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MiR-193-3p inhibits the malignant progression of atherosclerosis by targeting WDR5

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Abstract

Background: The aberrantly increased proliferation and migration of vascular smooth muscle cells (VSMCs) was critically associated with atherosclerosis (AS) progression. MiR-197-3p has been confirmed to regulate various biological processes, such as tumorigenesis; however, whether miR-197-3p is involved with the pathological development of AS remains largely unknown.
Methods: The serum levels of miR-197-3p in AS patients and healthy donors were determined by polymerase chain reaction (PCR) assay. The transfection efficacies of miR-197-3p mimic or inhibitor in VSMCs were evaluated by PCR assay. The effects of miR-197-3p on VSMC proliferation and migration were determined by EdU cell proliferation and Traswell migration assays. Western blotting was conducted to evaluate the effect of miR-197-3p on WDR5 expression in VSMCs.
Results: In the present study, we found that the expression of miR-197-3p was decreased in the serum of AS patients compared to healthy donors. Overexpression of miR-197-3p inhibited the proliferation and migration of VSMCs, while silencing miR-197-3p showed opposite effects. Mechanistical study revealed that WD Repeat Domain 5 (WDR5) was a target of miR-197-3p. Moreover, miR-197-3p was downregulated in VSMCs upon IL6 treatment and inhibited IL6-induced proliferation and migration in VSMCs.
Conclusions: These findings indicate that miR-197-3p could serve as a promising diagnostic marker for AS and that targeting IL6/miR-197-3p/WDR5 axis might be a potential approach to treat AS.

Keywords

atherosclerosis, vascular smooth muscle cells, miR-197-3p, WDR5, IL6

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Introduction

Atherosclerosis (AS), characterized by the formation of fatty plaques in the intima of arteries, has become the leading cause of morbidity and mortality all around the world.[1,2] The occurrence of luminal stenosis or thrombogenesis leads to the obstruction of blood flow to the heart, brain and lower extremities, resulting in coronary heart disease, ischemic stroke, and peripheral vascular disease, respectively.[1,2] It has been confirmed that the aberrantly increased proliferation and migration of vascular smooth muscle cells (VSMCs) critically contribute to the pathogenesis and progression of AS.[3,4] These VSMC behaviors can be induced by various growth factors, such as platelet-derived growth factor BB (PDGF-BB), as well as noncoding RNAs,[3,4] including microRNAs (miRNAs). Therefore, targeting VSMC dysfunctions might be a promising therapeutic strategy to manage AS-related vascular diseases.

Currently, increasing studies have demonstrated that a variety of miRNAs are dysregulated in AS and play an important role in AS progression via regulating VSMC functions.[5,6] For example, miR-186-5p regulated VSMC behaviors and can be served as diagnostic biomarker in AS.[7] MiR-128 targeted KLF4 to inhibit the proliferation and migration of VSMCs and reduce neointima formation in the injured carotids.[6] Of note, miR-197-3p functions as a tumor-suppressive miRNA in various types of tumors by inhibiting tumor cell proliferation

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and migration.[8,9] MiR-197-3p was also a target of circ_USP36 that was upregulated in AS patients,[10] indicating that miR-197-3p might be associated with AS progression. However, the expression of miR-197-3p in AS patients and the effects and mechanisms of miR-197-3p on VSMC proliferation and migration are largely unknown.

In the present study, we found that the expression of miR-197-3p was decreased in the serum of AS patients. MiR-197-3p targeted WD Repeat Domain 5 (WDR5) to inhibit the proliferation and migration of VSMCs. IL6 induced miR-197-3p downregulation in VSMCs and miR-197-3p inhibited IL6-induced VSMC behaviors. Our study indicated that miR-197-3p/WDR5 axis could serve as a diagnostic biomarker and therapeutic target in AS patients

Materials and Methods

Patient Study

The serum samples were obtained from 52 patients with stable AS (age, 42.6 ± 8.2, 32 male and 20 female) and the degree of AS was evaluated by the Gensini scoring system. The serum samples of healthy donors were obtained from 30 volunteers without AS (age, 40.2 ± 6.3, 17 male and 13 female). AS patients with diabetes or tumors, or with complications or infections of liver or kidney, were excluded. All the serum samples were obtained from January 2017 to December 2020 at WuHan Asia General Hospital.

Cell and Cell Culture

Human aortic smooth muscle cells (HASMCs) were purchased from ScienCell Research Laboratories and cultured in Smooth Muscle Cell Medium (SMCM) containing 2% fetal bovine serum, 1% Smooth Muscle Cell Growth Supplement (SMCGS), and 1% penicillin-streptomycin. The cells were maintained in a 37°C humidified atmosphere with 5% CO_2.

Cell Transfection

HASMCs were seeded in 6-well plates and cultured overnight. Then, miR-197-3p mimic, negative control (NC) mimic, miR-197-3p inhibitor, NC inhibitor, and WDR5 overexpression plasmid (GenePharma) were transfected into HASMCs using Lipofectamine™ 3000 Transfection Reagent according to the manufacturer's instructions. The miR-197-3p mimic and inhibitor sequences were showed as follow: miR-197-3p mimic (5′-UUCACCACCUUCUCCACCCAGC-3′, 5′-UGGGU GGAGAAGGUGGUGAAUU-3′), NC mimic (5′-UUCUCCGAA CGUGUCACGUTT-3′, 5′-ACGUGACACGUUCGGAGA ATT-3′), miR-197-3p inhibitor (5′-GCUGGGUGGAGAAG GUGGUGGUGA A-3′), and NC inhibitor (5′-GCUGGGUGG AGAAGGUGGUGGUGAA-3′). After transfection for 24 h, HASMCs were subjected to further experiments as indicated.

Quantitative polymerase chain reaction (PCR) (qPCR) Assay

The total RNA of transfected HASMCs was isolated with Total RNA Extraction Kit (Solarbio) according to the manufacturer's instructions. Then, cDNA was generated using All-in-One cDNA Synthesis SuperMix (Bimake). Primers and cDNA were mixed with 2 × SYBR Green PCR Mastermix (Solarbio), followed by amplification and quantification with LightCycler 480 real-time PCR system (Roche). $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of targeted genes, which was normalized to the expression of glyceraldehyde–3–phosphate dehydrogenase (*GAPDH*) in HASMCs.

Cell Proliferation Assay

Cell proliferation was determined by EdU cell proliferation and CCK8 assays. Briefly, HASMCs were seeded in 96-well plates and transfected for 48 h as indicated. For EdU cell proliferation assay, the culture medium was replaced by fresh medium containing 10 μM EdU for 2 h at 37°C, followed by EdU cell proliferation assay (Beyotime Biotechnology) according to the user guide. For CCK8 assay, the cells were further incubated with fresh medium containing 10% CCK8 solution (Beyotime Biotechnology) for 2 h and then the absorbances were detected with a microreader at a wavelength of 450 nm.

Transwell Migration Assay

Briefly, transfected HASMCs were trypsinized and resuspended in serum-free medium. 100 μl of serum-free medium containing HASMCs was seeded in the upper filters of chambers and 600 μl fresh medium was added into the lower chambers. After a 24-h incubation, the cells were fixed with 4% paraformaldehyde (Sigma) and stained with 0.2% crystal violet (Sigma). The migrated HASMCs were photographed and quantified.

Western Blot Assay

HASMCs after different transfections were harvested and total proteins were extracted with radio-immunoprecipitation assay lysis buffer containing protease inhibitor cocktails (Bimake). Then, the concentrations of proteins were determined and proteins were electrophoretically separated by 10% sodium dodecyl sulfate-polyacrylamide gels and analyzed with Western blot assay. Antibodies against WDR5 and GAPDH were purchased from Cell Signaling Technology.

Luciferase Reporter Assay

HASMCs were seeded into 6-well plates and cultured overnight. The wild type (wt) or mutant (mut) 3′UTR fragments of WDR5 containing miR-197-3p binding sites was cloned into a luciferase reporter vector (GenePharma). Then, the vectors were co-transfected with miR-197-3p or NC mimic into HASMCs. After 48 h, the cells were harvested and the relative luciferase activity was measured by

the Dual-Luciferase Reporter Assay System (Promega), which was normalized to the *Renilla* luciferase activity.

Statistical Analysis

The data are shown as mean ± SEM and analyzed with GraphPad Prism 7.0 software. The statistical differences between two groups were analyzed by unpaired two-tail Student's *t* test and the statistical differences among more than two groups were analyzed by One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. $P < .05$ indicated a significant difference.

Results

The Expression of miR-197-3p is Decreased in the Serum of Patients with AS

To investigate whether miR-197-3p is associated with AS progression, the levels of miR-197-3p in the serum from AS patients and healthy volunteers were determined. We found that the expression of miR-197-3p was significantly lower in patients with AS than in healthy donor (Figure 1). The result indicates that miR-197-3p might regulate the development of AS.

Ectopic Expression of miR-197-3p Inhibits the Proliferation and Migration of VSMCs

To functionally explore the effect of miR-197-3p on the proliferation and migration of VSMCs, the cells was transfected with miR-197-3p mimic. Our results showed that miR-197-3p mimic treatment significantly promoted the expression of miR-197-3p in VSMCs (Figure 2A). Then, cell proliferation and migration were determined. CCK8 assay showed that miR-197-3p mimic significantly suppressed the growth of VSMCs (Figure 2B). We found that overexpression of miR-197-3p inhibited the proliferation of VSMCs, as determined by EdU cell proliferation assay (Figure 2C). Moreover, ectopic expression of miR-197-3p also decreased the number of migrated VSMCs (Figure 2D). These results demonstrate that miR-197-3p negatively regulates the proliferation and migration of VSMCs.

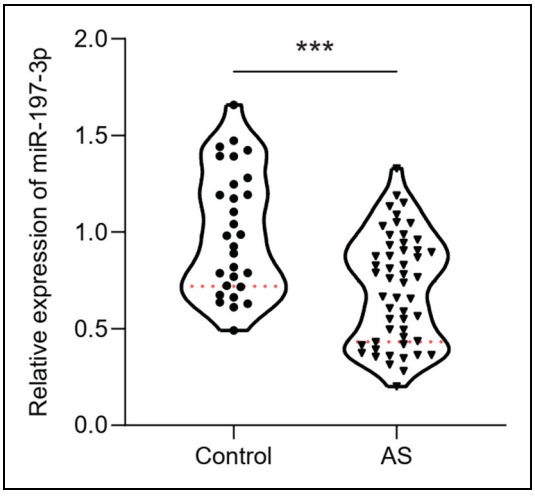

Figure 1. MiR-197-3p is downregulated in patients with AS. The expression of miR-197-3p in the serum of AS patients (n = 52) and healthy volunteers (n = 30) was determined by qPCR assay. Data are shown as mean ± SEM. ***$P < .001$.

Silencing miR-197-3p Promotes VSMC Proliferation and Migration

Then, VSMCs were transfected with miR-197-3p inhibitor to further confirm the effect of miR-197-3p on the proliferation and migration of VSMCs. PCR assay showed that miR-197-3p inhibitor downregulated miR-197-3p in VSMCs (Figure 3A). CCK8 (Figure 3B) and EdU cell proliferation (Figure 3C) assays showed that inhibition of miR-197-3p dramatically enhanced the proliferation of VSMCs. We also found that silencing miR-197-3p increased the migration capacity of VSMCs (Figure 3D). Our data further suggest that downregulation of miR-197-3p promote the proliferation and migration of VSMCs.

WDR5 is a Target of miR-197-3p

Next,we searched for candidate target genes of miR-197-3p by the databases, such as the Encyclopedia of RNA Interactomes and miRBD (Figure 4A). We found that a complementary miR-197-3p sequence was present in the 3′-UTR of WDR5 mRNA (Figure 4B), and studies have shown that WDR5 also critically regulates the phenotypic changes of VSMCs;[11–13] thus, WDR5 was selected for further study. Our result revealed that the luciferase activity of a reporter fused to the wild-type (WT) 3′-UTR of WDR5 was significantly decreased in the VMSCs transfected with miR-197-3p mimic, which was increased in the mutant (MUT) reporter (Figure 4C). Additionally, the expression of WDR5 mRNA was dramatically decreased in miR-197-3p-overexpressing VSMCs, while increased in VSMCs transfected with miR-197-3p inhibitor (Figure 4D). More importantly, similar results were also confirmed by Western blotting assay (Figure 4E). These results suggest that WDR5 might be a potential target of miR-197-3p.

Overexpression of WDR5 Reverses miR-197-3p-Mediated Suppression of Proliferation and Migration in VSMCs

Then, to further investigate whether overexpression of WDR5 reverses the miR-197-3p-mediated inhibition of proliferation and migration in VSMCs, VSMCs were co-transfected with WDR5 overexpression plasmid and miR-197-3p mimic. We found that the protein (Figure 5A) and mRNA (Figure 5B) expressions of WDR5 were increased in the VSMCs co-transfected with WDR5 overexpression plasmid and miR-197-3p mimic compared to cells transfected with miR-197-3p. Then, cell proliferation and migration were

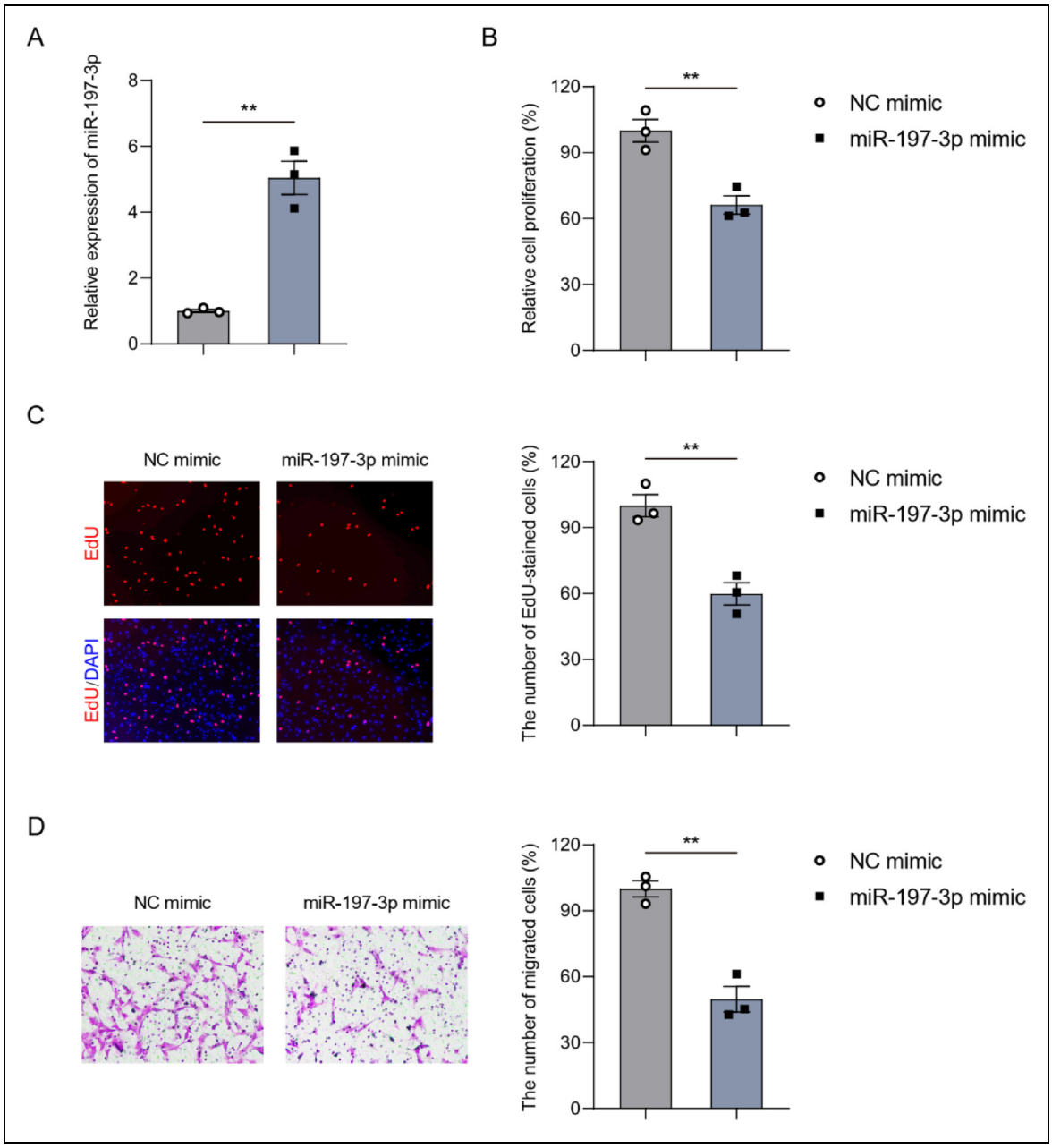

Figure 2. Ectopic expression of miR-197-3p inhibits the proliferation and migration of VSMCs. (A) HASMCs were transfected with miR-197-3p mimic and the expression of miR-197-3p was determined by qPCR assay. (B) The effect of miR-197-3p on HASMC growth was tested by CCK8 assay. (C) EdU cell proliferation assay for the proliferation of HASMCs transfected with miR-197-3p. Quantification of the EdU-stained HASMCs. (D) Transwell migration assay was conducted to determine the migration of HASMCs. Quantification of the number of migrated HASMCs. Data are shown as mean ± SEM. n = 3. $^{**}P < .01$.

explored. Our result showed that the proliferation rate was decreased in the miR-197-3p-overexpressing VSMCs, which was significantly increased in the VSMCs co-transfected with WDR5 overexpression plasmid and miR-197-3p mimic (Figure 5C and D). We also found that overexpression of WDR5 reversed the miR-197-3p-mediated inhibition of VSMC migration (Figure 5E). Our results further demonstrate that miR-197-3p inhibits the proliferation and migration of VSMCs by targeting WDR5.

IL6 Decreases the Expression of miR-197-3p in VSMCs

Next, we tried to figure out the mechanisms conferring miR-197-3p downregulation in VSMCs. Since pro-inflammatory factors are also crucial to AS progression, a well-known pro-inflammatory factor IL6 was selected. We found that IL6 treatment significantly downregulated miR-197-3p in VSMCs (Figure 6A). STAT3 is a key downstream effector of IL6-meidated inflammatory signaling

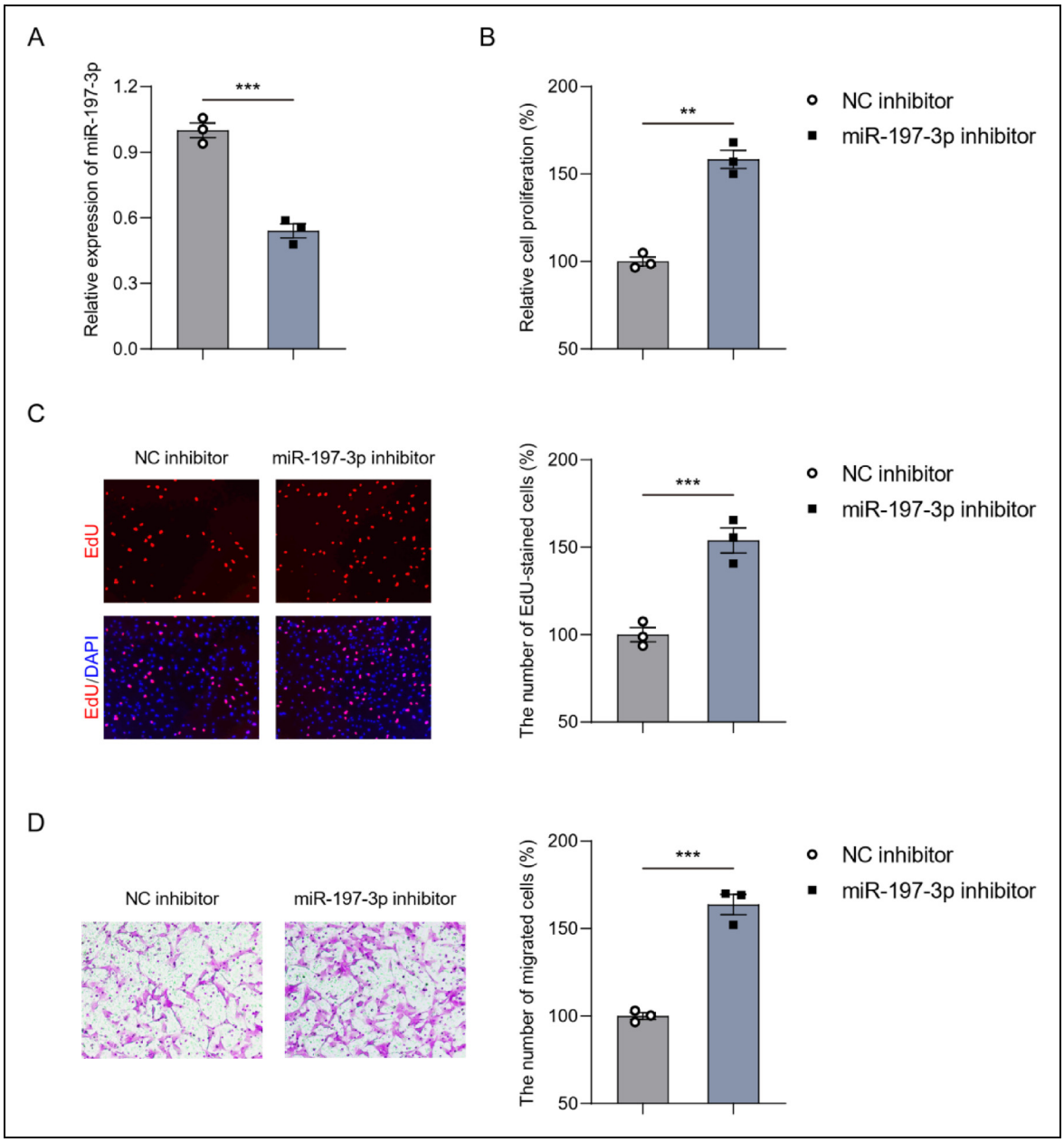

Figure 3. Inhibition of miR-197-3p promotes VSMC proliferation and migration. (A) HASMCs were transfected with miR-197-3p inhibitor and the expression of miR-197-3p was determined by qPCR assay. (B) CCK8 assay for the effect of miR-197-3p on HASMC growth. (C) The proliferation of HASMCs transfected with miR-197-3p was detected by EdU cell proliferation assay. Quantification of the EdU-stained HASMCs. (D) The migration of HASMCs were evaluated by Transwell migration assay. The number of migrated HASMCs was quantified. Data are shown as mean ± SEM. n = 3. $^{**}P < .01$, $^{***}P < .001$.

pathway, and then VSMCs were treated with a STAT3 inhibitor (STAT i) cryptotanshinone in the presence of IL6. Our results showed that the expression of miR-197-3p was increased in VSMCs treated with IL6 + cryptotanshinone compared to IL-6-treated VSMCs (Figure 6B). Then, we also determined whether ectopic expression of miR-197-3p affects the effect of IL6 on VSMC proliferation and migration. As expected, our results showed that IL6 treatment significantly promoted the proliferation (Figure 6C) and migration (Figure 6D) of HASMC, which was attenuated in cells transfected with miR-197-3p. These findings indicate that IL6/STAT3 signaling pathway might be responsible for miR-197-3p down-regulation in VSMCs and that miR-197-3p inhibits IL6-induced proliferation and migration in VSMCs.

Discussion

AS is one of the most common vascular diseases and is the underlying cause of clinical manifestation of various vascular

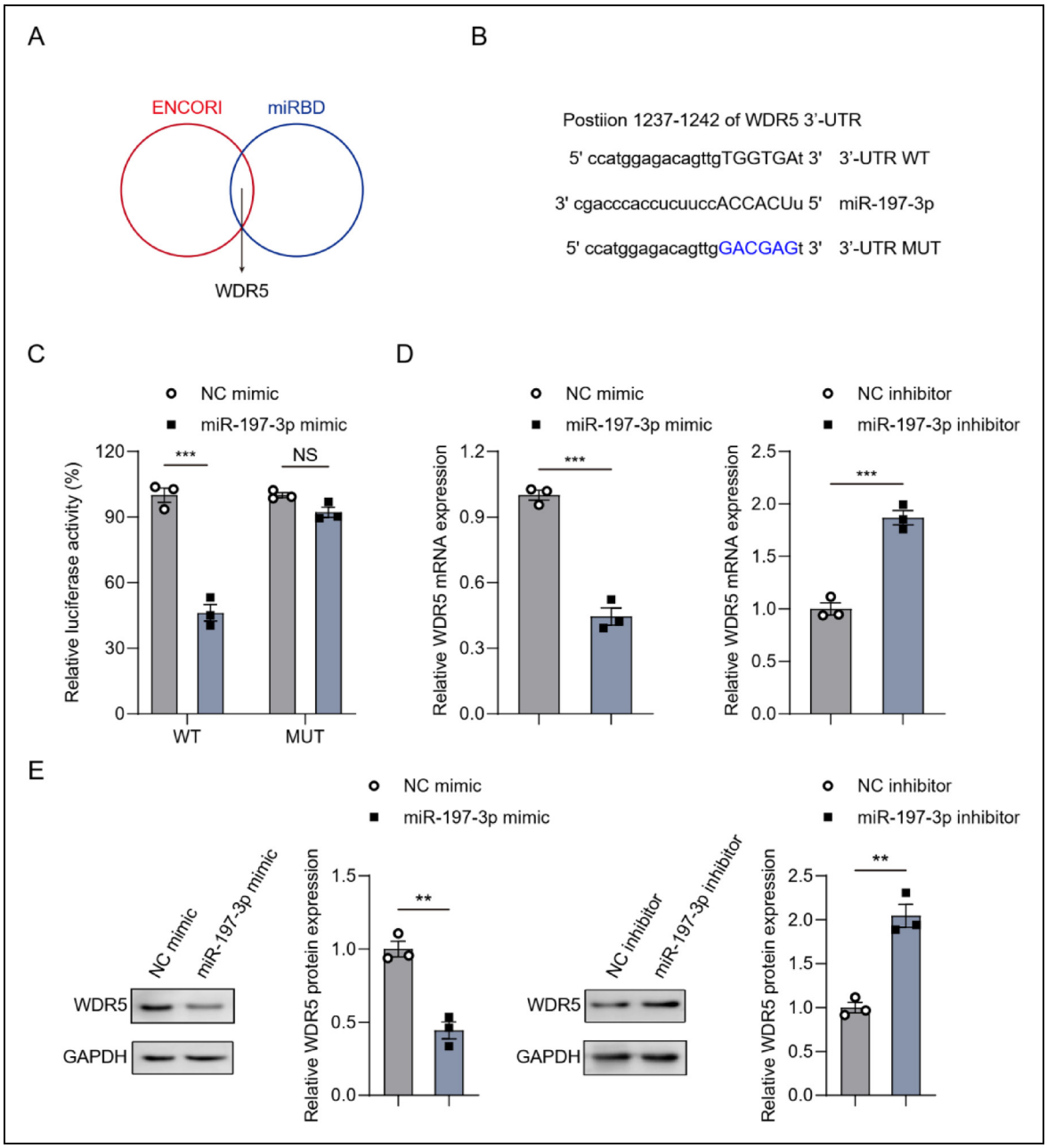

Figure 4. WDR5 might be a target of miR-197-3p. (A) The bioinformatic databases were used to predict the potential targets of miR-197-3p. (B) The predictive binding sites of miR-197-3p in the 3′-UTR of WDR5. (C) HASMCs were co-transfected with WT or MUT luciferase reporter vectors and miR-197-3p mimic, and the relative luciferase activity was determined. (D) The expression of WDR5 was measured by qPCR assay. (E) Western blot assay for the effect of miR-197-3p on WDR5 expression. Quantification of blots is shown. Data are shown as mean ± SEM. n = 3. $^{**}P < .01$, $^{***}P < .001$.

disorders, such as myocardial infraction and stroke. Endothelial cell damage, the accumulation of inflammatory cells and VSMCs, and the deposition of lipid and fibrous tissues are the key characteristics of AS, which are critically associated with AS progression.[14–16] VSMCs are the major type of cells present at all stages of an atherosclerotic plaque and aberrant proliferation and migration of VSMCs crucially regulate the development of AS.[3,4] In addition to known growth factors, including transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF)-BB, emerging evidence has showed that non-coding RNAs, such as miRNA[6,17] and lncRNA,[18,19] played an important role in AS progression via regulating VSMC behaviors. In our study, we found that miR-197-3p was downregulated in the serum of patients with AS. EdU cell proliferation assay showed that ectopic expression of miR-197-3p significantly inhibited the proliferation of VSMCs. Transwell migration assay revealed that overexpression of miR-197-3p suppressed the migration capacity of VSMCs. Whereas, silencing

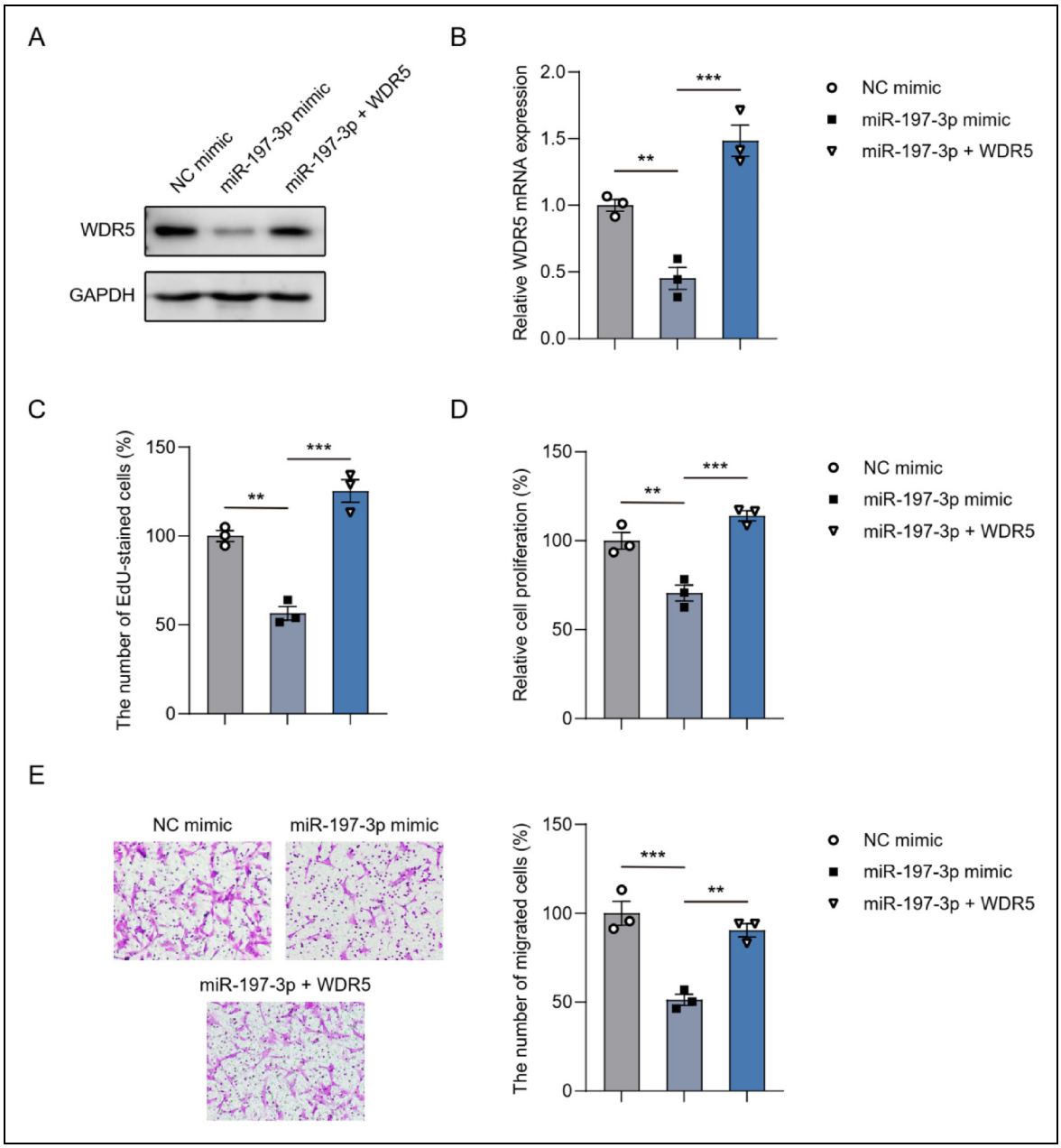

Figure 5. MiR-197-3p targets WDR5 to inhibit VSMC proliferation and migration. (A) HASMCs were co-transfected with miR-197-3p mimic and WDR5 overexpression plasmid, and the level of miR-197-3p and WDR5 were determined by PCR and Western blot assays. (B) qPCR assay for the expression of WDR5 in HASMCs co-transfected with miR-197-3p mimic and WDR5 overexpression plasmid. (C and D) The proliferation of HASMCs co-transfected with miR-197-3p mimic and WDR5 overexpression plasmid was determined by (C) EdU cell proliferation assay and (D) CCK8 assay. (E) Representative images of migrated HASMC and quantification of the number of migrated HASMCs are shown. Data are shown as mean ± SEM. n = 3. $^{**}P < .01$, $^{***}P < .001$.

miR-197-3p showed opposite effects. These results indicated that miR-197-3p might function as an atheroprotective miRNA and could serve as a novel diagnostic biomarker of AS progression.

MiRNA is a class of highly conserved small non-coding RNA with 19-25 nucleotides and regulates various cellular biological processes, such as proliferation and migration.[17,20] MiRNA mediates post-transcriptional regulation mainly by inhibiting mRNA translation or facilitating mRNA degradation through base-pairing with the 3′- UTR of target genes.[20] In the present study, we found that miR-197-3p negatively regulated the proliferation and migration of VSMCs. To explore the mechanisms of miR-197-3p on VSMC behaviors, we used several bioinformatic databases. We found that WDR5 was a target of miR-197-3p. WDR5, a highly conserved WD40-repeat protein, existed as part of several chromatin-regulatory complexes[21] and participated in numerous chromatin-centric processes.[22] WDR5 has been confirm to regulate the proliferation,[23,24] chemoresistance,[24] and metastasis[25] of tumor cells. Studies also

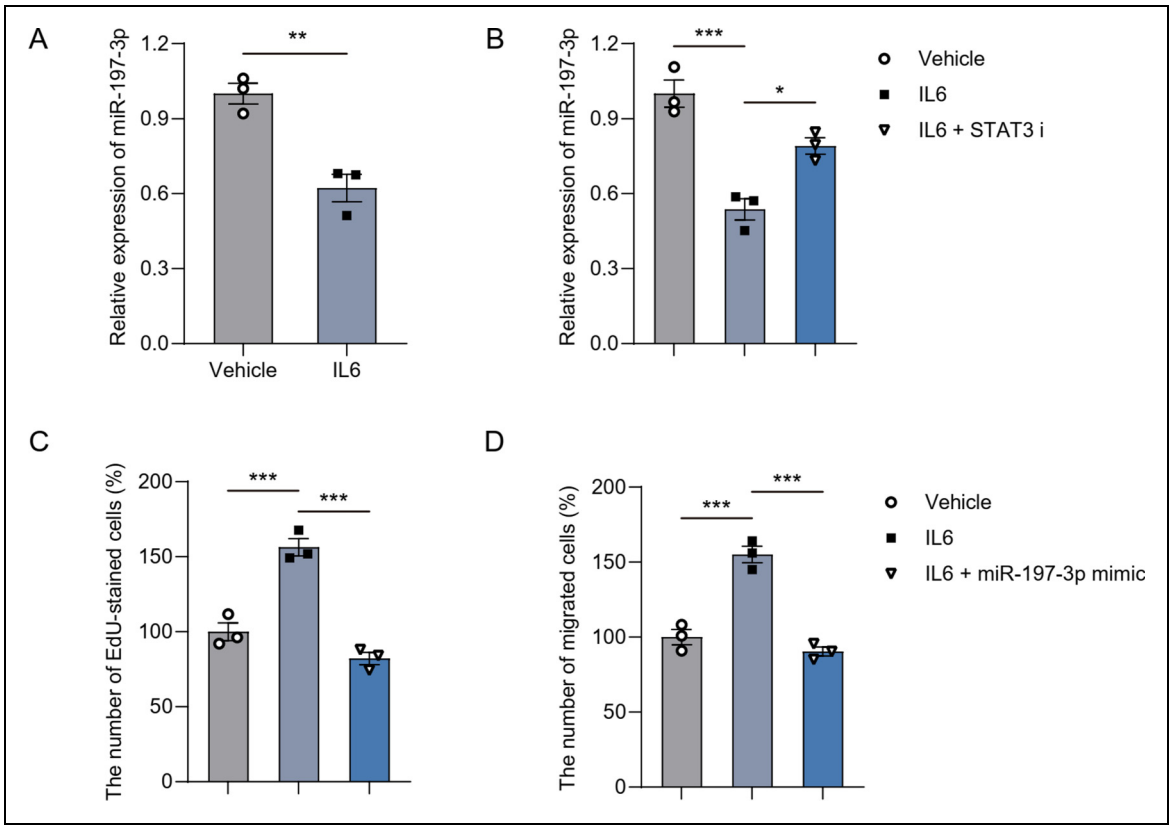

Figure 6. IL6/STAT3 axis decreases the expression of miR-197-3p in VSMCs. (A) HASMCs were treated with IL6 (50 ng/ml) for 24 h, and the expression of miR-197-3p was measured by qPCR assay. (B) HASMCs were treated with IL6 in the presence or absence of STAT3 inhibitor (STAT3 i) cryptotanshinone, and the level of miR-197-3p was determined by qPCR assay. (C) The effect of IL6 and miR-197-3p on HASMC proliferation was detected by EdU cell proliferation assay. (D) Transwell migration assay was used to evaluate the effect of IL6 and miR-197-3p on HASMC migration. Data are shown as mean ± SEM. n = 3. *$P < .05$, **$P < .01$, and ***$P < .001$.

showed that WDR5 critically participated in the development of AS, particularly the phenotypic changes of VSMCs, via various mechanisms. A previous study revealed that lncRNA NEAT1 sequestered WDR5 from vascular smooth muscle cell (VSMC)-specific gene loci, resulting in phenotypic switching of VSMCs.[12] LncRNA antisense non-coding RNA at the INK4 locus (ANRIL) acted as a potential modular scaffold that interacted with WDR5/HDAC3, which led to NOX1 upregulation by histone modification and consequently increased reactive oxygen species level and promoted the phenotypic alteration of VSMCs.[11] This study also showed WDR5 silence downregulated the levels of NOX1 and inhibited the ANRIL-induced NOX1 expression in VSMCs.[11] Thus, whether WDR5 was responsible for miR-197-3p-mediated inhibition of proliferation and migration in VSMCs was further investigated. Our results showed that miR-197-3p overexpression decreased the proliferation and migration in VSMCs, which was attenuated by WDR5 overexpression. These data confirmed that miR-197-3p might targets WDR5 to suppress VSMC proliferation and migration and our study further suggested that WDR5 could serve as a potential therapeutic target of AS.

It has been reported that the downregulation of atheroprotective miRNA was regulated by various factors, such as oxidative stress,[26] inflammation, excessive growth factor, and lncRNA upregulation. SNHG16 was upregulated by ox-low-density lipoprotein (LDL) treatment, and it promoted the formation of atherosclerotic plaque and enhanced ox-LDL-induced proliferation and migration in VSMCs by targeting miR-22-3p/HMGB2 axis.[27] PDGF-BB treatment decreased the expression of miR-233 in HASMCs and miR-233 inhibited PDGF-BB-induced proliferation and migration of HASMC.[28] IL6 has showed to regulate inflammation, atherogenesis,[29,30] and VSMC behaviors.[31] Inhibition of IL6 can alleviate atherosclerosis in $ApoE^{-/-}$ mice.[32,33] In our study, we found that IL6 treatment significantly downregulated the expression of miR-197-3p in VSMCs and that blockade of STAT3 inhibited IL6-mediated miR-197-3p downregulation as well as WD repeat domain upregulation in VSMCs. Notably, miR-197-3p abrogated the IL6-induced proliferation and migration in VSMCs. These finding indicated that an inflammatory factor IL6 might be responsible for the downregulation of atheroprotective miRNA miR-197-3p and suggested a potential role of IL6/STAT3 axis in the development of AS. However, whether the expression of miR-197-3p had a negative correlation with IL6 in the serum of AS patients needed to be further investigated.

Additionally, since miR-197-3p inhibited IL6-induced VSMC proliferation and migration, miR-197-3p might also suppress other inflammation-associated signaling pathways, such as nuclear factor-κB axis, and downregulate the levels of other pro-inflammatory factors, such as IL1β and TNFα. A prior study showed that oxidized low-density lipoprotein (ox-LDL) induced the expression of lncRNA ANRIL in VSMCs and that knockdown of lncRNA ANRIL inhibit the ability of ox-LDL to promote proliferation, migration, and phenotypic changes in VSMCs, which was associated with WDR5. Thus, miR-197-3p might also regulate the function of ox-LDL in AS induction. These issues were still remained largely unknown in the present study and were required to be further investigated in the near further, which will further unravel the roles and mechanisms of miR-197-3p in the development and progression of AS.

Conclusion

In conclusion, this study found that miR-197-3p was decreased in the serum of AS patients. MiR-197-3p inhibited the proliferation and migration of VSMCs by targeting WDR5. The IL-6/STAT3 axis might be critically associated with miR-197-3p downregulation in VSMCs. These finding indicated that miR-197-3p/WDR5 axis could serve as a diagnostic biomarker for AS and that IL6/STAT3/miR-197-3p/WDR5 pathway might be a potential therapeutic target to treat AS.

Author contributions

Kai Yang conceived the project and wrote the manuscript. Chunjun Yu, Lin Ruan, Shengpeng Hu, and Wenjie Zhu participated in experiments and data analysis. Feng Xia reviewed the manuscript.

Availability of Data and Materials

The data supporting the conclusions of this study are included in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics Approval and Consent to Participate

Prior patient consent and approval from WuHan Asia General Hospital were obtained for the use of these clinical tissues in the patient study. The present study was authorized by the Ethics Committees of WuHan Asia General Hospital.

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