

# CircRNA-0044073 is upregulated in atherosclerosis and increases the proliferation and invasion of cells by targeting miR-107

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**Abstract.** Circular RNAs (circRNAs) are endogenous non-coding RNAs implicated in atherosclerosis. The aim of the present study was to explore the function of circRNA-0044073 in atherosclerosis. Reverse transcription quantitative polymerase chain reaction assays were used to measure the expression levels of circRNA-0044073, microRNA (miRNA/miR)-107, janus kinase 1 (JAK1), signal transducer and activator of transcription 3 (STAT3), B-cell lymphoma 2 (Bcl-2) and v-myc avian myelocytomatosis viral oncogene homolog (c-myc) in blood cells from patients with atherosclerosis. RNA pull-down and luciferase reporter assays were then used to determine the association between circRNA and miR expression, and miR and gene expression, respectively. Matrigel invasion assay and flow cytometry were used to analyze cell invasion and cell cycle. Western blot analysis and ELISA were used to evaluate the expression levels of proteins. It was identified that the expression of circRNA-0044073 was upregulated and the expression of miR-107 was downregulated in atherosclerotic blood cells. Overexpression of circRNA-0044073 promoted the proliferation of human vascular smooth muscle cells (HUVSMCs) and human vascular endothelial cells (HUEVCs), while overexpression of miR-107 inhibited their proliferation. In addition, circRNA-0044073 suppressed the levels of miR-107 via a sponge mechanism. Lipopolysaccharide (LPS) affected the proliferation of HUVSMCs and HUEVCs, and also resulted in changes in circRNA-0044073 expression levels. CircRNA-0044073 promoted the proliferation and invasion of HUVSMCs and HUEVCs in spite of the opposite effect observed with LPS treatment. The JAK/STAT signaling

pathway was activated in patients with atherosclerosis. CircRNA-0044073 favored the activation of the JAK/STAT signaling pathway and inflammation in HUVSMCs and HUEVCs. These data indicate that circRNA-0044073 is upregulated in atherosclerosis and promotes the proliferation and invasion of cells by targeting miR-107 and activating the JAK/STAT signaling pathway, potentially offering a target for novel treatment strategies against atherosclerosis.

## Introduction

Atherosclerosis is a chronic inflammatory disorder of the arterial vessel walls characterized by lipid deposition and fibrous cap formation in the arterial intima. Atherosclerosis contributes to cardiovascular disease (CVD), a major cause of morbidity and mortality in Western countries. A total of ~80 million Americans have one or more forms of CVD (1). Growing evidence suggests that dysfunction of endothelial cells (ECs) and vascular smooth muscle cells (VSMC) is critical in the formation of atherosclerosis. Abnormal proliferation and migration of these cells contribute to the progression of atherosclerosis (2-4). VSMCs migrate and proliferate to form a stabilizing fibrous cap that encapsulates atherosclerotic plaques (5). During the initiation and development of inflammation, ECs and VSMCs produce numerous types of cytokines, including tumor necrosis factors (TNF), interleukin (IL), adhesion molecules, interferon, and adventitium-derived relaxing factors that are implicated in atherosclerosis (6-9).

Circular RNAs (circRNAs) are evolutionarily conserved non-coding RNAs produced by the scrambling of exons at the time of splicing. They are primarily produced in the brain and are present inside the cell. Previous data have suggested that the circRNA antisense non-coding RNA in the INK locus modulates ribosomal RNA maturation and atherosclerosis in humans, indicating that circularization of long non-coding RNAs may alter RNA function and therefore protect against disease (10). The great potential of circRNAs as biomarkers for the early detection of cardiovascular diseases has been highlighted in several patents and previous studies (11). Certain circRNAs, including circRNA-0003575 and circRNA-000595, are significantly associated with atherosclerosis (12,13). Furthermore, the role of circRNAs in atherosclerosis is associated with the

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regulation of the physiology of ECs and smooth muscle cells (SMCs) (10). MicroRNAs (miRNAs) are a group of highly conserved small non-coding RNA molecules measuring 18-22 nucleotides in length. By binding to the 3'-untranslated region of their target mRNAs, miRNAs regulate expression levels of downstream genes (14,15). In addition to participating in regulating cell-cycle and cell proliferation (16,17), miRNAs are associated with inflammatory processes involved in atherosclerosis (18). miR-107 is implicated in the pathogenesis of diseases, including renal disease, Alzheimer's disease, gastric cancer, colon cancer and hepatocellular carcinoma (19-22). Previous studies suggested that miR-107 is a regulator of atherosclerosis (23,24). In addition, binding of miR-107 to the Clock circadian rhythm gene resulted in the deregulation of the circadian rhythm of the cells (25,26). However, the exact mechanism is largely unknown.

Certain circRNAs have been described as regulatory transcripts as they behave like sponges to bind miRNAs. For example, a previous study examining the role of circular RNA-7 demonstrated that the circRNA was an inhibitor of miR-7, an miRNA known to regulate various diseases including, cancer, neurodegenerative diseases, diabetes and atherosclerosis (27). In this context, the present study aimed to investigate the role of circRNA in the regulation of atherosclerosis and to understand the underlying mechanism associated with miRNAs and signaling pathways.

## Materials and methods

**Patients and samples.** Human blood cells were collected from 20 patients with atherosclerosis and healthy controls at the Qi-Lu Hospital of Shandong University (Jinan, China). In total, 13 males and seven females (age range, 48-68 years) were enrolled in the study. Following sonographic examination, the patients with a carotid intima-media thickness  $\geq 1.3$  mm were considered to exhibit an atherosclerotic plaque. Informed consent was obtained from all patients and ethical approval was granted by The Ethics Committee of the Qi-Lu Hospital of Shandong University.

**Cell culture and treatment.** All reagents for cell culture were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and human umbilical vein smooth muscle cells (HUVSMCs) were purchased from iXCells Biotechnologies (San Diego, CA, USA). HUVECs and HUVSMCs cells were maintained in minimum essential medium-L-glutamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), smooth muscle cell growth medium and RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). The medium was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin-amphotericin. The cells were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The culture medium was changed twice weekly. HUVECs and HUVSMCs were sub-cultured subsequent to reaching 80% confluency. For the following assay, the cells initially received either 100 ng/ml lipopolysaccharide (LPS) or vehicle. The template DNA of circRNA-0044073 was synthesized and then inserted into the

multiple cloning site in the pAd-Track-cmv vector (Addgene, Inc., Cambridge, MA, USA). A total of 500 ng of plasmid DNA was subsequently transfected into HUVECs or HUVSMCs in 24-well plates using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to increase circRNA-0044073 expression, whereas, the empty vector was transfected as the negative control (NC). Following 48 h of incubation, the transfected cells were used in the following experiments.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and was reverse transcribed using the Bestar qPCR RT kit (DBI Bioscience, Ludwigshafen, Germany) at 65°C for 5 min. qPCR amplification was performed in triplicate using DBI Bestar® SybrGreen qPCR MasterMix (DBI Bioscience, Ludwigshafen, Germany) in a total volume of 20  $\mu$ l containing 2  $\mu$ l each cDNA. The amplification protocol consisted of initial denaturation for 2 min at 94°C, followed by 40 cycles of denaturation (94°C for 20 sec), annealing for 20 sec at 58°C and elongation for 20 sec at 72°C. The primers for circRNA-0044073, miR-107, U6, janus kinase 1 (JAK1), signal transducer and activator of transcription 3 (STAT3), B-cell lymphoma 2 (Bcl-2), v-myc avian myelocytomatosis viral oncogene homolog (c-myc) and GAPDH are summarized in Table I. Using GAPDH or U6 as endogenous control genes, the relative transcript levels were calculated using the 2<sup>- $\Delta\Delta C_q$</sup>  quantification method (28).

**Cell viability assay.** Cell viabilities were assessed by the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). In brief, cells were added into a 96-well plate at a density of  $2 \times 10^3$  cells/well and incubated overnight at 37°C. Then, LPS was added to each well and incubated at 37°C for 24, 48 and 72 h at the final concentration of 100 ng/ml. Thereafter, 10  $\mu$ l CCK-8 solution was added into each well and incubated for 2 h at 37°C. The absorbance was detected at 450 nm with the microplate reader. Optical density was defined as the relative number of viable cells. The assay was performed in triplicate.

**Western blot analysis.** Following treatments with LPS for 24 h, cells were washed with PBS and subsequently harvested. Total cellular proteins were extracted with radioimmunoprecipitation assay lysis buffer containing 1% phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Protein concentrations were determined with a BCA assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequent to denaturation, a total of 20  $\mu$ g protein per lane was separated by electrophoresis by 10% SDS-PAGE and analyzed. Proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) and then blocked with 5% dry milk in TBST (TBS with 0.1% Tween 20) for 1 h at room temperature, washed, and incubated overnight at 4°C with primary antibodies against JAK1 (1:1,000; cat. no. ab47435; Abcam, Cambridge, MA, USA), STAT3 (1:1,000; cat. no. ab68153; Abcam), phosphorylated (p)-STAT3 (1:800; cat. no. ab30647; Abcam), Bcl-2 (1:1,000; cat. no. 15071; Cell Signaling Technology, Inc., Danvers, MA, USA), c-myc (1:1,000; cat. no. 13987; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 2118; Cell Signaling Technology, Inc.). The membranes were then washed

Table I. Primers used in the reverse transcription quantitative polymerase chain reaction assay.

Genes	Sequence (5'-3')
hsa_circ_0044073F	ACAGGGGTGTTTGTGTGTGT
hsa_circ_0044073R	CTTCACGTTGCAGGTGTAGC
miR-107 F	ACACTCCAGCTGGGAGCAGCA UUGUACAGGG
miR-107 R	CTCAACTGGTGTCTGTGGA
U6 F	CTCGCTTCGGCAGCAC
U6 R	AACGCTTCACGAATTTGCGT
JAK1 F	CACCAGGATGCGGATAAATAAT
JAK1 R	CAAAGTTTCCAAGGTAGCCAAG
STAT3 F	TGGTGTCTCCACTGGTCTATCTC
STAT3 R	CATCAATGAATCTAAAGTGCGG
Bcl-2 F	ATCGCCCTGTGGATGACTG
Bcl-2 R	AGACAGCCAGGAGAAATCAAAC
c-myc F	CATACATCCTGTCCGTCCA
c-myc R	CGCACAAGAGTTCCGTAGC
GAPDH F	TGTTTCGTCATGGGTGTGAAC
GAPDH R	ATGGCATGGACTGTGGTCAT

F, forward; R, reverse; hsa, *Homo sapiens*; miR, microRNA; JAK1, janus kinase 1; STAT3, signal transducer and activator of transcription 3; Bcl-2, B-cell lymphoma 2; c-myc, v-myc avian myelocytomatosis viral oncogene homolog.

with TBST and then incubated with the secondary antibodies anti-rabbit and anti-mouse immunoglobulin G conjugated with horseradish peroxidase (HRP; 1:10,000; cat. no. 7074 and 7076; Cell Signaling Technology, Inc.). Finally, the immunoreactive bands were visualized by using an enhanced chemiluminescent substrate (ECL-Plus; GE Healthcare, Chicago, IL USA). Using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA; <http://imagej.nih.gov/ij/>), densitometric analysis of bands was performed.

**ELISA.** The IL-1 $\beta$  (cat. no. CSB-E08053h), IL-6 (cat. no. CSB-E04638h) and TNF- $\alpha$  (cat. no. CSB-E04740h) ELISA assay kits were purchased from CUSABIO (Wuhan, China). After centrifugation at 1,000  $\times$  g for 15 min at 4°C, the concentrations in the supernatants were assessed according to the manufacturer's protocols. In brief, the samples were added to wells in duplicate and then incubated at room temperature for 2 h. The liquid was removed, followed by the addition of biotin-antibody and then the plates were incubated at room temperature for 1 h. Following removal of the liquid and washing, HRP-avidin was added and incubated at room temperature for 1 h. Following additional wash steps, visualization was achieved by adding the TMB substrate. The absorbance was detected at 450 nm with a microplate reader (Thermo Fisher Scientific, Inc.). The experiments were performed in triplicate.

**Cell cycle analysis by flow cytometry.** The cell cycle distribution was analyzed as described previously (29). Following treatment with LPS for 24 h, the cells were collected and

washed with PBS and then stained with 50 mg/ml propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 30 min at 4°C. The data were collected with a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for cell-cycle distribution analysis using Cell Quest Software (version 5.1; Becton, Dickinson and Company).

**Matrigel invasion assay.** The cells were collected and suspended in medium without serum. Transwell inserts (24-well insert; pore size, 8 mm; Becton, Dickinson and Company) were inserted in the chamber and incubated at 37°C overnight with serum-free RPMI-1640 with matrigel (0.6 mg/ml; Becton, Dickinson and Company). Subsequently, 4 $\times$ 10<sup>4</sup> cells in 0.2 ml serum-free medium were seeded in the top chambers of the transwell inserts, and 10% FBS was used as an attractant. The cells were incubated with 1 mg/ml LPS or control treatment for 48 h at 37°C. The cells that did not invade through the pores were removed, and the filters were stained at room temperature with hematoxylin for 5 min, and with eosin for 1 min (cat. no. C0105; Beyotime Institute of Biotechnology, Haimen, China). Samples were visualized under a light microscope (magnification,  $\times$ 200) and counting.

**RNA pull-down assay.** The RNA pull-down assay was performed as previously described (30). In brief, either the pAd-Track-cmv-circRNA-0044073 or the control vectors were transfected into 293 cells (ATCC). Subsequently, 100  $\mu$ g total RNA was extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) from the cells and incubated with 500  $\mu$ g streptavidin magnetic beads (cat. no. S1421S; New England BioLabs, Inc., Ipswich, MA, USA), which were previously incubated for 2 h at 37°C with either 200 pmol biotin-miR-107 or 200 pmol biotin-miR-107-mut (Shanghai GenePharma Co., Ltd., Shanghai, China). Following RNA elution, RT-qPCR was performed to detect circRNA-0044073 as aforementioned.

**DNA construct and luciferase reporter assay.** As described previously (18), the psiCHECKTM-2 firefly luciferase reporter plasmids (Promega Corporation, Madison, WI, USA) were recombined with the potential miR-107 binding site sequences of JAK1 or its mutated sequence. 293 cells were seeded into a 12-well plate (3 $\times$ 10<sup>5</sup> cells/well) and transfected with 200 ng recombinant luciferase reporter plasmid using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection with 10 nM miR-107 mimics (5'-AGCAGCAUUGUACAGGGCUAUA-3') or NC miRNA (5'UUCUCCGAA CGUGUCACGUTT-3') for 24 h, the cells were collected, and luciferase activity was determined using the Dual-Luciferase Reporter Gene Assay kit (Beyotime Institute of Biotechnology). The relative luciferase activity was obtained by normalizing the firefly luciferase activity to the internal control *Renilla* luciferase activity.

**Statistical analysis.** Data in the present study are presented as the mean  $\pm$  standard deviation of three independent tests. Using GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA), two-tailed Student's t-tests were performed to compare data between two groups, whereas, one-way analysis of variance followed by Tukey's post hoc test was performed to compare data among three or more groups.



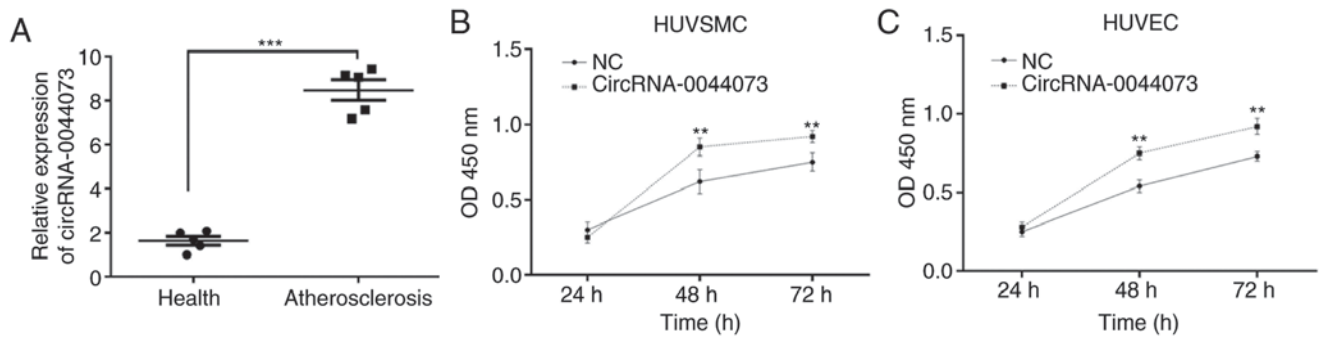


Figure 1. Dysregulated expression levels of circRNA-0044073 in blood cells of patients with atherosclerosis and its role in cell proliferation. (A) Expression levels of circRNA-0044073 from blood cells from 5 randomly selected patients with atherosclerosis and 5 healthy donor livers were detected. (B) CircRNA-0044073 significantly promoted the proliferation of HUVMCs. (C) CircRNA-0044073 significantly enhanced the proliferation of HUVECs. \*\*P<0.01 and \*\*\*P<0.001 vs. control. circRNA, circular RNA; HUVMCs, human vascular smooth muscle cells; HUVECs, human vascular endothelial cells; NC, negative control; Health, health donor samples.

## Results

*Levels of circRNA-0044073 are upregulated in blood cells of patients with atherosclerosis and are associated with the proliferation of HUVMCs and HUVECs.* The present study identified that the expression of circRNA-0044073 in blood cells was significantly increased in patients with atherosclerosis compared with that of healthy controls (P<0.001; Fig. 1A). Therefore, we hypothesized that circRNA-0044073 serves a key role in atherosclerosis. As the abnormal proliferation of ECs, SMCs and macrophages contribute to the progression of atherosclerosis, the *in vitro* protocols of the present study included HUVECs and HUVMCs to determine the effect of circRNA-0044073 on cell growth. It was observed that the overexpression of circRNA-0044073 significantly increased the proliferation of HUVECs and HUVMCs, compared with control groups, at 48 and 72 h (P<0.01; Fig. 1B and C). These results suggest a potential association between the upregulation of circRNA-0044073 and proliferation of atherosclerosis-associated cells.

*Levels of miR-107 are downregulated in blood cells of patients with atherosclerosis and is targeted by circRNA-0044073.* A previous study has suggested the involvement of miR-107 in the development of atherosclerosis (26). Therefore, the levels of miR-107 were detected and compared between patients with atherosclerosis and healthy individuals. Notably, the expression of miR-107 was significantly decreased in the blood cells of patients with atherosclerosis compared with the controls (P<0.05; Fig. 2A). In addition, miR-107 mimics significantly inhibited the proliferation of HUVECs and HUVMCs, compared with the control groups, at 48 and 72 h, which was in contrast to the observed effects of circRNA-0044073 (P<0.01; Fig. 2B and C). The association between circRNA-0044073 and miR-107 was then investigated; the data suggested that the overexpression of circRNA-0044073 significantly downregulated the levels of miR-107 in HUVECs and HUVMCs (P<0.01; Fig. 2D and E). Furthermore, RNA pull down assay demonstrated that miR-107 was a target of circRNA-0044073 (P<0.01; Fig. 2D-F). These results indicate that circRNA-0044073 counteracts the effect of miR-107 via a sponge mechanism.

*CircRNA-0044073 increases the proliferation and invasion of cells by regulating miR-107.* The regulatory mechanism of circRNA-0044073 on the invasive activity of HUVMCs and HUVECs was then explored. As an endotoxin, LPS is a potentially important stimulator and atherosclerosis risk factor. Therefore, an *in vitro* atherosclerosis model was established by treating cells with LPS. Compared with the control groups, treatment with LPS significantly increased the number of invasive HUVMCs and inhibited the proliferation of HUVECs, respectively (P<0.01; Fig. 3A and B). Additionally, LPS treatment significantly increased the levels of circRNA-0044073 in HUVMCs but downregulated the levels of circRNA-0044073 in HUVECs (P<0.001; Fig. 3C). It was additionally identified that in HUVMCs, the combined use of LPS and circRNA-0044073 significantly increased the proliferation of cells, compared with a single treatment of LPS or circRNA-0044073 overexpression vector (P<0.01; Fig. 3D and E). By contrast, miR-107 mimics significantly alleviated the effect of LPS + circRNA-0044073 treatment on the promotion the proliferation of cells (P<0.01; Fig. 3D-E). Although the invasive activity of HUVECs was suppressed by LPS, circRNA-0044073 transfection partially reversed the effect of LPS and induced the invasion of HUVECs. Similar to the effect in HUVMCs, miR-107 impeded the role of circRNA-0044073 in promoting cell proliferation. The effect of circRNA-0044073 on the cell cycle distribution was also examined. Despite the observation that LPS treatment affected the percentages of G2/M and S phase cells, with the addition of circRNA-0044073 transfection, the percentage of cells in G2/M phase was additionally increased, and was partially impaired by using miR-107 mimics (Fig. 4). These results support the hypothesis that circRNA-0044073 facilitates the proliferation and invasion of cells by inhibiting miR-107 in the development of atherosclerosis.

*CircRNA-0044073 activates the JAK/STAT signaling pathway of HUVMCs and HUVECs.* JAK1 is a critical contributor to the pathogenesis of a number of inflammatory diseases and has been identified as a potential therapeutic target (31,32). The expression levels of JAK1 and STAT3 were significantly increased in blood cells in patients with atherosclerosis, in addition to an increase in Bcl-2 and c-myc levels, compared with those in the healthy controls (P<0.001; Fig. 5A-D). Notably, in

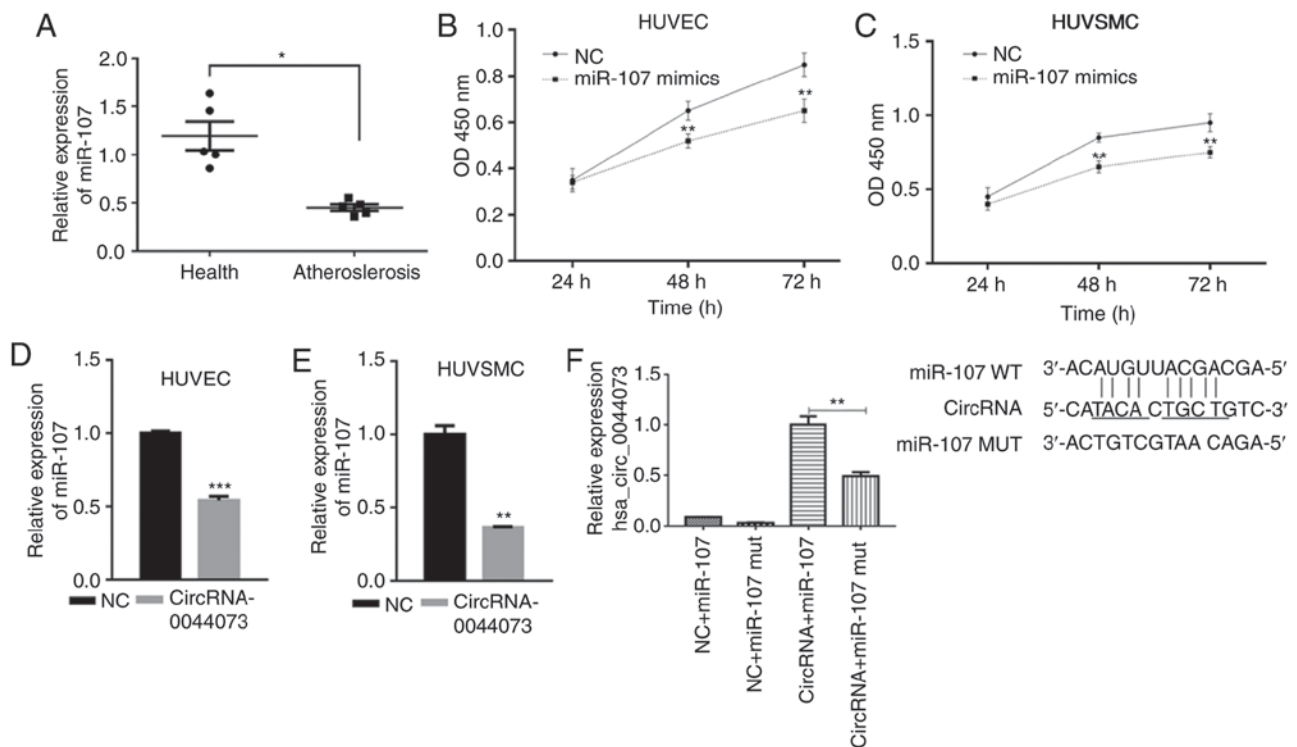


Figure 2. Effect of miR-107 in atherosclerosis and negative associations with circRNA-0044073. (A) Expression levels of miR-107 from blood cells from 5 randomly selected patients with atherosclerosis and 5 healthy-donor livers were detected. (B) miR-107 significantly inhibited the proliferation of HUVECs. (C) miR-107 significantly inhibited the proliferation of HUVECs. (D) CircRNA-0044073 significantly downregulated the level of miR-107 in HUVECs. (E) CircRNA-0044073 significantly decreased the level of miR-107 in HUVECs. (F) Targeting of miR-107 by circRNA-0044073 was determined by RNA pull-down assay. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control. miR, microRNA; circRNA, circular RNA; HUVECs, human vascular smooth muscle cells; HUVECs, human vascular endothelial cells; NC, negative control; Health, health donor samples.

the LPS treatment group, the Bcl-2 and c-myc levels were markedly increased in HUVECs and decreased in HUVECs, which is consistent with the distinct effect of LPS on the proliferation of these two types of cells (33,34). However, the overexpression of miR-107 significantly inhibited the levels of JAK1 in HUVECs and HUVECs (Fig. 5E-F). The luciferase reporter assay additionally confirmed that JAK1 was a target of miR-107 (Fig. 5G). In contrast to the inhibitory role of miR-107 on JAK1 expression, overexpression of circRNA-0044073 activated the JAK/STAT signaling pathway, along with upregulation of Bcl-2, c-myc, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels, compared with those in control group, which is in accordance with the *in vivo* data concerning the changes in expression of JAK1, p-STAT3, Bcl-2 and c-myc (Fig. 5H-N).

## Discussion

As the primary cause of myocardial infarction, heart failure, myocardial ischemia and stroke, atherosclerosis accounts for high mortality and morbidity rates worldwide (35). Although a number of treatments have been developed and widely applied for atherosclerosis, certain subgroups of patients remain at high risk for the development and progression of atherosclerosis, creating an urgent requirement to explore novel therapeutic targets or/and more effective treatments for atherosclerosis. A previous study demonstrated that circRNAs are crucial contributors to the pathogenesis of atherosclerosis (36). Therefore, increasing interest in the treatment of atherosclerosis is being focused on circRNAs at present. In the

present study, to the best of our knowledge, it was observed for the first time that circRNA-0044073 levels were negatively associated with atherosclerosis and that circRNA-0044073 may directly target miR-107 and thereby lead to the upregulation of JAK1/STAT3 signaling.

As a chronic inflammatory disease, atherosclerosis constitutes a dysregulation of a variety of cytokines. A previous study demonstrated that the JAK/STAT signaling pathway promotes vascular cell inflammation, proliferation, migration and adhesion (37). JAK1 is expressed in all cell types, including ECs and SMCs (38,39). Among the STAT proteins, STAT3 is an activator of systemic inflammatory genes and is present in the inflammatory regions of human atherosclerotic lesions in an activated form. Previous data demonstrated an important role for the JAK/STAT pathway in oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine-induced IL-8 transcription *in vitro* and in atherosclerosis *in vivo* (1). It has also been indicated that suppressors of cytokine signaling modulate the JAK/STAT-dependent responses in vascular cells, which are associated with atherosclerotic plaque development (40). In the present study, it was observed that the expression levels of JAK1 and STAT3 were significantly increased in the blood cells from patients with atherosclerosis, which was consistent with previous studies (18,41). Bcl-2 proteins represent the major regulators of extrinsic and intrinsic apoptosis signaling pathways and target apoptosis of vascular cells in atherosclerotic lesions. The decrease in c-myc oncogene levels alleviates the proliferation in rat VSMCs and induces apoptosis (42). Therefore, Bcl-2 and c-myc serve important roles in the deregulation of cell apoptosis and

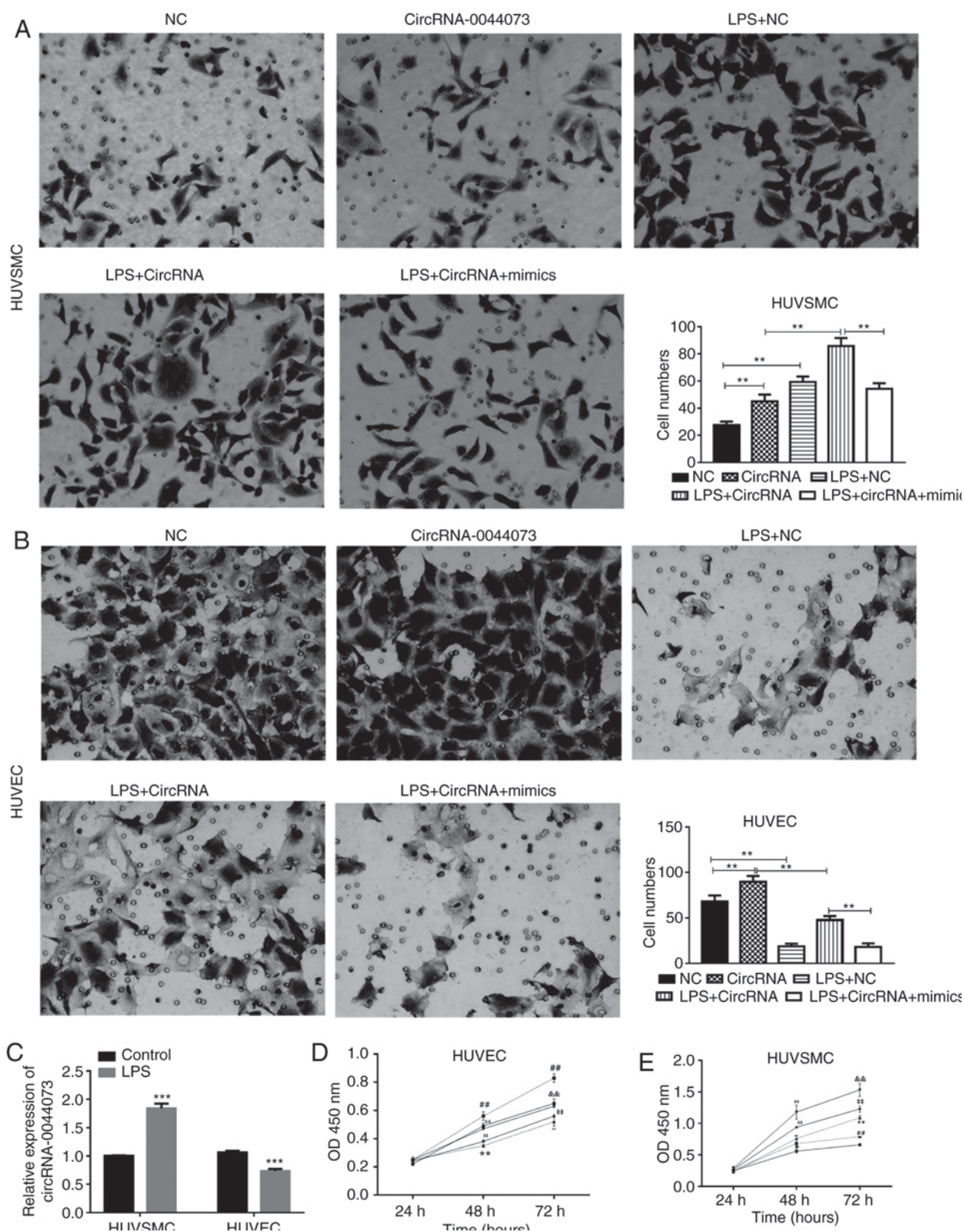


Figure 3. Effect of circRNA-0044073 on cell proliferation and invasion of HUVMSCs and HUVECs. (A) LPS induced the proliferation of HUVMSCs. (B) LPS facilitated the proliferation of HUVECs. (C) LPS affected the level of circRNA-0044073 in the HUVMSCs and HUVECs. (D) The representative images and histograms indicate the numbers of the migrated HUVMSCs (magnification, 100x). (E) The representative images and histogram indicate the numbers of the migrated HUVECs (magnification, 100x). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control; &\* $P < 0.01$  LPS+circRNA vs. LPS+NC; ## $P < 0.01$  CircRNA vs. NC; \*\* $P < 0.01$  LPS+circRNA+mimics vs. LPS+circRNA. circRNA, circular RNA; HUVMSCs, human vascular smooth muscle cells; HUVECs, human vascular endothelial cells; NC, negative control; LPS, lipopolysaccharide.

proliferation, contributing to the pathogenesis of atherosclerosis (43). The data presented in the present study indicated that the expression levels of Bcl-2 and c-myc were significantly

increased in the blood cells from patients with atherosclerosis compared with the healthy controls. Consistent with these results, a previous study revealed that c-myc was overexpressed



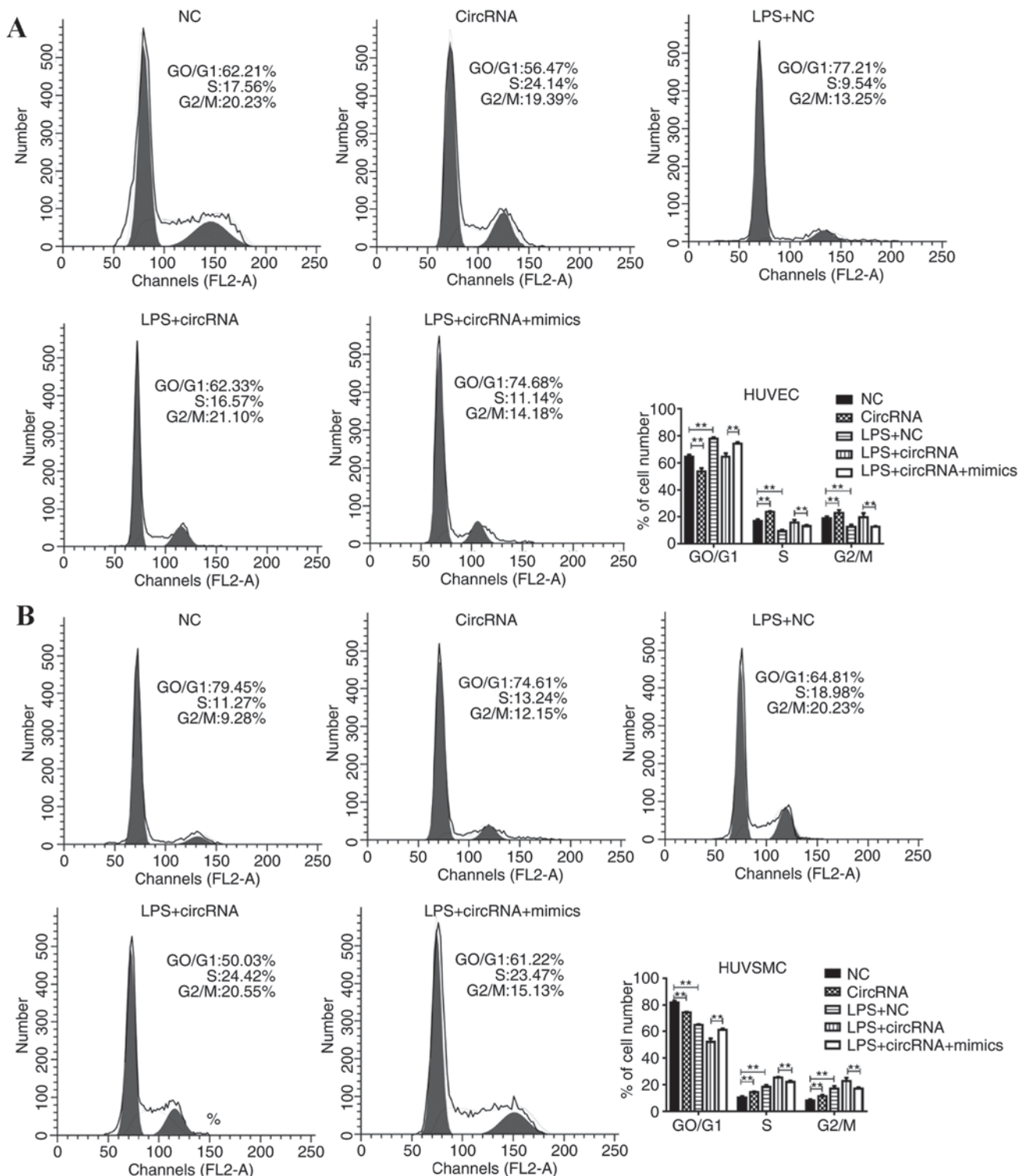


Figure 4. Effect of circRNA-0044073 on cell cycle. Cell cycle phases of (A) HUVECs and (B) HUVSMCs. \*\*P<0.01 vs. NC. circRNA, circular RNA; HUVSMCs, human vascular smooth muscle cells; HUVECs, human vascular endothelial cells; NC, negative control.

in plaque SMCs (44). However, a small number of studies contradict the Bcl-2 data from the present study (45,46). This may be due to differences in the test samples and the stage of atherosclerosis investigated.

HUVECs have been used as *in vitro* models for the study of atherosclerosis (47). As an endotoxin, LPS is a potentially important stimulator and risk factor for atherosclerosis (48). LPS has been implicated in endothelial injury and has induced

apoptosis and proliferation in endothelial and VSMCs, respectively (34,35,49). Consistently, the present study observed that LPS exposure resulted in the apoptosis of HUVECs and the proliferation of HUVSMCs, and the increased expression of circRNA-0044073 in the cells. With the proliferative effect of circRNA-0044073, the apoptosis of HUVECs by LPS was significantly attenuated, and the proliferation of HUVSMCs was significantly improved. Furthermore, it was also observed

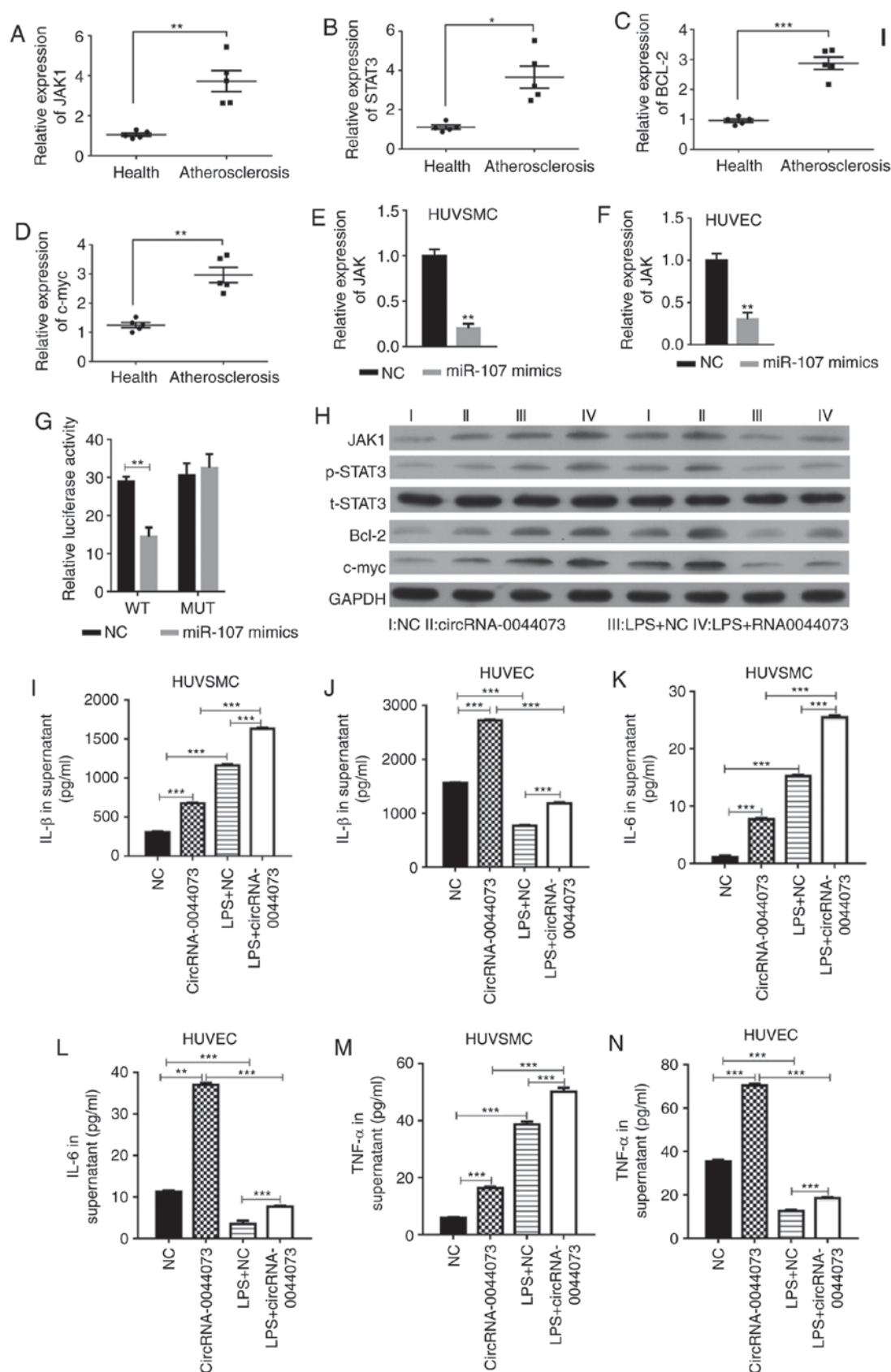


Figure 5. Effects of circRNA-0044073 on the JAK/STAT pathway and inflammation in atherosclerosis. Expression levels of (A) JAK1, (B) STAT3, (C) Bcl-2 and (D) c-myc from blood cells from 5 randomly selected patients with atherosclerosis and 5 healthy donor livers were detected. miR-107 significantly downregulated the expression levels of JAK1 in (E) HUVMSCs and (F) HUVECs. (G) The role of JAK1 as a target of miR-107 was identified by using a luciferase reporter assay. (H) JAK1, t-STAT3, p-STAT3, Bcl-2, c-myc levels were analyzed by western blot analysis. Expression levels of (I) IL-1 $\beta$  in HUVMSCs, (J) IL-1 $\beta$  in HUVECs, (K) IL-6 in HUVMSCs, (L) IL-6 in HUVECs, (M) TNF- $\alpha$  in HUVMSCs and (N) TNF- $\alpha$  in HUVECs were analyzed by ELISA. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. NC. circRNA, circular RNA; HUVMSCs, human vascular smooth muscle cells; HUVECs, human vascular endothelial cells; NC, negative control; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; JAK1, janus kinase 1; STAT3, signal transducer and activator of transcription 3; Bcl-2, B-cell lymphoma 2; c-myc, v-myc avian myelocytomatosis viral oncogene homolog; t-STAT3, total STAT3; p, phosphorylated; Health, health donor samples.



that the invasion levels of HUVECs and HUVSMCs were promoted on treatment with LPS. Therefore, we hypothesized that circRNA-0044073 is associated with the initiation and development of the disease. However, additional studies are required to validate this.

miR-107 is associated with inflammation (23). Previously, it was demonstrated that the expression of miR-107 is decreased in macrophages following LPS stimulation (23,50). A consistent change in miR-107 expression in the blood cells from patients with atherosclerosis was observed in the present study. However, it was also demonstrated that the miR-107 expression was upregulated in HUVECs and HUVSMCs following LPS treatment. This variation may be due to the different cells examined. Based on the actual pro-inflammation function of miR-107, the decrease in miR-107 expression may be a result of the regenerative feedback (23). Consistent with a previous study, the present study confirmed that miR-107 directly targeted JAK1 (51). Additionally, it was demonstrated that miR-107 is also a direct target of circRNA-0044073. Furthermore, it was observed that in HUVSMCs and HUVECs, circRNA-0044073 overexpression decreased miR-107 levels and resultantly increased the levels of JAK1 and p-STAT3, and the levels of downstream proteins including Bcl-2 and c-myc. As JAK1/STAT3 serve critical roles in inflammation, we hypothesized that circRNA-0044073 may be associated with inflammation, which is a key contributor to atherosclerosis. In the present study, it was observed that circRNA-0044073 significantly induced the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . These pro-inflammatory cytokines are pro-atherogenic. However, the underlying mechanisms by which circRNA-0044073 regulates these inflammatory cytokines requires additional investigation.

Taken together, the data from the present study suggests that circRNA-0044073 is a potential therapeutic target for the treatment of atherosclerosis, as it directly targets miR-107 and resultantly increases STAT3 activation, and upregulates the expression of the downstream proteins that contribute to atherosclerosis.

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## Authors' contributions

LS, YH and WWu designed the study. LS, YH, JL, SY, WWa and YW performed the experiments. LS, YX and WWa performed the data analysis and drafted the manuscript. All authors read and approved the final manuscript.

## Availability of data and materials

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

## Ethics approval and consent to participate

Informed consent was obtained from all patients and ethical approval was granted by The Ethics Committee of the Qi-Lu Hospital of Shandong University.

## Patient consent for publication

Informed consent was obtained from all patients.

## Competing interests

The authors declare that they have no competing interests.

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