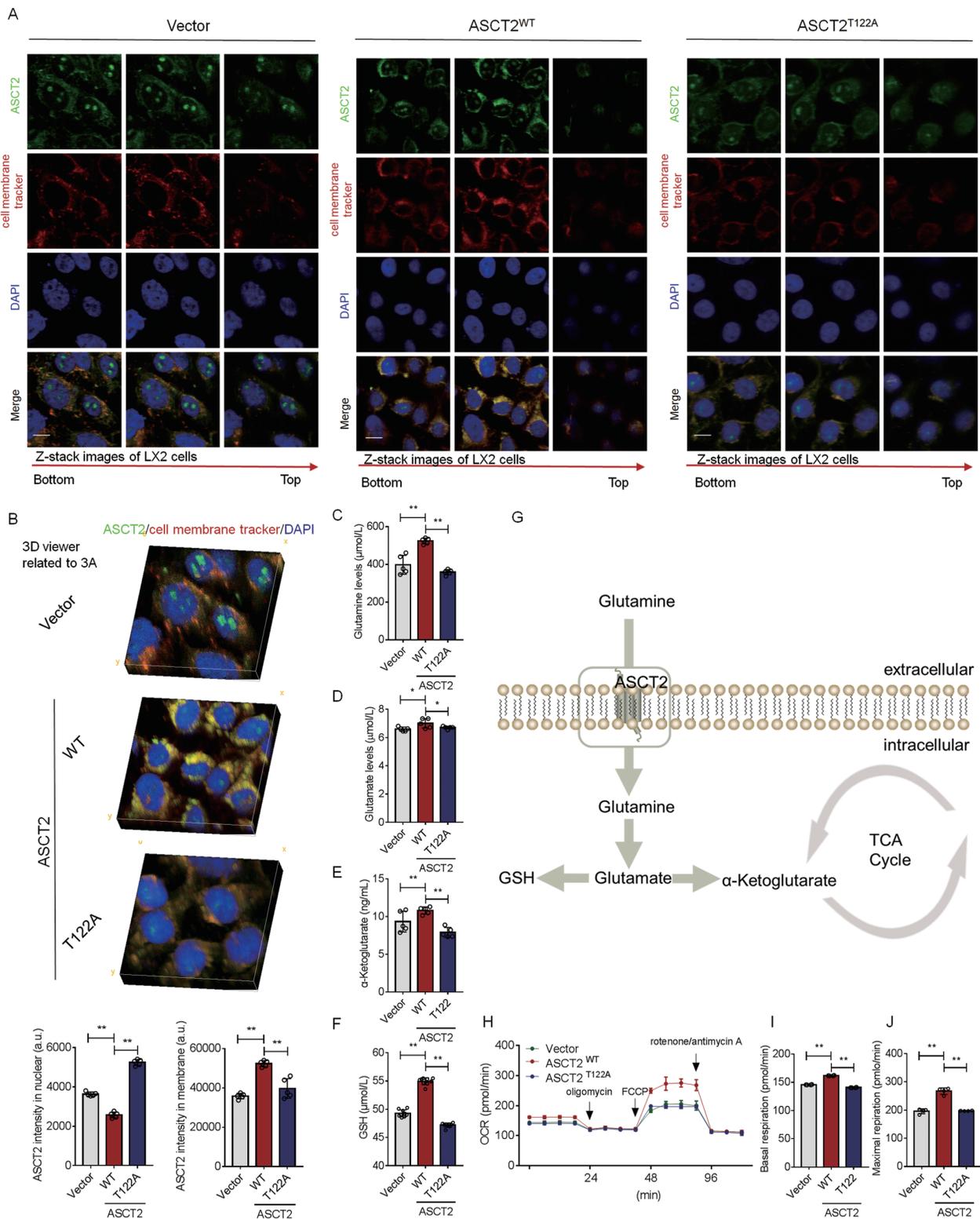


Fig. 4. Thr122 O-GlcNAcylation mediates membrane trafficking of ASCT2 for glutaminolysis in hepatic stellate cells. *In vitro* studies were conducted in HSCs; wild-type or T122A were transfected into LX2 cells, and cells were treated as indicated for 24 h before analysis. (A) Z-stack image of immunofluorescence staining of Dil tracker (red) and anti-ASCT2 (green) in LX2 cells. Scale bar = 10 μm. (B) Confocal immunofluorescence staining of Dil tracker (red) and anti-ASCT2 (green) in LX2 cells. DAPI-stained nuclei (blue). Scale bar = 10 μm. (C-F) Glutamine, Glutamate, α-ketoglutarate and GSH assay in LX2 cells. Data are means ± standard error of the mean, n=5. (G) Schematic illustration of glutaminolysis in hepatic stellate cells. (H-J) OCR was determined by Seahorse assay. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vector. ASCT2, alanine, serine, cysteine transporter 2; GSH, glutathione; OCR, oxygen consumption rate.

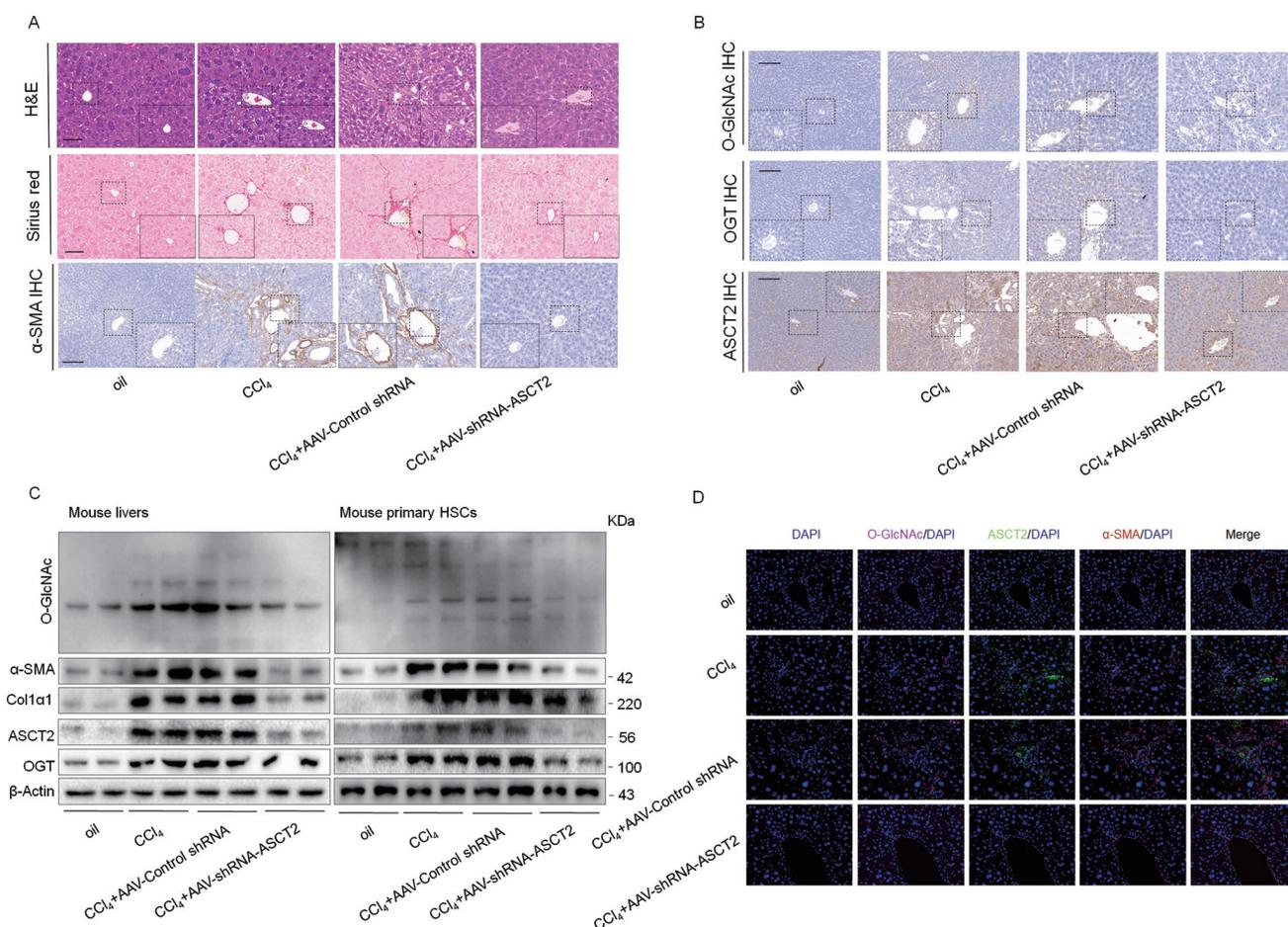


Fig. 5. O-GlcNAcylation of ASCT2 is increased in carbon tetrachloride-induced mice liver fibrosis and positively correlated with fibrogenesis. *In vivo* studies were conducted in liver. Carbon tetrachloride-induced mice liver fibrosis model was used for analysis. ASCT2 AAV-shRNA was used for ASCT2 depletion in mice. (A) 4 μ m liver sections were stained with hematoxylin and eosin, Sirius red, and immunohistochemistry of α -SMA for histopathological study. Scale bars = 50 μ m; (boxes): 25 μ m. Representative images are shown. (B) O-GlcNAcylation, OGT and ASCT2 immunohistochemistry in mouse liver. Scale bars = 50 μ m; (boxes): 25 μ m. Representative images are shown. (C) Western blot analysis of protein expression in mouse liver or mouse primary HSCs. (D) Triple immunohistofluorescence of α -SMA, O-GlcNAcylation, and ASCT2 in mouse liver sections. Nuclei were counterstained with DAPI. α -SMA, alpha smooth muscle actin; ASCT2, alanine, serine, cysteine transporter 2; OGT, O-GlcNAc transferase; DAPI, 4',6-Diamidino-2-phenylindole.

effects were abolished by ASCT2 deletion (Fig. 5C). Furthermore, triple immunohistofluorescence for α -SMA, O-GlcNAcylation, and ASCT2 showed increased O-GlcNAcylation modification of ASCT2 in activated HSCs that was reduced by ASCT2 depletion (Fig. 5D). Overall, the results confirmed the importance of ASCT2 and O-GlcNAcylation in controlling liver fibrogenesis and support the potential application of ASCT2 as a novel antifibrotic target.

Discussion

Previous studies have identified the key regulators of HSC activation and function. Positive and negative regulators of profibrotic activity have emerged as potential therapeutic targets for liver fibrosis.^{21–23} In that context, our work identified a previously unrecognized PTM in activated HSCs. ASCT2 O-GlcNAcylation at Thr122 promoted its stability, cell membrane localization, and interaction with OGT, thus coordinating glutaminolysis in activated HSCs, and was positively correlated with HSC activation and liver fibrogenesis (Fig. 6).

ASCT2, an alanine, serine, cysteine-preferring trans-

porter, mediated the uptake of glutamine, a conditionally essential amino acid in rapidly proliferating tumor cells.²⁴ There has been an increased focus on glutamine transport and metabolism as a putative therapeutic target owing to its high expression in many cancers. Previous studies have shown that glutaminolysis is a key component of the metabolic reprogramming of myfibroblasts and fibrosis progression in mice with liver fibrosis, which provides clues for understanding the role of ASCT2 in HSC activation and reversion. In doing so, it could unearth new therapeutic targets.^{6,25} O-GlcNAcylation is a dynamic and inducible PTM on serine and/or threonine residues of nuclear and cytosolic proteins.²⁶ N-glycosylation of ASCT2 has been investigated as a canonical pathway to determine the definitive localization of a protein in the plasma membrane.⁹ In our study, we aimed to characterize the O-GlcNAcylation expression pattern of the key glutamine transporter ASCT2 and its role in HSC activation. We found increased expression of O-GlcNAcylation and ASCT2 in a mouse model of liver fibrosis. Inhibition of ASCT2 by ASCT2 siRNA transfection and overexpression of ASCT2 using ASCT2 plasmid transfection showed a different effect on O-GlcNAcylation expression pattern. Co-immunoprecipitation of ASCT2 and

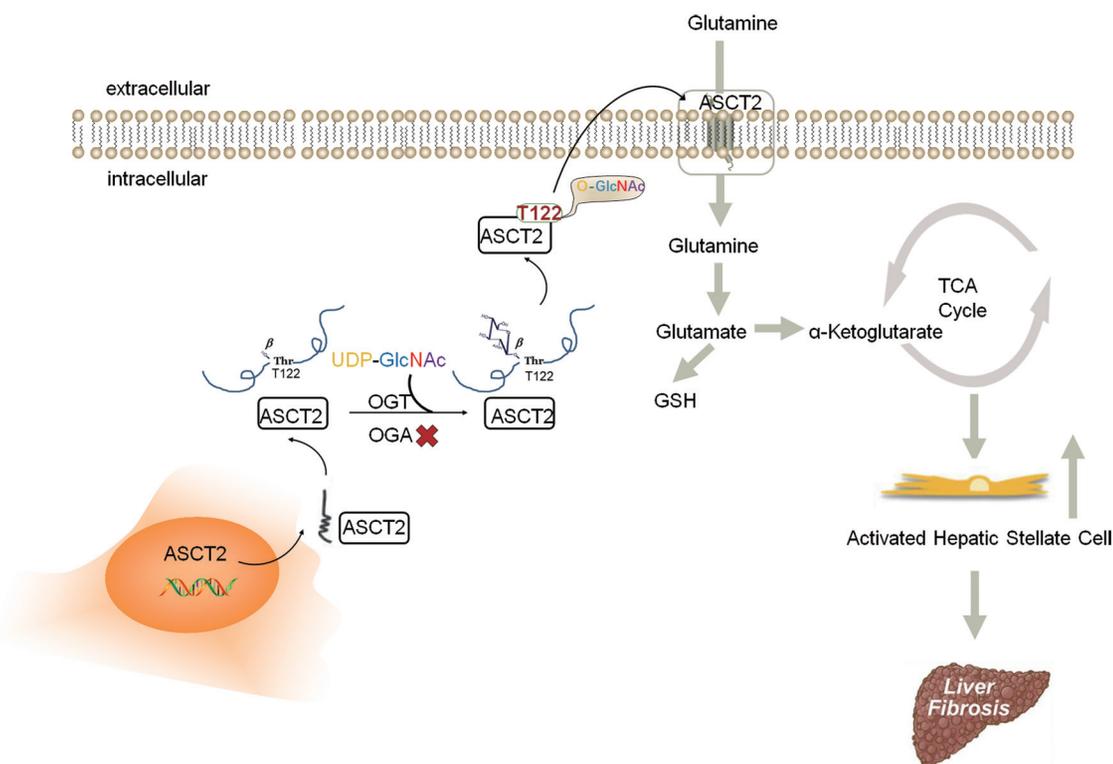


Fig. 6. Schematic illustration of the molecular mechanism underlying the role of ASCT2 O-GlcNAcylation in HSC activation and liver fibrosis. O-GlcNAcylation of ASCT2 at Thr122 promotes stability, cell membrane localization, and interaction with OGT not OGA, which thus coordinates glutaminolysis in activated HSCs. Increased ASCT2 O-GlcNAcylation is positively correlated with mice liver fibrogenesis and thereby has potential therapeutic benefits for liver fibrosis. ASCT2, alanine, serine, cysteine transporter 2; OGA, O-GlcNAc hydrolase; OGT, O-GlcNAc transferase.

O-GlcNAcylation confirmed that ASCT2 was modified by O-GlcNAcylation and had broader effects on the expression of α -SMA and Col1a1 for HSCs activation (Fig. 1). Going further, we focused on the specific mechanism of O-GlcNAcylation of ASCT2. The results showed that the interaction of ASCT2 with OGT and PTMs regulated ASCT2 protein stability (Fig. 2). We next sought to identify the site(s) of O-GlcNAcylation on ASCT2 using an online prediction tool. We identified the putative O-GlcNAcylation site (Thr122) on ASCT2. Further, we mutated Thr122 to Ser122 and found that the mutation reduced the O-GlcNAcylation signal to a large degree, suggesting that T122 was the glycosylation site on ASCT2. We examined the effect of O-GlcNAcylation of ASCT2 at T122 on the activation of HSCs and observed that mutation of ASCT2 at T122A also inhibited the activation of HSCs (Fig. 3). Nevertheless, the correlation between O-GlcNAcylation of ASCT2 and its translation remains largely unknown. The positioning of the ASCT2 and cell membrane trafficking results demonstrated that Thr122 O-GlcNAcylation mediated membrane trafficking of ASCT2. Given that ASCT2 participates in glutaminolysis, OCR measurement by the Seahorse assay showed that the Thr122 O-GlcNAcylation of ASCT2 increased mitochondrial respiration. Our results demonstrated that Thr122 O-GlcNAcylation of ASCT2 contributed to glutaminolysis in HSCs (Fig. 4). To probe the clinical relevance of ASCT2 glycosylation, we examined O-GlcNAcylation levels of ASCT2 in mice liver fibrosis tissues and vehicle tissues. We found that O-GlcNAcylation of ASCT2 was increased in CCl₄-induced mice liver fibrosis and had a positive correlation with the activation of HSCs. For validation, we used AAV-shRNA ASCT2 to knock down the expression of ASCT2 and found that the increased O-GlcNAcylation levels were decreased by

ASCT2 deletion. We also isolated primary HSCs from mice, and obtained consistent results. The results suggest that induced O-GlcNAcylation of ASCT2 in HSCs contributed to liver fibrogenesis (Fig. 5). Overall, the current work suggests that ASCT2 is a potential therapeutic target against HSC activation and liver fibrogenesis. Further testing of ASCT2 blocking antibodies or inhibitors will be critical to assess whether ASCT2 inhibition is a viable anti-fibrosis strategy for clinical application.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study and supervised the data collection (FW), analyzed and interpreted the data (FW, LC, BZ), provided material and technique support (ZL, MS, LT, ZZ, JS), reviewed the draft of the manuscript and made critical revisions (ST, FZ, SZ). All authors have read and approved the manuscript.

Ethical statement

The animal procedures were approved by the Institutional and Local Committee on the Care and Use of Animals of Nanjing University of Chinese Medicine. All animals were given humane care following the guidelines of the National Institutes of Health (USA).

Data sharing statement

The raw data used to support the findings of this study are available from the corresponding author upon request.

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