Astaxanthin inhibits proliferation and induces apoptosis of LX-2 cells by regulating the miR-29b/Bcl-2 pathway

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Abstract. The aim of the present study was to investigate the role of microRNAs (miRNAs/miRs) in the anti-fibrotic effect of astaxanthin (AST), using the human hepatic stellate cell (HSC) line LX-2 as the research model. LX-2 cells were treated with various concentrations of AST (10, 20 and 40 μM) for 24 or 48 h. miR-29b was selected based on existing literature, and its targeting gene B cell lymphoma (Bcl)-2 was predicted by TargetScan and miRanda databases for further analysis. Interactions between miR-29b and Bcl-2 in the AST treated LX-2 cells were evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. MTT analysis was used to analyze cell viability. Overexpression of miR-29b decreased the expression of Bcl-2 in AST-treated LX-2 cells, and silencing of it had the opposite effect. Additionally, Annexin V-fluorescein isothiocyanate/propidium iodide double staining and flow cytometry were used to evaluate the cell apoptosis, and overexpression of miR-29b increased cell apoptosis rates in AST-treated LX-2 cells; however, silencing of it had the opposite effect. RT-qPCR and western blotting demonstrated that AST induced LX-2 cells apoptosis which may be by regulating miR-29b, as indicated by inhibited Bcl-2 expression levels and elevated Bax and Caspase-3 expression levels. These results highlight an important role of miR-29b in the AST modulating LX-2 cells proliferation and apoptosis and implicate a potential mechanism of miR-29b and AST preventing liver fibrosis.

Introduction

Liver fibrosis is a reversible, wound healing process which derives from excessive accumulation of the extracellular matrix (ECM) (1). A previous epidemiological and animal study indicated that the consistent fibrosis eventually leads to cirrhosis resulting in hepatocellular carcinoma (HCC) and liver failure (2). The occurrence of liver fibrosis is associated with hepatic stellate cell (HSC) activation, ECM synthesis and degradation, abnormal expression of inflammatory factors and fiber-associated factors and liver cells apoptosis (3-5). Previous studies demonstrated that the activation of HSCs and their conversion to a myofibroblasts-like phenotype is responsible for the deposition of excessive ECM in the fibrotic liver (6,7). Therefore, it is of paramount importance to develop anti-fibrotic therapies.

MicroRNAs (miRNAs/miRs) are highly conserved, non-coding, small RNAs (21-23 nt) that directly regulate gene expression at the post-transcriptional level by binding the 3'-untranslated regions (3'-UTRs) of specific mRNA (8). They serve important regulatory roles in cell proliferation, apoptosis and development of human diseases (9,10). Dysregulated miRNAs are associated with various human diseases. As an example, miR-146a was demonstrated to inhibit the proliferation of HSCs by regulating the tumor growth factor-β/Smad4 signaling pathway (11), and miR-150 was demonstrated to inhibit the activation of HSCs (12,13). Previous studies identified that miRNAs may be an important diagnostic and therapeutic target of liver fibrosis by regulating the activation of HSCs (14,15).

Additionally, it was identified that astaxanthin (AST), a xanthophyll carotenoid, was able to inhibit the activation of HSCs in the progression of liver fibrosis (16,17). It is separated from other molecules of the carotene subclass, which contains 13 conjugated double polyunsaturated bonds responsible for the unique chemical properties of AST (18). Due to biological implications, anti-oxidant and anti-inflammatory effects of

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AST allow it to protect against oxidative stress-associated and inflammatory disease (17). In addition, AST may have potential effects on various diseases, including cancer, obesity, hypertriglyceridemia, hypercholesterolemia, cardiovascular, gastrointestinal, liver, neurodegenerative, ophthalmologic, bone, reproductive system and skin diseases (19,20). However, the anti-fibrotic mechanism of AST is not fully understood. Furthermore, the miRNAs in HSCs involved in the anti-fibrotic mechanism of AST remain unknown. Previous studies demonstrated that miR-29 family members are downregulated in mouse models of liver fibrosis and in human fibrotic livers, and its downregulation is negatively correlated with the activation of HSCs (10,21). However, at present, the role of miR-29b in AST regulation of HSCs has not been reported, to the best of the authors' knowledge. In the present study, in order to identify the role of AST in anti-fibrotic effect and its effect on promoting HSCs apoptosis and inhibiting HSCs proliferation, the role of miR-29b and specific apoptosis-associated genes in the AST-treated HSCs was investigated.

Materials and methods

Cell culture and treatment with AST. The human HSC line LX-2 was provided by the Central Laboratory of Central South University Xiangya Cell Library (Hunan, China). Cells were cultured in low-glucose Dulbecco's modified Eagle's medium (HyClone; GE Heathcare Life Sciences, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a 37°C cell culture incubator providing 5% CO_2 and 55% humidity.

AST standard (purity ≥98%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Prior to use, AST was completely dissolved in dimethyl sulfoxide (DMSO; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) to obtain AST stock solution (8 mM) and stored at -20°C. A filter membrane (0.45 μm) was used to get rid of bacterium. Prior to cell treatment, AST stock was incubated at 37°C for 30 min and dissolved in the cell culture medium to obtain preferred concentrations. The final FBS concentration in AST containing medium was 10%, and subsequently an equal amount of FBS and DMSO were added to controls. LX-2 cells were treated with AST at various concentrations (10, 20 and 40 μM) or DMSO as a control in 37°C for 24 or 48 h. Cells were collected to prepare total RNA and protein for subsequent experiments.

Transfection. LX-2 cells were seeded in 6-well plates and subsequently serum-starved overnight when cells reached 30-50% confluency. The miR-29b mimics (5'-UAGCACCAU UUGAAAUCAGUGUU-3'; antisense, 5'-AUCGUGGUAAAC UUUAGUCACAA-3'), miR-29b inhibitors (5'-UAGCACCAU UUGAAAUCAGUGUU-3') were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Subsequent to being starved overnight, LX-2 cells were transfected with either miR-29b mimics (miR-29b; 100 nmol/l) or mimic negative control (Con miR; 100 nmol/l) or miR-29b inhibitors (anti-miR-29b; 100 nmol/l) or inhibitor negative control (Con Inh; 100 nmol/l) at 37°C for 48 h using ribo*FECT*™

CP reagent (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's protocol. When treated with AST (DMSO, 10, 20 and 40 μM) for 24 and 48 h, miR-29b was upregulated in a dose-dependent manner, particularly at 40 μM. Therefore, 40 μM was selected for subsequent experiments. Subsequent to replacing the cell culture medium, LX-2 cells were immediately treated with AST (40 μM) or the vehicle (DMSO) in 37°C for 48 h. Cells were collected to prepare total RNA and protein.

MTT analysis. Cell proliferation was detected by MTT assay, and LX-2 cells were seeded in 96-well plates (Costar; Corning, Inc., Corning, NY, USA) in medium containing 10% FBS at ~2,000 cells/well. LX-2 cells were only treated with AST (ranging between 5 and 80 μM) in 37°C for 12, 24, 48 and 72 h. On the other hand, LX-2 cells were transfected with miR-29b mimics or mimic negative control or inhibitors or inhibitor negative control in 37°C for 48 h and subsequently AST (40 μM) or the vehicle (DMSO) were added to the refreshed medium for 48 h. To assess cell viability, 20 μl MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each well for 4 h at 37°C. Following removal of culture medium, 150 μl DMSO was added to each well. After 10 min, absorbance (A) at a wavelength of 450 nm (A_{450}) was detected by a multifunctional microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The proliferation inhibition rate was calculated from the following model: Proliferation inhibition rate=[1-(A $_{experimental\ group}$-A $_{blank\ group}$)/(A $_{control\ group}$-A $_{blank\ group}$)]x100.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells of each group using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA quality and quantity were determined with a Nanodrop spectrophotometer (Nanodrop 2000c; Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and the RNA purity was determined by gel electrophoresis. RNA (2 μg) was reverse-transcribed by Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Bazel, Switzerland). RT-qPCR was performed to determine the original number of specific transcripts associated with fibrotic markers using FastStart Universal SYBR Green Master (Roche Diagnostics). The miRNAs expression was normalized to U6 as a housekeeping gene and the mRNA expression was normalized against β-actin. RT-qPCR was conducted using the PikoReal™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientifc, Inc.) with the following reaction conditions: Initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 60 sec and extension at 65°C for 30 sec; followed by melting curve analysis. Each test was performed in triplicate and the $2^{-\Delta\Delta Cq}$ method (22) was used to calculate the expression of miRNAs and mRNA in LX-2 cells. The primers sequences used in the present study are listed in Table I.

Western blot analysis. LX-2 cells were lysed by radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) to obtain total protein, and a bicinchoninic protein assay kit (CWBIO Corporation, Allston, MA, USA) was used to detect the protein concentration.

Table I. Primer sequences.

Gene	Primer	Sequence (5'-3')
hsa-miR-29b	RT	CCTGTTGTCTCCAGCCACAAA AGAGCACAATATTTCAGGAGA CAACAGGAACACTG
	Forward	CGGGCTAGCACCATTTGAAAT
	Reverse	CAGCCACAAAAGAGCACAAT
U6	RT	AAAATATGGAACGCTTCACG
	Forward	CGCTTCGGCAGCACATATACT AAAATTGGAAC
	Reverse	GCTTCACGAATTTGCGTGTCA TCCTGC
α-SMA	Forward	GGCTCTGGGCTCTGTAAGG
	Reverse	CTCTTGCTCTGGGCTTCATC
Col1a1	Forward	CCCGGGTTTCAGAGACAAC TTC
	Reverse	TCCACATGCTTTATTCCAGCA ATC
Bcl-2	Forward	GTGCCTGCTTTTAGGAGAC CGA
	Reverse	GAGACCACACTGCCCTGTTG ATC
Bax	Forward	TTTGCTTCAGGGTTTCATCCA
	Reverse	GAGACACTCGCTCAGCTTC TTG
Caspase-3	Forward	GTAGAAGTCTAACTGGAAAA CCCAA
	Reverse	CATGTCATCATCAACACCACT GTCT
β-actin	Forward	TCCTCCCTGGAGAAGAGCTA
	Reverse	TCAGGAGGAGCAATGATC TTG

α-SMA, α-smooth muscle actin; Bax, Bcl-2-associated X protein; Bcl, B cell lymphoma; Col1a1, collagen α-1(I) chain; miR, micro RNA; PI3K, phosphoinositide 3-kinase; RT, reverse transcription.

Total protein samples (30-50 μg) were electrophoresed on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). After the proteins were fully transferred, the membranes were blocked with 5% bovine serum albumin (w/v) in Tween-20/Tris-buffered saline (TBST) at room temperature for 2 h, followed by 2 h incubation at room temperature with antibodies against B cell lymphoma (Bcl)-2 (1:1,000; cat. no. ab32124; Abcam, Cambridge, UK), α-SMA (1:3,000; cat. no. ab32575; Abcam), collagen α-1(I) chain (Col1a1; 1:5,000; cat. no. ab138492; Abcam) and Bax (1:5,000; cat. no. ab32503; Abcam) and β-tubulin (1:1,000; cat. no. 10094-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at room temperature for 1.5 h. The specific protein was detected with an enhanced chemiluminescence (ECL-plus; Thermo Fisher Scientific, Inc.). Band densities were quantified using an image analyzer with Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). All protein quantifications were adjusted according to their corresponding β-tubulin level, which was not varied with different treatment conditions.

Target prediction. TargetScan (www.targetscan.org/vert_72/) and miRanda (www.microrna.org) databases were used to predict the target genes of miR-29b. The predicted target genes were subjected to Gene Ontology enrichment analysis and biological pathway enrichment analysis by the Database for Annotation Visualization and Integrated Discovery database (david.ncifcrf.gov/).

Apoptosis assay. An Annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was used to detect the apoptosis of LX-2 cells, according to the manufacturer's protocol. LX-2 cells were seeded in 6-well plates at ~5x10^4 cells/ml and subsequently serum-starved overnight when cells reached 30-50% confluence. LX-2 cells were only treated with AST (ranging between 5 and 80 μM) at 37˚C for 12, 24, 48 and 72 h. However, LX-2 cells were transfected with miR-29b mimics or mimic negative control or inhibitors or inhibitor negative control at 37˚C for 48 h and subsequently AST (40 μM) or the vehicle (DMSO) were added to the refreshed medium for 48 h. Following treatment, LX-2 cells were washed with cold PBS, collected by centrifugation (2,000 x g), and suspended in 500 μl 1x binding buffer and subsequently incubated with 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. Subsequently, apoptosis was analyzed with a FACS Calibur flow cytometer (BD FACSCalibur™; BD Biosciences, San Jose, CA, USA). A minimum of 10,000 cells per sample were acquired and analyzed using FlowJo software (version 7.6.1; Tree Star, Inc., Ashland, OR, USA). The experiments were repeated three times.

Statistical analysis. All experiments were repeated in triplicate and results are expressed as the mean ± standard deviation and were analyzed using unpaired Student's t-test and one-way analysis of variance tests with Duncan's post hoc test. SPSS for Windows software (version 13.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. $P<0.05$ was used to indicate a statistically significant difference.

Results

AST increases miR-29b expression in the LX-2 cells. To investigate whether AST altered miR-29b expression levels in LX-2 cells, RT-qPCR was performed following 24 and 48 h of treatment with AST (DMSO, 10, 20 and 40 μM). After 24 or 48 h treatment, miR-29b was upregulated in a dose-dependent manner compared with the DMSO control group (Fig. 1).

Successful experimental transfection efficiency. The transfection efficiency of miR-29b mimics (miR-29b; 100 nmol/l) and Con miR group by RT-qPCR were 1.00±0.04 and 22.35±0.84, respectively. Compared with the Con miR
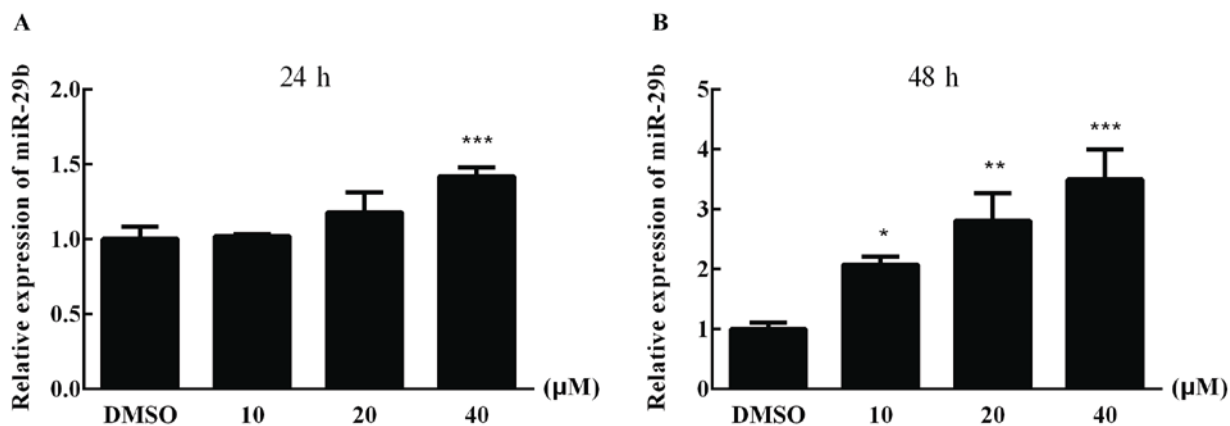
Figure 1. AST elevates the expression of miR-29b. LX-2 cells were treated with various concentration of AST (10, 20, 40 μM) or the vehicle (DMSO) for (A) 24 or (B) 48 h . *P<0.05, **P<0.01, ***P<0.001 vs. DMSO. AST, astaxanthin; DMSO, dimethyl sulfoxide; miR, microRNA.
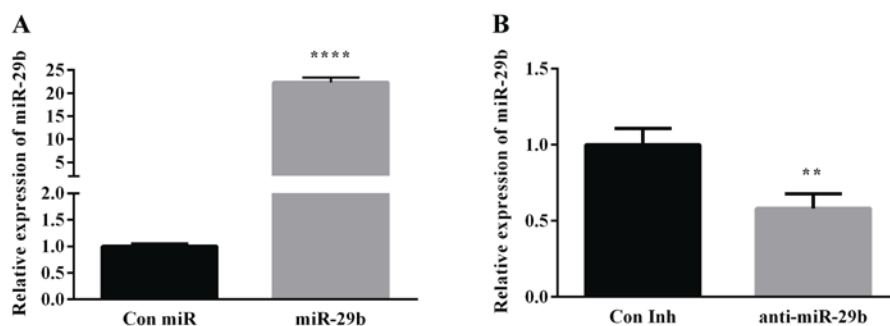

Figure 2. The expression efficiency of miR-29b. LX-2 cells were transfected with (A) miR-29b mimics or (B) miR-29b inhibitors for 48 h. Results are expressed as the mean ± standard deviation of fold. **P<0.01, ****P<0.0001 vs. Con miR or Con Inh group. AST, astaxanthin; Con, control; Inh, inhibitor; miR, microRNA.

group, the expression of the miR-29b group was significantly increased (P<0.05; Fig. 2A). The transfection efficiency of miR-29b inhibitors (anti-miR-29b; 100 nmol/l) and Con Inh group by RT-qPCR were 1.01±0.11 and 0.58±0.08, respectively. Compared with the Con Inh group, the expression of anti-miR-29b group was significantly decreased (P<0.05; Fig. 2B). The recommended dose of 100 nmol/l was used as the final concentration for transfection, according to the manufacturer's protocol.

AST inhibits the proliferation of LX-2 cells by the miR-29b. To investigate the effect of AST on LX-2 cells, MTT cell viability assay was performed. After treatment with AST for 24, 48 and 72 h, the absorbance in the AST treated groups was decreased in a dose-dependent manner compared with the DMSO control group. Results suggested that AST could inhibit the viability of LX-2 cells and the cell viability decreased by increasing AST concentration (Fig. 3A).

The MTT cell viability assay was additionally used to examine the effect of miR-29b on viability inhibition of AST on LX-2 cells. Following transfection with miR-29b mimics (miR-29b), mimic negative control (Con miR), miR-29b inhibitors (anti-miR-29b) and inhibitor negative control (Con Inh), 40 μM AST were added to the culture medium for 48 h. The results demonstrated that the cell viability in the miR-29b+AST group was significantly decreased compared with the Con miR group, and the cell viability of the miR-29b

group was additionally significantly decreased compared with the Con miR group (Fig. 3B). Conversely, the cell viability of the anti-miR-29b+AST and anti-miR-29b groups was significantly increased compared with the Con-Inh group (Fig. 3B). Therefore, miR-29b may serve a key role in AST anti-viability effect on the LX-2 cells.

To clarify the roles of AST in collagen deposition, RT-qPCR and western blot analysis were used to examine the expression of α-SMA and Col1a1 (Fig. 4). The results demonstrated that AST significantly reduced the mRNA expression of α-SMA (P<0.05; Fig. 4A) and Col1a1 (P<0.05; Fig. 4B). Using Quantity One software, the band densities were quantified and it was demonstrated that the protein expression levels of α-SMA (P<0.05; Fig. 4A and C) and Col1a1 (P<0.05; Fig. 4B and D) were significantly decreased in the AST treated group. These results suggested a notable inhibitory effect of AST. To further determine the role of miR-29b in collagen deposition, LX-2 cells were transfected with miR-29b mimics or inhibitors and treated with AST. As presented in Fig. 5, overexpression of miR-29b significantly suppressed mRNA and protein expression of α-SMA (Fig. 5A), which was reversed by miR-29b inhibition (Fig. 5B). Similarly, over-expression of miR-29b significantly suppressed mRNA and protein expression of Col1a1 (Fig. 5C), and AST aggravated this condition. However, this result was reversed by miR-29b inhibition (Fig. 5D) suggesting that AST can suppress ECM deposition possibly through miR-29b.
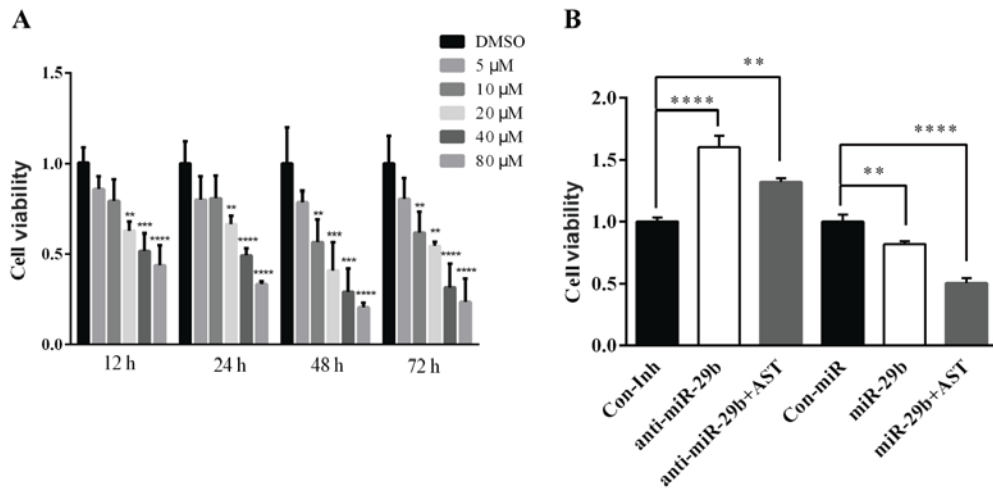
Figure 3. AST inhibits the LX-2 cells proliferation by regulating miR-29b. (A) LX-2 cells were treated with various concentration of AST (5, 10, 20, 40 and 80 μM) or the vehicle (DMSO) for 12, 24, 48 and 72 h. **P<0.01, ***P<0.001, ****P<0.0001 vs. DMSO. (B) LX-2 cells were transfected with either miR-29b mimics or miR-29b mimic negative control or miR-29b inhibitors or miR-29b inhibitor negative control for 48 h, and then treated with AST (40 μM) or the vehicle for 48 h. Data are expressed as the mean ± standard deviation. **P<0.01, ****P<0.0001 vs. Con miR or Con Inh group. AST, astaxanthin; Con, control; DMSO, dimethyl sulfoxide; Inh, inhibitor; miR, microRNA.

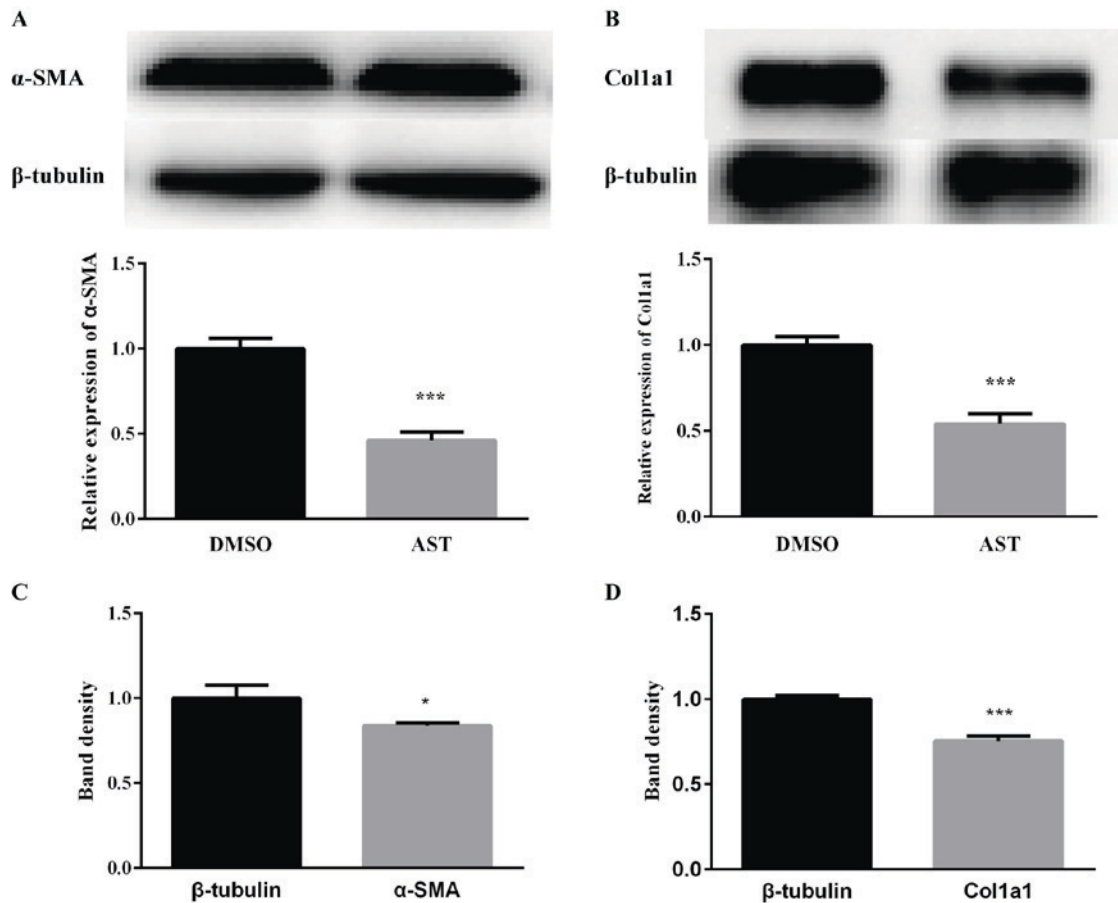

Figure 4. AST inhibits the expression of α-SMA and Col1a1 in LX-2 cells. After treating cells with AST or the vehicle for 48 h, mRNA and protein expression levels of (A) α-SMA and (B) Col1a1 were measured by RT-qPCR and western blotting. Band densities of α-SMA (C) and Col1a1 (D) were quantified using Quantity One software. Data are expressed as the mean ± standard deviation. *P<0.05, ***P<0.001 vs. DMSO. α-SMA, α-smooth muscle actin; AST, astaxanthin; Col1a1, collagen α-1(I) chain; DMSO, dimethyl sulfoxide; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

AST induces apoptosis through miR-29b in LX-2 cells. TargetScan and miRanda databases predicted that miR-29b had ~1,000 target genes, some of which are involved in cell apoptosis (data not shown). It was identified that the 3'-untranslated region (UTR) of Bcl-2 contains putative binding sites for miR-29b (Fig. 6A). Therefore, the effect of miR-29b on Bcl-2
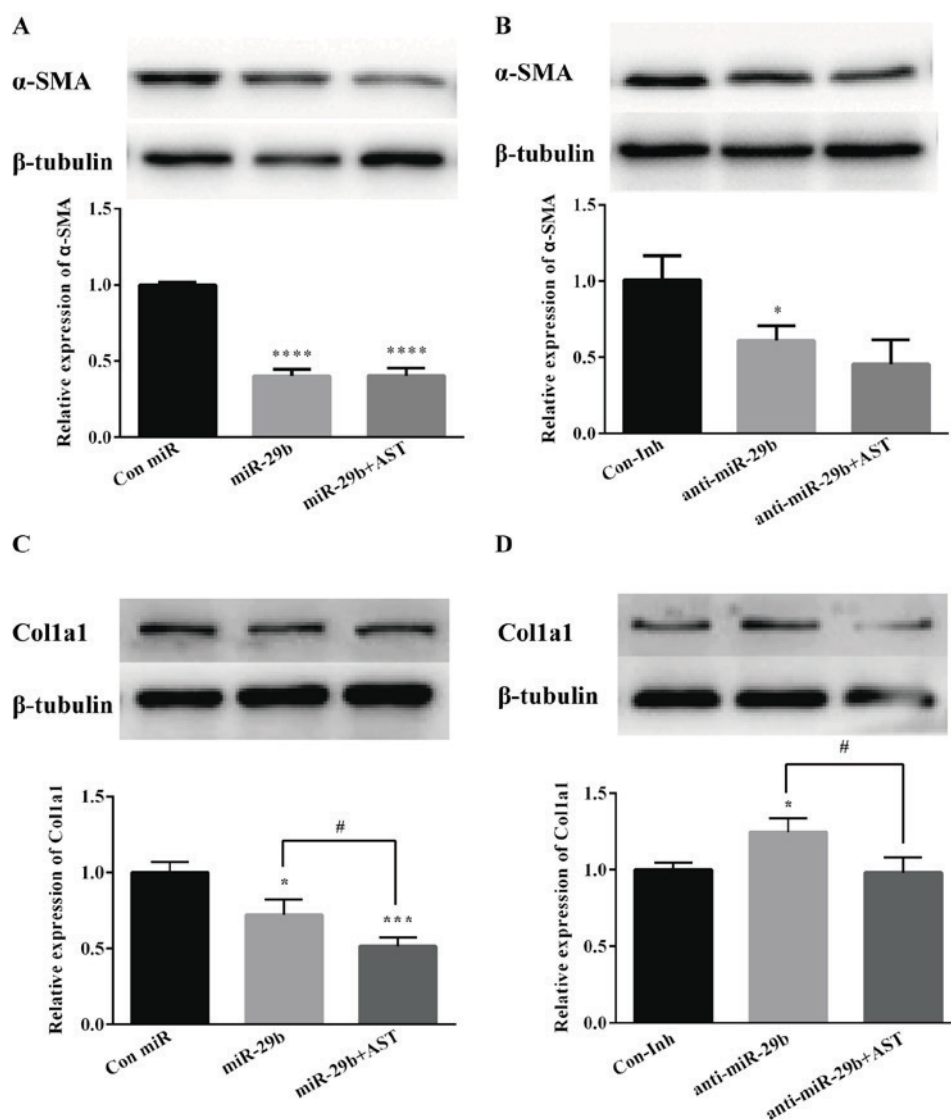
Figure 5. AST inhibits the expression of α-SMA and Col1a1 in LX-2 cells. LX-2 cells were transfected with either miR-29b mimics or miR-29b mimic negative control or miR-29b inhibitors or miR-29b inhibitor negative control for 48 h, and treated with AST (40 μM) or the vehicle for 48 h. (A and B) Protein and mRNA expression levels of α-SMA and (C and D) protein and mRNA expression levels of Col1a1 as measured by RT-qPCR and western blotting. Data are presented as the mean ± standard deviation. *P<0.05, ***P<0.001, ****P<0.0001 vs. Con miR or Con Inh group; #P<0.05 vs. miR-29b or anti-miR-29b group. α-SMA, α-smooth muscle actin; AST, astaxanthin; Col1a1, collagen α-1(I) chain; Con, control; DMSO, dimethyl sulfoxide; Inh, inhibitor; miR, microRNA.

gene expression in the AST-treated LX-2 cells was further examined. The Bcl-2 family serves a key role in apoptosis (23), and RT-qPCR and western blot analysis were used to detect the mRNA and protein expression levels of Bcl-2, Bax and Caspase-3, respectively (Fig. 6B-D). AST treatment resulted in a significant decreased expression of Bcl-2 (Fig. 6B), together with increased expression of Bax (Fig. 6C) and Caspase-3 (Fig. 6D). Accompanied with the upregulation of miR-29b, the mRNA and protein expression levels of Bcl-2 were decreased (Fig. 7A), whereas following miR-29b inhibition, AST treatment reversed this (Fig. 7B). Additionally, the mRNA and protein expression levels of Bax were increased (Fig. 7C) in the miR-29b upregulation group, whereas, following miR-29b inhibition, AST treatment reversed this (Fig. 7D). Similarly, the mRNA and protein expression levels of caspase-3 were increased when miR-29b was upregulated (Fig. 7E), whereas following miR-29b inhibition, AST treatment reversed this (Fig. 7F).

In order to determine the role of miR-29b in the observed suppressive effect of cell growth by AST, the Annexin V-FITC/PI double staining and flow cytometry was used to evaluate the cell apoptosis. When miR-29b is overexpressed, the AST-treated group demonstrated increased expression of Annexin-V compared with the Con miR and miR-29b alone groups (Fig. 8A and C). However, following miR-29b inhibition AST treatment increased the expression of Annexin-V compared with the Con Inh and anti-miR-29b alone groups (Fig. 8B and D). These findings indicated that AST induces cell apoptosis potentially through miR-29b.

Discussion

Liver fibrosis is a common cause of chronic liver disease and HSCs serve an important role in the development of liver fibrosis (3). Subsequent to exposing HSCs to various injurious agents, such as pro-inflammatory cytokines and irradiation, the
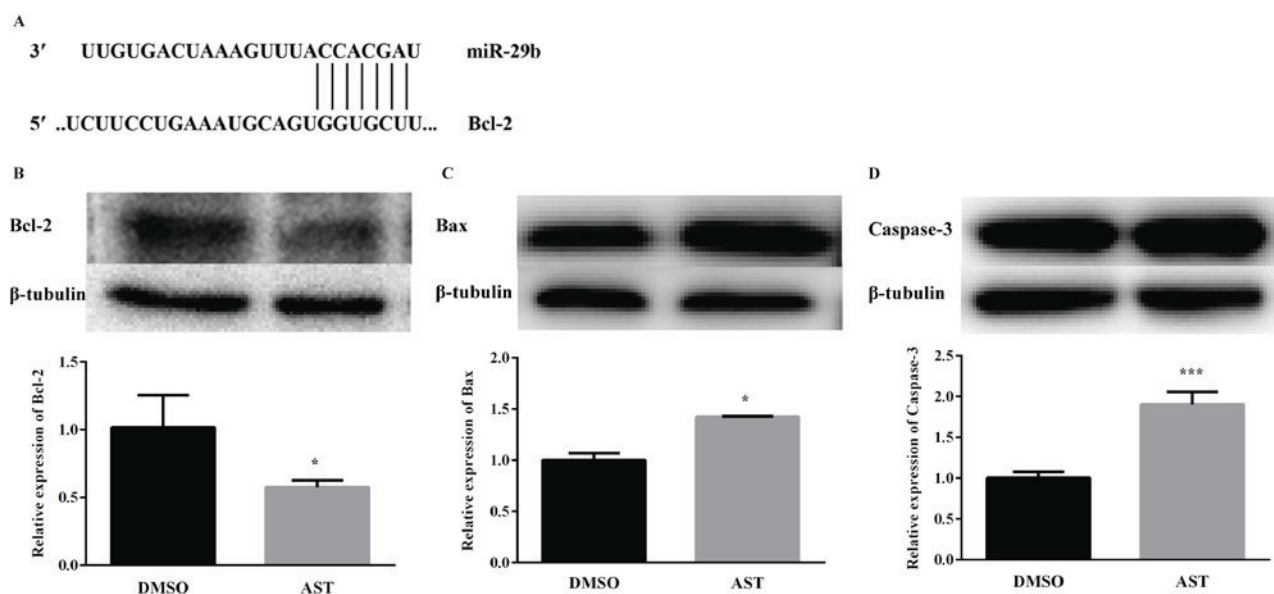
Figure 6. AST inhibits the expression levels of apoptosis-associated proteins in LX-2 cells. (A) TargetScan and miRanda databases predicted that the 3'-UTR of Bcl-2 contain putative binding sites for miR-29b. After treated with AST or the vehicle for 24 and 48 h, mRNA and protein expression levels of (B) Bcl-2, (C) Bax and (D) Caspase-3 were investigated by RT-qPCR and western blotting. Data are expressed as the mean ± standard deviation. *P<0.05, ***P<0.001 vs. DMSO. Bax, Bcl-2-associated X protein; Bcl, B cell lymphoma; DMSO, dimethyl sulfoxide; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; UTR, untranslated region.

cells may be transformed into myofibroblasts, and expression of α-SMA and Col1a1 may be increased followed by the accumulation of extracellular matrix (24). Therefore, α-SMA and col1a1 are markers of HSC activation. It was identified that AST inhibits the activation of HSCs by reducing the expression of α-SMA and Col1a1 (25). Although the abnormal expression of miRNAs has been reported on various diseases, the molecular mechanisms by which miRNAs and AST modulate the process of liver fibrosis and the activation of HSCs is still unknown. The current study demonstrated that AST may promote apoptosis of HSCs and inhibit their proliferation through upregulation of miR-29b. In the present study, it was shown that miR-29b positively regulates the HSCs apoptosis by suppressing the expression of Bcl-2, whereas AST increased miR-29b expression levels, leading to enhanced HSCs apoptosis.

AST is a non-vitamin A carotenoid which can be found in *Haematococcus pluvialis*, shrimp, crab and salmon (16,26). AST is beneficial as a therapeutic agent for various diseases due to its anti-oxidative property (27-29). Yang *et al* (25) observed that AST may be used as a preventive or therapeutic agent to prevent liver fibrosis by blocking the tumor growth factor-β1 (TGF-β1) signaling pathway. Additionally, AST inhibited the activation of HSCs and development of ECM via decreasing the expression of nuclear factor-κB and TGF-β1. It also reduced energy production of HSCs by downregulating the level of autophagy (30). However, the specific anti-fibrotic mechanism of AST remains unknown. To date, natural chemical-based drugs, including AST, in particular, are the main research direction for the treatment of liver fibrosis (31-33). Despite the protective effect of AST against liver fibrosis, however, the mechanism needs to be further explored. Therefore, AST is a crucial clinical component that requires consider and greater knowledge of the molecular mechanisms involved in its anti-fibrotic effect will assist the development of

novel treatment targets for eradicating liver fibrosis and other chronic liver diseases.

Dysregulation of miRNAs contribute to drug resistance in various cancer types (34), including gastric cancer, non-small-cell lung cancer, myeloid leukemia and breast cancer (35-38), as well as hepatocellular carcinoma. Thus, to determine the mechanism of miRNAs and AST in liver fibrosis is important. The miRNA-29 family includes miR-29a, miR-29b, and miR-29c (39). Previous studies demonstrated that the expression of miR-29b was decreased in activated HSCs (40,41).

Several studies have revealed that deviant expression of miR-29b is widespread in the majority of human cancers and serve as a tumor suppressor affecting the cancer progression (42). Wang *et al* (41) found that miR-29b can prevent liver fibrogenesis by inhibiting HSC activation and inducing HSC apoptosis via inhibiting Phosphoinositide 3 kinase (PI3K)/Akt pathway. Additionally, Li *et al* (43) reported that AST induces hepatocellular cells apoptosis through negative activation of PI3K/Akt. It may be inferred that AST may prevent liver fibrogenesis by regulating miR-29b/PI3K/Akt (43) (Fig. 9). Bcl-2 and myeloid cell leukemia-1 (Mcl-1) protein, a potent, multidomain anti-apoptotic protein of the Bcl-2 family, is downregulated by miR-29b (44,45). In addition, miR-29b may sensitize HCC cells to apoptosis by directly targeting the anti-apoptotic molecules Bcl-2 and Mcl-1 using luciferase reporter gene assay (44). These results support that apoptosis may be reinforced by miR-29 via a mitochondrial pathway involving Mcl-1 and Bcl-2, and implicate the potential application of miR-29 in prognosis prediction and in cancer therapy, but this needs to be investigated further. It is important to consider that miR-29b demonstrated an ability to target apoptosis regulators in the AST treated HSCs, and it was demonstrated that Bcl-2 serves as a crucial effector of miR-29b in the AST treated HSCs (Fig. 9).
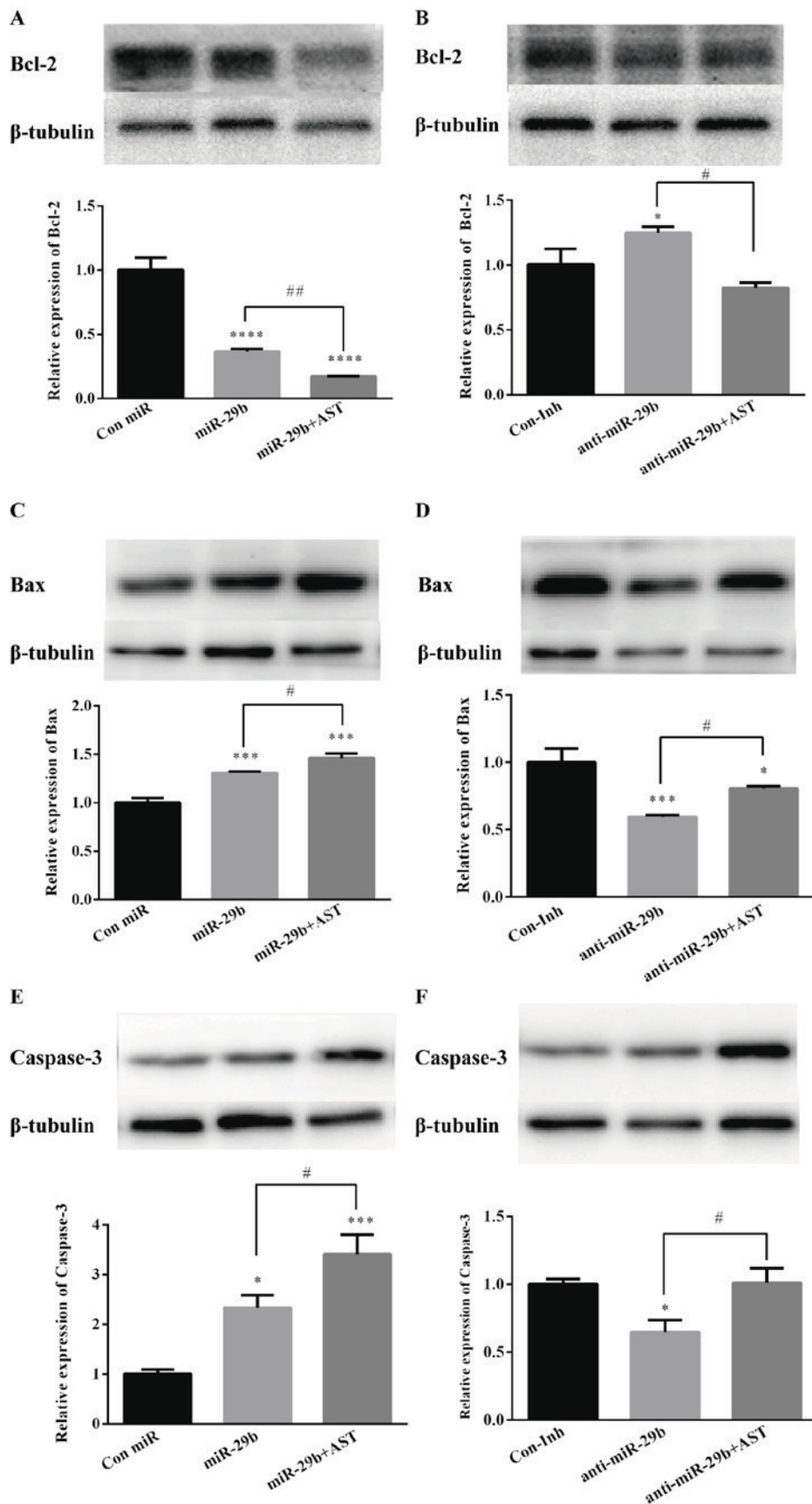
Figure 7. AST regulates the expression levels of apoptosis-associated proteins by regulating miR-29b in LX-2 cells. LX-2 cells were transfected with either miR-29b mimics or miR-29b mimic negative control or miR-29b inhibitors or miR-29b inhibitor negative control for 48 h, and then treated with AST (40 μM) or the vehicle for 48 h. mRNA and protein expression levels of (A and B) Bcl-2, (C and D) Bax and (E and F) Caspase-3 were investigated by RT-qPCR and western blotting. Data are expressed as the mean ± standard deviation. *P<0.05, ***P<0.001, ****P<0.0001 vs. Con miR or Con Inh group. $^{#}$P<0.05; $^{##}$P<0.01 vs. miR-29b or anti-miR-29b group. AST, astaxanthin; Bax, Bcl-2-associated X protein; Bcl, B cell lymphoma; Inh, inhibitor; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
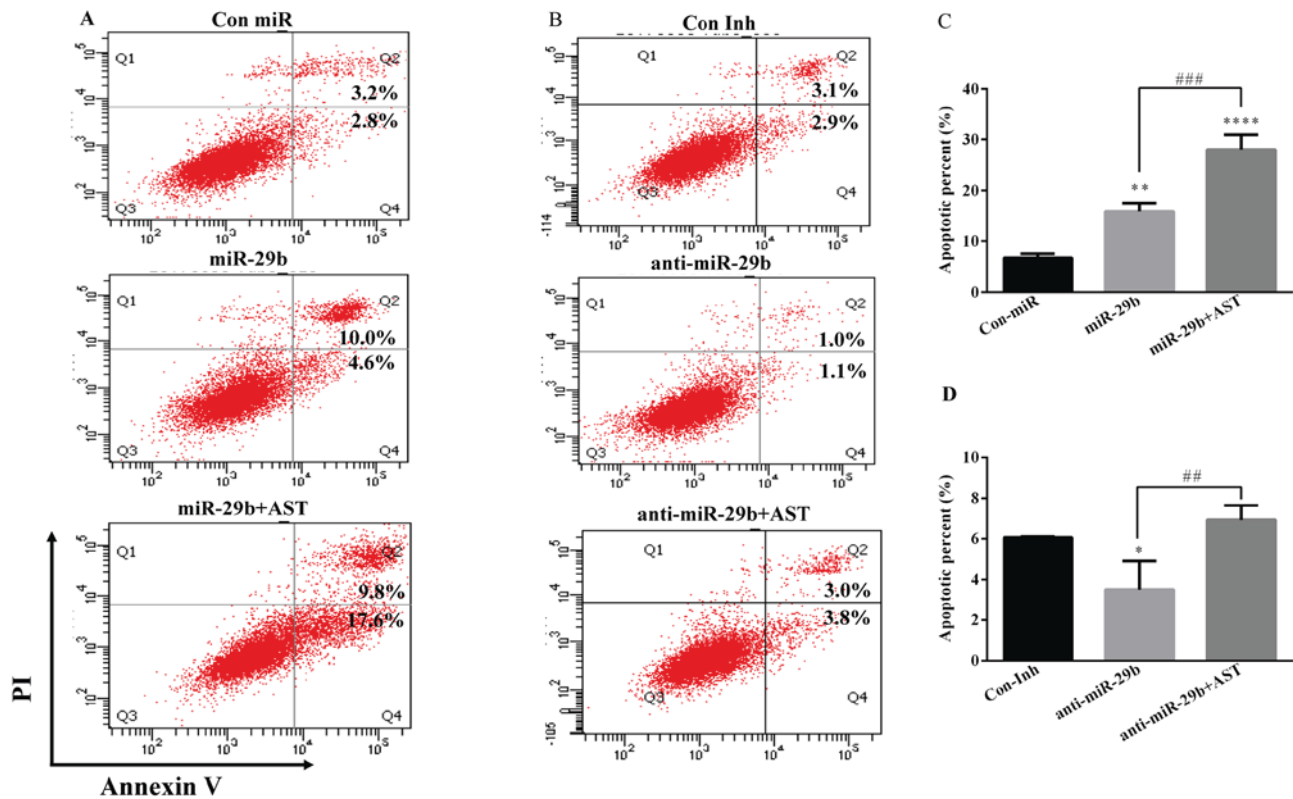
Figure 8. AST promotes LX-2 cells apoptosis by regulating miR-29b. LX-2 cells were transfected with either miR-29b mimics or miR-29b mimic negative control or miR-29b inhibitors or miR-29b inhibitor negative control for 48 h, and treated with AST (40 μM) or the vehicle for 48 h. (A and B) Apoptosis of LX-2 cells and (C and D) its quantification, as measured by flow-cytometry. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, ****P<0.0001 vs. Con miR or Con Inh group; ##P<0.01, ###P<0.001 vs. miR-29b or anti-miR-29b group. AST, astaxanthin; Inh, inhibitor; miR, microRNA.
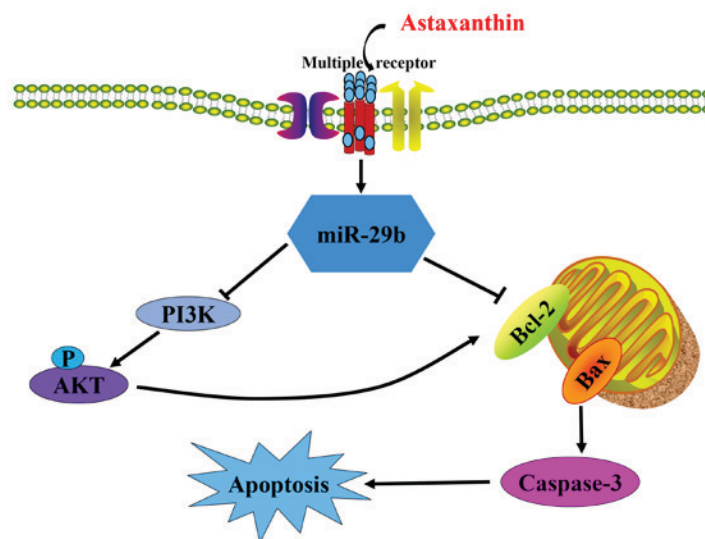

Figure 9. Schematic illustration showing the regulation of AST on liver fibrosis. AST reduces PI3K/Akt activity and Bcl-2 by elevating expression levels of miR-29b, which in turn increases Bax and Caspase-3 activity promoting HSCs apoptosis. AST, astaxanthin; Bax, Bcl-2-associated X protein; Bcl, B cell lymphoma; miR, microRNA; PI3K, phosphoinositide 3-kinase.

In the present study, miR-29b was a possible therapeutic marker for liver fibrosis, and it was identified that miR-29b is upregulated by AST in LX-2 cells compared with the vehicle control group. Furthermore, upregulation of miR-29b by AST prevented LX-2 cells proliferation and induced the LX-2 apoptosis through modulating expression of Bcl-2.

However, the possibility that the observed effects of AST and miR-29b are additive, as opposed to that AST is a regulator of miR-29b, requires further examination. In conclusion, the present data provided evidence that AST modulates miR-29b in promoting apoptosis and inhibiting proliferation of HSCs *in vitro*. The experimental data could offer a way to pinpoint

the miR-29b/Bcl-2 interaction as a novel therapeutic application to care for people suffering from liver fibrosis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SZ, TZ and HW conceived and designed the experiments. SZ, TW and FL performed the experiments. QJ, HL and TH analyzed the data. SZ and TZ wrote the paper. HW helped to revise the manuscript. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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