

Construction of a recombinant pIRES2-EGFP-ARTS plasmid and its effect on LX-2 cells

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Abstract. The inhibition of the activation of hepatic stellate cells (HSCs) and the induction of their apoptosis have been investigated as potential strategies to counteract the development and progression of liver fibrosis. Previous research has suggested that apoptosis-related protein in the transforming growth factor- β signaling pathway (ARTS) may serve a significant role in numerous cell types; however, little is known regarding its roles in HSCs. Total RNA was extracted from LX-2 cells, and the human full-length ARTS gene was obtained by reverse transcription-polymerase chain reaction and inserted into the pIRES2-EGFP cloning vector. Subsequently, the recombinant pIRES2-EGFP-ARTS plasmid was transfected into LX-2 cells by FuGENE 6 transfection reagent, and the expression of ARTS was detected by western blotting and fluorescent microscopy. In addition, the effects of pIRES2-EGFP-ARTS on the activation, apoptosis, viability and migration of LX-2 cells were assessed by western blot analysis, TUNEL staining, an MTT assay, and scratch and Transwell assays, respectively. The present results demonstrated that the pIRES2-EGFP-ARTS vector expressing human ARTS was successfully constructed, and the overexpression of ARTS contributed to enhance the apoptosis and inhibit the activation of human LX-2 HSCs. The present findings

suggested that ARTS overexpression may have potential as a novel therapeutic strategy to reverse hepatic fibrosis.

Introduction

Liver fibrosis, which characterized by abnormal deposition of extracellular matrix (ECM) may be caused by various etiological factors, and is associated with a high mortality and morbidity worldwide (1). A previous study demonstrated that the pivotal pathogenic event in liver fibrosis is the activation of hepatic stellate cells (HSCs) (2). Therefore, inhibition of activated HSCs or induction of HSCs apoptosis may be an effective strategy in the treatment of hepatic fibrosis (3). LX-2 is an immortalized human HSC line that expresses a range of human hepatic fibrosis features: As hepatic fibrosis progresses, LX-2 cells are activated, the expression of α -smooth muscle actin (α -SMA) increases, whereas the expression of peroxisome proliferator-activated receptor- γ (PPAR- γ) decreases. Therefore, LX-2 cells have been used as the primary cell type for liver fibrosis research (4-6).

Apoptosis-related protein in the transforming growth factor- β (TGF- β) signaling pathway (ARTS), also termed septin (Sept4_i2), is a recognized splice variant encoded by the human Sept4 gene (7). Unlike other splice variants, ARTS exists in the cytomembrane and is released into the cytoplasm following stimulation by various molecules, including TGF- β , staurosporine, Fas, arabinoside and etoposide, and subsequently exhibits a proapoptotic function by binding to the X-linked inhibitor of apoptosis protein (8). Previous studies have indicated that ARTS regulates apoptosis in various cell types, including COS-7, Hela, K562, HL-60 and ALLs (9-11). Furthermore, Iwaisako *et al* (12) reported that Sept4 was downregulated in mice with liver fibrosis that was induced by bile duct ligation and carbon tetrachloride, which indicated that Sept4 may have an important role in liver fibrosis. However, to the best of our knowledge, it has not previously been reported whether ARTS has a role in HSC apoptosis and activation. Therefore, the current study aimed to construct the recombinant pIRES2-enhanced green fluorescent protein (EGFP)-ARTS plasmid and determine the effect of ARTS overexpression on LX-2 cells.

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Materials and methods

Materials. LX-2 cells were obtained from Xiang Ya Central Experiment Laboratory (Hunan, China). pIRES2-EGFP vector was purchased from Clontech Laboratories, Inc. (Mountainview, CA, USA) and *E. coli* DH5 α was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Transwell chambers were preserved in our laboratory. DNA marker was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), *Bam*HI and *Hind*III restriction enzymes, RevertAid First Strand cDNA Synthesis kit and dNTPs were purchased from Fermentas (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) staining kit to measure apoptosis was purchased from Roche Diagnostics (Basel, Switzerland). FuGENE6 transfection reagent was purchased from Promega Corporation (Madison, WI, USA). Dimethyl sulfoxide (DMSO) and MTT reagent was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The pEASY-T1 vector, TransStart® FastPfu DNA Polymerase, polymerase chain reaction (PCR) Stimulant and DNA ligase were purchased from Beijing Transgen Biotech Co., Ltd. (Beijing, China), and TRIzol and DNA gel extraction kit were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Primary antibodies for caspase-3 and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), primary antibodies for ARTS were purchased from ProSci, Inc. (Poway, CA, USA), primary antibodies for α -SMA and all secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Gene amplification, cloning and identification. Total RNA was extracted from LX-2 cells by TRIzol reagent according to the manufacturer's protocol. Extracted RNA (1 μ g) was used for cDNA synthesis by reverse transcription (RT) using the RevertAid First Strand cDNA synthesis kit, according to the manufacturer's protocol. The temperature protocol used was as follows: At 42°C for 60 min and at 70°C for 5 min. The following primer sequences were designed based on the ARTS mRNA sequence (GenBank reference, NM_080415.2): 5'-CCCTCGAGGCCACCATGATCAAGCGTTTCCTGG AGG-3' (forward) and 5'-CGGGATCCCTAGTGGCAGCCCTGCCCTGGTG-3' (reverse). Underlined letters indicate the restriction sites. The PCR reaction system was prepared in a total volume 50 μ l containing 10 μ l 5X TransStart FastPfu reaction buffer, 1.25 μ l dNTPs, 1 μ l TransStart® FastPfu DNA Polymerase, 5 μ l PCR Stimulant, 2 μ l primer mix and double distilled H₂O up to 50 μ l. The PCR program was performed at 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and completed at 72°C for 10 min. The full-length ARTS was 846 bp. PCR products were separated by 1% polyacrylamide gels and the target fragment was purified by an agarose gel extraction kit.

The purified fragment was inserted into the pEASY-T1 cloning vector and 100 ng recombined plasmid was transformed into *E. coli* DH5 α competent cells and verified by two pairs of PCR primers maintained in our laboratory. The primer sequences were as follows: First pair primers: 5'-ACACCAGGT TTTGGGGATG-3' (forward) and 5'-TTCCCTTGACAGCAC CCG-3' (reverse); and the second pair primers: 5'-CTGCTG

CCTGTACTTCATCTCA-3' (forward) and 5'-TGCTCTTGG CCTGTTCCCT-3' (reverse). Subsequently, pEASY-T1-ARTS and the eukaryotic expression vector pIRES2-EGFP were digested by *Hind*III and *Bam*HI restriction enzymes at 37°C for 30 min simultaneously, and ligated by DNA ligase at 16°C for 2 h. The recombinant plasmid was validated by double restriction enzyme digestion, separated by 1% PAGE and sequenced by Sanger sequencing by Sangon Biotech Co., Ltd. The correct recombinant plasmid was termed pIRES2-EGFP-ARTS. The construction image is presented in Fig. 1.

Transfection of pIRES2-EGFP-ARTS into LX-2 cells. LX-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), and 100 μ g/ml streptomycin solution and 100 U/ml penicillin at 37°C in a humidified incubator with 5% CO₂. The experiment was divided into the following three groups: Control, vector and ARTS over-expression groups. Plasmids (3–4 μ g) were transfected into LX-2 cells at 37°C for 48 h using FuGENE 6 with DMEM containing 2% FBS, according to the manufacturer's protocol. LX-2 cells (1 \times 10⁶ cells/ml) were fixed in 4% paraformaldehyde for 10 min at room temperature. The transfection efficiency was visualized by using an inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) at magnification, x200. The protein expression of ARTS was confirmed by western blotting.

Western blot analysis. For western blot analysis, after LX-2 cells were transfected with pIRES2-EGFP-ARTS for 48 h, culture media was removed and the cells were washed with PBS. Proteins were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and quantified according to the Bradford method. Equal amounts (30 μ g) of extracted protein samples were separated by 10 or 12% SDS-PAGE followed by electroblotting onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Following electrophoresis, the membranes were blocked with 5–10% non-fat milk for 2 h at room temperature and incubated with specific primary antibodies against ARTS (cat no. 3025; 1:1,000), caspase-3 (cat no. 9665; 1:1,000), α -SMA (cat no. 130617; 1:200) and GAPDH (cat no. 2118; 1:1,000) at 4°C overnight. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat no. sc-2004; 1:5,000) for 2 h at a room temperature. Membranes were visualized using an enhanced chemiluminescence kit (EMD Millipore). Blots were semi-quantified by densitometry using ImageJ software version 2.0 (National Institutes of Health, Bethesda, MD, USA).

TUNEL assay. Apoptosis was detected using a TUNEL kit according to the manufacturer's protocol. Briefly, cultured cells (1 \times 10⁶ cells/ml) following transfection were fixed with 4% paraformaldehyde (pH 7.4) for 1 h at a room temperature and subsequently permeabilized with 0.1% Triton-X-100 for 2 min on ice. The cells were then incubated with the TUNEL reaction mixture for 60 min and counterstained with Hoechst 33258 (0.5 μ g/ml) for 15 min at room temperature. Cells were

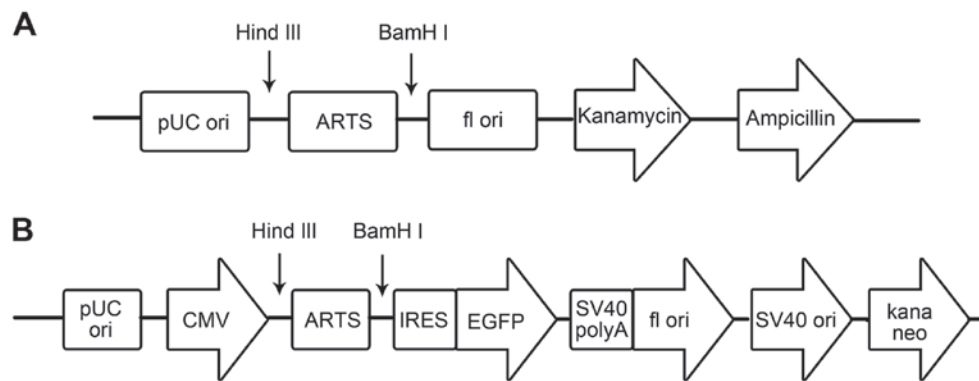


Figure 1. Schematic indicating construction of recombinant plasmids and restriction sites. The construction of the recombinant (A) pEASY-T1-ARTS and (B) pIRES2-EGFP-ARTS plasmids. ARTS, apoptosis-related protein in the transforming growth factor- β signaling pathway; EGFP, enhanced green fluorescent protein.

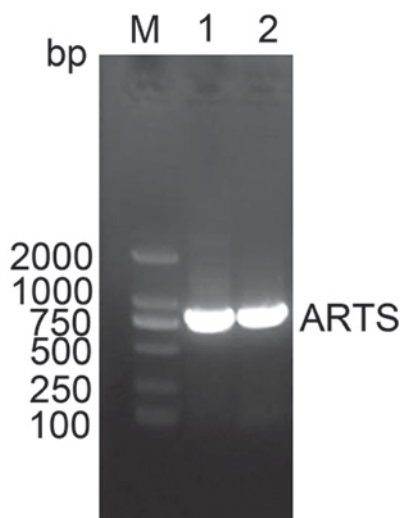


Figure 2. Amplification of ARTS gene by RT-PCR. Lane M, DL2000 DNA marker; lanes 1 and 2, RT-PCR products of ARTS gene (846 bp). ARTS, apoptosis-related protein in the transforming growth factor- β signaling pathway; RT-PCR, reverse transcription-polymerase chain reaction.

washed with 1X PBS. Apoptotic cells were visualized by fluorescence microscopy in at least 10 random fields of view per cover slip.

MTT assay. Cell survival and proliferation were detected by an MTT assay. Briefly, after transfection for 48 h, LX-2 cells were plated in a 96-well culture plate (5×10^3 cells/well) and were incubated with MTT reagent (0.5 mg/ml) for 4 h at room temperature. Dimethyl sulfoxide (0.1%) was subsequently added to dissolve the formazan products for 10 min at room temperature. An ELISA reader was used to determine the optical density at 490 nm wavelength.

Scratch assay. A scratch-wound test was performed to evaluate the effect of ARTS on LX-2 migration. LX-2 cells were seeded in 6-well plates at a density of 1×10^6 cells/ml and transfected for 48 h, as described above. A 200 μ l pipette tip was used to scrape and generate a scratch wound. Following 24 h, cell debris was removed by washing with 1X PBS. The migrated cells were counted with an inverted microscope

under magnification, $\times 50$ to evaluate the migrated proportion in at least 5 random fields of view per well.

Transwell assay. For the Transwell migration assay, cells were resuspended at a density of 1×10^6 cells/ml following transfection for 48 h. Subsequently, 200 μ l cell suspension (2×10^5 cells/ml) in DMEM was added into the upper chambers of the Transwell inserts, whereas the lower chambers were maintained with DMEM containing 10% FBS. Following incubation at 37°C for 24 h, the cell suspension in the upper chambers was removed and cells migrated to the lower chambers were stained with 0.1% crystal violet for 15 min at room temperature. The migrated cells were counted randomly in at least five fields each time under an inverted microscope.

Statistical analysis. All experiments were performed in triplicate and data are presented as the mean \pm standard deviation. The statistical significance of the differences between groups was assessed using one-way analysis of variance followed by a post hoc Tukey test for multiple comparisons. Statistical analysis was performed using SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Amplification of the human full-length ARTS gene in LX-2 cells. To investigate the effect of the overexpression of ARTS on LX-2 cells, the full-length ARTS was amplified. RT-PCR results demonstrated that there was a visible DNA band at ~ 800 bp, which correspond to the target gene ARTS, as determined by 1% agarose gel electrophoresis (full length ARTS, 846bp; Fig. 2).

Construction and identification of recombinant pEASY-T1-ARTS and pIRES2-EGFP-ARTS plasmids. The recombinant pEASY-T1-ARTS was confirmed by PCR as described in the materials and methods, and the results in Fig. 3A demonstrate that two PCR products were observed in lanes 2 and 3 with sizes that match the expected sizes (223 bp and 99 bp, respectively). In the construction of the

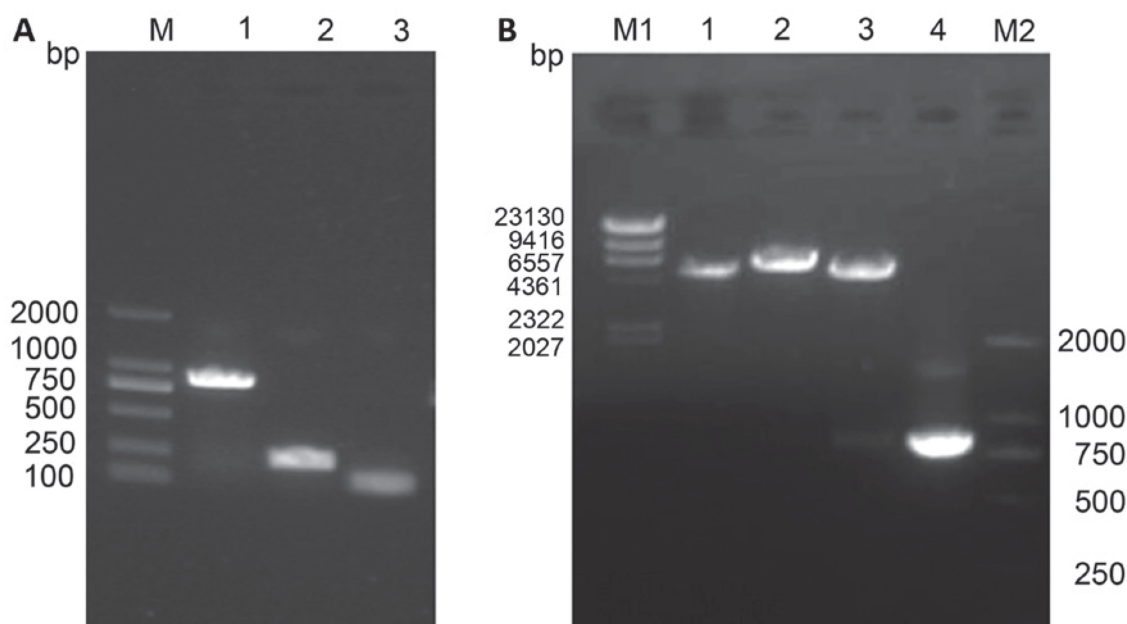


Figure 3. Identification of recombinant plasmids pEASY-T1-ARTS and pIRES2-EGFP-ARTS. (A) Identification of pEASY-T1-ARTS recombinant plasmid. Lane M, DL2000 DNA marker; lane 1, RT-PCR products of ARTS gene; lanes 2 and 3, PCR verified products (223 and 99 bp, respectively). (B) Identification of recombinant plasmid pIRES2-EGFP-ARTS. Lane M1, λ -HindIII DNA marker; lane 1, recombinant plasmid pIRES2-EGFP-ARTS; lane 2, recombinant plasmid single enzyme digestion; lane 3, recombinant plasmid double enzyme digestion with *Bam*HI and *Hind*III; lane 4, ARTS RT-PCR products; lane M2, DL2000 DNA marker. ARTS, apoptosis-related protein in the transforming growth factor- β signaling pathway; EGFP, enhanced green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction.

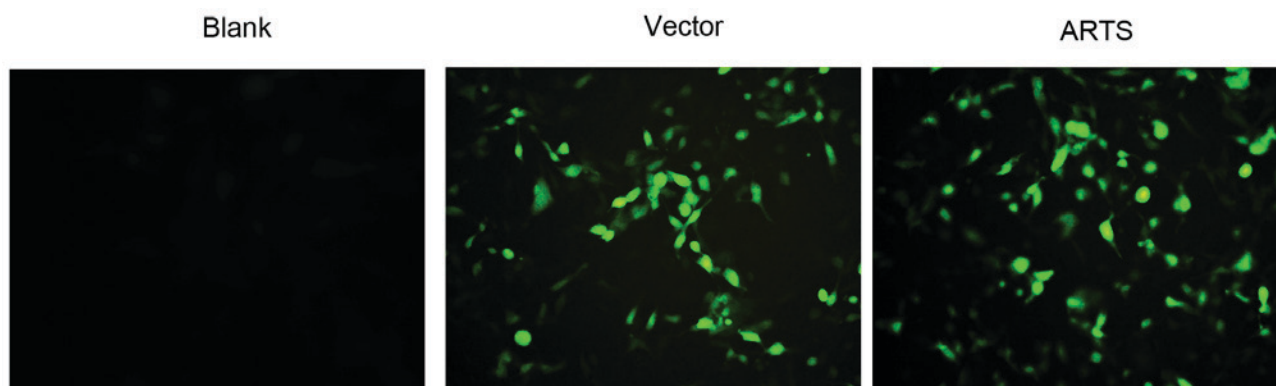


Figure 4. Transfection efficiency of pIRES2-EGFP vector and the recombinant pIRES2-EGFP-ARTS plasmid in LX-2 cells by fluorescence microscopy. Magnification, $\times 200$. EGFP, enhanced green fluorescent protein; ARTS, apoptosis-related protein in the transforming growth factor- β signaling pathway.

pIRES2-EGFP-ARTS plasmid, double enzyme digestion results demonstrated that a ~ 800 bp fragment was present in lane 4 (Fig. 3B). The correct recombinant plasmid was successfully constructed for sequencing verification and subsequent experiments.

ARTS induces apoptosis and inhibits activation of LX-2 cells. After 48 h transfection, EGFP expression was clearly observed in vector and ARTS overexpression groups (Fig. 4). To investigate the effect of ARTS in LX-2 cells, the expression levels of caspase-3 and α -SMA were detected by western blot analysis. As demonstrated in Fig. 5A, the results demonstrated that overexpression of ARTS upregulated the protein levels of cleaved-caspase-3 and downregulated the levels of α -SMA, compared with the vector group ($P < 0.05$). No significant differences were observed between the

vector-only group and the blank group ($P > 0.05$). TUNEL staining demonstrated that ARTS induced apoptosis in LX-2 cells (Fig. 5B). The apoptotic cells revealed typical morphological changes associated with apoptotic cells, including cell shrinking and rounding. Therefore, these results indicate that ARTS may inhibit the activation of LX-2 cells, based on the effects on α -SMA protein levels, and induce their apoptosis.

Overexpression of ARTS has no effect on the proliferation and migration of LX-2 cells. To investigate the effect of ARTS on cell proliferation in LX-2 cells, an MTT assay was performed to evaluate cell survival following transfection. As presented in Fig. 6A, no significant differences were observed among blank, vector and ARTS overexpression groups ($P > 0.05$). In addition, scratch and Transwell assays were performed to

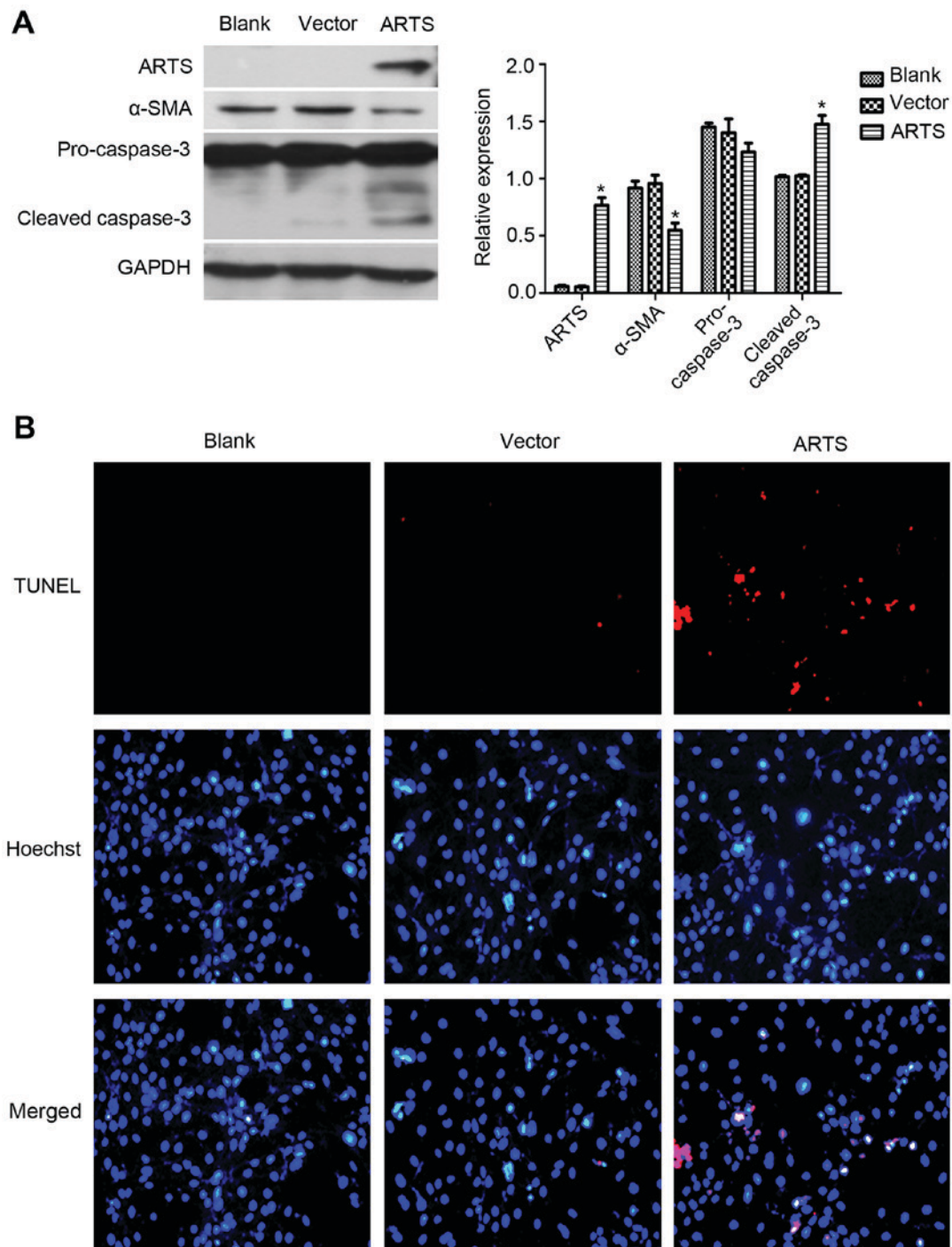


Figure 5. Overexpression of ARTS induced activation and inhibited apoptosis in LX-2 cells. (A) Protein expression of ARTS, α -SMA, pro-caspase-3 and cleaved-caspase-3 was detected by western blot analysis. Results were evaluated by densitometric analysis with GAPDH as a loading control. * $P < 0.05$ vs. vector group. (B) Overexpression of ARTS increased apoptosis in LX-2 cells as detected by TUNEL staining. Magnification, x200. Nuclei were stained blue and apoptotic cells were stained red. ARTS, apoptosis related protein in the transforming growth factor- β signaling pathway; α -SMA, α -smooth muscle actin.

determine the effect of ARTS overexpression on the migration of LX-2 cells. Results presented in Fig. 6B-D demonstrate that overexpression of ARTS had no effect on the migration of LX-2 cells ($P > 0.05$).

Discussion

Hepatic fibrosis, which involves the activation of HSCs, may be caused by various factors, including persistent liver

injuries, chemical ingestion, virus infection and alcoholism (13). Activated HSCs usually secrete ECM and express α -SMA, while quiescent HSCs primarily express PPAR- γ (14). In recent years, apoptosis, senescence, phenotype reversal and immune clearance have been reported to be involved in the clearance of activated HSCs, and apoptosis of activated HSCs may lead to the remission of hepatic fibrosis (2,13,15,16). Therefore, induction of apoptosis in activated HSCs may be a valid strategy for fibrotic reversion.

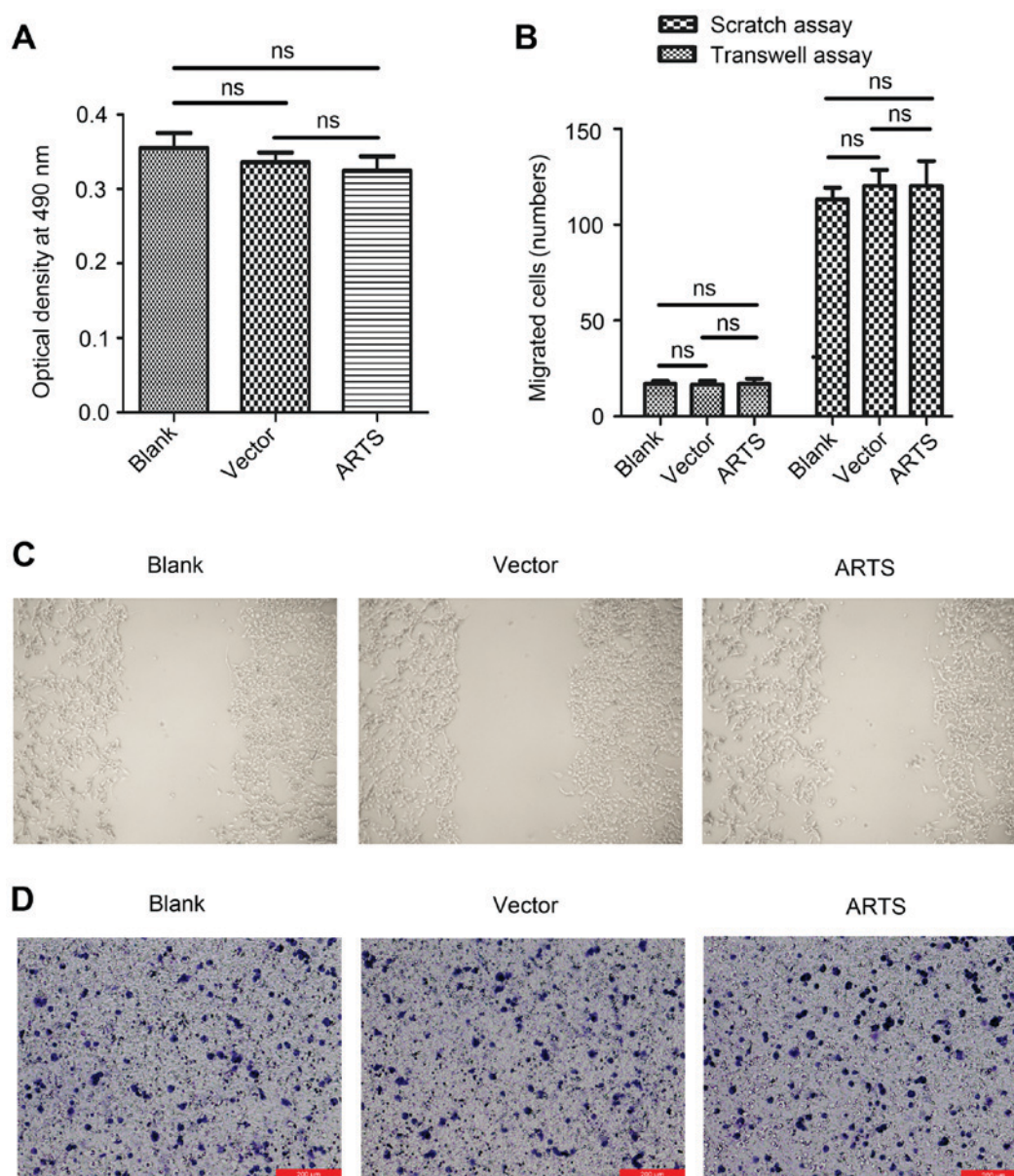


Figure 6. Effect of ARTS overexpression on proliferation and migration in LX-2 cells. (A) MTT assay results demonstrated that overexpression of ARTS had no effect on cell proliferation. (B) Quantified results for scratch and Transwell assays demonstrated that overexpression of ARTS had no effect on cell migration. Representative images for (C) scratch (magnification, x50) and (D) Transwell (magnification, x200) assays. Data are presented as the mean \pm standard deviation of at least three independent experiments. ARTS, apoptosis-related protein in the transforming growth factor- β signaling pathway; ns, no significant difference.

ARTS, a splice variant of Sept4 composed of 846 nucleotides, has been reported to have a role in apoptosis in various cell lines, including A549 and ALLs cells (8,17). Iwaisako *et al* (12) also reported that Sept4 has a role in hepatic fibrosis, however, the underlying mechanism remains unclear. In addition, Shen *et al* (18) demonstrated that Sept4_i2 sensitized hepatocellular carcinoma cells to serum starvation-induced apoptosis (18). The results of previous studies also indicated that Sept4_i2 may be involved in *Schistosoma japonicum*-induced hepatic fibrosis progression, and may regulate apoptosis in HSCs via the PPAR- γ /Akt/Bcl-2 pathway (19-21). Therefore, the present study constructed the eukaryotic recombinant pIRES2-EGFP-ARTS plasmid and determined the effects of ARTS overexpression on LX-2 cells. EGFP is a green fusion protein and has no influence on target

gene construction and function (22). EGFP is commonly used as a method for expression modality and gene transfection efficiency detection (23).

In the present study, in order to determine the effect of ARTS on LX-2 cells, the eukaryotic recombinant pIRES2-EGFP-ARTS plasmid was constructed and EGFP was used to observe transfection efficiency and expression. Western blotting and TUNEL staining results demonstrated that overexpression of ARTS induced apoptosis and inhibited activation in LX-2 cells. Therefore, ARTS may be a candidate molecule for treatment against hepatic fibrosis. Our preliminary work may provide the fundamental basis for further research regarding the intracellular signaling mechanisms underlying the effects of ARTS expression in LX-2 cells.

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