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CircRNA-PTPRA promoted the progression of atherosclerosis through sponging with miR-636 and upregulating the transcription factor SP1

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Abstract. – OBJECTIVE: Atherosclerosis (AS) is the leading cause of death for humans worldwide, and some circular RNAs (circRNAs) have been demonstrated to play important roles in its progression. In this study, we mainly investigated the functions and molecular mechanisms of circRNA-PTPRA (circPTPRA) in AS.

PATIENTS AND METHODS: The expressions of circPTPRA and miR-636 were detected in serum samples of AS patients (n=30) and healthy controls (n=30) by RT-PCR. Then levels of circPTPRA were detected after ox-LDL treatment into vascular smooth muscle cell (VSMCs), macrophage and endothelial cells. LV-sh circPTPRAs were constructed and infected into VSMCs. CCK-8 assay was performed to measure cell proliferation abilities, flow cytometry (FACS) was performed to measure cell-cycle distribution and TUNEL staining was performed to detect cell apoptosis. Western blot (WB) was performed to detect protein levels of SP1, Cyclin D1, Cyclin E, Bax, Bad, Cleaved Caspase3. Luciferase reporter assay was performed to verify the potential binding sites of circPTPRA and miR-636, miR-636 and SP1.

RESULTS: RT-PCR showed that circPTPRA was upregulated in serum samples of AS patients, which was increased by ox-LDL in VSMCs. CircPTPRA inhibition repressed cell proliferation, improved cell-cycle distribution in G0/G1 phase and promoted cell apoptosis. MiR-636, a potential target for circPTPRA, was reduced in serum samples of AS patients and Luciferase reporter assay confirmed that circPTPRA could directly sponge with miR-636 in VSMCs. Furthermore, miR-636 inhibition promoted proliferation and repressed apoptosis of VSMCs, while miR-636 overexpression reversed these results. SP1, a transcription factor that played some roles in the progression of AS, was predicted to be a target of miR-636. MiR-636 inhibition increased SP1 while miR-636 overexpression repressed SP1 expression, Luciferase reporter assay proved that miR-636 could target at SP1 in VSMCs. Moreover, the repressed cell proliferation and promoted cell apoptosis abilities in LV-sh SP1 were reversed following with miR-636 inhibitor transfection. In addition, the repressed cell proliferation and promoted cell apoptosis abilities in VSMCs with LV-sh circPTPRAs were reversed following with miR-636 inhibitor transfection, which suggested that circPTPRA regulated cell proliferation and apoptosis through miR-636/SP1 axis in AS.

CONCLUSIONS: According to the results, we found that circPTPRA was upregulated in serum samples of AS patients, which promoted cell proliferation and inhibited cell apoptosis through repressing miR-636 and upregulating SP1 signaling axis. Our results uncovered a potential role of circPTPRA, which might be a marker and therapeutic target for AS patients.

Key Words:
CircRNA-PTPRA, MiR-636, SP1, Vascular smooth muscle cell, Atherosclerosis.

Introduction

Atherosclerosis (AS) is the leading cause of death for humans worldwide, and it is responsible for coronary heart disease (CHD), which is a major problem for human health[1-3]. Thus, every year, more than 20 million people die because of AS and CHD, which are leading causes of mortality worldwide[3-5]. It has been demonstrated that LDL is one of the most important and dangerous factors for the development of AS[6-8]. Contact between various chemokines, vascular cells and non-vascular cells, promotes the formation of atherosclerotic lesions, such as vascular smooth muscle cells (VSMCs), macrophage and endothelial cells, etc.[9-12]. VSMCs have been proved to be an important component for blood vessels and play critical roles in the development of AS[13-15]. Endothelial cells (ECs) mainly form the inner walls of blood vessels, and the death of ECs has been described to cope with the

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progression of AS[13-15]. Macrophages have been revealed to secret inflammatory factors, which will promote plaque formation and the development of AS[13-15]. Ox-LDL has been demonstrated to promote the development of AS and increase the downstream chronic inflammatory responses; as a result, it has been widely used to conduct the simulation environment of AS[16,17].

Circular RNAs (circRNAs) are classes of non-coding RNAs that have recently become a hot topic and attracted great interest in the fields of cancers and cardiovascular diseases[18-21]. CircRNAs are a kind of closed-loop structures that have no terminal 5' caps and 3' polyadenylated tails[18,19]. Competing endogenous RNA (ceRNA) is a process that plays an important role in the progression of various diseases. CircRNAs may sponge with microRNAs (miRNAs) and repress the levels of miRNAs, which will then affect the expressions of downstream target genes and regulate different biological functions in various diseases[18-22].

CircRNAs play some roles in the development of AS[23-25]. Li et al[26] found that the circ-0003575 was upregulated in oxLDL -induced human umbilical vein endothelial cells (HUVECs), which could regulate cell proliferation and angiogenesis of HUVECs, thereby regulating the progression of AS. Yang et al[23] demonstrated that circCHFR was overexpressed in the ox-LDL-induced VSMCs, which promoted the proliferation and migration of VSMCs via interacting with miR-370/FOXO1/ Cyclin D1 pathway. And the functions of circRNAs in AS are largely unknown. CircPTPRA was firstly found in NSCLC in which it could suppress epithelial-mesenchymal transitioning and metastasis by sponging with miR-96-5p[27]. He et al[28] demonstrated circPTPRA could act as a tumor suppressor to inhibit bladder cancer cell proliferation and tumor growth through sponging with miR-636/ KLF9 axis. However, the level and roles of circPTPRA in AS remained unknown.

MiRNAs can directly bind with the 3′-UTR (untranslated region) of mRNA and inhibit expressions, thereby regulating functions in various diseases[29-32]. MiR-636 had been found to play key roles in modulating the inflammatory processes[33] and it could promote cell proliferation in bladder cancer[28]. However, the role of miR-636 in AS is unclear.

In this study, we aimed at exploring the level and functions of circPTPRA in AS patients. For the first time, we found that circPTPRA was significantly increased in AS. Therefore, we investigated the functions and mechanisms of circPTPRA in AS.

Patients and Methods

Serum Samples

Human serum samples were collected from AS patients (30 cases; 46.6± 10.5 years old; 18 males) and healthy controls (30 cases; 43.5± 11.8 years old; 16 males) in our hospital from March 2017 to September 2017. Samples were stored in liquid nitrogen at -80°C. No significant differences were found between AS patients and healthy controls in age and sex. Informed consent was received from patients and healthy controls. This study was approved by the Ethics Committee of our hospital, and it was done according to the principles of the Declaration of Helsinki. AS patients were diagnosed by two experienced doctors according to the 2013 ACC/AHA guideline on the assessment of cardiovascular risk[34]. Patients were excluded if they were suffering from active bleeding, severe diseases of heart, liver and kidney or other severe chronic diseases; pregnant or lactating women were also excluded.

Cell Culture

Human vascular smooth muscle cell line (VSMCs), human THP-1 macrophages and endothelial cells (ECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml), which were then cultured at an incubator with 37°C and 5% CO_2.

Construction of Lentivirus

The short hairpin circPTPRA (sh circPTPRA) and sh SP1 sequences were cloned into different lentivirus (Shanghai Genechem Co., LTD, Shanghai, China), resulted with circPTPRA or SP1 inhibition, which were named LV-sh circPTPRA-1, LV-sh circPTPRA-2 and LV-sh SP1. Lentivirus or negative control (NC) was respectively infected into VSMCs and the stable VSMCs with circPTPRA or SP1 inhibition were selected and constructed after 1 to 2 weeks according to its instructions.

Cell Transfection

Indicated cells were prepared and seeded in 6-well plates (1×10^6/well) until reaching about 50%; transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), serum-free

DMEM and miR-636 inhibitor or inhibitor NC miR-636 mimic or mimic NC were mixed together and transfected into prepared cells according to its instructions.

CCK8 Assay

Cell proliferation ability was measured by Cell Counting Kit 8 (CCK8, Dojindo, Kumamoto, Japan) assay. Cells were seeded on 96-well plates (2×10^3/well) and cultured with 100 ul DMEM for 24 h, 48 h, 72 h and 96 h. 10 ul CCK8 was added for each well and incubated at darkness for 2 h at 37°C with 5% CO_2. The absorbance (OD) value was measured at 450 nm with a microplate reader (Thermo Fisher, Waltham, MA, USA). Three replicate wells were used for each group, and every experiment was repeated for three times.

TUNEL Staining

Cell apoptosis was determined by using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (DeadEnd Colorimetric TUNEL System, Promega, Madison, WI, USA) according to manufacturer's instructions. Apoptotic nuclei exhibiting fragmented DNA were stained and the number of apoptotic cells was calculated in four separated 200×fields. Cells were exposed to RQ1 DNase (Promega, Madison, WI, USA), and were used as a positive control.

Flow Cytometry for Cell-Cycle Distribution

After treatments, cells were digested with trypsin and washed twice by phosphate-buffered saline (PBS), then cell precipitation was collected after centrifugation. It was mixed with 75% ethanol at 4°C for about 4 h, which was washed twice by cooled PBS and stained with FITC-Annexin V and Propidium iodide (PI) at room temperature for 10 mins. Finally, cells were subjected to a FACS Calibur system (BD Pharmingen, San Diego, CA, USA) to determine the cell-cycle status.

RNA Isolation and Quantitative Real-Time PCR

TRIzol LS (Invitrogen, Carlsbad, CA, USA) was used to extract total RNAs from serum samples and RNAiso Plus (TaKaRa, Otsu, Shiga, Japan) was used to extract total RNAs from VSMCs, macrophage and endothelial cells according to the protocols. PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) was used to make reverse transcription in accordance with the protocol. Primer sequences for RT-PCR were synthesized by Shanghai Genechem (Shanghai Genechem Co., LTD, Shanghai, China), which were showed in Table I. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect gene expressions and GAPDH or U6 was used as the reference, $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expressions.

Protein Extraction and Western Blot

Total proteins were extracted by using RIPA lysis buffer (Beyotime, Shanghai, China) and protein concentrations were measured by a BCA kit (Sigma-Aldrich, St. Louis, MO, USA) according to its protocol. 40 μg total proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which were transferred onto polyvinylidene fluoride (PVDF) membranes. After blocked at room temperature with 5% non-fat milk for 1 h, membranes were incubated with primary antibodies at 4°C overnight, all primary antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA), including Bax (ab32503, 1:1000, 21 kDa), Bad (ab32445, 1:2000, 23 kDa), Cleaved Caspase-3 (ab2302, 1:500, 17 kDa), Cyclin D1 (ab16663, 1:1000, 36 kDa), Cyclin E (ab33911, 1:1000, 50 kDa), SP1 (ab124804, 1:1000, 90 kDa), and GAP-

Table I. Primer sequences for RT-PCR.

Gene names	Primer sequences
circPTPRA	Forward: 5'-TAACCAGTTCACGGATGCCA-3' Reverse: 5'- AGCATTGTTGGCACTGACAC-3'
miR-636	Forward: 5'-ACACTCCAGCTGGGTGTGC-3' Reverse: 5'-TGGTGTCGTGGAGTCG-3'
GAPDH	Forward:5'-GGAGTCCACTGGTGTCTTCA-3' Forward:5'-GGGAACTGAGCAATTGGTGG-3'
U6	Forward:5'-CTCGCTTCGGCAGCACA-3' Forward:5'-AACGCTTCACGAATTTGCGT-3'

DH (ab8245, 1:5000, 36 kDa). Matched secondary antibodies were used to incubate at room temperature for 1 h. Protein bands were detected by an ECL Kit (Millipore, Billerica, MA, USA) in ECL detection system (Thermo Fisher Scientific, Shanghai, China).

Luciferase Reporter Assay

The wild type and mutant sequences in 3'UTR of circPTPRA and SP1 were respectively synthesized and constructed into different GLO plasmids (Promega, Madison, WI, USA), which named WT-circPTPRA, MUT-circPTPRA, WT-SP1 and MUT-SP1. Cells were seeded on 48-well plates until about 40%, miR-636 mimic or mimic NC was respectively added for 24 h. Plasmids were transfected into prepared cells with Lipofectamine 2000 according to the protocol. After 24 h, cells were lysed and Dual-Luciferase reporter assay (Promega, Madison, WI, USA) was used to measure Luciferase activities of firefly and Renilla. According to the protocol, Renilla Luciferase gene was used as the reference and the relative activities of Luciferase were analyzed.

Statistical Analysis

All data were performed by SPSS 19.0 (IBM, Armonk, BY, USA) and images were graphed by GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA); data were presented as the mean±SD. Data were calculated using Student's *t*-test or one-way ANOVA with SNK post-hoc test. Pearson's correlation analysis was performed to analyze the correlations. *p*-value <0.05 was considered statistically significant.

Results

CircPTPRA was Upregulated in AS Patients and was Increased by ox-LDL in VSMCs

To identify the expression and functions of circPTPRA in AS, we detected the levels of circPTPRA in serum samples from AS patients (n=30) and healthy controls (n=30), which revealed that it was obviously upregulated in AS patients, compared with the healthy controls (Figure 1A) ($p<0.001$). To find out which factor and cells affected the level of circPTPRA in serum samples, we used ox-LDL to treat vascular smooth muscle cells (VSMCs), macrophage and endothelial cells. Results showed that circPTPRA was increased by ox-LDL treatments in VSMCs (Figure 1B) ($p<0.01$), but not in macrophage and endothelial cells (Figure 1C, D) ($p>0.05$). As a result, these results suggested that circPTPRA was upregulated in AS patients and it was increased by ox-LDL treatment in VSMCs, and we chose VSMCs to explore the functions and potential mechanism of circPTPRA in AS.

CircPTPRA Inhibition Repressed Proliferation and Promoted Apoptosis in VSMCs

To explore the functions of circPTPRA in AS, sh circPTPRA-1 and sh circPTPRA-2 were constructed into lentivirus, which were then infected into VSMCs. Results showed that circPTPRA levels were significantly inhibited after LV-sh circPTPRA-1 and LV-sh circPTPRA-2 infection (Figure 2A) ($p<0.001$). First of all, we used CCK8 assay to measure the proliferation abilities of VSMCs; results showed that circPTPRA inhibition repressed proliferation abilities of VSMCs, compared with LV-NC group (Figure 2B) ($p<0.001$). Furthermore, we used flow cytometry (FACS) to measure the cell-cycle distribution, which showed that circPTPRA inhibition repressed cell growth by improving cell distribution in G0/G1 phase and reducing cell distribution in S phase (Figure 2C) ($p<0.01$). Moreover, we used TUNEL staining to detect cell apoptosis, which showed that circPTPRA inhibition increased apoptotic rates of VSMCs (Figure 2D) ($p<0.001$). Besides, we detected proliferation associated and apoptotic gene expressions, which revealed that Cyclin D1 and Cyclin E were significantly repressed, while Bax, Bad and Cleaved Caspase3 were increased after circPTPRA inhibition (Figure 2E, F) ($p<0.01$). These results suggested that circPTPRA inhibition repressed proliferation and promoted apoptosis in VSMCs.

CircPTPRA could Directly Bind with miR-636 in VSMCs

To further investigate the mechanism that circPTPRA regulated cell proliferation and apoptosis in AS, we used starBase v2.0 database to analyze downstream targets, which revealed that miR-636 might be a potential target miRNA for circPTPRA. As a result, we observed expressions of miR-636 in AS patients and healthy controls, which showed that miR-636 was repressed in AS patients (n=30) (Figure 3A) ($p<0.01$). Furthermore, correlation analysis revealed that miR-636 was negatively correlated with circPTPRA in AS patients (Figure 3B) ($p<0.01$), but not in healthy

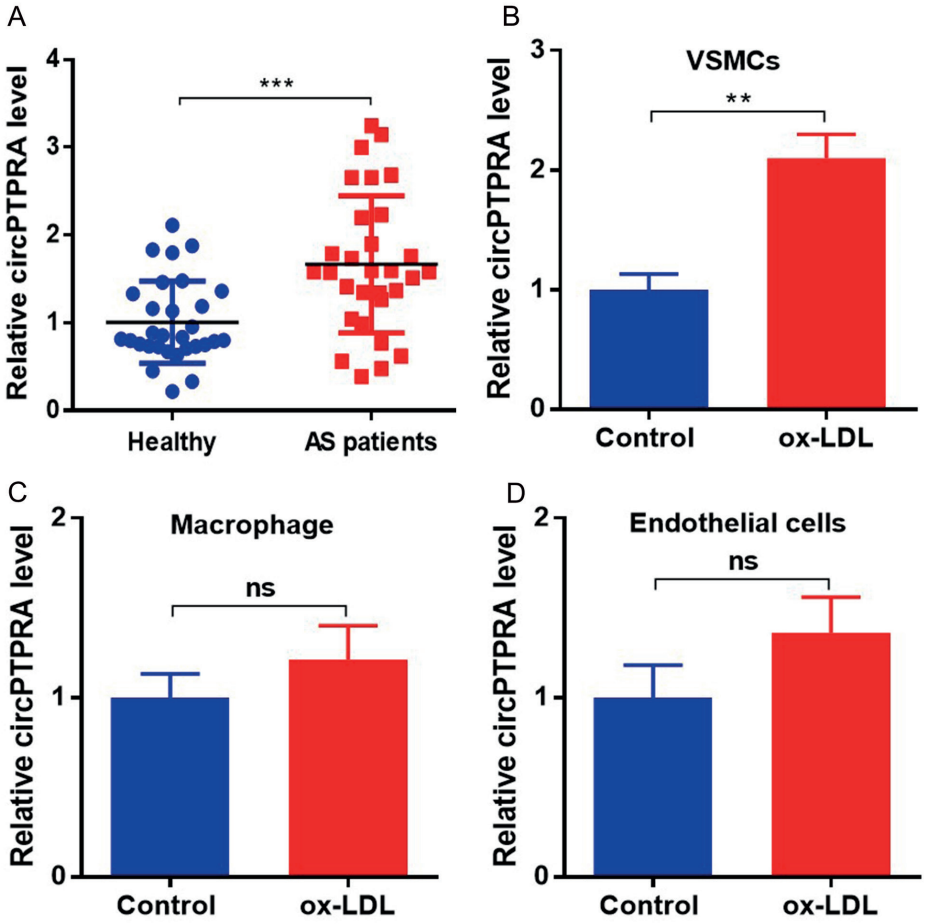

Figure 1. CircPTPRA was upregulated in AS patients and was increased by ox-LDL in VSMCs. **A,** Levels of circPTPRA in serum samples of AS patients (n=30) and healthy controls (n=30) were detected by RT-PCR. **B-D,** Expressions of circPTPRA were detected by RT-PCR after ox-LDL treatment into VSMCs, macrophage and endothelial cells. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$, $^{***}p<0.001$.

controls (Figure 3C) ($p>0.05$). Besides, we also detected miR-636 expressions in VSMCs infected with LV-sh circPTPRA-1 and LV-sh circPTPRA-2, which showed that circPTPRA inhibition increased miR-636 expressions (Figure 3D) ($p<0.001$). These results suggested that circPTPRA was negatively related to miR-636, and miR-636 was predicted to be a potential target miRNA for circPTPRA. To assess that miR-636 was downstream of circPTPRA, the wild type and mutant sequences of circPTPRA were synthesized and constructed in GLO plasmid (Figure 3E). Finally, Luciferase gene reporter assay was performed, which showed that the relative Luciferase activity in VSMCs co-transfected with WT-circPTPRA and miR-636 mimic was significantly repressed, while it was reversed in VSMCs co-transfected with MUT-circPTPRA (Figure 3F) ($p<0.01$). Above all, these results demonstrated that circPTPRA could directly bind with miR-636 in VSMCs, which might then regulate the progression of AS.

MiR-636 Repressed Cell Proliferation and Increased Apoptosis in VSMCs

To further explore whether miR-636 could regulate cell proliferation and apoptosis in AS, the mimic NC, miR-636 mimic, inhibitor NC or miR-636 inhibitor was respectively transfected into VSMCs. Results showed that miR-636 was inhibited after miR-636 inhibitor transfection and miR-636 was upregulated after miR-636 mimic transfection (Figure 4A) ($p<0.001$), which suggested that miR-636 inhibitor or mimic was successfully transfected into VSMCs. Then CCK-8 assay showed that the cell proliferation

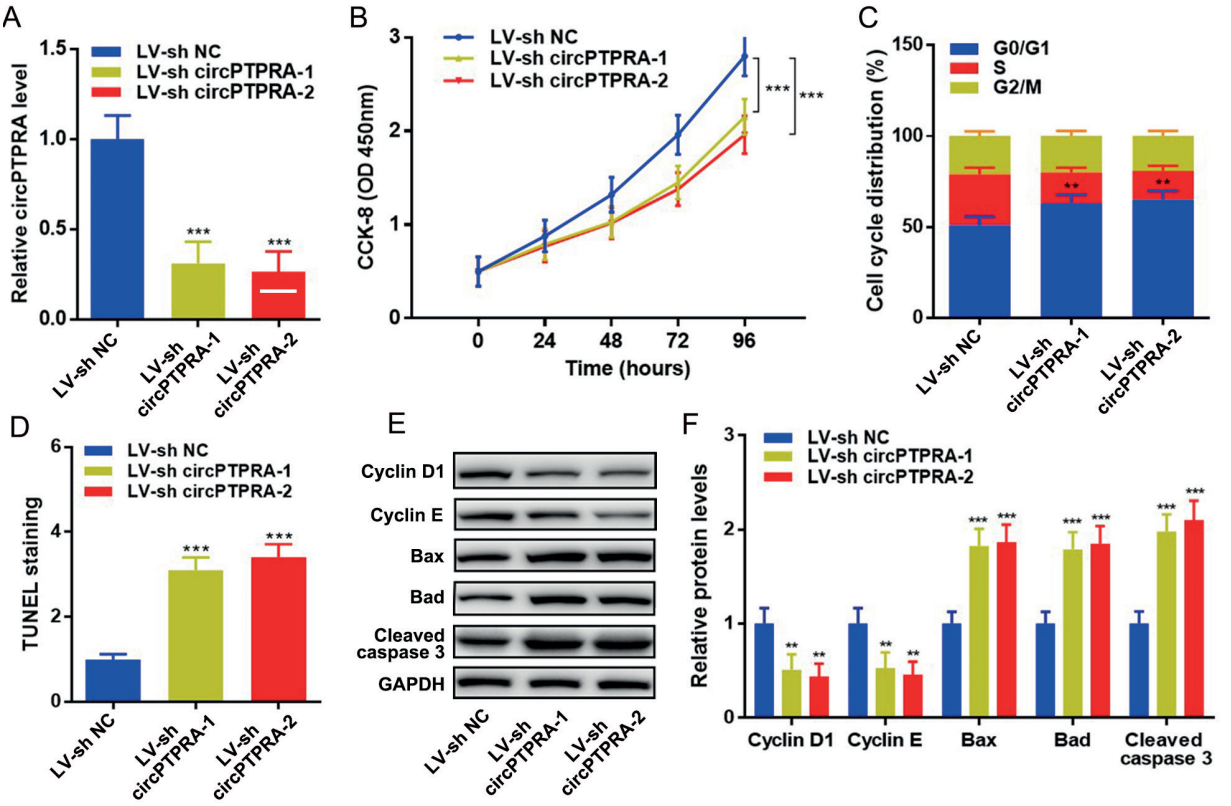

Figure 2. CircPTPRA inhibition repressed proliferation and promoted apoptosis in VSMCs. **A,** Expressions of circPTPRA were detected by RT-PCR in VSMCs after lentivirus infection. **B,** CCK8 assay was used to measure cell proliferation abilities. **C,** FACS was used to measure cell-cycle distribution of VSMCs. **D,** TUNEL staining was used to detect cell apoptosis. **E, F,** Protein expressions of Cyclin D1, Cyclin E, Bax, Bad and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01, ***p<0.001.

ability was increased following with miR-636 mimic transfection, while it was repressed following with miR-636 inhibitor transfection (Figure 4B) (p<0.001). Furthermore, FACS showed that miR-636 inhibition promoted cell growth by reducing cell distribution in G0/G1 phase and improving cell distribution in S phase (Figure 4C) (p<0.01). In the contrast, miR-636 overexpression repressed cell growth by improving cell distribution in G0/G1 phase and reducing cell distribution in S phase (Figure 4C) (p<0.01). Moreover, TUNEL staining indicated that miR-636 inhibition reduced cell apoptosis (Figure 4D) (p<0.01); however, miR-636 overexpression increased cell apoptosis (Figure 4D) (p<0.001). Besides, WB revealed that Cyclin D1 and Cyclin E were increased, Bax, Bad and Cleaved Caspase3 were reduced following with miR-636 inhibition, while these genes were opposite following with miR-636 overexpression (Figure 4E, F) (p<0.001). Collectively, these results suggested that miR-636 could repress cell proliferation and increased apoptosis in VSMCs.

MiR-636 Could Target at Binding with SP1 in VSMCs

MiRNAs have been reported to regulate the progression of AS through targeting at the 3′-UTR of target genes[29-32]. To further explore the mechanism that miR-636 regulates cell proliferation and apoptosis of VSMCs, we used the Targetscan database to predict the target genes of miR-636. Results showed that SP1 might be one of the potential target genes, which was a transcription factor that could promote cell proliferation and regulate the progression in AS[35,36]. And we found that protein expression of SP1 was upregulated following with miR-636 overexpression, while it was repressed following with miR-636 inhibition (Figure 5A) (p<0.001). Moreover, we observed that circPTPRA inhibition repressed the protein expressions of SP1 (Figure 5B) (p<0.01). These

results indicated that SP1 was negatively related to miR-636, while it was positively related to circPTPRA. To identify that miR-636 could directly target at repressing SP1, the wild type and mutant sequences of SP1 were synthesized and constructed in GLO plasmids (Figure 5C) and Luciferase gene reporter assay was performed. Results showed that the relative Luciferase activity in VSMCs co-transfected with WT-SP1 and miR-636 mimic was repressed, while it was reversed following with co-transfected with MUT-SP1 (Figure 5D) (p<0.01). These data indicated that miR-636 could target at binding with SP1 in VSMCs.

MiR-636/SP1 Axis Regulated Proliferation and Apoptosis of VSMCs

In order to confirm that miR-636 regulated cell proliferation and apoptosis through SP1 pathway in VSMCs, LV-sh SP1 was synthesized and constructed into a lentivirus and infected into VSMCs, which showed that SP1 expression was inhibited (Figure 6A) (p<0.001). Then miR-636 inhibitor or inhibitor NC was respectively transfected into VSMCs with LV-sh SP1. Results showed that SP1 protein expression was increased following with miR-636 inhibitor transfection (Figure 6A) (p<0.01). Furthermore, CCK8 assay showed that SP1 inhibition repressed cell proliferation, while it was repressed following with miR-636 inhibitor transfection (Figure 6B) (p<0.01). Moreover, FACS showed that SP1 inhibition improved cell distribution in G0/G1 phase and it was reduced following with miR-636 inhibitor transfection (Figure 6C) (p<0.01). Besides, SP1 inhibition promoted cell apoptosis and it was reversed following with miR-636 inhibition (Figure 6D) (p<0.01). In addition, protein levels of Cyclin D1 and Cyclin E were repressed, Bax, Bad and Cleaved Caspase3 were promoted following with SP1 inhibition (Figure 6E, F) (p<0.01). However, these proteins

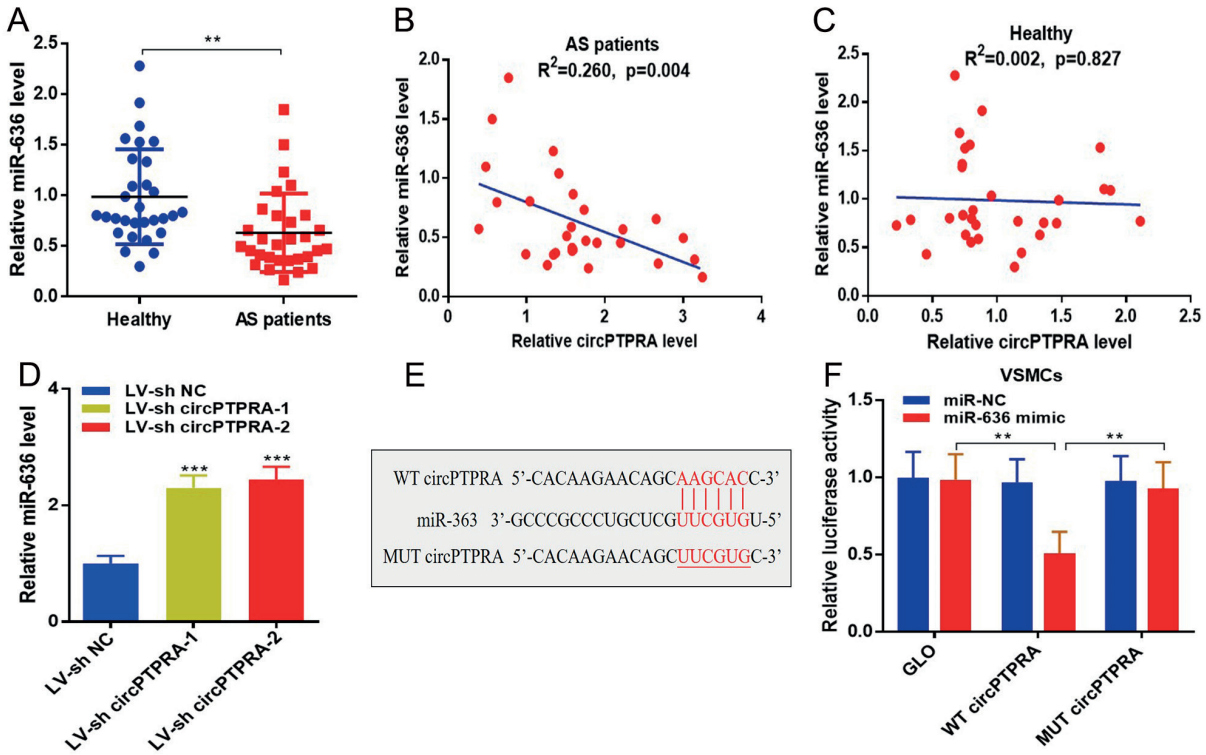

Figure 3. CircPTPRA could directly bind with miR-636 in VSMCs. **A,** Levels of miR-636 were detected by RT-PCR in AS patients (n=30) and healthy controls(n=30). **B, C,** Correlation analysis was performed to find relations between circPTPRA and miR-636 in AS patients and healthy controls. **D,** Levels of miR-636 were detected by RT-PCR in VSMCs after lentivirus infection. **E,** Wild type and mutant sequences of circPTPRA and miR-636 were constructed into GLO plasmid. **F,** Luciferase reporter assay was performed in VSMCs. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01, ***p<0.001.

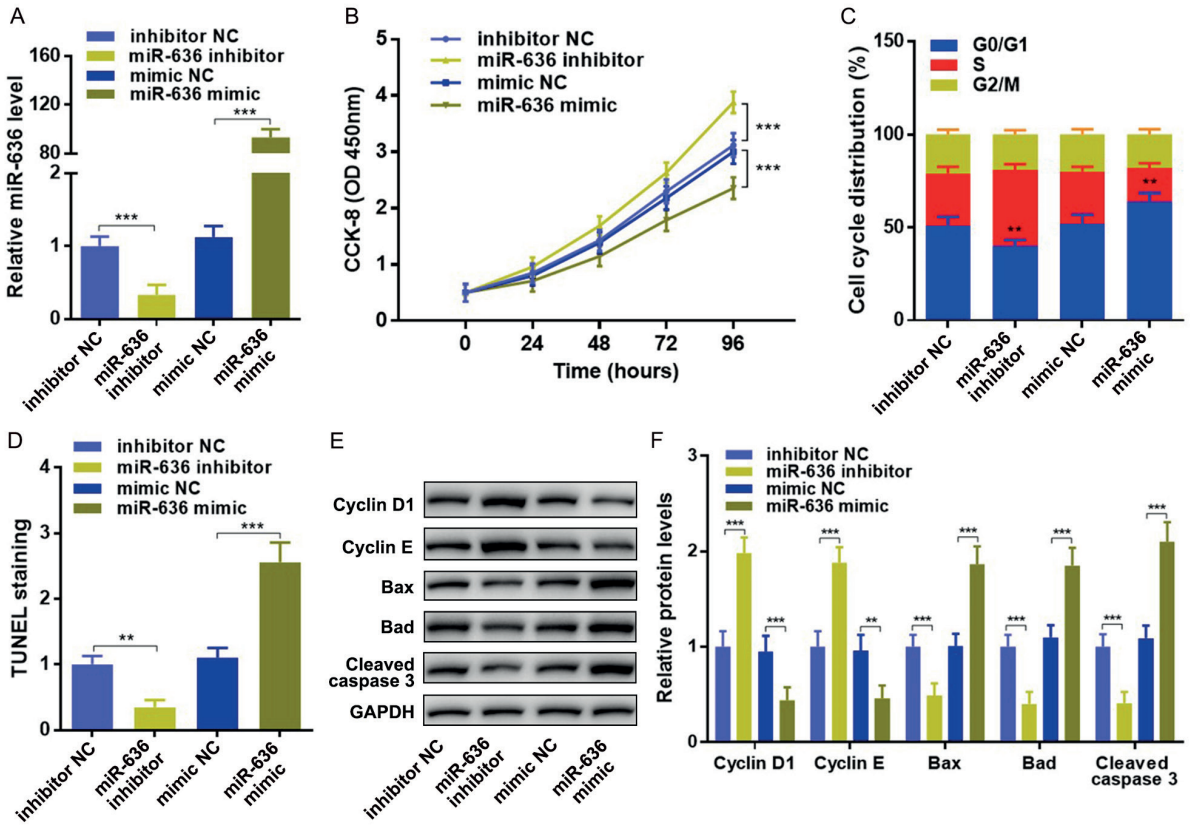

Figure 4. MiR-636 repressed cell proliferation and increased apoptosis in VSMCs. **A,** Expression of miR-636 was detected by RT-PCR after mimic NC, miR-636 mimic, inhibitor NC or miR-636 inhibitor transfection into VSMCs. **B,** CCK8 assay was used to measure cell proliferation abilities. **C,** FACS was used to measure cell-cycle distribution of VSMCs. **D,** TUNEL staining was used to detect cell apoptosis. **E, F,** Protein expressions of Cyclin D1, Cyclin E, Bax, Bad and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01, ***p<0.001.

were reversed following with miR-636 inhibition (Figure 6E, F) (p<0.01). These results suggested that miR-636/SP1 axis regulated proliferation and apoptosis of VSMCs. Collectively, these results had revealed that circPTPRA could sponge with miR-636, which targeted at repressing SP1 and regulated the progression of AS.

CircPTPRA Regulated Cell Proliferation and Apoptosis Through miR-636/SP1 Pathway in AS

To further confirm that circPTPRA regulated proliferation and apoptosis through miR-636/SP1 pathway, we transfected miR-636 inhibitor or inhibitor NC into VSMCs with circPTPRA inhibition. Results showed that circPTPRA expressions were repressed and miR-636 was increased following with circPTPRA inhibition, while circPTPRA expressions were promoted and miR-636 was reduced following with miR-636 inhibitor transfection (Figure 7A) (p<0.01). Furthermore, cell proliferation abilities were repressed following with circPTPRA inhibition, while it was reversed following with miR-636 inhibition (Figure 7B) (p<0.001). Besides, circPTPRA inhibition promoted cell apoptosis, while it was reversed following with miR-636 inhibition (Figure 7C) (p<0.01). Finally, protein levels of Cyclin D1 and Cyclin E were repressed, Bax, Bad and Cleaved Caspase3 were promoted following with circPTPRA inhibition, while they were reversed following with miR-636 inhibition (Figure 7D, E) (p<0.01). Collectively, our study suggested that circPTPRA was upregulated in serum samples of AS, which would sponge with miR-636 upregulating the transcription factor SP1, thereby promoting the proliferation of VSMCs and regulating the progression of AS.

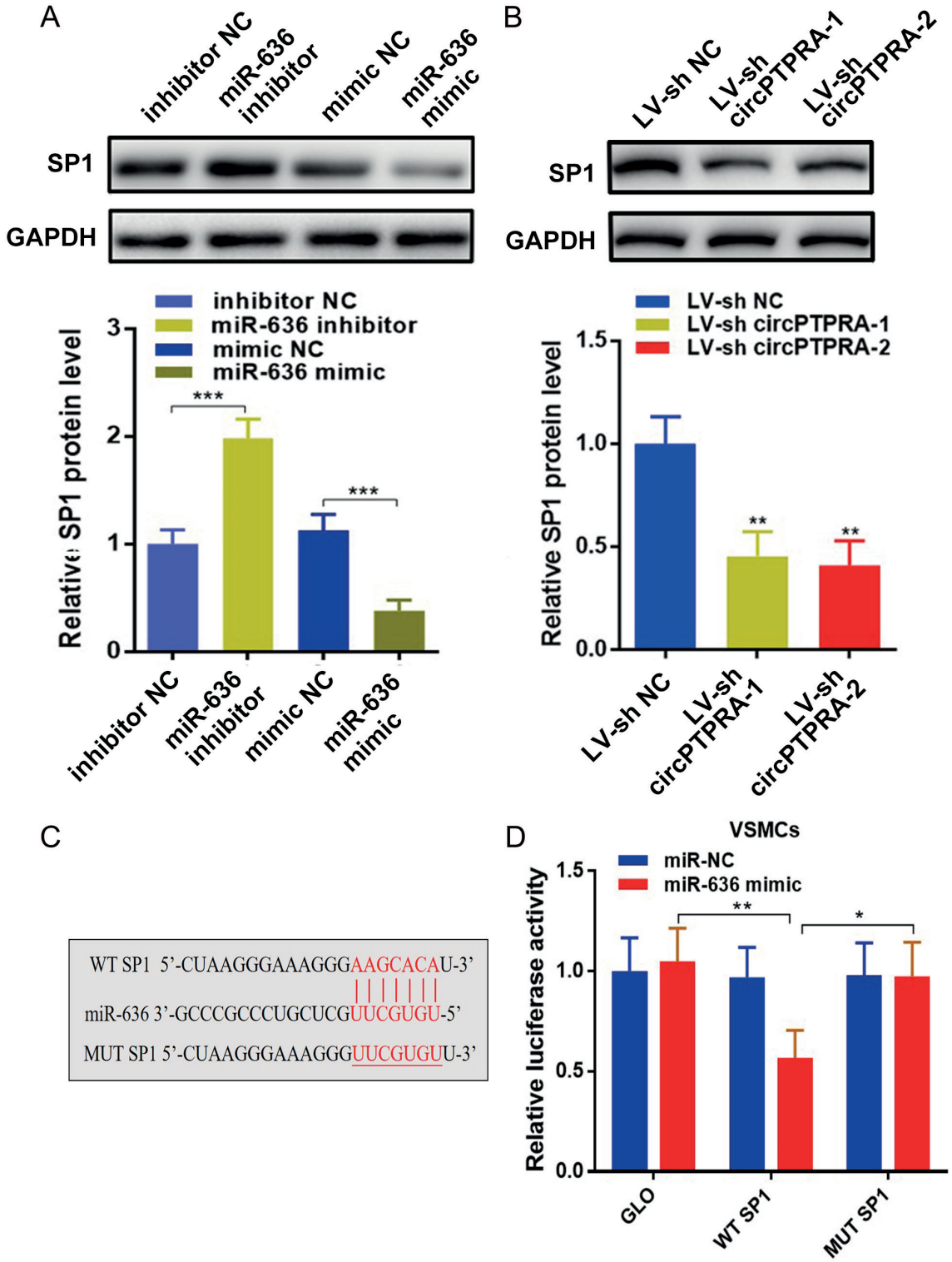

Figure 5. MiR-636 could target at binding with SP1 in VSMCs. **A,** Protein levels of SP1 were detected by WB after mimic NC, miR-636 mimic, inhibitor NC or miR-636 inhibitor transfection into VSMCs. **B,** Protein expressions of SP1 were detected by WB in VSMCs after lentivirus infection. **C,** Wild type and mutant sequences of SP1 were constructed into GLO plasmid. **D,** Luciferase reporter assay was performed in VSMCs. *p<0.05, **p<0.01, ***p<0.001.

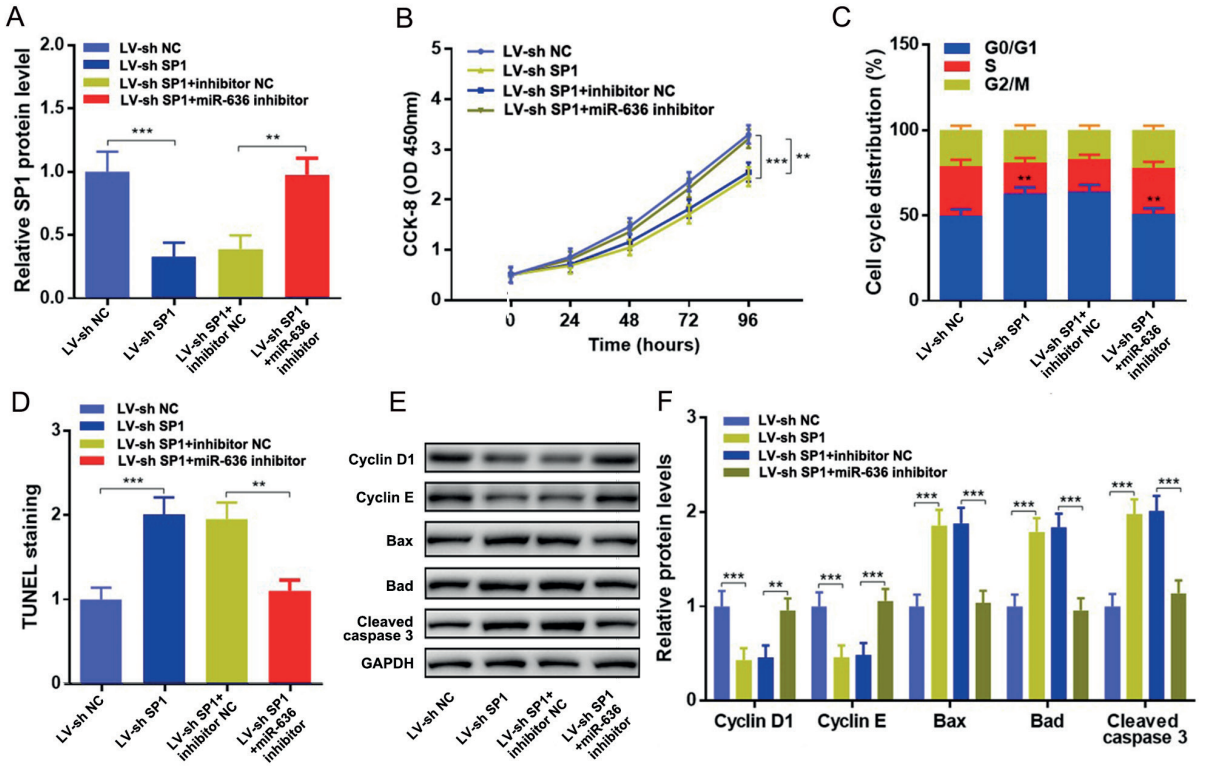

Figure 6. MiR-636/SP1 axis regulated proliferation and apoptosis of VSMCs. **A,** Protein expressions of SP1 were detected by WB after miR-636 inhibitor or inhibitor NC transfection into VSMCs with LV-sh SP1. **B,** CCK8 assay was used to measure cell proliferation abilities. **C,** FACS was used to measure cell-cycle distribution of VSMCs. **D,** TUNEL staining was used to detect cell apoptosis. **E, F,** Protein expressions of Cyclin D1, Cyclin E, Bax, Bad and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$, $^{***}p<0.001$.

Discussion

AS is a primary cause of death worldwide, leading to coronary heart disease (CHD), which is a major problem for human health[1-3]. More and more evidence demonstrated that circRNAs played some critical roles in the development of AS[23-25], which might provide novel markers and therapeutic targets for AS. However, the expressions and functions of circRNAs in AS remained little known. In this study, we found that circPTPRA was increased in serum samples of AS, which was upregulated by ox-LDL treatment in VSMCs, but not in macrophage and endothelial cells. These results suggested that circPTPRA might play some roles in AS, while the functions remained unclear.

To explore the functions of circPTPRA in AS, sh circPTPRAs were constructed into lentivirus and infected into VSMCs. We uncovered that circPTPRA inhibition repressed cell proliferation abilities of VSMCs, improved cell distribution in G0/G1 phase and reduced cell distribution in S phase and promoted cell apoptosis. Cyclin D1 and Cyclin E are kinds of genes that promote cell proliferation in cancers[37-39]. Bad, Bax and Cleaved caspase 3 are important apoptotic genes in the process of apoptosis[40-42]. After we detected these gene expressions, WB results showed that circPTPRA inhibition repressed Cyclin D1, Cyclin E and promoted Bad, Bax and Cleaved caspase 3, which further demonstrated that circPTPRA inhibition repressed proliferation and promoted apoptosis in VSMCs.

CircRNAs had been reported to act as a "sponge" to bind with miRNAs, which was called "competing endogenous RNA " (ceRNA)[18-22]. CircRNAs could competitively bind with miRNAs and repress the levels of miRNAs, which would target at binding with downstream target genes that could regulate biological functions in various diseases[18-22]. Then, we used starBase database to analyze targets of circPTPRA and miR-636 was predicted to be a target miRNA. We found that miR-636 was reduced and it was negatively related with circPTPRA in AS patients. Furthermore, circPTPRA inhibition increased miR-636 expres-

sion. Besides, Luciferase reporter assay proved that circPTPRA could directly bind with miR-636 in VSMCs. However, the detailed functions and mechanisms of miR-636 in AS remained unclear.

To further explore whether miR-636 could regulate cell proliferation and apoptosis in AS, miR-636 inhibitor or miR-636 mimic was respectively transfected into VSMCs. Results showed that miR-636 inhibition promoted cell proliferation and repressed cell apoptosis of VSMCs; however, miR-636 overexpression repressed cell proliferation and promoted cell apoptosis of VSMCs. These results suggested that miR-636 played important roles in regulating cell proliferation and apoptosis in VSMCs. However, the detailed mechanism of miR-636 in AS remained little known.

In order to explore the way miR-636 regulates cell proliferation and apoptosis of VSMCs, we used the TargetScan database to predict the target genes of miR-636. SP1 was predicted to be a potential target gene, which was a transcription factor that could promote cell proliferation and regulate the progression in AS[35,36]. We found that miR-636 inhibition increased SP1 protein expression; however, miR-636 overexpression repressed SP1 protein expression. Luciferase reporter assay proved that miR-636 could target at repressing SP1 expression in VSMCs.

To observe that miR-636 regulated cell proliferation and apoptosis through SP1 pathway in VSMCs, LV-sh SP1 was synthesized and constructed into a lentivirus and infected into VSMCs. We found that SP1 inhibition repressed cell proliferation and promoted cell apoptosis, while it was reversed after miR-636 inhibitor transfection, which suggested that miR-636/SP1 axis contributed to regulate cell proliferation and apoptosis of VSMCs. Furthermore, we transfected miR-636 inhibitor into VSMCs with LV-sh circPTPRAs. We found that the repressed cell proliferation ability and promoted cell apoptosis in circPTPRA inhibition groups were reversed following with miR-636 inhibitor transfection. Collectively, these results suggested that circPTPRA regulated cell proliferation and apoptosis via miR-636/SP1 axis in AS.

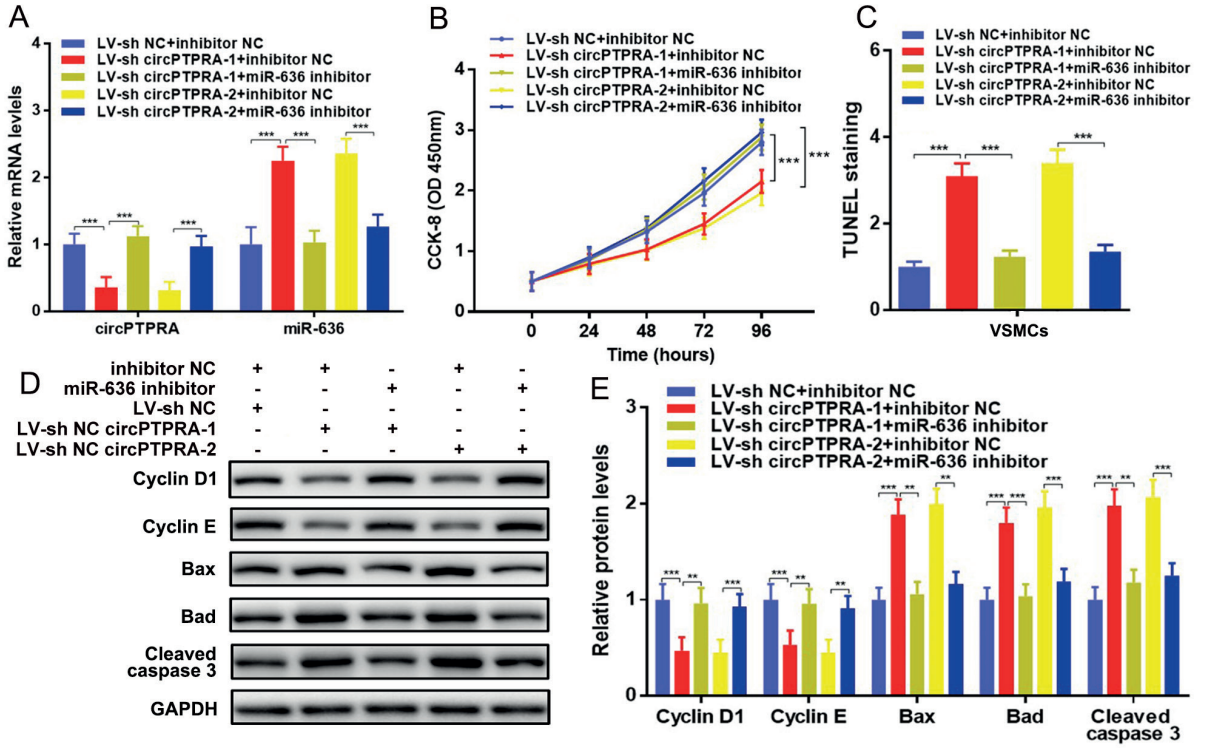

Figure 7. CircPTPRA regulated cell proliferation and apoptosis through miR-636/SP1 pathway in AS. **A,** Expressions of circPTPRA and miR-636 were detected by RT-PCR in VSMCs after miR-636 inhibitor or inhibitor NC transfection into VSMCs with LV-sh circPTPRA or LV-sh NC. **B,** CCK8 assay was used to measure cell proliferation abilities. **C,** TUNEL staining was used to detect cell apoptosis. **D, E,** Protein expressions of Cyclin D1, Cyclin E, Bax, Bad and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$, $^{***}p<0.001$.

Conclusions

We firstly found that circPTPRA was upregulated in serum samples of AS patients, which might be used as a biomarker for AS. We demonstrated that circPTPRA could promote cell proliferation and inhibit cell apoptosis via sponging with miR-636, which regulated SP1 signaling axis. Our results uncovered a novel role of circPTPRA, which might be a new therapeutic target for AS patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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