RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2023.100

ScienceAsia 49 (2023): **899–909**

Silencing circRNA TLK1 reduces endothelial cell injury in coronary heart disease

Jian Li[a], **Zhu Zhuo**[b], **Zhen Ren**[c], **GaiXia Li**[d,*]

[a] Department of Internal Medicine, Qingdao Shinan District People's Hospital, Qingdao City, Shandong 266005 China
[b] Department of Cardiology, Linyi Third People's Hospital, Linyi City, Shandong 276023 China
[c] Department of Nursing, Qingdao Shinan District People's Hospital, Qingdao City, Shandong 266005 China
[d] Department of ECG Room, Qingdao Women and Children Hospital, Qingdao City, Shandong 266034 China

*Corresponding author, e-mail: ligaixia@stu-edu.cn

Received 16 Feb 2023, Accepted 31 Oct 2023
Available online 29 Dec 2023

ABSTRACT: Circular RNAs (circRNAs) play important roles in the progression of many diseases including coronary heart disease (CHD), one of the leading causes of heart-related death worldwide. This study aimed to investigate the effect and mechanism of circTLK1 on vascular endothelial cell injury by targeting the miR-374a-5p/FOXO1 axis. Here, oxidized low-density lipoprotein (ox-LDL)-treated HUVECs and HFD-fed ApoE$^{-/-}$C57BL/6J mice were used as *in vitro* and *in vivo* models, and the expression levels of circTLK1, miR-374a-5p, and FOXO1 were examined by RT-qPCR or Western blot analysis. CircTLK1 and FOXO1 were found to be increased in blood of patients with coronary heart disease and ox-LDL-induced HUVECs, while miR-374a-5p was decreased. Silencing circTLK1 attenuated endothelial cell injury and atherosclerosis development *in vitro* and *in vivo*. miR-374a-5p can be sponged by circTLK1, and miR-374a-5p inhibitor can abolish the therapeutic effect of silencing circTLK1 on endothelial cell injury. In addition, miR-374a-5p bound to FOXO1, which mediated the therapeutic effect of circTLK1 silencing on vascular endothelial cell injury. Taken together, circTLK1 silencing reduces endothelial cell injury in CHD by targeting FOXO1 with miR-374a-5p, suggesting that circTLK1 may be a new target for CHD treatment.

KEYWORDS: circTLK1, coronary heart disease, FOXO gene, endothelial cell injury

INTRODUCTION

Coronary heart disease (CHD) is one of the leading causes of death worldwide [1]. Atherosclerosis is considered the main pathogenesis of CHD [2]. Factors such as lipid metabolism disorder, vascular endothelial cell damage, inflammation, and oxidative stress can stimulate the development of atherosclerosis [3]. At present, CHD is still treated by traditional treatment methods such as lipid-lowering therapy, antiplatelet drugs, nitroglycerin, and coronary artery bypass surgery [4, 5]. To make matters worse, CHD morbidity and mortality are still rising rapidly [6]. Therefore, there is still a need to increase the understanding of the pathogenesis of CHD.

Circular RNAs (circRNAs) are highly conserved and abundant and characterized by higher stability under physical conditions [7]. More and more studies have revealed that circRNAs are involved in the pathogenesis of various diseases including CHD [8]. CircTLK1, derived from TLK1 messenger RNA (mRNA), is associated with cardiovascular and cerebrovascular diseases [9, 10]. It has been reported that circTLK1 can exacerbate myocardial ischemia/reperfusion injury [11]. Recent studies have also revealed that circTLK1 regulates inflammatory and oxidative stress responses as well as cardiomyocyte apoptosis [9, 12]. However, the function of circTLK1 in CHD remains unclear.

Notably, circRNAs mainly regulate downstream target gene expression by sponging microRNAs (miRNAs) [13–15]. Importantly, miRNAs, as powerful regulators of biological processes, have been extensively studied in the inflammatory process and vascular endothelial cell dysfunction in CHD [16]. miR-374a-5p prevents myocardial ischemia-reperfusion injury in mice [17]. Interestingly, it is hypothesized that circTLK1/miR-374a-5p/FOXO1 functions as a competitive endogenous RNA (ceRNA) network in CHD. Therefore, we speculate that circTLK1 may be involved in the progression of CHD by competing with miR-374a-5p. This study revealed for the first time the role of circTLK1 in endothelial cell dysfunction and atherosclerosis and explored the potential regulatory network of circTLK1/miR-374a-5p/FOXO1, developing new insights into the treatment and diagnosis of CHD.

METHODS

Collection and processing of blood samples

Patients with CHD (coronary artery stenosis of not less than 50%) and controls were selected according to the results of diagnostic coronary angiography. Patients with lung, liver, kidney, or immune diseases, other cardiac diseases or underlying diseases were excluded. All participants were pretreated with conventional doses of aspirin and clopidogrel before percutaneous coronary intervention. The age, gender, diabetes history, smoking history, drinking his-

Table 1 Baseline characteristics of the patients.

Clinical data ($n = 42$)	
Age, y	60 [58–65]
Sex (male/female)	24/18 (57.1/42.9)
BMI, kg/m^2	24.6 [22.3–26.6]
Hypertension	27 (64.3)
TC, mmol/l	4.10 ± 1.05
TG, mmol/l	1.36 [1.19–1.85]
LDL-C, mmol/l	2.32 [1.8–2.71]
HDL-C, mmol/l	1.03 [0.88–1.25]

Counting data were expressed as n (%); continuous variables are presented as mean (SD) or median (IQR, quartile range). BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol; and HDL-C, high-density lipoprotein cholesterol.

tory, blood pressure, triglyceride, cholesterol, LDL-C, and HDL-C were obtained from the electronic medical record system (Table 1). Peripheral blood mononuclear cells (PBMCs) were isolated using peripheral blood mononuclear cell isolation medium (Solarbio, Beijing, China) and centrifuged twice for a total of 40 min. Trizol LS (Invitrogen, CA, USA) was then added to PBMCs immediately and stored. The study received approval from the Ethics Committee of Qingdao Women and Children Hospital (approval number: 201603QD11), and all experimental procedures adhered to the regulations set forth by the Ethics Committee. Prior to surgery, this study obtained informed consent from all patients or their respective family members, who willingly signed a written consent form.

Cell culture and treatment

HUVECs (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM-high glucose medium (Hyclone, USA) containing penicillin (100 units/ml, Hyclone) and 10% FBS (Hyclone). As previously described for atherosclerosis [18], HUVECs were treated with 100 μg/ml oxidized low-density lipoprotein (ox-LDL, Yeasen, Shanghai, China) for 24 h to simulate the endothelial injury model of CHD *in vitro*. All plasmids and oligonucleotides (GenePharma, Shanghai, China) were transfected into HUVECs with Lipofectamine 3000 (Invitrogen), including shRNA carrying circTLK1 (sh-circTLK1), FOXO1 overexpression plasmid (pcDNA-FOXO1), miR-374a-5p mimic and inhibitor, and their negative controls (sh-NC, pcDNA3.1, miR-NC and in-NC).

ELISA

Human or mouse vWF, sTM, sE-selectin, sVCAM-1 ELISA kit (Beijing Wintersong Biotech, China) as well as human or mouse IL-6, IL-1β, and TNF-α ELISA kits (Abcam, Wuhan, China) were applied for checking vWF, sTM, sE-selectin, and sVCAM-1, IL-6, IL-1β, and TNF-α.

RT-qPCR

Total RNA was extracted from peripheral blood samples and HUVECs using Trizol reagent (Invitrogen), followed by reverse transcription performed with PrimeScript RT reagent kit and miRNA First Strand Synthesis kit (Takara, Tokyo, Japan), respectively. RT-qPCR was performed using the SYBR Green kit (Thermo Fisher Scientific, Massachusetts, USA) and the Mx3005P QPCR system (Agilent Technologies, California, USA). The primer sequences are shown in Table 2.

Western blot analysis

Total protein was extracted with RIPA lysis buffer (Beyotime, China), loaded on 8% SDS-PAGE gels (Solarbio), transferred to PVDF membranes (Invitrogen), and blocked with 5% skim milk for 1 h. It was then incubated with primary antibody of rabbit anti-FOXO1A (1:1000, ab39470, Abcam) and specific secondary antibody (1:1000, Beyotime). Densitometric analysis was performed using ImageJ software after signal visualization based on ECL kit (34080, Thermo Fisher Scientific).

RNase R assay

CircTLK1 and TLK1 total RNA (2 μg) were incubated with 3 U/μg RNase-R (07250, Epicentre Technologies, Thane, India) and checked by RT-qPCR [19].

Subcellular localization analysis

Cytoplasmic and nuclear RNAs of HUVECs were isolated using the PARIS Kit (Ambion, Texas, USA), checked by RT-qPCR, and normalized to GAPDH and U6, respectively.

Cell proliferation assay

CCK-8 assay: HUVECs seeded at 2×10^3 cells/well were combined with CCK8 reagent at 10 μl/well (Dojindo, Kumamoto, Japan) for 2 h, after which the optical density ($OD_{450\ nm}$) value was examined.

Colony formation assay: Incubation of HUVECs seeded at 2×10^3 cells/well lasted for 2 weeks, then HUVECs were fixed with 4% formaldehyde and stained with GIMSA for 20 min to count colonies ($geq100$ μm) under a microscope (Olympus, Tokyo, Japan).

Apoptosis assay

HUVECs were stained with 5 μl FITC (BD Biosciences, New Jersey, USA) and 5 μl propidium iodide (BD Biosciences) and loaded on a flow cytometer (BD Biosciences) for analysis [20].

Detection of H_2O_2 and CAT activity levels

Fe^{2+} is oxidized by H_2O_2 to form Fe^{3+}, addition of xylenol orange to a specific solution can form a purple product, and the concentration of H_2O_2 can be determined by colorimetry. HUVECs (1×10^6) were dissolved with lysis buffer, centrifuged at 12000g, and

Table 2 Sequences of primers.

Gene	Sequence of primer (5′ − 3′)
CircTLK1 (human)	Forward: 5′-ACAGTTTTGGAAGCTTGGGATCT-3′ Reverse: 5′-TGCTCCCACTTGCAACTCCA-3′
CircTLK1 (mouse)	Forward: 5′-GACAAAGAATCAGAGGTGCAATGG-3′ Reverse: 5′-ACTCCCAGTACTCCCAGTAGC-3′
Has-miR-374a-5p (human)	Forward: 5′-TCCGATATAATACAACCT-3′ Reverse: 5′-GTGCAGGGTCCGAGGT-3′
mmu-miR-374b-5p (mouse)	Forward: 5′- CCCGGGTTATAATACAACCTG -3′ Reverse: 5′- CTCAACTGGTGTCGTGGAGTC -3′
FOXO1 (human)	Forward: 5′-AACCT GGCATTACAGTTGGCC-3′ Reverse: 5′-AAATGCAGGAGGCATGACTACGT-3′
FOXO1 (mouse)	Forward: 5′-GCACAGTGAACTCCAGGAAAGG-3′ Reverse: 5′-CACCAAAGGAAATGAATCAAACAAG-3′
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3′ Reverse: 5′-AACGCTTCACGAATTTGCGT-3′
GAPDH	Forward: 5′-TCCCATCACCATCTTCCA-3′ Reverse: 5′-CATCACGCCACAGTTTTCC-3′

CircTLK1, Circular RNA TLK1; miR-374a-5p, microRNA-374a-5p; FOXO1, forkhead box O1; and GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

liquefied with H_2O_2 reagent. $OD_{560\ nm}$ value was measured, and a standard curve was drawn.

CAT activity in cells was detected based on CAT detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and measured at 405 nm [21].

Dual luciferase reporter assay

CircTLK1 or FOXO1 3′UTR fragments containing the wild-type (WT) and mutant (MUT) binding site of miR-374a-5p were cloned into pGL3 reporter vector (Promega, Wisconsin, USA) to obtain circTLK1 WT/-MUT or TLR4 3′UTR WT/MUT reporter vector. miR-374a-5p mimic was co-transfected into HUVECs with the reporter vector and conditioned to measurements of luciferase activity using the Dual-Lucy Assay Kit (Solarbio) [18].

RNA pull-down

For RNA pull-down assay, Pierce RNA 3′ End Dethiobiotinylation Kit (Thermo Fisher Scientific) was utilized. Biotinylated miR-NC (Bio-miR-NC) probe, Bio-miR-374a-5p probe, or Bio-miR-374a-5p mutant (Bio-miR-330-5p MUT; binding site mutated to complementary sequences) probes were transfected into HUVECs for 48 h, and the cell lysate was mixed with magnetic beads to determine circTLK1 and FOXO1.

Atherosclerosis animal model

Male ApoE$^{-/-}$C57BL/6J mice (4 weeks old, 20 ± 5 g) obtained from the Basic Research Institute of Peking Union Medical College were placed in a controlled environment. Mice were fed a high-fat diet (15% fat and 0.25% cholesterol) for 12 weeks to induce atherosclerosis while those were fed a normal diet (4% fat, 0% cholesterol) were considered normal mice. At week 8 after HFD feeding, 20 mice fed a high-fat diet were randomly selected and injected with lentivirus (200 µl, 1×10^9 TU/ml) into the left ventricle weekly for 8–12 weeks. CircTLK1 shRNA and its negative control (sh-NC) were loaded into lentiviral vector PHY-lV-KD5.1 (ThermoFisher Scientific) and then into lentiviral particles. sh-circTLK1: 5′-GAATCAGAGGTGCAATGGA-3′. Five animals were used in each group ($n = 5$). No mice died in the experiment, and all animals were euthanized by anesthesia after 12 weeks. Renal artery puncture was performed to collect 1 ml of blood, which was cryopreserved in liquid nitrogen. In addition, the coronary artery (about 1 cm long) was excised from the inferior border of the coronary artery, fixed with 4% paraformaldehyde, embedded in paraffin, and serially sectioned [22]. All animal experiments followed the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, being approved by the Animal Ethics Committee of Qingdao Shinan District People's Hospital (approval number: 20160922C).

Vascular Oil Red O staining

Coronary artery sections were dyed with staining solution for 3 h and immersed in 70% ethanol until the plaque is red and the background is white [22].

H&E staining

After paraffin embedding, coronary arteries were sliced to 4 µm, dewaxed using xylene, and hydrated with

gradient ethanol, followed by hematoxylin staining and differentiation with 1% hydrochloric acid alcohol. After 30 s in 1% ammonia blue, eosin staining was done. Following dehydration, sections were cleared twice with xylene and sealed with neutral glue until microscopic observation (Olympus).

Statistical analysis

Data analysis was completed with SPSS 21.0, and data normality was checked by Kolmogorov-Smirnov test. Values shown as mean ± standard deviation were evaluated by One-way ANOVA and Fisher's least significant difference t-test. Enumeration data shown as rate or percentage were assessed by chi-square test. p was a two-sided test, and $p < 0.05$ was considered statistically significant.

RESULTS

CircTLK1, miR-374a-5p, and FOXO1 levels in blood of patients with CHD and ox-LDL-induced HUVECs

Both circTLK1 and FOXO1 in blood of patients with CHD showed high expression levels while miR-374a-5p showed low expression (Fig. 1A-C). It was further found that the expression patterns of circTLK1, miR-374a-5p, and FOXO1 in ox-LDL-induced HUVECs were consistent with those detected in the blood of patients with CHD (Fig. 1D). Subsequently, the circular character of circTLK1 was confirmed by RNase R analysis, which showed that circTLK1 was resistant to RNase R digestion and was more stable than linear TLK1 (Fig. 1E,F). Furthermore, subcellular localization analysis presented that circTLK1 was mainly distributed in the cytoplasm of HUVECs (Fig. 1F), confirming the characterization of circTLK1 as a circRNA and suggesting that its function might benefit from the biological stability of this molecule.

Knockdown of circTLK1 attenuating ox-LDL-induced endothelial injury

CircTLK1 was knocked down after transfection of sh-circTLK1 in ox-LDL-treated HUVECs (Fig. 2A). As measured by CCK-8 assay and colony formation assay, ox-LDL hampered the proliferation of HUVECs, which could be reversed by knockdown of circTLK1 (Fig. 2B,C). Furthermore, flow cytometry demonstrated that ox-LDL induced the apoptosis of HUVECs, whereas silencing of circTLK1 reversed this phenomenon (Fig. 2D). Meanwhile, silencing circTLK1 restrained the expression of ox-LDL-induced inflammatory factors and vascular endothelial injury factors (Fig. 2E-I). H_2O_2 is an active molecule that mediates oxidative stress-induced endothelial cell damage, and CAT is an antioxidant enzyme that is widely present in almost all organisms and can reflect the level of oxidative stress. Here, inhibiting circTLK1 suppressed ox-LDL-induced H_2O_2 levels and CAT activity in HUVECs (Fig. 2J,K).

CircTLK1 as a sponge for miR-374a-5p

To reveal the underlying mechanism by which circTLK1 works, starBase website predicted the target miRNAs of circTLK1. miR-374a-5p was found to have a targeted binding site for circTLK1 (Fig. 3A). Next, the interaction between circTLK1 and miR-374a-5p was validated by dual-luciferase reporter assay, as reflected by the fact that miR-374a-5p inhibited the luciferase activ-

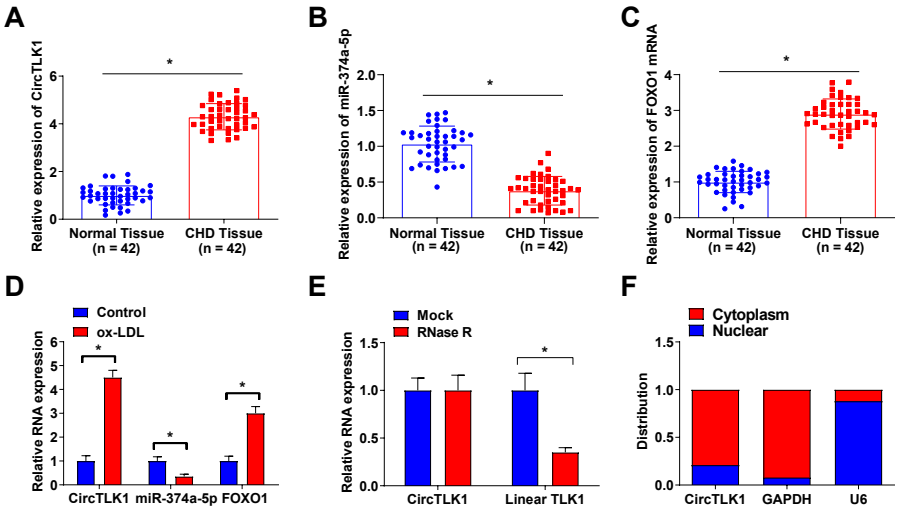

Fig. 1 CircTLK1, miR-374a-5p, and FOXO1 levels in blood of patients with CHD and ox-LDL-induced HUVECs. CircTLK1, miR-374a-5p, and FOXO1 in serum of patients with CHD and in HUVECs induced by ox-LDL (A–D) ($n = 42$). RNase R analysis confirming the circular character of circTLK1 (E). Distribution of circTLK1 in HUVECs (F). Mean ± SD, $n = 3$, * $p < 0.05$.

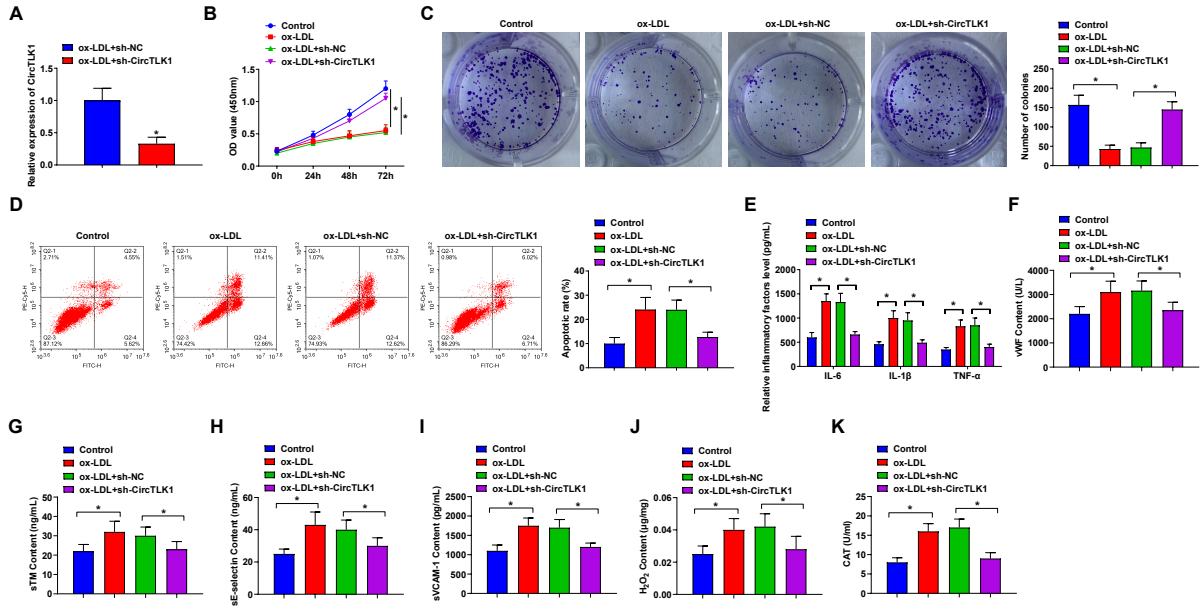

Fig. 2 Knockdown of circTLK1 attenuating ox-LDL-induced endothelial injury. CircTLK1 knockdown efficacy in HUVECs (A) and effects on HUVECs proliferation (B,C), apoptosis (D), inflammatory factors (E), vascular endothelial injury factors (F–I), H_2O_2 (J), and CAT activity (K). Mean ± SD, $n = 3$, * $p < 0.05$.

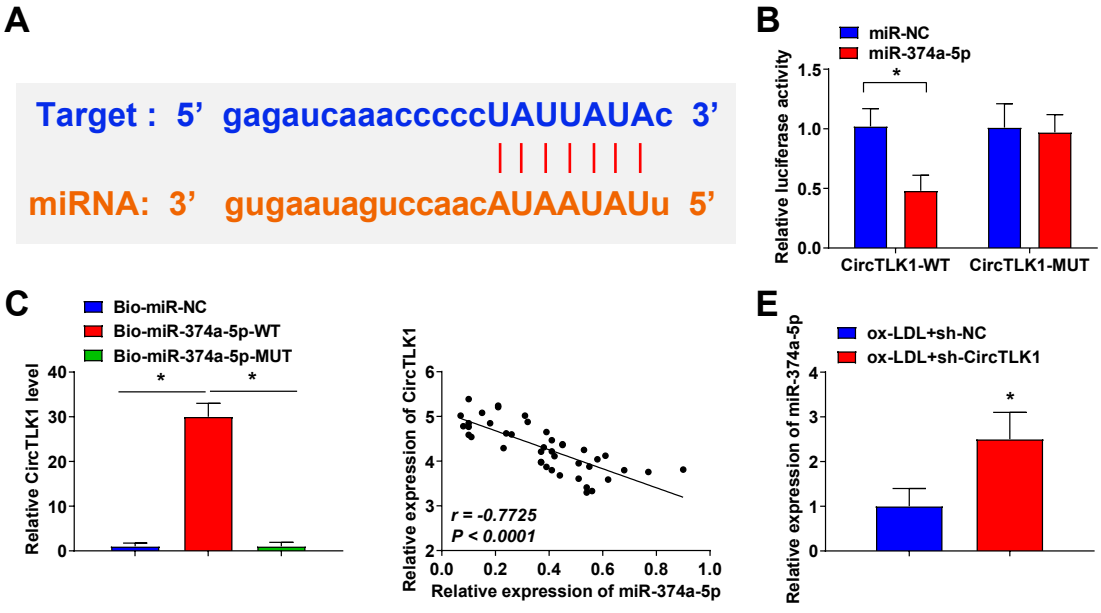

Fig. 3 CircTLK1 as a sponge for miR-374a-5p. Starbase prediction results (A), evaluation of the interaction between circTLK1 and miR-374a-5p (B,C), correlation between miR-374a-5p and circTLK1 (D), and miR-374a-5p expression after down-regulation of circTLK1 in HUVECs (E). Mean ± SD, $n = 3$, * $p < 0.05$.

ity of circTLK1-WT but not circTLK1-MUT (Fig. 3B). Meanwhile, RNA pull-down experiments showed that circTLK1 was pulled down when bio-miR-374a-5p-WT was used instead of bio-miR-NC and bio-miR-374a-5p-MUT (Fig. 3C). Clinical correlation analysis revealed that circTLK1 expression was negatively correlated with miR-374a-5p (Fig. 3D) and silencing circTLK1 increased miR-374a-5p expression in HUVECs (Fig. 3E).

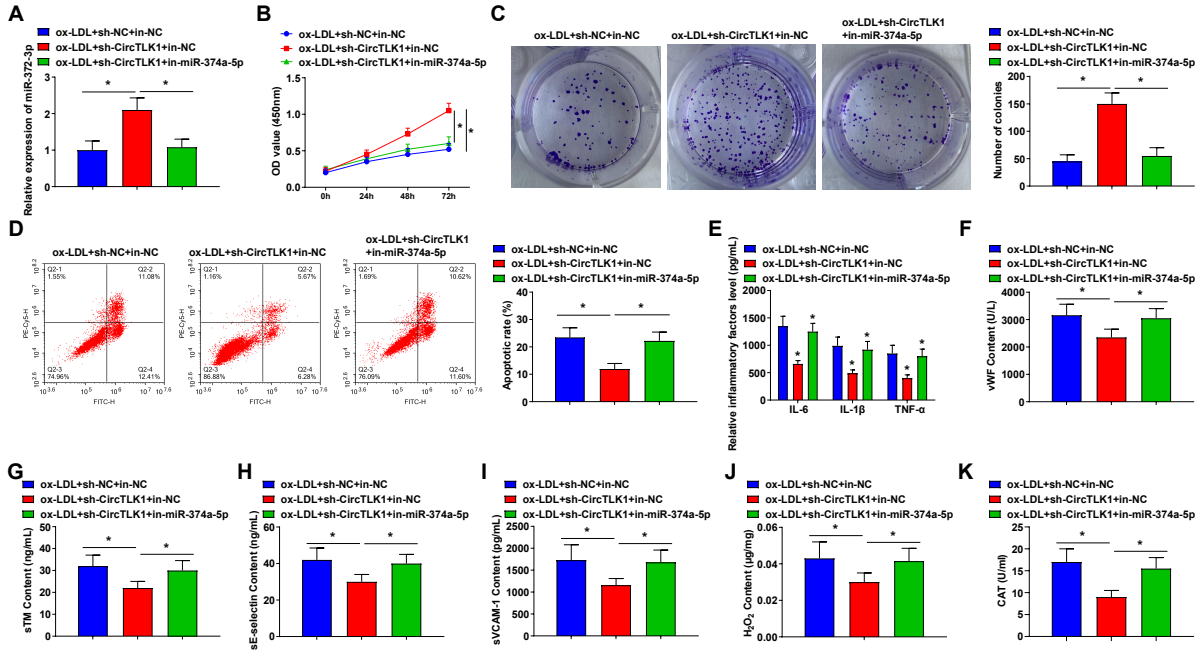

Fig. 4 CircTLK1/miR-372-3p axis collaborating in ox-LDL-induced HUVECs. miR-372-3p knockdown efficacy in HUVECs (A) and effects on HUVECs proliferation (B,C), apoptosis (D), inflammatory factors (E), vascular endothelial injury factors (F–I), H_2O_2 (J), and CAT activity (K). Mean ± SD, $n = 3$, * $p < 0.05$.

CircTLK1/miR-372-3p axis collaborating in ox-LDL-induced HUVECs

By co-transfecting sh-circTLK1 and in-miR-372-3p into ox-LDL-induced HUVECs, their regulation on vascular endothelial cell injury was investigated. miR-372-3p expression was enhanced in ox-LDL-induced HUVECs after transfection with sh-circTLK1, whereas miR-372-3p expression was decreased after co-transfection with in-miR-372-3p (Fig. 4A). Functional experiments discovered that in ox-LDL-induced HUVECs, after silencing circTLK1, proliferation ability (Fig. 4B,C), apoptosis ability (Fig. 4D), inflammatory factor expression (Fig. 4E), vascular endothelial injury factor expression (Fig. 4F-I), H_2O_2 level (Fig. 4J), and CAT activity (Fig. 4K) were mitigated by co-transfection with in-miR-372-3p.

miR-374a-5p directly targeting FOXO1

Bioinformatics analysis of the target gene mRNA of miR-374a-5p showed that there are multiple complementary binding sites in miR-374a-5p and FOXO1 (Fig. 5A). Dual-luciferase reporter gene assay showed that overexpression of miR-374a-5p effectively reduced the luciferase activity in the FOXO1-WT 3′UTR (Fig. 5B). Meanwhile, the results of RNA pull-down experiments suggested that FOXO1 enrichment in Bio-miR-374a-5p-WT group was significantly enhanced (Fig. 5C). In addition, FOXO1 expression was negatively correlated with miR-374a-5p and positively correlated with circTLK1 by clinical correlation analysis (Fig. 5D,E), and inhibiting circTLK1 or enhancing miR-374a-5p lowered FOXO1 expression in HUVECs (Fig. 5F,G).

Therapeutic effect of circTLK1/FOXO1 on ox-LDL-induced HUVECs

FOXO1 expression was hindered in ox-LDL-induced HUVECs after transfection with sh-circTLK1, whereas FOXO1 expression was reduced after co-transfection with pcDNA-FOXO1 (Fig. 6A,B). Functional experiments exhibited that in ox-LDL-induced HUVECs, after silencing circTLK1, proliferation ability (Fig. 6C,D), apoptosis ability (Fig. 6E), inflammatory factor expression (Fig. 6F), vascular endothelial injury factor expression (Fig. 6G-J), H_2O_2 level (Fig. 6K), and CAT activity (Fig. 6L) were reversed after co-transfection with pcDNA-FOXO1.

CircTLK1 silencing inhibiting atherosclerotic plaque formation and endothelial cell injury

An animal model of atherosclerosis was constructed and intervened with sh-circTLK1 for infection. Indeed, sh-circTLK1 effectively reduced circTLK1 expression in coronary artery tissue of atherosclerosis mice (Fig. 7A). In addition, H&E staining and Oil Red O staining showed that the plaque area and lipid deposition area of coronary artery tissue were increased in atherosclerosis mice, which were attenuated by sh-

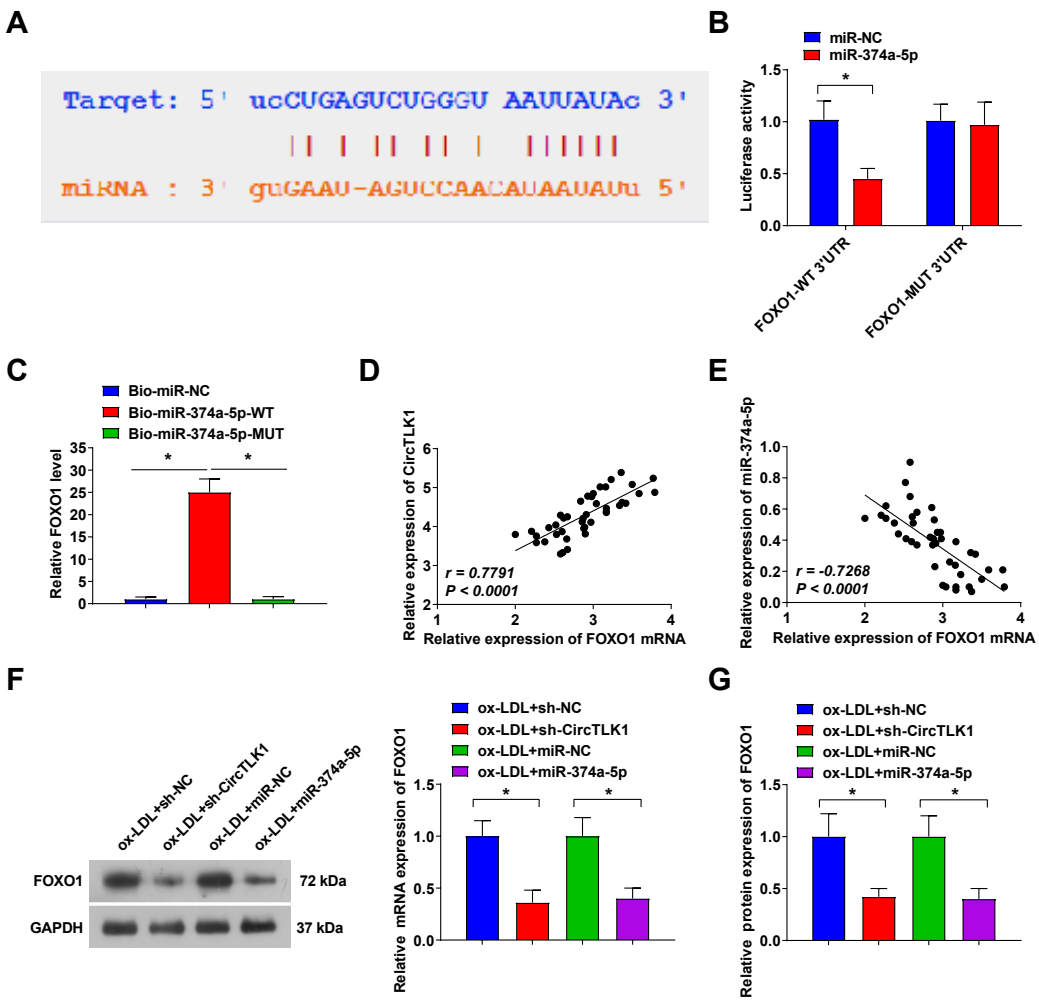

Fig. 5 miR-374a-5p directly targeting FOXO1. Starbase prediction results (A), evaluation of the interaction between FOXO1 and miR-374a-5p (B,C), correlation between FOXO1 and miR-374a-5p and circTLK1 (D,E), and FOXO1 expression patterns after down-regulation of circTLK1 or up-regulation of miR-374a-5p in HUVECs (F,G). Mean ± SD, $n = 3$, $^*p < 0.05$.

circTLK1 (Fig. 7B,C). At the same time, TNF-α, IL-1β, IL-6, vWF, sTM, sE-selectin, and sVCAM-1 in the serum of atherosclerosis mice were increased, while they were decreased in mice infected with sh-circTLK1 (Fig. 7D-H). CD34 is a surface marker of mature endothelial cells, and CD34 immunostaining in coronary sections can be used to reflect the integrity of the vascular endothelium. Here, HFD damaged endothelial cells, resulting in increased CD34 levels, while silencing circTLK1 attenuated endothelial cell damage and decreased CD34 levels (Fig. 7I).

DISCUSSION

CHD is an increasingly serious cardiovascular disease in recent years, and the acute myocardial infarction caused by it is also one of the main causes of disability or death in patients with cardiovascular disease, which brings more and more harm to patients [23]. The plight of CHD treatment makes it necessary to further explore its mechanism and find new treatment strategies. It is currently known that CHD is caused by coronary atherosclerotic lesions, which mainly start with functional changes in endothelial cells [24]. CircRNAs will be a promising diagnostic and therapeutic target in CHD [8]. Here demonstrates for the first time that circTLK1 and FOXO1 are up-regulated in CHD patients and cells, whereas miR-374a-5p is down-regulated. Also, related functional experiments were about to determine whether circTLK1 could inhibit the formation of atherosclerotic plaques and alleviate endothelial cell injury.

CircTLK1 has been shown to downregulate its expression in myocardial ischemia-reperfusion injury [11]. However, the relevant function of circTLK1 in

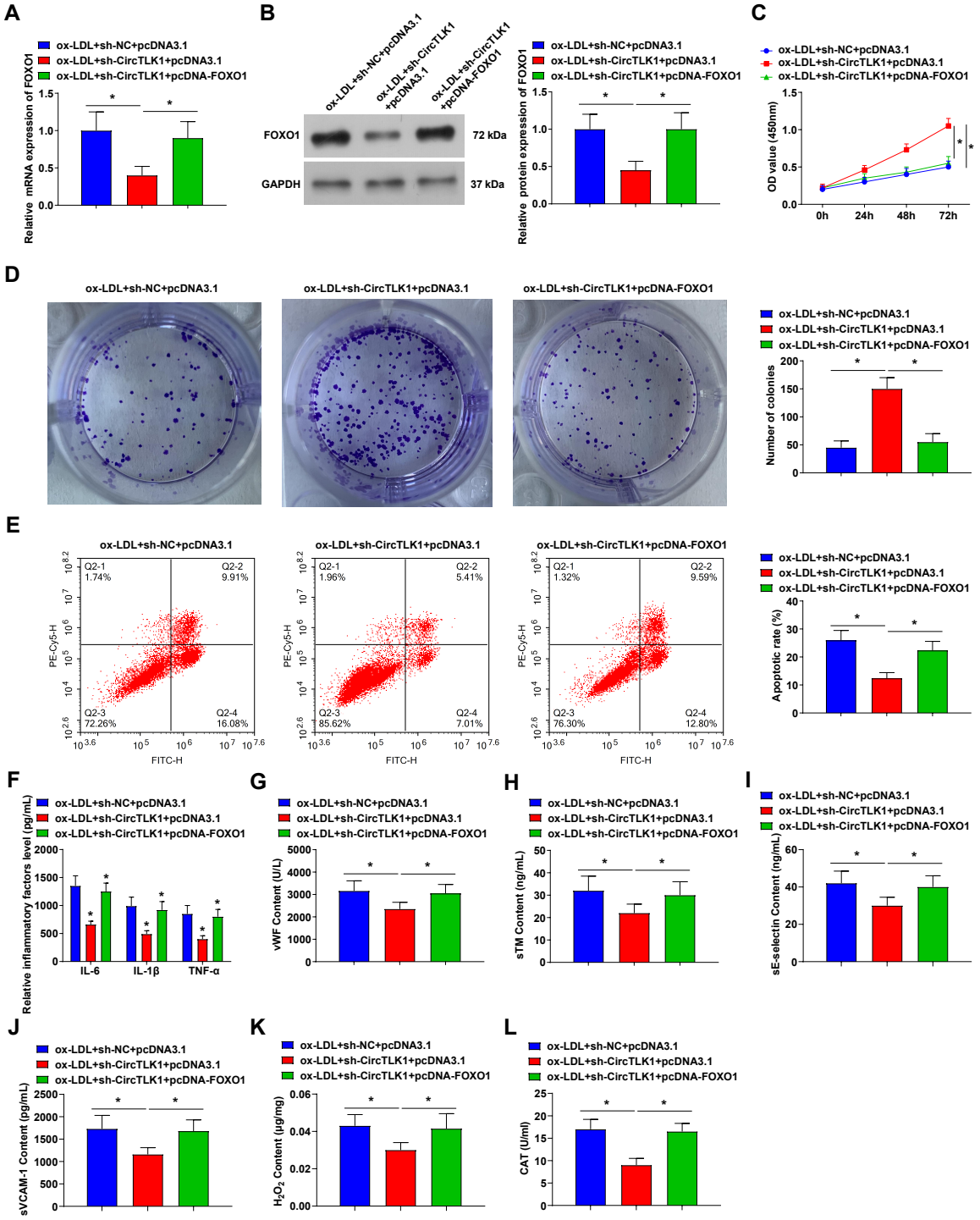

Fig. 6 Therapeutic effect of circTLK1/FOXO1 on ox-LDL-induced HUVECs. FOXO1 overexpression efficacy in HUVECs (A,B) and effects on HUVECs proliferation (C,D), apoptosis (E), inflammatory factors (F), vascular endothelial injury factors (G–J), H_2O_2 (K), and CAT activity (L). Mean ± SD, $n = 3$, * $p < 0.05$.

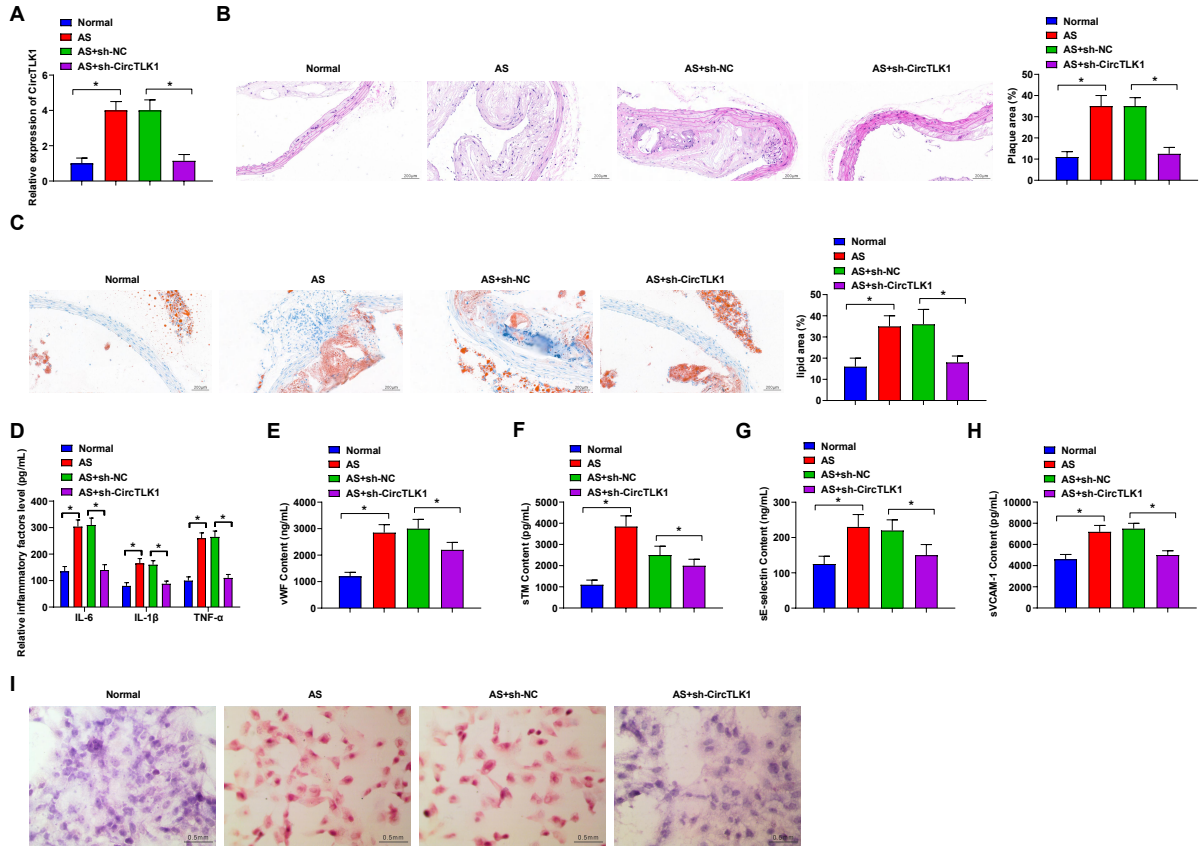

Fig. 7 CircTLK1 silencing inhibiting atherosclerotic plaque formation and endothelial cell injury. CircTLK1 expression pattern in mouse coronary tissue (A), H&E staining and Oil red O staining in the coronary arteries of mice (B,C), inflammatory factors (D) in the serum of mice, vascular endothelial injury factors (E-H) in mouse serum, and CD34 immunohistochemical staining (I). Mean ± SD, $n = 5$, $^*p < 0.05$.

CHD has not yet been discovered. Atherosclerosis is the basis of the pathological process of CHD, the inflammatory response runs through the entire process of atherosclerosis, and inflammation can activate oxidative stress damage [25, 26]. TNF-α, IL-1β, and IL-6 are all pro-inflammatory factors produced by inflammatory cells that work to promote the formation of atherosclerotic plaques, thereby causing endothelial dysfunction [23]. Notably, silencing circTLK1 has been elucidated to inhibit oxidative stress, inflammation, and apoptosis in sepsis-related acute kidney injury [12]. Here, silencing circTLK1 promoted the viability of ox-LDL-induced HUVECs and inhibited apoptosis, inflammation, and oxidative stress injury, thereby alleviating endothelial cell dysfunction. *In vivo* experiments presented that circTLK1 silencing inhibited atherosclerotic plaque formation and endothelial cell injury and decreased production and release of inflammatory factors.

The target miRNA (miR-374a-5p) of circTLK1 was predicted and then confirmed. miRNAs are crucial regulators of the complex biological progression of various cardiovascular diseases [27], including CHD. In particular, miR-374a-5p, a highly conserved sequence, has been described to be closely associated with the progression of many cardiovascular diseases. miR-374a-5p prevents myocardial ischemia-reperfusion injury in mice [17] and inhibits the release of pro-inflammatory factors in neonatal hypoxic-ischemic encephalopathy [28]. The present study proved that miR-374a-5p upregulation partially mitigated the therapeutic effect of silencing circTLK1 on endothelial cell injury.

Furthermore, miR-374a-5p directly targeted the 3′UTR of FOXO1, leading to the degradation of FOXO1 and repressing its transcription. FOXO1 is a key molecular pro-inflammatory transcription factor [29] that has been illustrated to be associated with various diseases such as cardiovascular disease and cancer. Targeting FOXO1 can control the development of cerebral ischemia-reperfusion injury [29]. We found a report studying miR-374a-5p/FOXO1 axis. The authors suggest that lncTTTY15 affects the expression and

autophagy of FOXO1 in myocardial cells during I/R by targeting miR374a-5p, thereby inhibiting myocardial I/R injury [30]. Moreover, FOXO1 is abnormally expressed in patients with coronary artery disease, and its expression level is correlated with inflammatory factors [31, 32]. As expected, FOXO1 overexpression could abolish the suppressive effects of silencing circTLK1 on ox-LDL-induced apoptosis, inflammation, and oxidative stress injury in HUVECs as well as the promotion of viability. Furthermore, numerous studies have been conducted on FOXO1 on other programmed cell death inductions, including necroptosis, pyroptosis, autophagy, and ferroptosis associated with FOXO1. Li Xiudan et al [33] have demonstrated that FOXO1 plays a crucial role in regulating autophagy and cell ptosis in pancreatic β cells, which have been implicated in the pathogenesis and progression of type 2 diabetes. Additionally, it has been observed that CD73 mitigates microglial cell apoptosis by suppressing the expression of FOXO1 [34]. In addition, N6-methyladenosine modification of FOXO1 can aggravate endothelial inflammation and atherosclerosis by increasing the N6-methyladenosine modification of FOXO1 [35]; therefore, it is imperative to conduct additional investigations to ascertain the potential association between FOXO1 and other programmed cell death inductions as well as the impact of N6-methyladenosine modification on FOXO1, in relation to endothelial cell injury and atherosclerosis.

CONCLUSION

In short, circTLK1 upregulates FOXO1 by binding to miR-374a-5p, which in turn promotes endothelial cell injury in CHD. These findings provide new insights into new target therapy for CHD and highlight a role in the circTLK1/miR-374a-5p/FOXO1 pathway.

REFERENCES

1. Zhang L, Lou D, He D, Wang Y, Wu Y, Cao X, Qu P (2021) Dysregulated circulating apoptosis- and autophagy-related lncRNAs as diagnostic markers in coronary artery disease. *Biomed Res Int* **2021**, 5517786.
2. Zhang F, Cheng N, Du J, Zhang H, Zhang C (2021) MicroRNA-200b-3p promotes endothelial cell apoptosis by targeting HDAC4 in atherosclerosis. *BMC Cardiovasc Disord* **21**, 172.
3. Zhou Y, Zhu X, Cui H, Shi J, Yuan G, Shi S, Hu Y (2021) The role of the VEGF family in coronary heart disease. *Front Cardiovasc Med* **8**, 738325.
4. Wu L, Tan G, Li X, Jiang X, Run B, Zhou W, Liao H (2021) LncRNA TONSL-AS1 participates in coronary artery disease by interacting with miR-197. *Microvasc Res* **136**, 104152.
5. Malakar AK, Choudhury D, Halder B, Paul P, Uddin A, Chakraborty S (2019) A review on coronary artery disease, its risk factors, and therapeutics. *J Cell Physiol* **234**, 16812–16823.
6. Wang X, Gao M, Zhou S, Wang J, Liu F, Tian F, Jin J, Ma Q, et al (2017) Trend in young coronary artery disease in China from 2010 to 2014: a retrospective study of young patients ⩽ 45. *BMC Cardiovasc Disrd* **17**, 18.
7. Tian M, Cao Z, Pang H (2021) Circular RNAs in sudden cardiac death related diseases: Novel biomarker for clinical and forensic diagnosis. *Molecules* **26**, 1155.
8. Ghafouri-Fard S, Gholipour M, Taheri M (2021) The emerging role of long non-coding RNAs and circular RNAs in coronary artery disease. *Front Cardiovasc Med* **8**, 632393.
9. Qiu Y, Yu Y, Qin XM, Jiang T, Tan YF, Ouyang WX, Xiao ZH, Li SJ (2021) CircTLK1 modulates sepsis-induced cardiomyocyte apoptosis via enhancing PARP1/HMGB1 axis-mediated mitochondrial DNA damage by sponging miR-17-5p. *J Cell Mol Med* **25**, 8244–8260.
10. Li J, Zhao Z, Wang X, Ma Q, Ji H, Wang Y, Yu R (2021) PBX2-mediated circTLK1 activates JAK/STAT signaling to promote gliomagenesis via miR-452-5p/SSR1 axis. *Front Genet* **12**, 698831.
11. Song YF, Zhao L, Wang BC, Sun JJ, Hu JL, Zhu XL, Zhao J, Zheng DK, et al (2020) The circular RNA TLK1 exacerbates myocardial ischemia/reperfusion injury via targeting miR-214/RIPK1 through TNF signaling pathway. *Free Radic Biol Med* **155**, 69–80.
12. Xu HP, Ma XY, Yang C (2021) Circular RNA TLK1 promotes sepsis-associated acute kidney injury by regulating inflammation and oxidative stress through miR-106a-5p/HMGB1 axis. *Front Mol Biosci* **8**, 660269.
13. Lin F, Chen HW, Zhao GA, Li Y, He XH, Liang WQ, Shi ZL, Sun SY, et al (2020) Advances in research on the circRNA-miRNA-mRNA network in coronary heart disease treated with traditional Chinese medicine. *Evid Based Complement Alternat Med* **2020**, 8048691.
14. Pan RY, Liu P, Zhou HT, Sun WX, Song J, Shu J, Cui GJ, Yang ZJ, et al (2017) Circular RNAs promote TRPM3 expression by inhibiting hsa-miR-130a-3p in coronary artery disease patients. *Oncotarget* **8**, 60280–60290.
15. Ji P, Song X, Lv Z (2021) Knockdown of circ_0004104 alleviates oxidized low-density lipoprotein-induced vascular endothelial cell injury by regulating miR-100/TNFAIP8 axis. *J Cardiovasc Pharmacol* **78**, 269–279.
16. Cao C, Zhen W, Yu H, Zhang L, Liu Y (2021) lncRNA MALAT1/miR-143 axis is a potential biomarker for instent restenosis and is involved in the multiplication of vascular smooth muscle cells. *Open Life Sci* **16**, 1303–1312.
17. Huang ZQ, Xu W, Wu JL, Lu X, Chen XM (2019) MicroRNA-374a protects against myocardial ischemia-reperfusion injury in mice by targeting the MAPK6 pathway. *Life Sci* **232**, 116619.
18. Su Q, Dong X, Tang C, Wei X, Hao Y, Wu J (2021) Knockdown of circ_0003204 alleviates oxidative low-density lipoprotein-induced human umbilical vein endothelial cells injury: Circulating RNAs could explain atherosclerosis disease progression. *Open Med (Wars)* **16**, 558–569.
19. Tan J, Pan W, Chen H, Du Y, Jiang P, Zeng D, Wu J, Peng K (2021) Circ_0124644 serves as a ceRNA for miR-590-3p to promote hypoxia-induced cardiomyocytes injury via regulating SOX4. *Front Genet* **12**, 667724.
20. Liang G, Chen S, Xin S, Dong L (2021) Overexpression of hsa_circ_0001445 reverses oxLDL induced inhibition of HUVEC proliferation via SRSF1. *Mol Med Rep* **24**, 507.

21. An R, Li D, Dong Y, She Q, Zhou T, Nie X, Pan R, Deng Y (2021) Methylcobalamin protects melanocytes from H_2O_2-induced oxidative stress by activating the Nrf2/HO-1 pathway. *Drug Des Devel Ther* **15**, 4837–4848.
22. Bai J, Liu J, Fu Z, Feng Y, Wang B, Wu W, Zhang R (2021) Silencing lncRNA AK136714 reduces endothelial cell damage and inhibits atherosclerosis. *Aging* **13**, 14159–14169.
23. Xu H, Zhang X, Yu K, Zhang G, Shi Y, Jiang Y (2020) Analysis on the expression and prognostic value of lncRNA FAF in patients with coronary heart disease. *Biomed Res Int* **2020**, 9471329.
24. Manjula G, Pranavchand R, Kumuda I, Reddy BS, Reddy BM (2020) The SNP rs7865618 of 9p21.3 locus emerges as the most promising marker of coronary artery disease in the southern Indian population. *Sci Rep* **10**, 21511.
25. Li P, Li Y, Chen L, Ma X, Yan X, Yan M, Qian B, Wang F, et al (2021) Long noncoding RNA *uc003pxg.1* regulates endothelial cell proliferation and migration via miR 25 5p in coronary artery disease. *Int J Mol Med* **48**, 160.
26. Wang Y, Jiao J, Wang D, Song J, Huang K (2021) Effects of ticagrelor on proliferation, apoptosis, and inflammatory factors of human aortic vascular smooth muscle cells through lncRNA KCNQ1OT1. *Am J Transl Res* **13**, 13462–13470.
27. Wang D, Yan C (2022) MicroRNA-208a-3p participates in coronary heart disease by regulating the growth of hVSMCs by targeting BTG1. *Exp Ther Med* **23**, 71.
28. Chen Z, Hu Y, Lu R, Ge M, Zhang L (2020) MicroRNA-374a-5p inhibits neuroinflammation in neonatal hypoxic-ischemic encephalopathy via regulating NLRP3 inflammasome targeted Smad6. *Life Sci* **252**, 117664.
29. Li W, Zhu Q, Xu X, Hu X (2021) MiR-27a-3p suppresses cerebral ischemia-reperfusion injury by targeting FOXO1. *Aging* **13**, 11727–11737.
30. Lee J, Lee S, Zhang H, Hill MA, Zhang C, Park Y (2017) Interaction of IL-6 and TNF-α contributes to endothelial dysfunction in type 2 diabetic mouse hearts. *PloS One* **12**, e0187189.
31. Babaee M, Chamani E, Ahmadi R, Bahreini E, Balouchnejadmojarad T, Nahrkhalaji AS, Fallah S (2020) The expression levels of miRNAs- 27a and 23a in the peripheral blood mononuclear cells (PBMCs) and their correlation with FOXO1 and some inflammatory and anti-inflammatory cytokines in the patients with coronary artery disease (CAD). *Life Sci* **256**, 117898.
32. Kazemi Fard T, Ahmadi R, Akbari T, Moradi N, Fadaei R, Kazemi Fard M, Fallah S (2021) Klotho, FOXO1 and cytokines associations in patients with coronary artery disease. *Cytokine* **141**, 155443.
33. Li X, Bai C, Wang H, Wan T, Li Y (2022) LncRNA MEG3 regulates autophagy and pyroptosis via FOXO1 in pancreatic β-cells. *Cell Signal* **92**, 110247.
34. Xu S, Wang J, Zhong J, Shao M, Jiang J, Song J, Zhu W, Zhang F, et al (2021) CD73 alleviates GSDMD-mediated microglia pyroptosis in spinal cord injury through PI3K/AKT/Foxo1 signaling. *Clin Transl Med* **11**, e269.
35. Jian D, Wang Y, Jian L, Tang H, Rao L, Chen K, Jia Z, Zhang W, et al (2020) METTL14 aggravates endothelial inflammation and atherosclerosis by increasing FOXO1 N6-methyladeosine modifications. *Theranostics* **10**, 8939–8956.