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FTY720 inhibits the activation of pancreatic stellate cells by promoting apoptosis and suppressing autophagy via the AMPK/mTOR pathway

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ABSTRACT

Aims: Pancreatic stellate cells (PSCs) play a critical role in the development of pancreatic fibrosis. Any agents that can affect PSC activation could become potential candidates for treating pancreatic fibrosis. FTY720 can attenuate chronic pancreatic fibrosis by suppressing T-cell infiltration, but its effect on PSCs remains unknown. This study was conducted to investigate the effects of FTY720 on PSC activation in cultured rat PSCs.

Main methods: The viability of PSCs after FTY720 treatment was detected by MTT. Cell proliferation and migration analysis was performed using the iCELLigence System and a Transwell assay. Cell apoptosis was assessed by flow cytometry, western blot and an activity assay. The mitochondrial membrane potential (MMP) was assessed by JC-1 staining. The expression of α-SMA, collagen I, fibronectin, Beclin-1, Atg5, P62 and LC3B were analysed by immunofluorescence, quantitative real-time PCR and western blot. Rapamycin and phenformin hydrochloride were used to determine whether FTY720 inhibits PSC autophagy by the AMPK/mTOR pathway.

Key findings: FTY720 supressed PSC viability, proliferation and migration. FTY720 inhibited PSC activation, induced PSC apoptosis and supressed PSC autophagy. We also confirmed that FTY720 inhibited PSC autophagy via the AMPK/mTOR pathway.

Significance: Our results indicated that FTY720 inhibited PSC activation by promoting cell apoptosis and inhibiting PSC autophagy by suppressing AMPK and activating the mTOR pathway. These findings may explain the therapeutic mechanisms of FTY720 in treating pancreatic fibrosis and further suggest that targeting autophagy and the related signalling pathways may provide new strategies for the treatment of pancreatic fibrosis.

1. Introduction

Pancreatic stellate cells (PSCs), which are located between the pancreatic lobules and the area surrounding the acinus, play a critical role in the development of pancreatic fibrosis [1]. In a healthy pancreas, PSCs are inactive and accompanied by lipid droplets that contain vitamin A. When inflammation or necrosis occurs in the pancreas, PSCs are activated and begin to proliferate rapidly and synthesize and secrete collagen type I (Col I), fibronectin (FN), laminin, and other extracellular matrix (ECM) components. A number of studies have suggested that activated PSCs are the main source of extracellular matrix (ECM) proteins that accumulate under pathological conditions, which lead to pancreatic fibrosis in chronic pancreatitis and pancreatic cancer [2].

Autophagy is an important process for maintaining cellular homeostasis [3], which participates in the occurrence of multiple diseases [4]. Many autophagy-related proteins (ATGs) (including ATG13, ATG5 and ATG12) participate in the process of autophagy, promoting the formation of autophagosomes expressing LC3-II. Sequestosome 1 (P62/SQSTM1) facilitates the autophagic degradation of ubiquitinated protein in lysosomes [5]. The formation of the autophagosomal membrane is controlled by an ATGL complex containing Bcl-2-interacting protein-1 (beclin-1) and the ULK complex (including ULK1). It has been reported that autophagy is required for PSC activation, which promotes pancreatic cancer growth and metastasis by tumour stromal

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interactions [6]. Our previous study confirmed that autophagy participates in PSC activation and showed that inhibiting autophagy could suppress PSC activation and increase ECM degradation in primary cultured PSCs [7]. Any agents that can inhibit PSC autophagy could be potential inhibitors of PSCs activation.

FTY720 is a structural analogue of the sphingosine-1-phosphate (S1P) and is derived from myriocin, a fungal derivative used in Chinese medicine [8]. FTY720 has been approved by the FDA for the treatment of multiple sclerosis [9]. The dominant action of FTY720 is largely attributed to auto-reactive T-cell sequestration in lymph nodes [8]. Recent studies have reported that FTY720 treatment can inhibit various animal models of fibrosis, such as hepatic fibrosis, renal fibrosis and pulmonary fibrosis [10,11]. A study also showed that FTY720 attenuates chronic pancreatitis in rats by suppressing T-cell infiltration and reducing myeloperoxidase activity and pancreatic hydroxyproline content in the pancreas [12]. However, the effect of FTY720 on PSC activation was unclear until now.

In this study, we investigated the effect of FTY720 on PSC activation by cell apoptosis and cell autophagy. Our research can provide more groundwork for FTY720-related clinical treatment strategies of pancreatic diseases, such as pancreatic cancer and chronic pancreatitis.

2. Materials and methods

2.1. Reagents

FTY720 was purchased from MedChem Express (Shanghai, China). Anti-α-SMA (A5228) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anti-collagen I (ABT123) was from purchased from Millipore (Billeric, MA, USA). Anti-LC3B was obtained from Novus Biologicals (Littleton, CO, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 5174), Atg5 (12994), Beclin-1 (3495), P62 (5114), p-AKT (4060s), p-mTOR (2971), p-AMPKα (2535), Caspase-3 (14220s), Caspase-9 (9508s) and Bax (2772) antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Fibronectin (ab2413) and Bcl-2 (ab196495) antibodies were purchased from Abcam (Cambridge, MA, USA). Rapamycin was purchased from Selleck Chemicals (Shanghai, China). Phenformin hydrochloride was provided by MedChem Express.

2.2. Isolation, identification and culture of pancreatic stellate cells

Pancreatic stellate cells (PSCs) were isolated from rat pancreases by Nycodenz density gradient centrifugation as previously described [7]. PSCs cultured over 3 generations were used for the experiments.

2.3. Cell viability assay

Cell viability was determined by MTT assay. PSCs were seeded in 96-well plates with 5×10^3 cells/well. After incubation with FTY720 (0, 0.5, 1, 5, 10 or 20 μM) for 24 h or 48 h, 20 μl of MTT (5 mg/ml) was added to the wells, and the cells were incubated at 37 °C for 4 h. Dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals. The absorbance (570 nm) was measured using a microplate reader (Kehua, China). The percentage of viable cells was determined using the following formula: cell viability (%) = (A570 treated / A570 control) × 100.

2.4. Real-time cell proliferation analysis

Real-time cell proliferation analysis (RTCA) was performed using iCELLigence System as described previously [13]. Briefly, 5×10^3 PSCs were seeded onto E-plates, integrated with gold microelectrode arrays, and RTCA was carried out with the iCELLigence System (ACEA, California, USA). After an 18 h initial incubation period on the E-plates, cells were treated with 0.5 μM, 1 μM or 2 μM FTY720. Non-treated samples were used as controls. The cell index was monitored for 72 h, with measurements taken every 5 min.

2.5. Cell migration assay

Cell migration was assessed by a Transwell assay (Millipore, Germany). First, 5×10^4 cells were seeded into the cell culture inserts with a pore size of 8.0 μm in a 24-well companion plate. After 24 h of incubation, the PSC migration towards the lower chamber was evaluated. Cells in the upper chamber were carefully removed by wiping with cotton-tipped swabs, and cells at the bottom of the membrane were fixed and stained with haematoxylin. The cells were imaged using a Leica DM4000B (Leica, Germany) and were counted in 5 randomly chosen fields (100× magnification).

2.6. Immunofluorescence staining

PSCs were seeded in 6-well plates with 5×10^4 cells/well. PSCs were treated with 0.5 μM FTY720, 1 μM FTY720 and 2 μM FTY720 separately for 24 h. The cells were fixed with neutral formalin and stained with α-SMA (dilution 1:200) and LC3B (dilution 1:200), and the nucleus was stained with propidium iodide (PI). The images were captured by fluorescence microscope.

2.7. Apoptosis analysis

PSCs were seeded in 6-well plates (2×10^5 cells per well) and treated with FTY720 (0.5, 1, 2 μM) for 24 h. The Annexin-V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Sungene, Tianjin, China) was used to evaluate cell apoptosis according to the manufacturer's instruction. The samples were detected by NovoCyte flow cytometry (ACEA Biosciences, California USA). The experiments were repeated three times.

2.8. Detection of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) is an indicator of mitochondrial function. MMP was measured using the lipophilic cationic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide). PSCs were seeded in 24 well plates and treated with FTY720 for 24 h. The cells were incubated with JC-1 staining buffer for 2 h and were then detected with a fluorescence microscope (Leica, Germany).

2.9. Caspase-3, Caspase-9 activity assay

PSCs were seeded in 6 well plates and treated with FTY720 for 24 h. Caspase-3 and Caspase-9 activities were detected using commercially available kits (KGA204 and KGA404) from KeyGen BioTech (Jiangsu, China) according to the manufacturer's instructions. Caspase activity was expressed as a ratio of the absorbance of control cells.

2.10. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted by TRIzol (Promega, China). Then, 1 μg of total RNA of each sample was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo, USA). cDNA templates were then amplified with specific primers in a Bio-Rad real-time PCR system (Bio-Rad, USA) using a DyNAmo Flash SYBR Green RT-qPCR Kit (cat. no. F-415L; Thermo). The relative amounts of mRNA of the target genes were normalized to the endogenous control, GAPDH, and presented as $2^{-\triangle\triangle Ct}$. Primer sequences for RT-qPCR analysis are listed in Table 1.

Table 1
Primer sequences used for RT-qPCR analysis.

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
α-SMA	AGGGAGTGATGGTTGGAATG	GATGATGCCGTGTTCTATCG
Collagen I	GGATAGGGACTTGTGTGA	GCTGGAAGAGTGAAGAGG
Fibronectin	GATTCTTCTGGCGTCTGCAC	GCCCCGGAACATGAGGATAG
Beclin-1	TGTTTGGAGATGTTGGAGCA	ATGGAAGGTCGCATTGAAGA
Atg5	TGAAGGAAGTTGTCTGGATAG	AAGTCTGTCCTTCCGCAGTC
LC3B	CGGAGCTTCGAACAAAGAGTG	CTTGGTCTTGTCCAGGACGG
GAPDH	AGATGGTGAAGGTCGGTGTG	CTGGAAGATGGTGATGGGTT

2.11. Western blot analysis

Total protein was extracted with cold RIPA lysis buffer (Millipore, USA) containing protease inhibitor and phosphatase inhibitor (MCE, China). The concentration of protein was measured using a BCA protein assay kit (Pierce, USA). Then, 10 μg of total protein of each sample was used for western blotting. Protein bands were visualized by the West Pico Chemiluminescent Substrate Kit (Pierce, USA) and quantified using a Chemidoc XRS system (Bio-Rad, CA).

2.12. Statistical analysis

All data are expressed as the mean ± standard derivation (S.D.). The significant differences were determined using two-way ANOVA in MTT analysis, one-way ANOVA (nonparametric), and the Tukey test in other analyses using Prism software (Graph Pad). A P value of < 0.05 was considered statistically significant.

3. Results

3.1. FTY720 reduced PSC cell viability, proliferation and migration

Cell viability was detected by MTT after PSCs were treated with different doses of FTY720 for 24 h or 48 h. As shown in Fig. 1A, the viability of PSCs decreased in a dose-dependent manner in response to FTY720 treatment. Therefore, 0.5 μM, 1 μM and 2 μM FTY720 were applied in subsequent experiments. Due to the importance of PSC proliferation and migration in pancreatic fibrosis [14], a real-time cell proliferation analysis (RTCA) of PSCs was performed using an iCELLigence System. As shown in Fig. 1B, FTY720 suppressed PSC proliferation, especially at a concentration of 2 μM. The migration of PSCs was also reduced significantly compared with that of the control (Fig. 1C–D).

3.2. FTY720 reduced PSC activation

The high expression level of α-SMA and ECM indicates the activation of PSCs [15]. Levels of α-SMA, collagen I (COLI) and fibronectin (FN) were measured in PSCs treated with FTY720 (0.5 μM, 1 μM and 2 μM) for 24 h. As shown in the immunofluorescence staining results (Fig. 2A), the α-SMA-positive cells were notably decreased after FTY720 treatment. The mRNA levels of α-SMA, COLI and FN were downregulated significantly by FTY720 compared with those of the control (Fig. 2B). The protein levels of α-SMA, COLI and FN also demonstrated the same results as those obtained with mRNA (Fig. 2C–D). These results suggest that FTY720 can inhibit PSC activation.

3.3. FTY720 promoted the apoptosis of PSCs

To assess the role of cell apoptosis in the inhibitory effect of FTY720 in PSCs, cell apoptosis was analysed by flow cytometry and western blotting. As shown in Fig. 3A–B, FTY720 significantly increased apoptosis in a dose-dependent manner in PSCs. Moreover, FTY720 significantly increased the expression of the pro-apoptotic protein Bax, while reducing the expression of the anti-apoptotic protein Bcl-2 (Fig. 3C). The protein levels of caspase-3 and caspase-9 were markedly decreased after FTY720 treatment, while the expression levels of cleaved caspase-3 and cleaved caspase-9 were too weak (data not

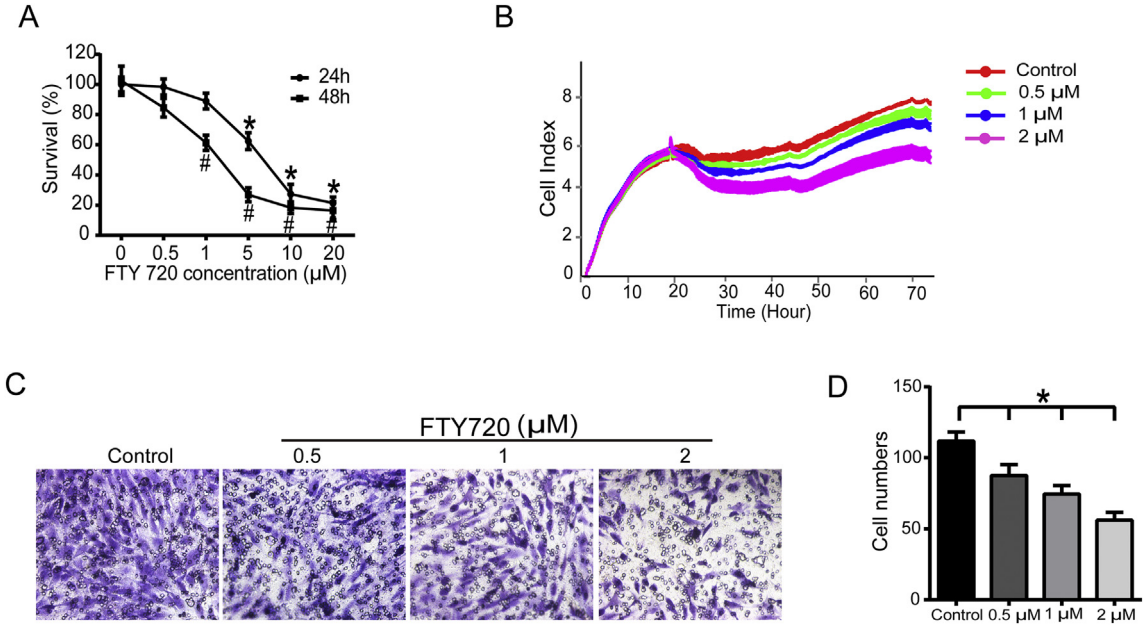

Fig. 1. FTY720 reduced PSC cell viability, proliferation and migration. (A) MTT assay of PSC cell viability after PSCs were treated with FTY720 (0, 0.5, 1, 5, 10, or 20 μM) for 24 h or 48 h. The data are expressed as fold changes over the values of the control group. The data are expressed as the mean ± S.D. from 5 independent experiments. *P $<$ 0.05 versus the control group at 24 h and $^{\#}$P $<$ 0.05 versus the control group at 48 h. (B) The real-time cell proliferation analysis (RTCA) of PSCs (treated with 0 μM, 0.5 μM, 1 μM or 2 μM of FTY720) was performed using the iCELLigence System. The cell index was monitored for 72 h, with measurements taken every 5 min. (C) PSCs treated with 0 μM, 0.5 μM, 1 μM or 2 μM of FTY720 were assessed for cell migration by a Transwell assay. The migrated PSCs were staining with haematoxylin and photographed by Leica microscope (100× magnification). (D) The numbers of migrated PSCs were analysed by counting in 5 randomly chosen fields. The data is expressed as the mean ± S.D. from 3 independent experiments. *P $<$ 0.05 versus the control group.

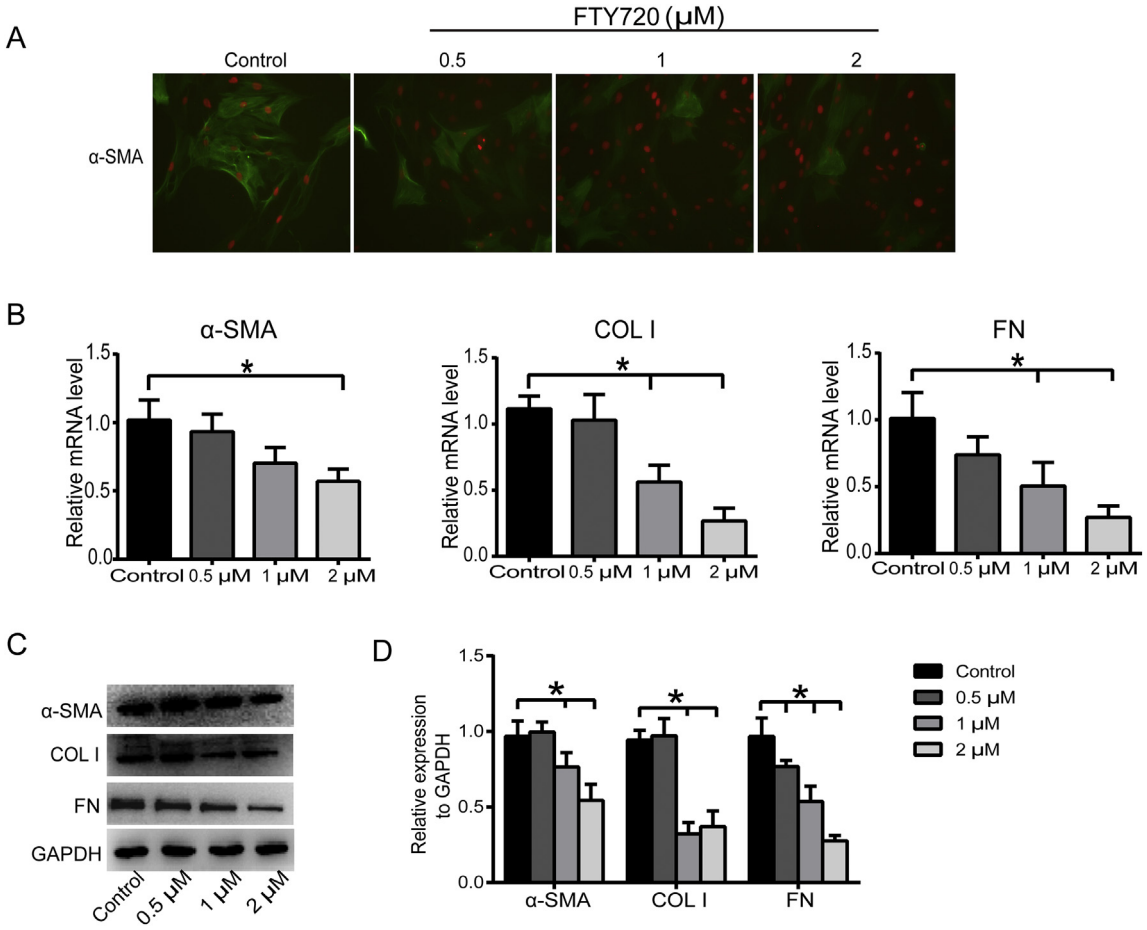

Fig. 2. FTY720 reduced PSC activation. (A) Immunofluorescence staining of α-SMA (green) after PSCs were treated with 0.5 μM, 1 μM and 2 μM FTY720 for 24 h. The nucleus was staining with PI (red) (original magnification, 200×). (B) RT-qPCR analysis of α-SMA, collagen I (COLI) and fibronectin (FN) after PSCs were treated with FTY720 for 24 h. The data were expressed as fold changes over the values of the control group. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (C) Western blot analysis of α-SMA, COLI and FN after PSCs were treated with FTY720 for 24 h. GAPDH was used as a loading control. (D) Density analysis of the western blot bands of α-SMA, COLI and FN. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

shown) to detect after treatment with FTY720 (Fig. 3C). Then, we investigated the activities of caspase-3 and caspase-9 and found that the activities of caspase-3 and caspase-9 were significantly increased after FTY720 treatment (Fig. 3D). The disruption of mitochondrial integrity is one of the key events in apoptosis [16]. The mitochondrial membrane potential (MMP) was assessed by JC-1 staining. As shown in Fig. 3E, FTY720 treatment significantly decreased red fluorescence intensity and increased green fluorescence intensity. That means that FTY720 reduced the MMP, therefore inducing mitochondrial dysfunction. These results indicated that FTY720 significantly induced PSC apoptosis partially through the modulation of apoptosis-related protein expression, caspase activity and mitochondrial function.

3.4. FTY720 suppressed the autophagy of PSCs

It has been reported that autophagy is required for PSC activation [6]. We analysed the important markers of autophagy after PSCs were treated with FTY720 for 24 h. The immunofluorescence staining showed that FTY720 decreased the level of LC3B (Fig. 4A). The mRNA and protein levels of Atg5 and LC3B were decreased in a dose-dependent manner after FTY720 treatment. The Beclin-1 level did not change significantly in any treatment group. The protein level of P62 increased significantly in the FTY720-treated groups compared with that of the control (Fig. 4B–D). The amount of LC3-II reflects the number of autophagosome [17], and our results indicated that the level of LC3B-II reduced significantly after FTY720 treatment compared with that of the control (Fig. 4C, E). These results indicated that FTY720 can inhibit PSC autophagy.

3.5. FTY720 inhibited PSCs autophagy through the AMPK/mTOR pathway

Mammalian target of rapamycin (mTOR) is a suppressive regulator of autophagy, which inhibits autophagy initiation by controlling mammalian Atg1 (ULK1) function [4,18]. In this study, p-mTOR was increased in a dose-dependent manner after PSCs were treated with FTY720 (Fig. 5A–B). Then, the level of p-ULK1 was downregulated by FTY720 (Fig. 5A–B). These results indicated that FTY720 inhibited PSC autophagy by mTOR. To confirm the role of mTOR signalling in the suppressive effect of FTY720 on autophagic activity, we treated PSCs with or without rapamycin (inhibitor of mTOR). The upregulation of p-mTOR by FTY720 was inhibited after administration of rapamycin (Fig. 5C–D). The reduction of LC3B by FTY720 was weakened after PSCs were treated with rapamycin. These results indicated that mTOR plays an important role in FTY720-regulated PSC autophagy.

Studies have revealed that mTOR can be regulated by upstream PI3K/Akt or the AMPK pathway [19,20]. We detected p-AKT and p-AMPK after PSCs were treated with FTY720 for 24 h. The p-AKT level did not change significantly after FTY720 treatment compared with

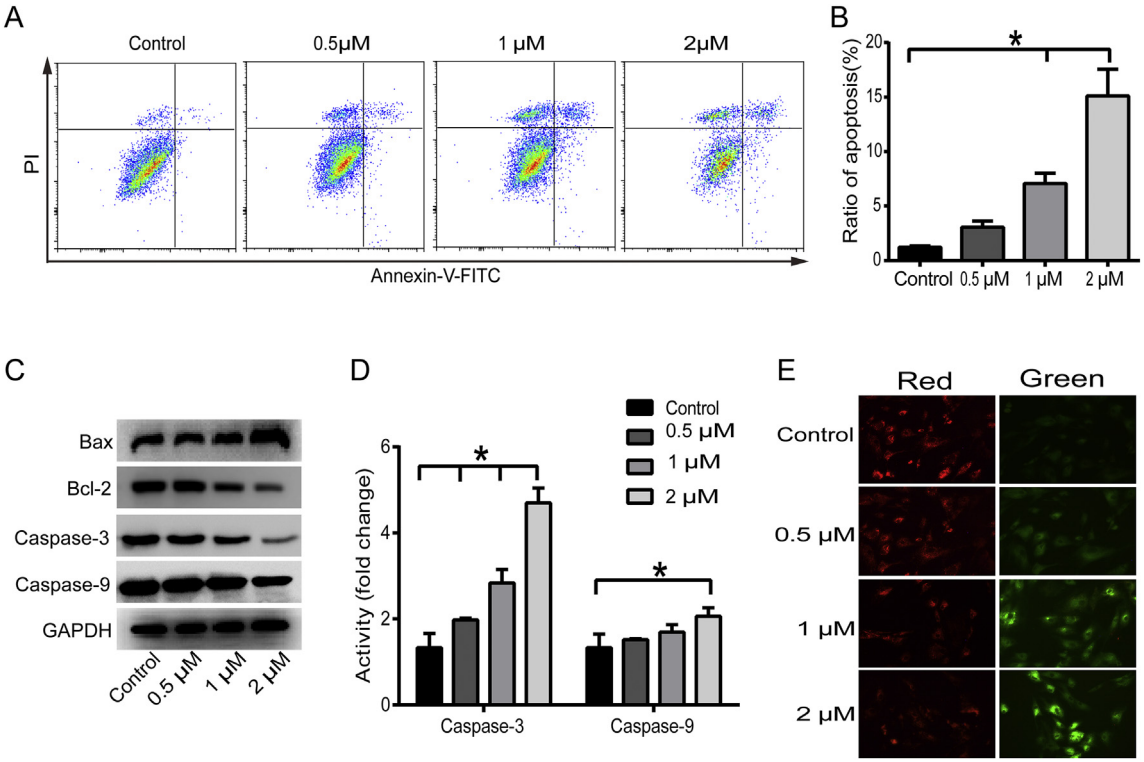

Fig. 3. FTY720 promotes apoptosis in PSCs. PSCs were treated with FTY720 (0.5 μM, 1 μM and 2 μM) for 24 h. (A) PSCs were stained with the Annexin-V-FITC/propidium iodide (PI) apoptosis detection kit and detected by flow cytometry. (B) The analysis of the ratio of apoptosis. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (C) Western blot analysis of Bax, Bcl-2, Caspase-3, and Caspase-9. GAPDH was used as a loading control. The experiments were repeated at least three times, and representative images are shown. (D) Caspase-3 and Caspase-9 activity assay. Caspase activity was expressed as a ratio of the absorbance of the control cells. The data are expressed as the mean ± S.D. *P < 0.05 versus the control group. (E) The change in MMP (mitochondrial membrane potential) was detected by JC-1 staining. Red fluorescence represents the mitochondrial aggregate form of JC-1, indicating an intact MMP; green fluorescence represents the monomeric form of JC-1, indicating MMP dissipation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that of the control. The level of p-AMPK decreased after FTY720 treatment (Fig. 5A–B). Then, we tested the role of AMPK by using phenformin hydrochloride, a commonly used AMPK activator. The inhibition of p-AMPK by FTY720 was weakened by phenformin hydrochloride (Fig. 5E–F). In addition, the decreased level of LC3B-II caused by FTY720 was upregulated by phenformin hydrochloride. These results showed that FTY720 inhibited PSC autophagy through the AMPK/mTOR signalling pathway.

4. Discussion

Pancreatic stellate cells (PSCs) are the primary effector cells in pancreatic fibrosis [21]. When PSCs are activated by various mediators and inflammatory cytokines, they increase the production of extracellular matrix (ECM) components. A large portion of the ECM is deposited in the pancreas, which causes pancreatic fibrosis. Thus, insight into the mechanisms that regulate PSC activation is important for the treatment of pancreatic fibrosis. FTY720, an FDA-approved medicine for treatment of multiple sclerosis [6], has recently been found to have different functions in various organs [22,23]. Studies have revealed that FTY720 can attenuate acute pancreatitis and acute necrotizing pancreatitis [24,25]. Okamoto showed that FTY720 attenuates chronic pancreatitis in rats by suppressing T-cell infiltration [12]. However, the effect of FTY720 on PSC activation remained unknown. In this study, FTY720 treatment significantly reduced PSC cell viability, proliferation and migration. FTY720 also inhibited PSC activation, as evidenced by the decreased level of α-SMA, collagen I, and fibronectin. This provided another explanation of how FTY720 inhibits PSC activation in pancreatic fibrosis, which was different from that of the previous report [12].

It is well-known that apoptosis plays an important role in cell proliferation, differentiation, and death [26]. The potential to induce PSC apoptosis has also become an important strategy in the treatment of pancreatic fibrosis. In this study, our results first showed that FTY720 markedly promoted PSC apoptosis. To further examine the role of FTY720 in cell apoptosis, apoptosis-related proteins (Bax and Bcl-2), caspase activity (caspases-3 and -9), and mitochondria membrane potential (MMP) were also assessed in PSCs. The results showed that FTY720 significantly increased the ratio of Bax/Bcl-2 and the activities of caspase-3 and -9, while decreasing the MMP, suggesting that FTY720 significantly induces PSC apoptosis via modulation of apoptosis-related protein expression, caspase activity and MMP levels. Taken together, our results indicate that FTY720 inhibits PSC activation, partly by promoting cell apoptosis.

Autophagy participates in various types of organ fibrosis, such as pulmonary fibrosis [27] and renal fibrosis [28]. A previous study showed that autophagy releases lipids to promote fibrogenesis by activating hepatic stellate cells [29]. PSC activation is related to autophagy and can promote pancreatic cancer growth and metastasis [6]. Our previous study indicated that autophagy participates in PSC activation and showed that inhibiting autophagy could suppress PSC activation and increase ECM degradation in primary cultured PSCs [7]. Whether FTY720 inhibits PSC activation by regulating autophagy is still unknown. In this study, the autophagy-related markers Atg5, LC3B-II and ULK1 were decreased in PSCs after FTY720 treatment, indicating that FTY720 can inhibit PSC autophagy. To confirm which pathway plays a major role in PSC autophagy, p-Akt, p-AMPK and p-mTOR levels were detected after FTY720 treatment. Our results indicated that the

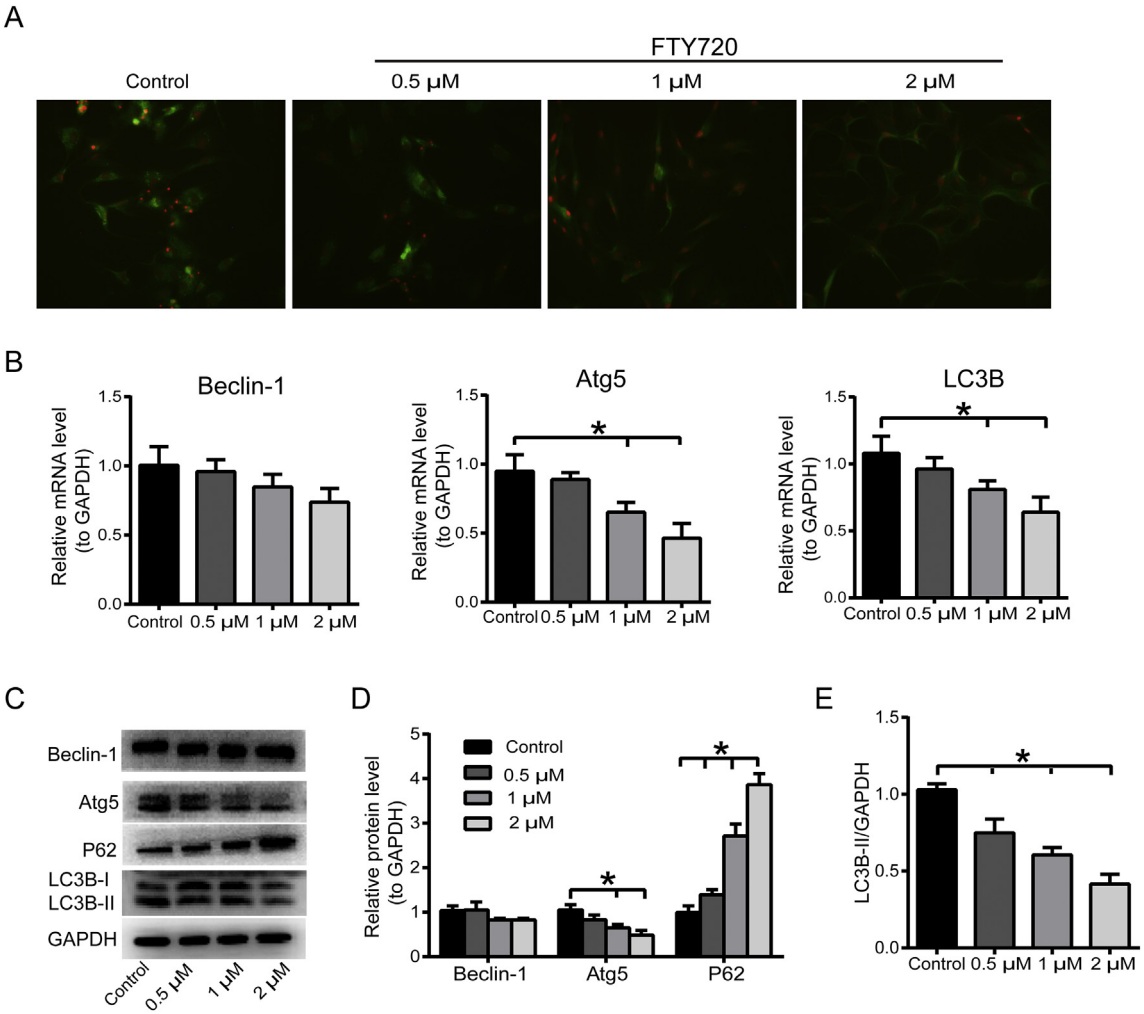

Fig. 4. FTY720 inhibited the autophagy of PSCs. PSCs were treated with 0.5 μM, 1 μM and 2 μM FTY720 for 24 h. (A) Immunofluorescence staining of LC3B (green). The nucleus was stained with PI (red) (original magnification, 200 ×). (B) RT-qPCR analysis of Beclin-1, Atg5 and LC3B. The data are expressed as fold changes over the value of the control group. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (C) Protein levels of Beclin-1, Atg5, P62 and LC3B-I/II in PSCs were detected by western blot. GAPDH was used as a loading control. (D) Density analysis of the western blot bands of Beclin-1, Atg5 and P62. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (E) The relative ratio of LC3B-II was normalized to GAPDH. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PI3K/Akt pathway was not involved in the suppressive effect of FTY720 on autophagy. In contrast, FTY720 could activate the mTOR pathway and inhibit the AMPK pathway. Rapamycin (mTOR inhibitor) and phenformin hydrochloride (AMPK activator) were applied to further confirm that FTY720 inhibited PSC autophagy via the AMPK/mTOR pathway. These may be the potential mechanisms by which FTY720 suppresses PSC activation.

Autophagy and apoptosis, which are two important aspects of maintaining cell status, play different roles in different states. Previous studies have shown that autophagy induced by FTY720 in U266 cells promotes apoptosis [30]. FTY720 induces necrotic cell death and autophagy in ovarian cancer cells, but autophagy plays a protective role in ovarian cancer [31]. In this study, we found that FTY720 induced PSC apoptosis and inhibited PSC autophagy. This may be because PSCs are phenotypically altered cells. FTY720 promotes apoptosis and reduces cell viability to reduce the proliferation of PSCs. The inhibition of autophagy by FTY720 can suppress PSC activation and reduce ECM deposition. The consistent result of FTY720 treatment was the inhibition of the activation of PSCs.

In summary, our results indicated that FTY720 inhibited PSC activation. FTY720 exerted this effect, on the one hand, by promoting cell apoptosis by increasing the ratio of Bax/Bcl-2, increasing the activity of caspase-3 and caspase-9 and decreasing the MMP level, and on the other hand by inhibiting PSC autophagy by suppressing AMPK and activating the mTOR pathway. These findings may partly explain the therapeutic mechanisms of FTY720 in treating pancreatic fibrosis and further suggest that targeting autophagy and the related signalling pathways may provide new strategies for the treatment of pancreatic fibrosis.

Conflicts of interest

We declare that we have no conflict of interest.

Author contributions

Lihua Cui, Caixia Li, Ge Gao, Yuzhen Zhuo and Lei Yang performed the experiments and analysed the data. Naiqiang Cui and Shukun Zhang participated in experimental design. Lihua Cui wrote the manuscript. Lihua Cui and Caixia Li revised the manuscript and all other authors had final approval of the submitted version.

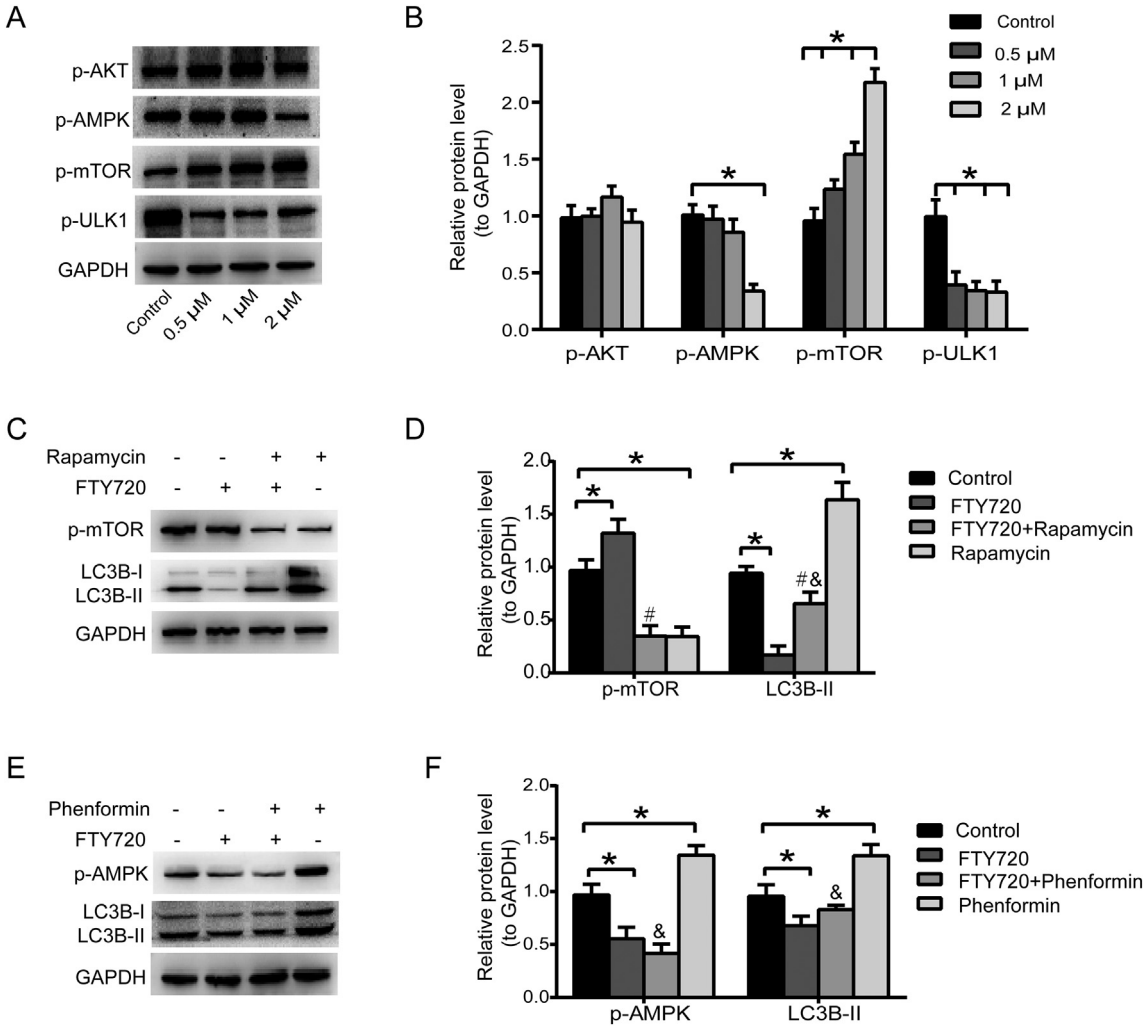

Fig. 5. FTY720 inhibited PSC autophagy through the AMPK/mTOR pathway. (A) PSCs were treated with 0.5 μM, 1 μM and 2 μM FTY720 for 24 h and subjected to western blot analysis of p-AKT, p-AMPK, p-mTOR and p-ULK1. GAPDH was used as a loading control. (B) Density analysis of p-AKT, p-AMPK, p-mTOR and p-ULK1. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. (C) PSCs were treated with 2 μM FTY720 with or without rapamycin (mTOR inhibitor) for 24 h and subjected to western blot analysis of p-mTOR and LC3B-I/II. (D) Density analysis of western blot bands of p-mTOR and LC3B-II. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. #P < 0.05 versus treatment with FTY720. & P < 0.05 versus treatment with rapamycin. (E) PSCs were treated with 2 μM FTY720 with or without phenformin (AMPK activator) for 24 h and subjected to western blot analysis of p-AMPK and LC3B-I/II. (F) Density analysis of western blot bands of p-AMPK and LC3B-II. The data are expressed as the mean ± S.D. from 3 independent experiments. *P < 0.05 versus the control group. &P < 0.05 versus treatment with phenformin.

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