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Circ_0006251 mediates the proliferation and apoptosis of vascular smooth muscle cells in CAD via enhancing TET3 and PPM1B expression

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ABSTRACT

Coronary artery disease (CAD) is a serious global problem that has been considered to be a major cause of death. Circular RNAs (circRNAs) as a key player in the regulation of cardiac progression and disease. Nevertheless, most circRNAs are poorly understood. In our research, we discussed the circ_TET3 (circ_0006251) function in the development of CAD. Firstly, circ_0006251 expression was measured through RT-qPCR analysis. Functional results prove the clear functionality of circ_0006251 for CAD. In addition, mechanism experiments including RIP, RNA pull-down and luciferase reporter results were applied to delve into the mechanisms of regulation of circ_0006251 in CAD. Results showed that Circ_0006251 expression was notably increased in PDGF-BB-induced VSMCs cells. Moreover, circ_0006251 interference mitigated the VSMCs cells proliferation and stimulated apoptosis after being treated with PDGF-BB. Furthermore, circ_0006251 targeted TET3 and PPM1B via sponging miR-361-3p, thereby contributing to CAD occurrence. In conclusion, Circ_0006251 could be identified as a biomarker for CAD which might shed light on the diagnosis and therapy of CAD.

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Introduction

As one of the major cardiovascular diseases, coronary artery disease (CAD) also greatly threatens human health all over the world (1). It is commonly acknowledged that the onset of the development of CAD remains silent which makes it difficult to cure (2). The risk factors of CAD include smoking, hypertension, obesity, exercise, insulin resistance and so on (3). Also, it is non-negligible that great improvements in the treatment of CAD have been made (4). Nevertheless, the mortality of CAD remains high. Thence, exploring novel biomarkers to improve the treatment and prognosis of CAD is of great value.

Circular RNAs (circRNAs) have been identified as crucial regulators in manyCarcinoma (5). For instance, Liu et al illustrated that circRNA_100367 influences esophageal squamous cell carcinoma cells radio-sensitivity by sequestering miR-217 and mediating Wnt3 the expression of Wnt3 (6). Yang et al proposed that circRNA_100876 induced breast cancer cell proliferation via sponging miR-361-3p (7). Chen et al demonstrated that circRNA cRAPGEF5 is a tumor suppressor in RCC as a competing endogenous RNA (ceRNA) (8). At the same time, circRNAs have also been confirmed to participate in CAD which includes atherosclerosis, ischemia/reperfusion injury and ischemic heart failure (9). For instance, circRNA 010567 is promoting myocardial infarction via regulating TGF-β1 expression (10). Wu et al have exposed that circ_0005540 is closely associated with coronary artery disease (11). Peng et al have clarified that circDHCR24 contributes to vascular restenosis through modulation of the miR-149-5p/MMP9 axis (12).

As Yu et al reported, circ_0006251 has been reported to be dramatically up-regulated in coronary heart disease (13). However, its concrete functions in the progression of CAD still remain elusive. In this research, we focus on measuring the function of circ_0006251 for CAD and investigating the latent regulatory mechanism.

Materials and Methods

Cell culture

VSMCs) were from ATCC and incubated in the F-12K Medium containing 10% FBS (Gibco) in 5% CO_2 at 37°C. To establish a CAD model, treatment of VSMCs cells with PDGF-BB.

Cell transfection

pcDNA3.1/PPM1B, pcDNA3.1/TET3 all from GenePharma Company. The shRNAs targetingcirc_0006251, miR-361-3p mimics/inhibitors and NC were all from RiboBio (Guangzhou, China). The transfection reagent is lipofectamine 3000 (Invitrogen).

RT-qPCR

RNA was extracted as TRI reagent, cDNA reverse transcription reagent (Toyobo, Osaka, Japan). Using Real-Time PCR Kit (TIANGEN), RT-qPCR was performed. GAPDH or U6 as internal reference controls. and gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

CCK-8 assay

Inoculate 5×10^3cells in 96-well plates for culture, added CCK-8 solution at the same time on four consecutive

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days, and the cells were incubated for 2 hours protected from light before cell proliferation levels were detached with a spectrophotometer (DeTie).

EdU staining assay

Cells were maintained in 96-well plates for preparation. Under the fluorescence microscope (Olympus), cell proliferation was observed.

Transwell assay

In brief, inoculate 2 × 10^4 cells in serum-free medium in Transwell chambers (Corning, NY, USA) for testing cell migration. Using complete medium in the lower chamber. day, the migrated cell number was observed using a light microscope after 24 hours.

TUNEL assay

After treatment with 4% PFA, transfected VSMCs cells were added to the TUNEL reagent (MedChemExpress, USA). After DAPI staining, the labelled samples were analyzed by light microscopy (Olympus).

Flow cytometry analysis

1×10^6cells were taken and treated with PBS three times. propidium iodide (PI) and Annexin-V-FITC and were then added and after 15 minutes of incubation, apoptosis was analysed by flow cytometry.

Subcellular fractionation

The cytoplasmic and nuclear RNA of the cells were purified separately using the PARIS kit (MedChemExpress, USA). GAPDH or U6 was used as an internal reference control. The assay was performed three times independently.

FISH

After fixation and permeabilization, VSMCs cells were hybridized with the RNA FISH probe specifically designed for circ_0006251 in the buffer. Hoechst solution was utilized for the counterstain of cell nuclei. The confocal laser microscope (Olympus) was used to observe the images.

RNA pull-down (RPD) assay

VSMCs cells were treated with biotin-labeled circ_0006251, miR-361-3p-Wt or miR-361-3p-Mut probe, added magnetic beads, then washed and purified for PCR analysis. The assay was independently implemented in triplicate.

RIP

Experiments were performed using the RIP Kit (Millipore, Burlington, MA, USA), lysed VSMCs cells in RIPA (Solarbio, Beijing, China) and immunoprecipitated using anti-Ago2 (Abcam) or anti-IgG (Abcam) antibody. RNA precipitates were extracted for analysis.

Luciferase reporter assay

The DUXAP8 or POSTN mRNA 3'UTR) sequences containing miR-361-3p Wt and Mut were inserted into the pmirGLO dual luciferase vector. After miR-361-3p mimics were co-transfected with the reporter gene into VSMCs cells. 2 days later, luciferase activity was assayed using the Dual-Luciferase Reporter Gene Assay KitWB.

Cells were lysed and protein samples were produced. Sample concentrations were assayed and then separated by SDS-PAGEn and transferred to PVDF membranes (Millipore, Billerica, MA, USA) and closed with skimmed milk powder for two hours. Afterward, the primary antibody was incubated overnight at 4°C in the refrigerator. After three washes with PBST, incubated secondary antibody for 1 hour. The antibodies were purchased from Abcam, and the contrast solution was prepared for protein quantification by a chemiluminescence system.

Statistical analysis

SPSS 16.0 (IBM, Chicago, IL) software was used for data analysis and is expressed as mean ± standard deviation (SD). Significant differences between multiple groups were analysed using t-tests or ANOVA. $p<0.05$ were considered to be statistically significant. All experiments were repeated three times.

Results

The characterization of circ_0006251

As Yu et al reported, circ_0006251 is confirmed to be up-regulated in coronary heart disease and correlated to the progression of coronary heart disease (13). To identify the function of circ_0006251 for CAD, we first utilized PDGF-BB to mimic CAD-like injury. As demonstrated in Figure 1A, By PDGF-BB treatment, the proliferation of VSMCs cells was observably promoted. Besides, flow cytometry results exposed that the apoptotic of PDGF-BB-induced VSMCs cells was attenuated (Figure 1B). Similarly, WB analyzed that by PDGF-BB treatment, the BCL2 level was enhanced while Bax and Cleaved caspase-3 were increased (Figure 1C). Subsequently, by RT-qPCR report, circ_0055412 was continuously elevated with the increasing of PDGF-BB concentrations and time (Figure 2A-B). As exhibited in Figure 2C, circ_0006251 was cyclized from TET3 and predicts the genomic location of circ_0006251 schematically. In addition, we observed that after being treated with RNase R, the expression of linear-TET3 was reduced and circ_0006251 expression remained unchanged, suggesting that RNase R can digest

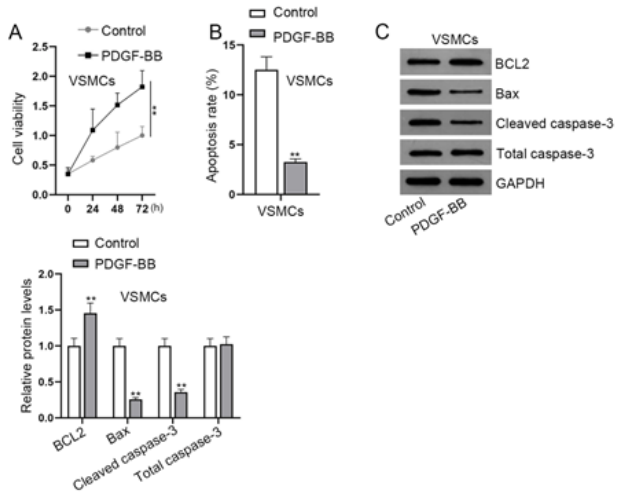

Figure 1. The establishment of CAD model. (A) The proliferative capacity of VSMCs cells after being induced by PDGF-BB was evaluated by CCK-8 assay. (B-C) Flow cytometry and western blot analysis assessed cell apoptosis in the control group and PDGF-BB group. **P<0.01.

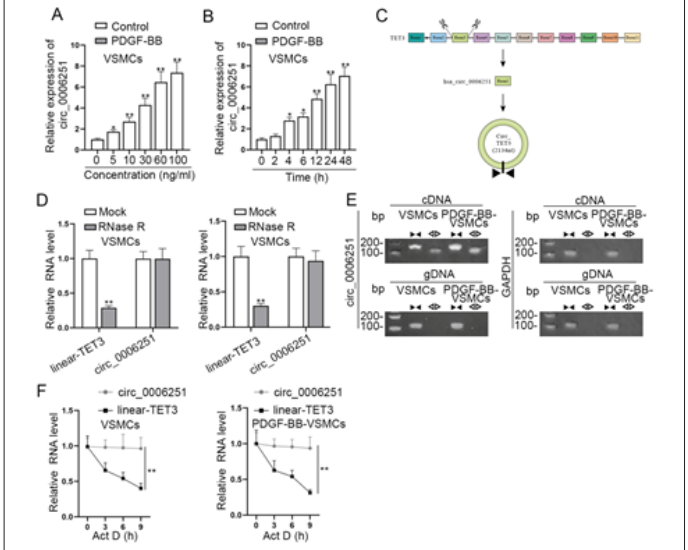

Figure 2. The characterization of circ_0006251. (A) RT-qPCR examined circ_0006251 expression in VSMCs cells with the increasing PDGF-BB concentration. (B) RT-qPCR examined circ_0006251 expression in VSMCs cells after being treated with PDGF-BB with increasing time. (C) Sequence diagram of cyclization site analysis of circ_0006251. (D) Analysis of the expression of linear-TET3 and circ_0006251 after being treated with RNase R. (E) Agarose gel electrophoresis confirmed the existence of circ_0006251. (F) Act D was added to detect the stability of circ_0006251 and linear-TET3. *P<0.05, **P<0.01.

linear-TET3, while RNase R does not affect circ_0006251. (Figure 2D). Also, we discovered that circ_0001665 could only be amplified cDNA but not gDNA, whereas linear-TET3 could be amplified with convergent primers for cDNA and gDNA in VSMCs cells without PDGF-BB (Figure 2E). Besides, after adding actinomycin D (Act D), the stability of linear-TET3 became weakened while no observed changes were observed in circ_0006251 (Figure 2F). Briefly, circ_0006251 is up-regulated in PDGF-BB-induced VSMCs cells.

Inhibition of circ_0006251 abrogates the proliferation, migration, and invasion while facilitating the apoptosis of PDGF-BB-induced VSMCs cells

Before the implementation of functional assays, circ_0006251 was knocked down in VSMCs cells after being treated with PDGF-BB and the interference efficiency was examined (Figure 3A). Through CCK-8 assay, cell proliferation was observed and we discovered that the viability of PDGF-BB-induced VSMCs cells was impeded after circ_0006251 was silenced (Figure 3B). Down-regulation of circ_0006251 led to a decrease in cell proliferation (Figure 3C). Similarly, the transwell assay disclosed that the migratory and invasive abilities of PDGF-BB-induced VSMCs cells were both attenuated when circ_0006251 was depleted (Figure 3D-E). In addition, after circ_0006251 was knocked down, the apoptosis of PDGF-BB-induced VSMCs cells was exacerbated (Figure 3F-G). Additionally, WB results indicated that BCL2 was decreased while the levels of Bax and Cleaved caspase-3 were increased in the sh-circ_0006251#1/2 group (Figure 3H). In summary, circ_0006251 may contribute to the development of CAD.

Circ_0006251 modulates TET3 expression at the post-transcriptional level

circ_0006251 is highly abundant in the cytoplasm of VSMCs cells (Figure 4A-B), suggesting that circ_0006251 may exert its roles in CAD through ceRNA mechanism. Moreover, when circ_0006251 was down-regulated the TET3 expression was distinctly reduced on account of circ_0006251 deficiencies (Figure 4C-D). In a word, TET3 is positively modulated by circ_0006251.

Circ_0006251 binds to miR-361-3p to target TET3

Considering the past research, we speculated that circ_0006251 may interact with miRNAs to function as miRNA sponges and modulate TET3 expression. Through

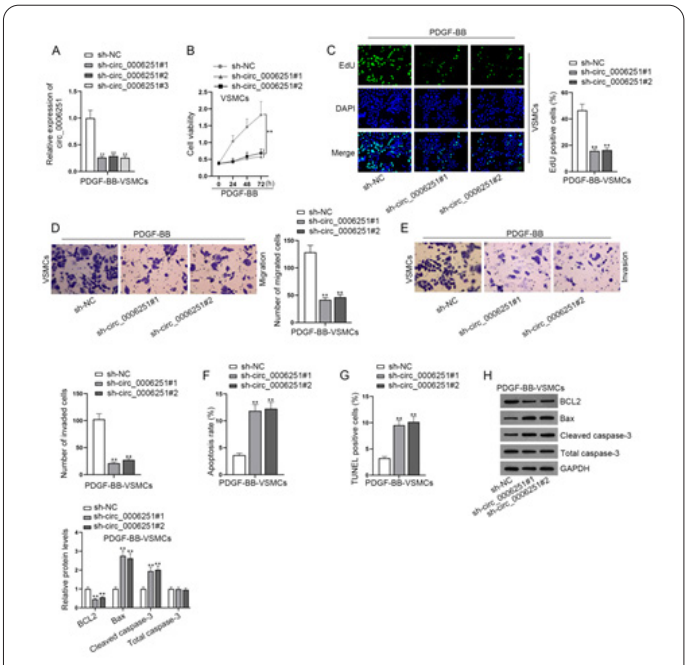

Figure 3. Inhibition of circ_0006251 abrogates the proliferation, migration, and invasion while facilitating the apoptosis of PDGF-BB-induced VSMCs cells. (A) Circ_0006251 expression was cut down in PDGF-BB-induced VSMCs cells. (B-C) CCK-8 and EdU assays evaluated cell proliferation. (D-E) The migratory and invasive abilities of PDGF-BB-induced VSMCs cells were assessed by transwell assays. (F-G) Cell apoptosis was observed through flow cytometry analysis and TUNEL assay. (H) Western blot analyzed the protein levels of apoptosis-related factors. **P<0.01.

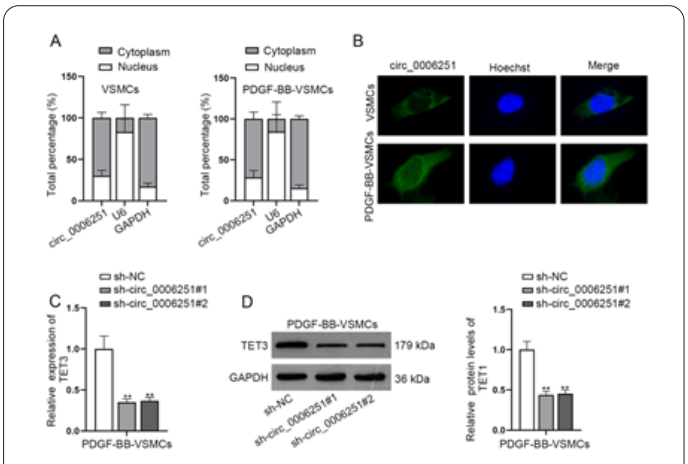

Figure 4. Circ_0006251 modulates TET3 expression at the post-transcriptional level. The distribution of circ_0006251 in VSMCs cells was found by subcellular fractionation (A), and FISH (B). (C-D) TET3 was tested after circ_0006251 was silenced. **P<0.01.

starBase, 5 potential miRNAs were predicted and displayed by the Venn diagram (Figure 5A). Further, miR-361-3p was enriched in the biotin-labeled circ_0006251 probe (Figure 5B). Using starBase, miR-361-3p had a potential site targeting circ_0006251 or TET3 (Figure 5C). RIP assay also exhibited a high concentration of circ_0006251, miR-361-3p, and TET3 in the Ago2 antibody (Figure 5D). After transfection of miR-361-3p mimics, (Figure 5E), luciferaseenzyme activity in the circ_0006251-Wt, circ_0006251-Mut, TET3 3'UTR-Wt and TET3 3'UTR-Mut and the binding between miR-361-3p and circ_0006251 or TET3 was verified (Figure 5F). MiR-361-3p inhibitor-induced VSMCs cells to cut down miR-361-3p expression (Figure 5G). Interestingly, reduced the expression of TET3 caused by circ_0006251 was restored by miR-361-3p down-regulation (Figure 5H-I). To testify whether circ_0006251 participated in the progression of CAD via targeting TET3, rescue assays were performed. Firstly, TET3 expression was elevated in PDGF-BB-induced VSMCs cells (Figure 6A). Then, CCK-8 and EdU proved that circ_0006251 interference impeded cell proliferation, however, this effect was indeed fully restored by miR-361-3p inhibition and rescued by TET3 overexpression (Figure 6B-C). Likewise, the experimental results from transwell results unmasked that the inhibited ox-LDL metastasis-induced VSMCs cells were completely offset by down-regulation of miR-361-3p and partially counteracted by overexpression TET3 (Figure 6D-E). Eventually, flow cytometry analysis, TUNEL assay and WB analysis exposed that circ_0006251 down-regulation induced cell apoptosis was counteracted by miR-361-3p inhibitor and even rescued by up-regulation of TET3 (Figure 6F-H). Overall, circ_0006251 could sponge miR-361-3p and modulates TET3 expression.

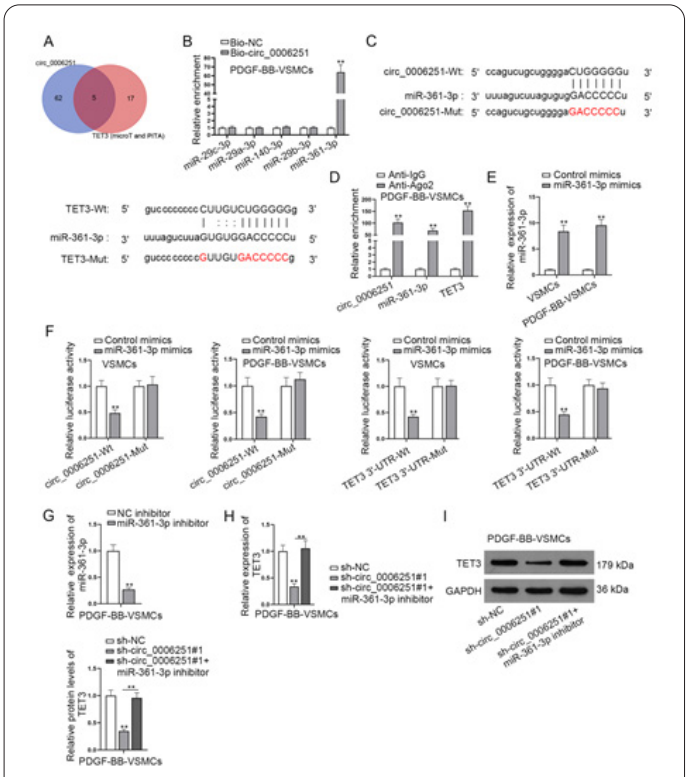

Figure 5. Circ_0006251 binds to miR-361-3p to target TET3. (A) Venn diagram displayed the predicted miRNAs. (B) RPD assay detected the pulldown miRNAs enrichment by biotin-labeled circ_0006251 probe. (C) Binding regions between circ_0006251 or TET3 and miR-361-3p. (D) Ago2 RIP assay exhibited the accumulation of circ_0006251, miR-361-3p and TET3 in Ago2 antibody. (E) miR-361-3p expression detached by qPCR. (F) luciferase reporter assay detached the interaction between miR-361-3p and circ_0006251 or TET3. (G) miR-361-3p expression detached by qPCR. (H-I) RT-qPCR and western blot checked TET3 expression. **P<0.01.

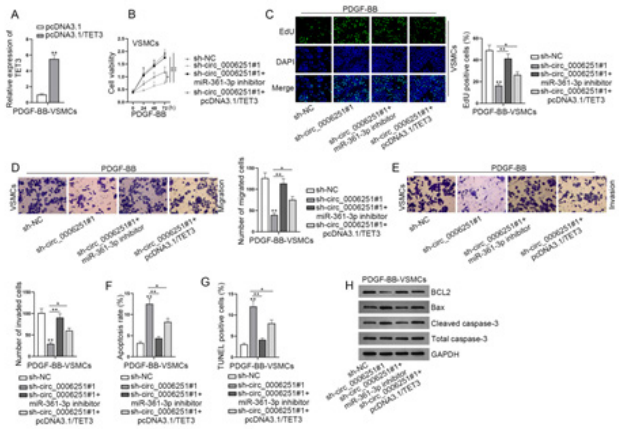

Figure 6. TET3 up-regulation partially rescues the suppressive role of circ_0006251 in CAD. (A) TET3 was overexpressed in PDGF-BB-induced VSMCs cells. (B-C) Cell proliferation was observed in VSMCs cells after transfection of sh-NC, sh-circ_0006251#1, sh-circ_0006251#1+miR-361-3p inhibitor and sh-circ_0006251#1+pcDNA3.1/TET3 plasmids respectively. (D-E) Transwell assay examined cell migration and invasion in different groups. (F-H) Cell apoptosis was observed in different groups. *P<0.05, **P<0.01.

PPM1B is a target gene of miR-361-3p

According to the previous findings, we supposed that there was another correlated gene of miR-361-3p. Then screen out the downstream correlated of miR-361-3p, starBase was utilized and 10 potential mRNAs (RC3H2, HOXC6, KDM5C, CHTF8, PODXL, ZDHHC9, SMG1, PPM1B, LPGAT1 and CADM4) were predicted by means of microT, miRmap, PITA and PicTar on the condition of CLIP Data ≥ 5. RT-qPCR analyzed that only PPM1B was found to be distinctly down-regulated with miR-361-3p mimics transfection (Figure 7A). The powerful affinity of miR-361-3p with PPM1B (Figure 7B). The binding sequences between them were exhibited in Figure 7C. Up-regulation of miR-361-3p decreased the luciferase activity in the PPM1B 3'UTR-Wt (Figure 7D). Moreover, miR-361-3p inhibitor rescued the decreased PPM1B expression due to circ_0006251 insufficiency (Figure 75E-F). Collectively, PPM1B is also targeted by miR-361-3p.

Circ_0006251 aggravates the process of CAD by enhancing TET3 and PPM1B expression

To certify the circ_0006251/miR-361-3p/TET3/PPM1B axis in CAD, rescue results were found. First of all, overexpressed the PPM1B in PDGF-BB-induced VSMCs cells (Figure 8A). Cell proliferation assays attested that in the PDGF-BB induced VSMCs cells transfected with sh-circ_0006251#1 plasmid, miR-361-3p inhibition accelerated cell proliferation while PPM1B overexpression partially restored this effect and up-regulation of PPM1B and TET3 completely restored this effect (Figure 8B-C). In the same way, the attenuated migratory and invasive abilities of PDGF-BB-induced VSMCs cells after trans-

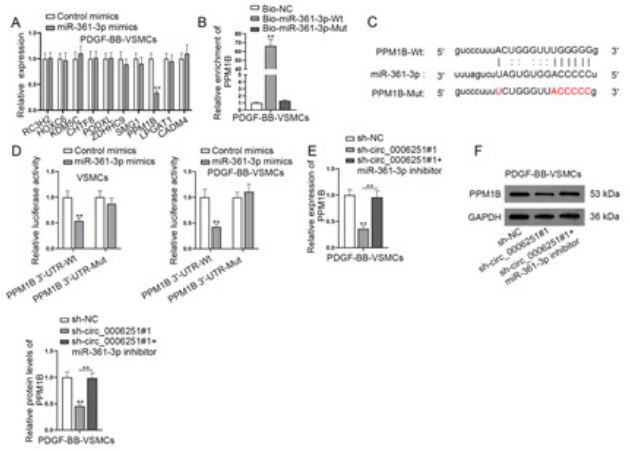

Figure 7. PPM1B is also a target gene of miR-361-3p. (A) The expression of predicted 10 target genes was examined after transfection of miR-361-3p mimics. (B) The binding between miR-361-3p and PPM1B was confirmed by the RPD assay. (C) Predicted binding sites between miR-361-3p and PPM1B. (D) Luciferase reporter assay tested the luciferase activity in the PPM1B 3'UTR-Wt group and PPM1B 3'UTR-Mut group after miR-361-3p was overexpressed. (E-F) RT-qPCR and western blot analyzed the expression of PPM1B in different groups. **P<0.01.

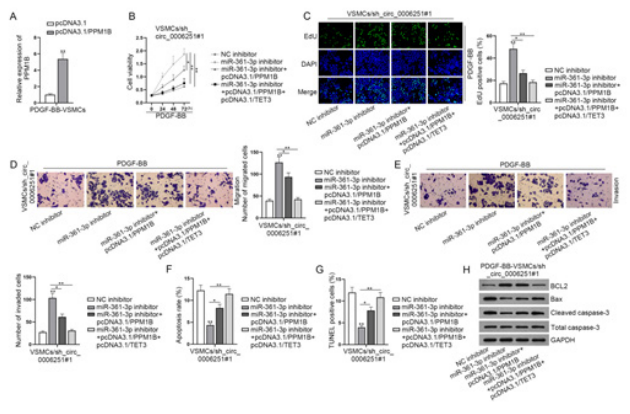

Figure 8. Circ_0006251 aggravates the process of CAD by enhancing TET3 and PPM1B expression. (A) Transfection of pcDNA3.1/PPM1B to up-regulate PPM1B expression. (B-C) Cell apoptosis was observed in VSMCs cells transfected with sh-circ_0006251#1 plasmid in the NC inhibitor group, miR-361-3p inhibitor group, miR-361-3p inhibitor +pcDNA3.1/PPM1B group and miR-361-3p inhibitor+pcDNA3.1/PPM1B+pcDNA3.1/TET3. (D-E) Transwell assay measured cell migration and invasion in VSMCs cells transfected with sh-circ_0006251#1 plasmid in different groups. (F-H) The apoptotic rate of VSMCs cells transfected with sh-circ_0006251#1 plasmid in different groups was analyzed through flow cytometry analysis, TUNEL assay, and western blot analysis. *P<0.05, **P<0.01.

fection of sh-circ_0006251#1 plasmids were strengthened by miR-361-3p inhibitor, and this effect was partially offset by PPM1B up-regulation and completely countervailed by overexpression of PPMB1 and TET3 (Figure 8D-E). Conversely, the promoted apoptosis of PDGF-BB-induced VSMCs cells was hampered by miR-361-3p inhibitor, and this effect was partially countervailed by PPM1B overexpression while completely counteracted by up-regulation of PPM1B and TET3 (Figure 8F-H). Taken together, circ_0006251 plays the promoting role in AS via modulation of TET3 and PPM1B.

Discussion

The importance of circRNAs in human cancers and diseases has been highlighted (5,14). For example, Yu et al have reported that circ_0006251 is one of the up-regulated circRNAs in coronary heart disease (13). Here, circ_0006251 was overexpressed in VSMCs cells after being treated with PDGF-BB. Functionally, interference of circ_0006251 hindered cell function while promoting the apoptosis of PDGF-BB-induced VSMCs cells.

CeRNA) network with circRNAs, miRNAs and mRNAs have been determined to influence the processes of cardiovascular diseases (15). Through our investigation, we first observed that circ_0006251 was chiefly distributed in the cytoplasm of VSMCs cells. Subsequently, the host gene of circ_0006251, TET3 expression was discovered to be positively regulated by circ_0006251. It is widely reported that miR-361-3p plays a suppressive role in lung cancer (16), cervical cancer (17), thyroid cancer (18) and so on. Moreover, Zhong et al manifested that miR-361-3p was down-regulated in CAD (19). Likewise, our findings validated that circ_0006251 and TET3 competitively had a binding with miR-361-3p. The interaction among circ_0006251, miR-361-3p and TET3 was also verified by mechanism assays. Rescue assays also proved that the inhibited CAD progression on account of circ_0006251 depletion was rescued by miR-361-3p inhibition and partially restored by TET3 overexpression.

PPM1B has been investigated in necroptosis (20), bladder cancer (21) and hepatocellular carcinoma (22). Furthermore, PPM1B participates in the progression of coronary atherosclerosis (23). Our study also attested that PPM1B was also a target gene of miR-361-3p. Eventually, rescue assays demonstrated that overexpression of TET3 and PPM1B completely offset the suppressive role of circ_0006251 inhibition in CAD which indicated that circ_0006251 aggravated the development of CAD via elevating TET3 and PPM1B expression.

All in all, circ_0006251 was testified overexpressed in PDGF-BB induced VSMCs cells. Functionally, reducing circ_0006251 restrained the cell function while inducing the apoptosis of PDGF-BB-induced VSMCs cells. In terms of mechanism, circ_0006251 could be sponging miR-361-3p to modulate the TET3 and PPM1B expression, thereby getting involved in the progression of CAD. Briefly, circ_0006251 was recognized to be a potential biomarker for the diagnosis and therapy of CAD.

However, there also have limitations in our research, other regulatory mechanisms of circ_0006251in CAD need to be explored. In addition, whether signaling pathways participate in the progression of CAD also needs to be found.

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Conflicts of interest

No conflicts of interest were declared.

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