

# Antisense long non-coding RNA WEE2-AS1 regulates human vascular endothelial cell viability via cell cycle G<sub>2</sub>/M transition in arteriosclerosis obliterans

BAOHONG JIANG<sup>1-3</sup>, RUI WANG<sup>1-3</sup>, ZEFEI LIN<sup>4</sup>, JIEYI MA<sup>4</sup>, JIN CUI<sup>1-3</sup>, MIAN WANG<sup>1-3</sup>,  
RUI MING LIU<sup>2-4</sup>, WEIBIN WU<sup>1-3</sup>, CHUNXIANG ZHANG<sup>5</sup>, WEN LI<sup>2-4</sup> and SHENMING WANG<sup>1-3</sup>

<sup>1</sup>Division of Vascular Surgery, The First Affiliated Hospital;

<sup>2</sup>National-Local Joint Engineering Laboratory of Vascular Disease Diagnosis and Treatment;

<sup>3</sup>Guangdong Key Engineering Laboratory of Diagnosis and Treatment of Vascular Disease;

<sup>4</sup>Laboratory of General Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510080, P.R. China; <sup>5</sup>Department of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, AL 35233, USA

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**Abstract.** Long non-coding RNAs (lncRNAs) affect atherosclerosis by regulating the physiological and pathological processes of endothelial cells; however, the role of lncRNA WEE2 antisense RNA 1 (WEE2-AS1) in arteriosclerosis obliterans (ASO) is not completely understood. The present study aimed to explore the function of lncRNA WEE2-AS1 in human vascular endothelial cells. The results indicated that lncRNA WEE2-AS1 was significantly elevated in plasma and artery tissue samples of patients with ASO compared with healthy controls. The fluorescence *in situ* hybridization results suggested that lncRNA WEE2-AS1 was expressed in the cytoplasm and nuclei of primary human umbilical vein endothelial cells (HUVECs). The Cell Counting Kit-8 assay results suggested that lncRNA WEE2-AS1 knockdown significantly promoted HUVEC viability, whereas lncRNA WEE2-AS1 overexpression inhibited HUVEC viability compared with the negative control groups. Furthermore, analysis of the cell cycle by flow cytometry indicated that lncRNA WEE2-AS1 knockdown significantly decreased the proportion of cells in the G<sub>0</sub>/G<sub>1</sub> phase and

significantly increased the proportion of cells in the G<sub>2</sub>/M phase compared with the negative control group. However, lncRNA WEE2-AS1 overexpression had no significant effect on cell cycle distribution compared with the negative control group. The western blotting results indicated that lncRNA WEE2-AS1 knockdown significantly reduced the expression levels of phosphorylated cyclin dependent kinase 1, WEE1 homolog 2 and myelin transcription factor 1, but increased the expression level of cell division cycle 25B compared with the negative control group. lncRNA WEE2-AS1 overexpression displayed the opposite effect on protein expression. Collectively, the present study suggested that lncRNA WEE2-AS1 was significantly upregulated in ASO and may serve a role in regulating human vascular endothelial cell viability. Further investigation into lncRNA WEE2-AS1 may broaden the current understanding of the molecular mechanism underlying ASO, and aid with the identification of specific probes and precise targeted drugs for the diagnosis and treatment of ASO.

## Introduction

Atherosclerosis is the leading cause of disability and death worldwide, resulting in ~17.3 million deaths, which is expected to exceed 23.6 million each year by 2030 (1-3). Atherosclerosis is the pathological basis of the majority of cardiovascular diseases and poses a serious threat to human health (4). After cardiovascular and cerebrovascular diseases, peripheral arterial disease (PAD) is the third most common cause of morbidity among individuals with atherosclerosis (5). PAD frequently occurs in the arteries of the lower extremities and occlusive atherosclerosis impairs the blood supply to the lower extremities (6). Accumulating evidence has revealed that a variety of long non-coding RNAs (lncRNAs) participate in the onset and progress of atherosclerosis, and are involved in multiple pathological processes and signaling pathways, suggesting that lncRNAs may serve a vital role in atherosclerosis (7-12).

**Correspondence to:** Professor Shenming Wang, Division of Vascular Surgery, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhong Shan Er Road, Guangzhou, Guangdong 510080, P.R. China  
E-mail: shenmingwang@hotmail.com

Professor Wen Li, Laboratory of General Surgery, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhong Shan Er Road, Guangzhou, Guangdong 510080, P.R. China  
E-mail: wenli28@163.com

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For example, silencing the lnc RNA myocardial infarction associated transcript inhibits endothelial cell proliferation, migration and tube formation (13). Another example is that lncRNA-DYNLRB2-2 not only accelerates cholesterol efflux, but also decreases the levels of cellular inflammatory cytokines [such as interleukin (IL)-6, IL-1 $\beta$  and TNF- $\alpha$ ] under hyperlipidemic stress (14).

lncRNAs, a class of non-coding RNAs, contain >200 nucleotides and were previously considered to be transcriptional noise (15,16); however, the extensive functions of lncRNAs have gradually been identified. lncRNAs contribute to a wide range of vital processes, such as chromatin remodeling, genomic imprinting, dosage compensation effects, gene expression, post-transcriptional modification of mRNA and regulation of proteins (17,18). lncRNAs provide a novel layer of regulation in the mechanism underlying atherosclerosis (8-11). However, the full repertoire of the physiological and pathological functions of lncRNAs has not been identified.

lncRNA WEE2 antisense RNA 1 (WEE2-AS1) is the antisense RNA of the gene encoding WEE1 homolog 2 (WEE2) (19). Although the sequence of lncRNA WEE2-AS1 has been characterized, its subcellular localization and biological function are not completely understood (19). M-phase or maturation promoting factor (MPF) activation is a prerequisite for the cell cycle transition from the G<sub>2</sub> phase to the M phase. WEE2 protein inhibits MPF activity by phosphorylating the Tyr15 residue of its regulatory subunit, cyclin dependent kinase 1 (CDK1) (20-22). At present, the identified role of WEE2 is primarily based on the findings of germ cell research (23,24). Antisense RNAs may exert positive or negative influences on sense mRNAs at the post-transcriptional, processing and mRNA stability levels (25-31). Therefore, it was hypothesized that altering the expression level of lncRNA WEE2-AS1 may affect the expression of WEE2, leading to alterations in WEE2-related functions, especially those associated with the cell cycle.

Vascular endothelial cells are the primary regulators for maintaining vascular stability. Endothelial dysfunction, which is the initiation event of a series of pathological cascades in atherosclerosis, serves a crucial role in the pathogenesis of the disease, especially in the initial stage (1,2). When exposed to various atherosclerosis risk factors (for example, hyperlipidemia, hypertension, shear stress and diabetes), the function of vascular endothelial cells is impaired, leading to certain pathological alterations, including inhibition of endothelial cell viability and alterations to the cell cycle (3,5). It has also been suggested that atherosclerosis is a response of the vascular wall to endothelial damage (6). Nitric oxide is an effective vasodilator and anti-inflammatory substance, and endothelial dysfunction inhibits the production of nitric oxide, leading to a loss of inhibition of vascular smooth muscle cell (VSMC) proliferation (32). Extensive proliferation of VSMCs can cause thickening of blood vessel walls, which narrows the vascular lumen (33). Endothelial cells, which also experience dysfunction, express a large number of adhesion molecules, which can recruit and bind inflammatory cells to exacerbate vascular damage (34). Local inflammation induced by endothelial injury promotes plaque formation and thrombosis (35-40). A large number of lncRNAs are

expressed in endothelial cells and serve crucial regulatory roles (41). For instance, antisense non-coding RNA CDKN2B antisense RNA 1 in the INK4 locus preserves the normal phenotype of endothelial cells by inhibiting the expression of Kruppel-like factor 2 (11). Therefore, it was hypothesized that lncRNA WEE2-AS1 may be associated with endothelial dysfunction.

In the present study, the expression level of lncRNA WEE2-AS1 in an ASO group and a normal control group was measured. The function and molecular mechanism underlying lncRNA WEE2-AS1 during the regulation of the cell cycle were also assessed. In addition, whether antisense lncRNA WEE2-AS1 could influence the expression of the corresponding protein-coding gene was investigated.

## Materials and methods

**Sample acquisition.** The present study was approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University and was conducted in accordance with the Declaration of Helsinki. The participants or their guardian provided written informed consent before the tissue and blood samples were obtained.

Arterial samples were obtained from the main artery of amputated lower limbs at The First Affiliated Hospital of Sun Yat-sen University (China) between October 2015 and May 2017. A total of 5 atherosclerotic samples were obtained from patients with severe lower extremity ASO who had undergone amputation (4 male patients and 1 female patient; mean age, 67.60 years; age range, 59-75 years). All patients with severe lower extremity ASO were diagnosed with arteriosclerosis obliterans according to the guidelines issued by the European Society of Cardiology in 2011 (42). A total of 5 healthy arterial samples were obtained from donors without atherosclerosis who had undergone amputation (3 male donors and 2 female donors; mean age, 52.20 years; age range, 42-62 years) at The First Affiliated Hospital of Sun Yat-sen University between October 2015 and May 2017.

In addition, blood samples were collected from patients with severe lower extremity ASO (mean age, 65.93 years; age range, 53-80 years; 9 male patients and 6 female patients) and healthy subjects (mean age, 66.13 years; age range, 52-82 years; 9 male patients and 6 female patients). Blood samples were obtained from the superficial veins of the upper limbs at The First Affiliated Hospital of Sun Yat-sen University (China) between January 2015 and November 2017. The blood samples were collected into tubes containing EDTA as an anticoagulant, and were centrifuged at 3,000  $\times$  g for 10 min at 4°C. The upper plasma was transferred to the EP tube and immediately frozen at -80°C until RNA extraction.

Human umbilical cords were obtained from healthy women (mean age, 30.14 years; age range, 25-35 years) post-delivery at The First Affiliated Hospital of Sun Yat-sen University between June 2015 and March 2017.

**Isolation and culture of human umbilical vein endothelial cells (HUVECs).** Human umbilical cords were obtained from healthy post-delivery women and endothelial cells were immediately isolated from the umbilical veins as previously described (43). HUVECs were cultured in Endothelial Cell

Basal Medium (EBM-2; Lonza Group, Ltd.) supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and endothelium growth medium kit (Lonza Group, Ltd.). Cells were cultured at 37°C in a humidified environment with 5% CO<sub>2</sub>. The medium was changed every 3 days. Passage 1-10 cells were used for subsequent experiments.

**RNA isolation and reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from HUVECs and plasma samples (250 µl) using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and TRIzol® LS reagent (Invitrogen; Thermo Fisher Scientific, Inc.), respectively. An equal volume of plasma sample was used as the internal control (44). The ultraviolet absorbance of each sample at a wavelength of 260 and 280 nm was measured to assess RNA purity and concentration, respectively. Total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. Subsequently, qPCR was performed using SYBR-Green PCR Master Mix (Roche Diagnostics GmbH) according to the manufacturer's instructions. The thermocycling conditions used for qPCR consisted of pre-incubation followed by 40 amplification cycles. Each cycle comprised 95°C for 10 sec, 60°C for 20 sec, and 72°C for 20 sec. The following primers were used for qPCR: lncRNA WEE2-AS1 forward, 5'-AGAAATCACCAACCG GCTCA-3' and reverse, 5'-GAACTTCGCTTTCCCCCT GT-3; and GAPDH forward, 5'-GCACCGTCAAGGCTG AGAGAAC-3 and reverse, 5'-TGGTGAAGACGCCAGTGG A-3'. The qPCR products were resolved by agarose gel (1%) electrophoresis to confirm the specificity of the primers. mRNA expression levels were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (45) and normalized to the internal reference gene GAPDH.

**Hematoxylin and eosin (H&E) staining.** Artery tissue samples were immediately fixed in 4% paraformaldehyde at room temperature for 1 day, embedded in paraffin and cut into 4-µm thick sections. The sections were mounted on slides and heated at 62°C for ~4 h. Subsequently, the slides were deparaffinized, air-dried and stained with hematoxylin at room temperature for ~5 min. The slides were washed in running water, rinsed with ammonia water and washed again in running water. After counterstaining with eosin at room temperature for ~5 min, the slides were dehydrated using ascending ethanol concentrations (95-100%). The coverslips were positioned using a xylene-based mountant. Slides were observed under a light microscope and scanned by the Panoramic MIDI scanner (3DHISTECH Kft.). Images were captured using CaseViewer (Version 2.0; 3DHISTECH Kft.) at x35 or x200 magnification.

**Oil Red O Staining.** Artery tissue samples preserved in liquid nitrogen were cut into 4-µm thick sections and mounted onto slides. The slides were air-dried at room temperature and fixed in 4% paraformaldehyde for 15 min at room temperature. The slides were rinsed in distilled water and air-dried. Subsequently, the slides were stained with Oil Red O solution for 8-10 min at room temperature and rinsed in distilled water. Slides were differentiated in 75% ethanol for ~2 sec at room temperature, washed in distilled water and stained with

hematoxylin for 3-5 min at room temperature. Subsequently, the slides were rinsed in running water for 3 min and mounted using glycerin jelly. Slides were observed under a light microscope and scanned by the Panoramic MIDI scanner. Images were captured using CaseViewer at x35 or x200 magnification.

**Fluorescence in situ hybridization (FISH).** The sequence of the lncWEE2-AS1 probe used for FISH was 5'-GCCCCGCTTC TTGCACATCTTACTC-3'. The lncWEE2-AS1 probe, which was labeled with Cy3, was synthesized by Servicebio. Artery tissue samples were immediately fixed in 4% paraformaldehyde at room temperature for 1 day, and embedded in paraffin. The artery tissue samples imbedded in paraffin wax were cut into 4-µm thick sections. The sections were mounted on slides and baked. At room temperature, the slides were deparaffinized at room temperature as follows: 100% xylene for 15 min, 100% xylene for 15 min, 100% ethanol for 5 min, 100% ethanol for 5 min, 85% ethanol for 5 min, 75% ethanol for 5 min and diethyl pyrocarbonate-treated water for 5 min. Subsequently, the slides were air-dried. The slides were boiled in antigen retrieval buffer (citrate) for 10 min and cooled naturally. The tissues were digested with protease K (Servicebio, Inc.) at 37°C for 30 min. After washing with pure water, the slides were washed three times in PBS for 5 min each time. After preheating to the hybridization temperature, the probes (8 ng/µl) were applied to the slides and incubated at 37°C overnight. Post-hybridization washes were performed using sodium chloride-sodium citrate buffer at room temperature for 10 min. The slides were then incubated with DAPI for 10 min at room temperature to stain the nuclei and then mounted with anti-fade fluorescence mountant. Images were obtained using a fluorescence microscope at x400 or x900 magnification and analyzed using ImageJ software (version 6.0; National Institutes of Health).

For FISH using cells, the cell suspension (1-2x10<sup>4</sup> cells) was added to a slide and cultured at 37°C for 24 h. The supernatant was removed and the slides were washed twice with PBS, and then incubated in 4% formaldehyde for 20 min at room temperature. The slides were washed three times with PBS. The subsequent steps were as performed as aforementioned from the protease K digestion step.

**Construction of a protein-protein network.** The protein-protein interaction network was constructed and mapped by analyzing data obtained from Kyoto Encyclopedia of Genes and Genomes (genome.jp/kegg/), Pfam (pfam.xfam.org/), Search Tool for the Retrieval of Interacting Genes/Proteins (string-db.org/) and Gene Ontology (geneontology.org/) databases (46-48).

**Immunofluorescence.** Slides containing cells were prepared as described above (up to the 4% formaldehyde incubation step). The slides were washed with PBS and blocked with 3% bovine serum albumin (Servicebio, Inc.) for 30 min at room temperature. The slides were incubated at 4°C overnight with the following primary antibodies: Mouse anti-platelet-endothelial cell adhesion molecule-1 (CD31; 1:3,000; cat. no. 3528; Cell Signaling Technology, Inc.) and rabbit anti-von Willebrand Factor (vWF; 1:1,000; cat. no. GB11020; Servicebio, Inc.). Following washing in PBS three times for 5 min each, the slides were incubated with fluorophore-tagged secondary antibodies

for 50 min at room temperature in the dark. The secondary antibodies included FITC-conjugated Goat Anti-Mouse (1:200; cat. no. GB22301; Servicebio, Inc.) and Anti-Rabbit IgG (1:200; cat. no. GB22303; Servicebio, Inc.). Subsequently, the slides were incubated with DAPI for 10 min at room temperature and mounted with anti-fade fluorescence reagent. Stained slides were observed using a fluorescence microscope. Slides were scanned by the Panoramic MIDI scanner. Images were captured using CaseViewer at x200 or x900 magnification.

**Small interfering (si)RNA transfection.** Cells were seeded into 6-well plates and incubated for 24 h. At 60% confluence, cells were transfected for 10 h with 50 nmol/l lncRNA WEE2-AS1 siRNA (siR-lnc WEE2-AS1) or the negative control (NC) siRNA (siR-NC) using Lipofectamine<sup>®</sup> RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.). To knockdown lncRNA WEE2-AS1, two siRNAs (Suzhou GenePharma Co., Ltd.) were used with the following sequences: siR-lnc WEE2-AS1#1, 5'-GCCCAUCACAUUUCUCAUUTT-3'; and siR-lnc WEE2-AS1#2: 5'-GCAGCAAGCGACGUUCU ATT-3'. The siR-NC (Suzhou GenePharma Co., Ltd.) used as the negative control had the following sequence: 5'-UUCUCC GAACGUGUCACGUTT-3'.

**Lentivirus preparation and infection.** Lentiviruses expressing lncRNA WEE2-AS1 (lv-lnc WEE2-AS1) and negative control (lv-lnc WEE2-AS1-NC) sequences were prepared by GeneCopoeia, Inc. Cells were infected with lv-lnc WEE2-AS1 or lv-lnc WEE2-AS1-NC according to the manufacturer's protocol. Following infection with lv-lnc WEE2-AS1 or lv-lnc WEE2-AS1-NC at 37°C for 10 h, HUVECs were cultured in selection medium containing puromycin. Stably infected cells were cultured in EBM-2 containing puromycin.

**Cell viability assay.** Cell suspensions (100  $\mu$ l per well) were added to a 96-well plate. Following culture at 37°C for 72 h, cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). Briefly, 10  $\mu$ l CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The absorbance of each well was measured at a wavelength of 450 nm using a spectrophotometer.

**Cell cycle analysis.** Cells were seeded at  $1 \times 10^5$  cells/well in 6-well plates. Following siRNA transfection or lentivirus infection, flow cytometry was performed to assess the cell cycle distribution. Following trypsinization, cells were centrifuged at 100 x g for 3 min at room temperature. Then cells were washed with PBS. The cell concentration was adjusted to  $1 \times 10^6$  cells/ml. Subsequently, cells were fixed in 70% cold ethanol at 4°C overnight. Fixed cells were washed with PBS, centrifuged at 100 x g for 3 min at room temperature and resuspended. Subsequently, cells ( $1 \times 10^6$ /ml) were stained using RNase and propidium iodide buffer (Nanjing KeyGen Biotech Co., Ltd.) for 60 min at room temperature in the dark. Cell cycle distribution was assessed using a CytoFLEX flow cytometer (Beckman Coulter, Inc.) and analyzed using ModFit LT software (version 4.1; Verity Software House, Inc.).

**Western blotting.** Total protein was extracted from HUVECs using lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) and

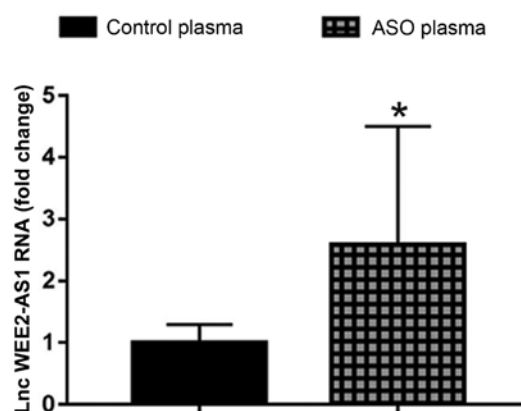


Figure 1. lncRNA WEE2-AS1 expression is upregulated in the plasma of patients with ASO. Reverse transcription-quantitative PCR was performed to detect the expression level of lncRNA WEE2-AS1 RNA in plasma samples obtained from patients with ASO and healthy control subjects. \* $P < 0.05$  vs. control plasma. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; ASO, arteriosclerosis obliterans.

quantified using a BCA protein assay kit (Nanjing KeyGen Biotech Co., Ltd.). Equal amounts of protein (40–50  $\mu$ g) were separated via 8–12% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The membranes were blocked in TBS containing 5% non-fat dry milk for  $\geq 1$  h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with the following primary antibodies: Anti-cell division cycle 25B (CDC25B; 1:1,000; cat. no. 9525; Cell Signaling Technology, Inc.), anti-cyclin dependent kinase 1 (CDK1; 1:1,000; cat. no. ab32594; Abcam), anti-myelin transcription factor 1 (MYT1; 1:1,000; cat. no. 4282; Cell Signaling Technology, Inc.), anti-CDK1 (phospho Y15; 1:1,000; cat. no. ab47594; Abcam), anti-WEE2 (1:500; cat. no. ab138162; Abcam),  $\beta$ -actin (1:100,000; cat. no. AC026; ABclonal Biotech Co., Ltd.) and GAPDH (1:100,000; cat. no. AC036; ABclonal Biotech Co., Ltd.). Following primary incubation, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) secondary antibody for 2 h at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence (EMD Millipore) and photographed using an Amersham Imager 600 (Cytiva). Protein expression levels were quantified using ImageJ software (version 6.0; National Institutes of Health) with  $\beta$ -actin and GAPDH as the loading controls.

**Statistical analysis.** Data are expressed as the mean  $\pm$  SD. Statistical analyses were performed using SPSS software (version 21.0; IBM Corp.). The paired Student's t-test was used to compare differences between two groups. One-way ANOVA followed by Tukey's post hoc test was used to compare differences among multiple groups.  $P < 0.05$  was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

## Results

**lncRNA WEE2-AS1 expression levels are upregulated in ASO plasma and artery samples.** RT-qPCR was performed to evaluate the expression levels of lncRNA WEE2-AS1 in



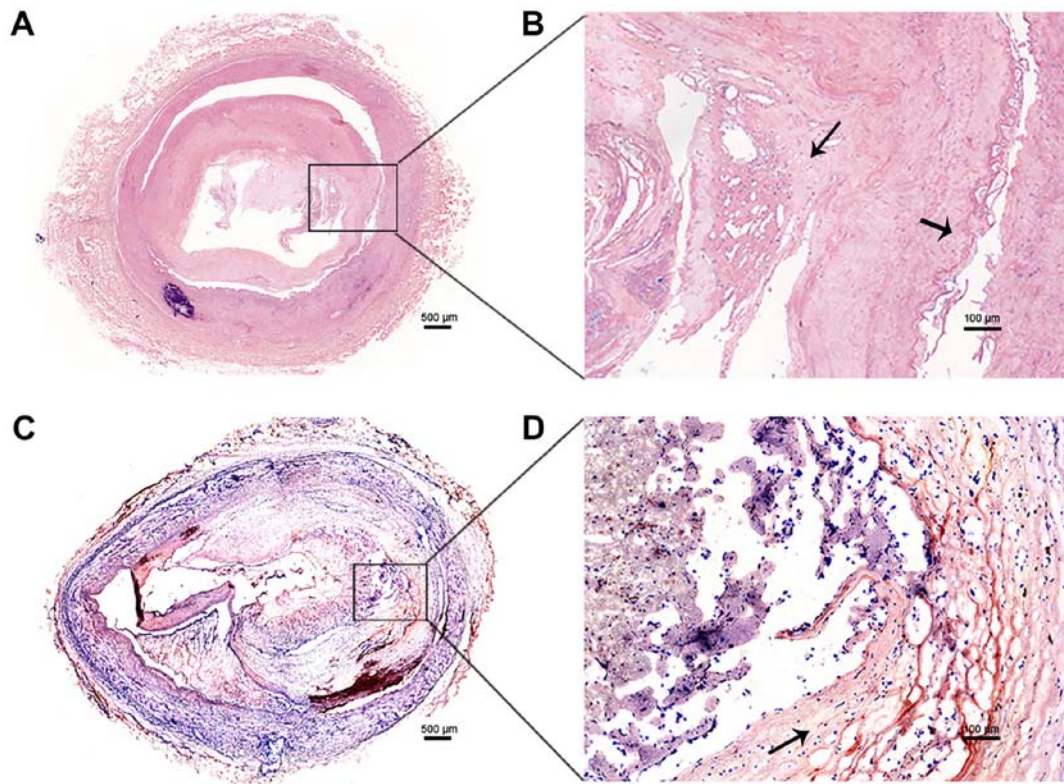


Figure 2. Histopathology of artery tissue samples obtained from patients with ASO. (A and B) Representative photomicrographs of ASO artery samples stained with H&E. (C and D) Representative photomicrographs of ASO artery samples stained with Oil Red O. Arrows indicate pathological changes associated with ASO. ASO, arteriosclerosis obliterans.

plasma samples obtained from patients with ASO and healthy control subjects. The results indicated that the expression level of lncRNA WEE2-AS1 was significantly upregulated in the ASO group compared with the healthy control group (Fig. 1).

The expression level of lncRNA WEE2-AS1 was further evaluated between tissue samples obtained from 5 patients with ASO and 5 healthy subjects. The results of H&E (Fig. 2A and B) and Oil Red O staining (Fig. 2C and D) indicated that the ASO tissue samples displayed pathological alterations consistent with ASO, including thickening and hardening of the blood vessel walls, artery stenosis, occlusion, endothelial cell injury, VSMC proliferation, atheromatous plaques, lipid deposition and rupture of the fiber cap. By contrast, the results of H&E staining showed no pathological alterations in the healthy artery tissue samples. The FISH analysis results suggested that, compared with healthy control arteries, the signal intensity for lncRNA WEE2-AS1 in the ASO artery group was significantly higher (Fig. 3). Collectively, the results suggested that the expression level of lncRNA WEE2-AS1 was significantly upregulated in plasma and artery tissue samples obtained from patients with ASO compared with healthy control subjects, which indicated that there might be an association between the expression level of lncRNA WEE2-AS1 and ASO. Moreover, FISH and immunofluorescence assays indicated that lncRNA WEE2-AS1 co-localized with CD31 in endothelial cells, which suggested that lncRNA WEE2-AS1 was expressed by human arterial endothelial cells (Fig. 4).

**Subcellular localization of lncRNA WEE2-AS1 in HUVECs.** HUVECs are one of the most important cell models

for the *in vitro* study of alterations to endothelial cell function in ASO (49,50); therefore, the present study investigated the functions of lncRNA WEE2-AS1 in HUVECs. Immunofluorescence was performed to characterize cells isolated from human umbilical veins. The cells stained positively for endothelial cell markers, including CD31 and vWF (Fig. 5A and B) (49,51,52), which indicated that the isolated cells were HUVECs.

FISH was performed to investigate the subcellular localization of lncRNA WEE2-AS1 in HUVECs. The results indicated that lncRNA WEE2-AS1 was expressed in the cytoplasm and nuclei of HUVECs, implying that it may participate in a variety of physiological and pathological processes (Fig. 5C).

**Bioinformatic analysis of the WEE2 protein interaction network.** lncRNA WEE2-AS1 is the antisense RNA of the WEE2 gene (19). The protein encoded by the sense strand has important significance for the prediction of antisense lncRNAs, which indicates a potential association between the biological functions of the antisense and sense strands (53-55). To explore the biological function of lncRNA WEE2-AS1, a protein-protein interaction network of WEE2 was constructed and mapped (Fig. 6). Analysis of the network suggested that WEE2 participated in several critical pathways, including cell viability, mitotic cell cycle and the G<sub>2</sub>/M checkpoint.

**lncRNA WEE2-AS1 regulates HUVEC viability and cell cycle progression.** RT-qPCR was performed to evaluate the effect of lncRNA WEE2-AS1 knockdown and overexpression

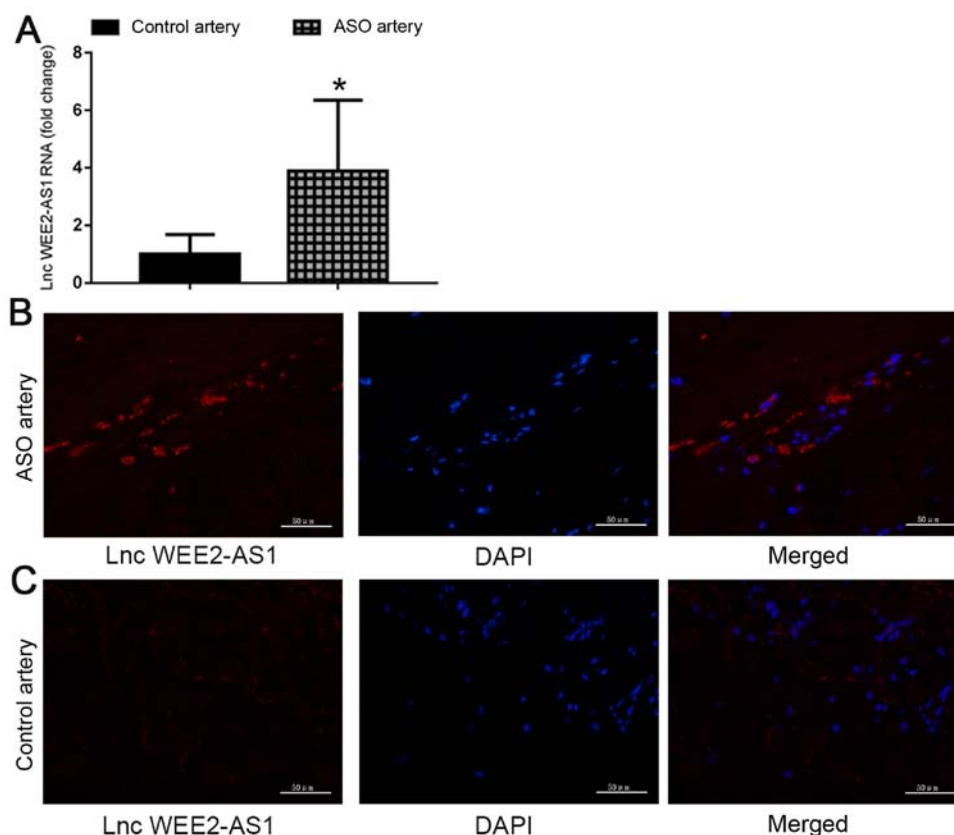


Figure 3. Signal intensity of lncRNA WEE2-AS1 is higher in ASO artery tissue samples. (A) FISH analysis was conducted to detect the expression level of lncRNA WEE2-AS1 in ASO artery and healthy artery tissue samples. Representative FISH photomicrographs of (B) ASO artery and (C) healthy artery tissue samples. Scale bar, 50  $\mu$ m \* $P$ <0.05 vs. control artery. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; ASO, arteriosclerosis obliterans; FISH, fluorescence *in situ* hybridization.

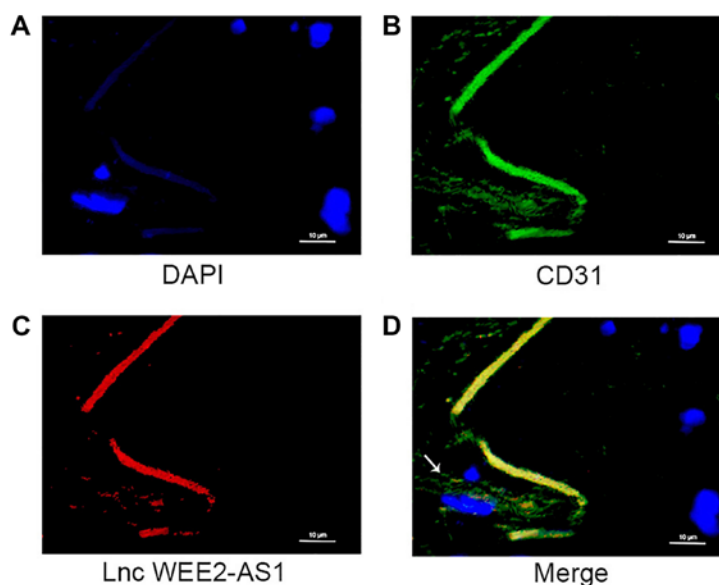


Figure 4. lncRNA WEE2-AS1 is expressed in endothelial cells of human artery tissue samples. (A) Nuclei were stained blue with DAPI. (B) Endothelial cells of human artery tissue samples were immunostained for CD31 (green), an endothelial cell marker. (C) Human artery tissue samples were analyzed by FISH a specific lncRNA WEE2-AS1 probe (red). (D) Merged image of immunofluorescence and FISH analysis of human artery tissue samples. Arrow indicates WEE2-AS1 expression. Scale bar, 10  $\mu$ m. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; CD31, platelet-endothelial cell adhesion molecule-1; FISH, fluorescence *in situ* hybridization.

on HUVECs. Compared with the corresponding negative control groups, lncRNA WEE2-AS1 expression levels were significantly reduced by siRNA transfection and significantly

increased by lentivirus infection. The results indicated that the siRNA transfection and lentivirus infection were successful (Fig. 7A and B).

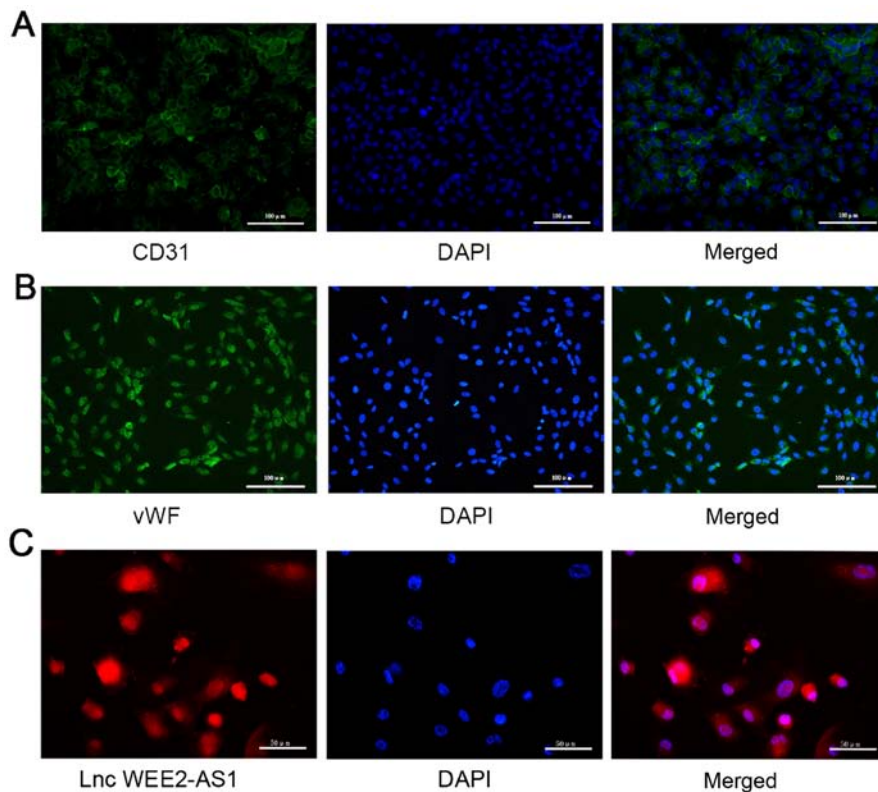


Figure 5. Subcellular localization of lncRNA WEE2-AS1 in HUVECs. Representative immunofluorescence photomicrographs of HUVECs positively stained DAPI (blue) and an (A) anti-CD31 (green) or (B) anti-vWF (green) antibody. Scale bars, 100  $\mu$ m. (C) Representative photomicrographs of fluorescence *in situ* hybridization analysis indicating the localization of lncRNA WEE2-AS1 (red) in HUVECs. Scale bar, 50  $\mu$ m. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; HUVEC, human umbilical vein endothelial cell; CD31, platelet-endothelial cell adhesion molecule-1; vWF, von Willebrand Factor.

The CCK-8 assay was conducted to explore the effect of lncRNA WEE2-AS1 on vascular endothelial cell viability. lncRNA WEE2-AS1 knockdown significantly enhanced cell viability compared with the negative control (siR-NC) group, whereas lncRNA WEE2-AS1 overexpression significantly inhibited cell viability compared with the negative control (lv-lnc WEE2-AS1-NC) group (Fig. 8).

To investigate the function of lncRNA WEE2-AS1 during mitosis, the cell cycle distribution was assessed via flow cytometry. The results indicated that lncRNA WEE2-AS1 knockdown significantly decreased the proportion of cells in the G<sub>0</sub>/G<sub>1</sub> phase, but significantly increased the proportion of cells in the G<sub>2</sub>/M phase compared with the negative control (siR-NC) group (Fig. 9A and B). However, no significant difference in the proportion of cells in the different cell cycle phases was detected between lncRNA WEE2-AS1-overexpression cells and negative control (lv-lnc WEE2-AS1-NC) cells (Fig. 9C and D).

**lncRNA WEE2-AS1 modulates WEE2 expression and MPF activity.** Antisense lncRNAs are widespread in human cells and have emerged as regulators of the corresponding sense strand genes (56). However, the mechanism underlying how antisense lncRNAs affect the expression of the neighboring coding gene remains unclear. Therefore, whether lncRNA WEE2-AS1 regulated the corresponding sense gene expression was investigated. The western blotting results indicated that lncRNA WEE2-AS1 knockdown significantly decreased WEE2 protein expression levels compared with the negative control (siR-NC) group,

whereas lncRNA WEE2-AS1 overexpression significantly increased WEE2 protein expression levels compared with the negative control (lv-lnc WEE2-AS1-NC) group (Fig. 10A).

MPF activation is a prerequisite for the cell cycle transition from the G<sub>2</sub> phase to the M phase, and is regulated by a variety of cell cycle-related proteins via both negative and positive loops (57). WEE2 inhibits MPF activity by phosphorylating the Tyr15 residue of CDK1 to regulate the cell cycle (20,21). To identify the regulatory mechanism underlying lncRNA WEE2-AS1, the effect of lncRNA WEE2-AS1 expression on MPF activity was investigated. The western blotting results indicated that lncRNA WEE2-AS1 knockdown significantly decreased the expression level of MYT1 and the phosphorylation of CDK1, but increased the expression level of CDC25B compared with the negative control (siR-NC) group. By contrast, lncRNA WEE2-AS1 overexpression displayed the opposite effect on CDK1 phosphorylation, and the expression of MYT1 and CDC25B (Fig. 10B and C). Therefore, the western blotting results indicated an association between lncRNA WEE2-AS1 and the regulation of cell viability.

## Discussion

The molecular mechanism underlying atherosclerosis is not completely understood; however, numerous studies have indicated that lncRNAs serve pivotal roles in the pathological processes of atherosclerosis (7-12).

The present study suggested that lncRNA WEE2-AS1 expression levels were significantly upregulated in the plasma



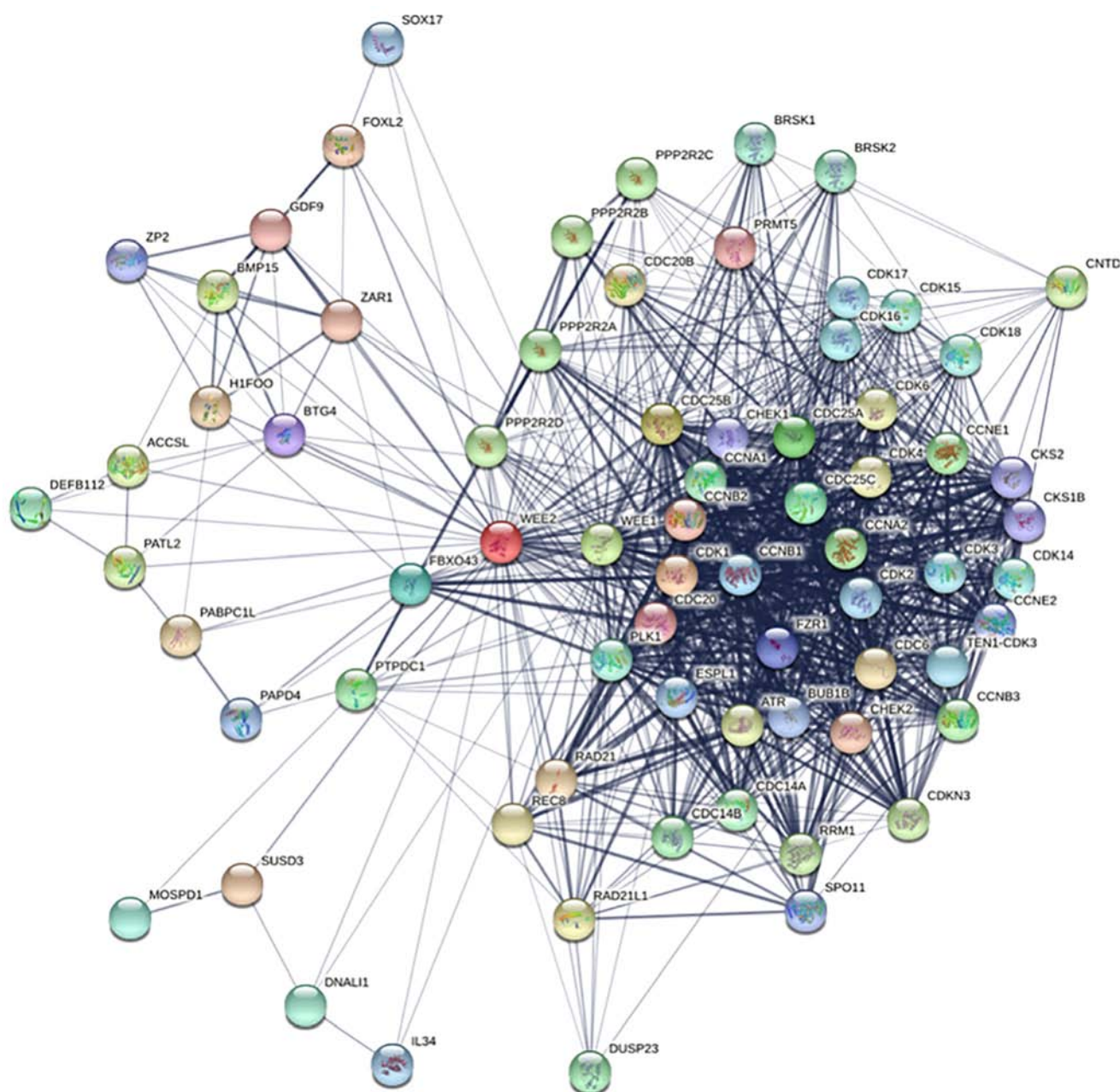


Figure 6. Protein-protein interaction network of WEE2. WEE2, WEE1 homolog 2.

and artery tissue samples of patients with ASO compared with healthy control subjects, which indicated that lncRNA WEE2-AS1 may be involved in ASO. The results also suggested that lncRNA WEE2-AS1 was expressed in the cytoplasm and nuclei of human vascular endothelial cells, which indicated that lncRNA WEE2-AS1 may be involved in a variety of physiological and pathological processes.

Endothelial dysfunction can lead to alterations to synthetic and secretory functions, causing disruption of the local microbalance, which represents the beginning of atherosclerosis. Identifying factors that control endothelial dysfunction is essential for the treatment of atherosclerosis (58). Increasing evidence suggests that various lncRNAs are involved in endothelial dysfunction. For instance, silencing the lncRNA myocardial infarction associated transcript inhibits endothelial cell proliferation, migration and tube formation (13).

Abnormal alterations to the viability and cell cycle progression of endothelial cells are important features of atherosclerosis. Therefore, whether lncRNA WEE2-AS1 could affect endothelial cell viability was investigated in the present study. lncRNA WEE2-AS1 knockdown significantly promoted endothelial cell viability, whereas lncRNA WEE2-AS1 overexpression significantly inhibited endothelial cell viability compared with the negative control groups. Cell cycle dysregulation is implicated in various diseases, including atherosclerosis. Therefore, the effect of lncRNA WEE2-AS1 on the cell cycle was assessed. lncRNA WEE2-AS1 knockdown significantly decreased the proportion of cells in the G<sub>0</sub>/G<sub>1</sub> phase and significantly increased the proportion of cells in the G<sub>2</sub>/M phase compared with the negative control group. However, there was no significant difference in the proportion of cells in the different cell cycle phases between lncRNA WEE2-AS1-overexpression



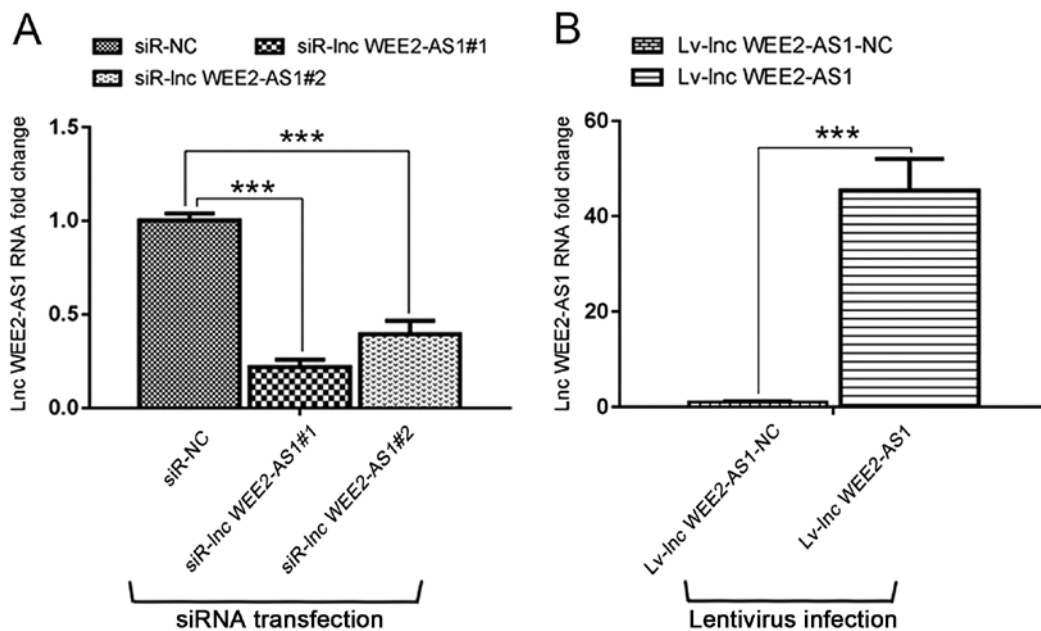


Figure 7. Effects of lncRNA WEE2-AS1 knockdown and overexpression. (A) lncRNA WEE2-AS1 expression was knocked down by siRNA transfection. (B) lncRNA WEE2-AS1 expression was overexpressed via lentivirus infection. \*\*\*P<0.001 vs. siR-NC or lv-lnc WEE2-AS1-NC. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; siR, small interfering RNA; NC, negative control; lv, lentivirus.

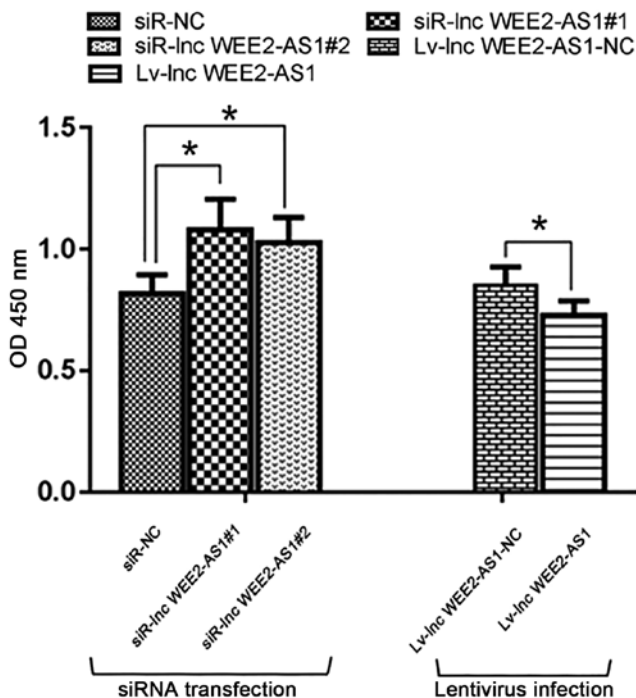


Figure 8. Effects of lncRNA WEE2-AS1 on HUVEC viability. \*P<0.05 vs. siR-NC or lv-lnc WEE2-AS1-NC. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; HUVEC, human umbilical vein endothelial cell; siR, small interfering RNA; NC, negative control; lv, lentivirus; OD, optical density.

cells and negative control cells. lncRNAs commonly contain multiple splice variants (59); however, the present study only overexpressed the single consensus gene model of lncRNA WEE2-AS1 via lentivirus transfection, which may provide an explanation for the minimal effects of lncRNA WEE2-AS1 overexpression on cell viability and the cell cycle compared

with lncRNA WEE2-AS1 knockdown. Moreover, the results also indicated that only overexpressing the single consensus gene model of lncRNA WEE2-AS1 via lentivirus transfection makes it difficult to assess the full extent of its biological functions. The transcription and activation of lncRNAs involves complex post-transcriptional processing, modification and regulation processes (60); therefore, the current understanding of these potential mechanisms remains in its infancy. Further studies are required to identify the splice variant of lncRNA WEE2-AS1 that is most closely related to the viability and cell cycle of endothelial cells, as well as the underlying mechanisms.

In eukaryotic cells, MPF is an indispensable inducer for entry into the mitosis phase of the cell cycle. MPF is a complex comprised of a catalytic subunit, Cyclin B, and a regulatory subunit, CDK1 (20-22). WEE2 and MYT1 perturb the G<sub>2</sub>/M transition via specific phosphorylation of residues of CDK1 (61,62). In opposition to the inhibitory phosphorylation of WEE2 and MYT1, the stimulatory dephosphorylation of CDC25B restores MPF activity (63,64). Once activated MPF reaches the threshold for G<sub>2</sub>/M transition, cells are triggered to undergo mitosis (65,66). The balance between WEE2 and CDC25B participates in the regulation of MPF activity and maintains the orderly progression of mitosis (67,68). The present study investigated the regulatory role of lncRNA WEE2-AS1 on cell viability and the cell cycle. lncRNA WEE2-AS1 was expressed in the nuclei and cytoplasm of vascular endothelial cells, and increased the synthesis of WEE2 protein, which indicated that antisense lncRNA WEE2-AS1 might positively regulate the expression of WEE2 encoded by the sense strand gene and could be involved in gene transcription and post-transcriptional regulation. Furthermore, the results indicated that lncRNA WEE2-AS1 knockdown significantly decreased WEE2, MYT1 and pCDK1 expression levels, but increased CDC25B expression levels compared with the negative control

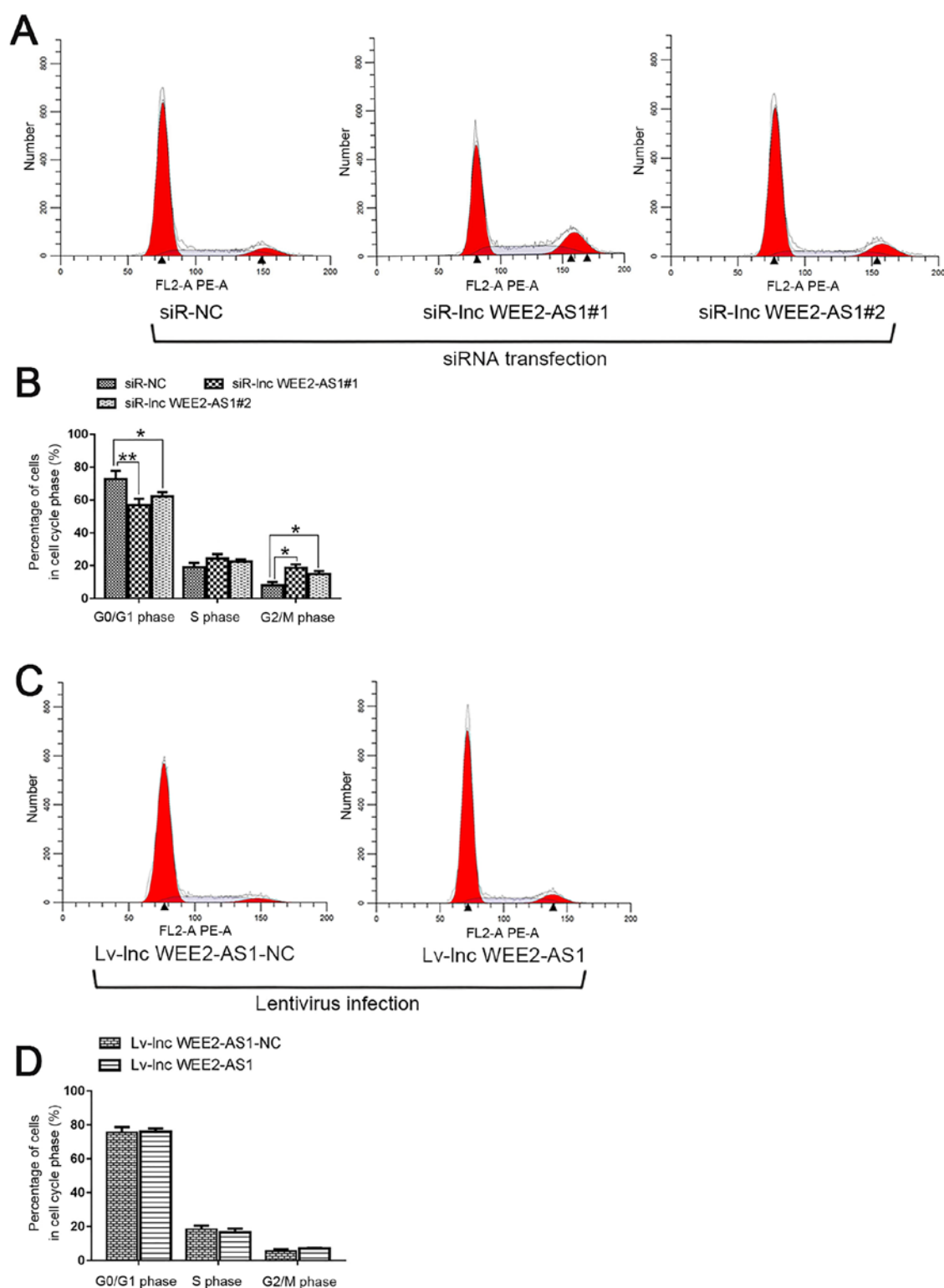


Figure 9. IncRNA WEE2-AS1 regulates HUVEC cell cycle progression. The effect of IncRNA WEE2-AS1 knockdown on HUVEC cell cycle progression was (A) determined by flow cytometry and (B) quantified. The effect of IncRNA WEE2-AS1 overexpression on HUVEC cell cycle progression was (C) determined by flow cytometry and (D) quantified. \* $P < 0.05$  and \*\* $P < 0.01$  vs. siR-NC or lv-lnc WEE2-AS1-NC. IncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; HUVEC, human umbilical vein endothelial cell; siR, small interfering RNA; NC, negative control; lv, lentivirus.

group. By contrast, IncRNA WEE2-AS1 displayed the opposite effects on protein expression. The results also suggested that IncRNA WEE2-AS1 may modulate the G<sub>2</sub>/M transition of the cell cycle and serve a role in cell cycle progression. A large number of antisense lncRNAs have been identified to upregulate the expression of their sense gene. For example,

PDCD4-AS1 and PDCD4 are positively correlated (22). The results of the present study implied that IncRNA WEE2-AS1 regulated the expression of WEE2 gene. Although a series of models have been proposed to illustrate how lncRNAs regulate sense RNA, there is no convincing molecular explanation. Further research is required to reveal the molecular mechanism

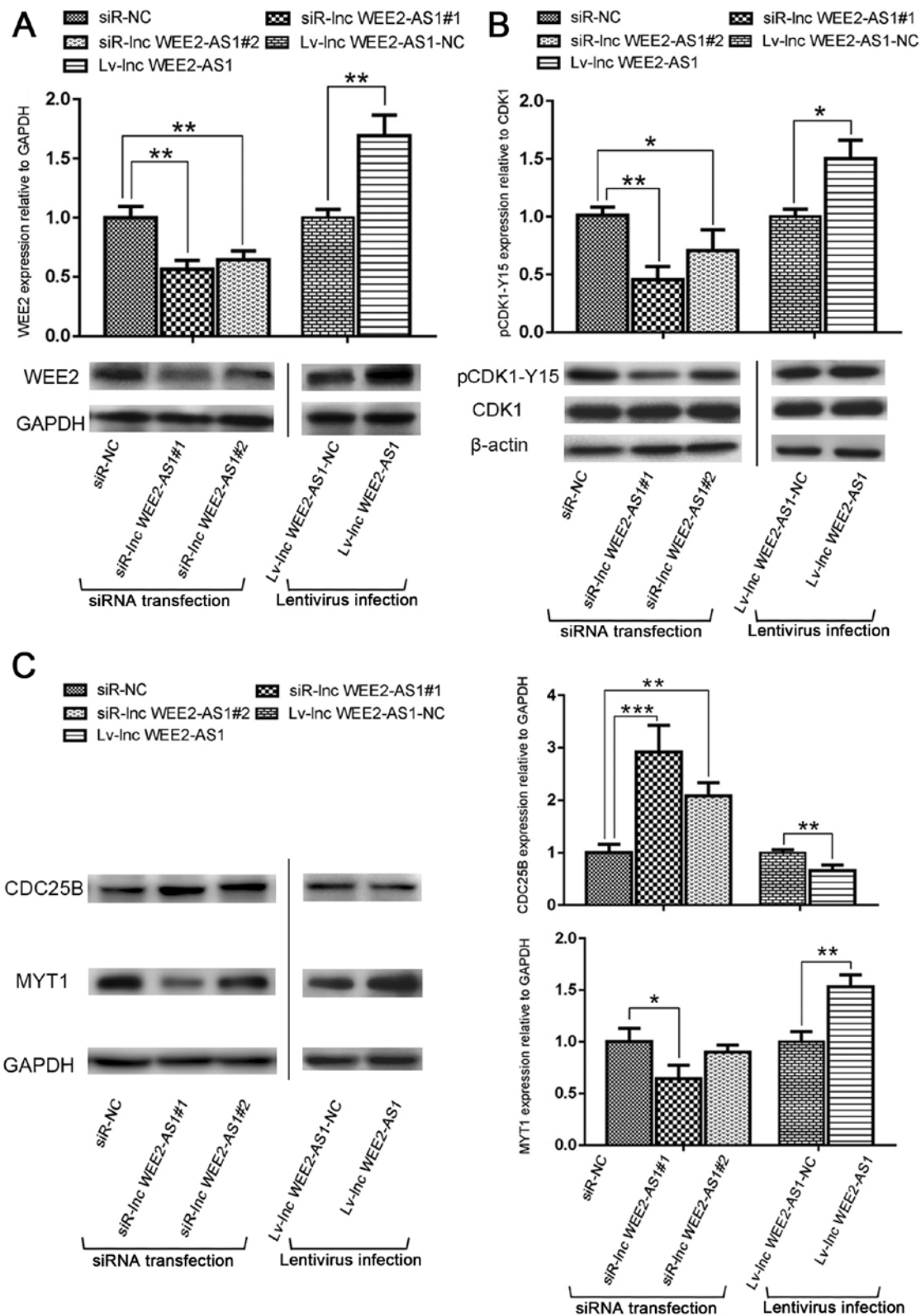


Figure 10. Western blotting was performed to assess the expression of G2/M transition-related proteins. The effects of lncRNA WEE2-AS1 knockdown and overexpression on (A) WEE2, (B) the ratio of pCDK1-Y15 to CDK1, (C) CDC25B and MYT1 protein expression levels were determined by western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. siR-NC or Lv-lnc WEE2-AS1-NC. lncRNA/lnc, long non-coding RNA; WEE2-AS1, WEE2 antisense RNA 1; WEE2, WEE1 homolog 2; p, phosphorylated; CDK1-Y15, cyclin dependent kinase 1-phosphorylated Y15; CDC25B, cell division cycle 25B; MYT1, myelin transcription factor 1; siR, small interfering RNA; NC, negative control; lv, lentivirus.

underlying lncRNA WEE2-AS1-mediated regulation of WEE2 expression. lncRNAs can regulate gene transcription in the nucleus or the activity of other noncoding and coding

RNAs in the cytoplasm (69-72). Moreover, lncRNAs can form secondary, tertiary and even more complex and higher order structures, which can tether functionally interacting or



unrelated proteins together (73,74). Thus, further research is required to determine the molecular mechanisms underlying lncRNA WEE2-AS1-mediated regulation of crucial proteins in the G<sub>2</sub>/M transition pathway. Hu *et al* (75) demonstrated that lncRNA WEE2-AS1 accelerated hepatocellular carcinoma cell proliferation in hepatitis B virus-related HCC. Therefore, whether the role of lncRNA WEE2-AS1 depends on the specificity of tissue and cell type requires further investigation (75).

In conclusion, the present study indicated that lncRNA WEE2-AS1 expression was significantly upregulated in the plasma and artery tissue samples of patients with ASO compared with healthy control subjects. Moreover, lncRNA WEE2-AS1 may regulate endothelial cell viability via the G<sub>2</sub>/M transition pathway of the cell cycle. The results of the present study may further the current understanding of the molecular mechanism underlying ASO and may aid with the identification of specific probes and targeted drugs for the diagnosis and treatment of ASO.

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### Availability of data and materials

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

### Authors' contributions

SW, WL, BJ and CZ conceived and designed the study. BJ performed the majority of the experiments, analyzed the data and drafted the manuscript. SW, WL and CZ revised the manuscript. RW, JM and RL performed some experiments and analyzed some the data. ZL, RW, JC, MW and WW participated in the collection of clinical specimens and performed some experiments. SW supervised the project. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University [approval no. (2013)70] and conducted in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants or their guardians

provided written informed consent before the tissue and blood samples were obtained.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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