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Mechanisms of miR-29a-5p involvement in osteogenic phenotype transformation and cellular regulation of vascular smooth muscle and thus influencing calcification in VSMCs in chronic kidney disease

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ABSTRACT

Vascular calcification is one of the major complications of chronic kidney disease (CKD), which could be further accelerated by the osteogenic transition and apoptosis of smooth muscle cells, thereby advancing the progression of renal diseases and increasing the mortality rate of cardiovascular events. MicroRNA is a kind of key regulator in the phenotypic transition of vascular smooth muscle cells (VSMCs), but its role remains unclear in VSMCs. In this study, VSMCs were stimulated by platelet-derived growth factors – BB (PDGF-BB) in varying concentrations to establish the VSMC dysfunction models. The relative expression of miR-29a-5p was quantified via the quantitative real-time polymerase chain reaction (qRT-PCR). The proliferation of VSMCs was determined via the BrdU method, analysis of cell cycle via flow cytometry, and the migration of VSMCs via Transwell assay. Expression of γ-secretase activating protein (GSAP) and markers of VSMC differentiation, including α-SMA, SM-22α, SMMHC and Calponin, was quantified via the Western blot. The targeting relationship between the 3'-UTR of miR-29a-5p and GSAP was validated through the dual-luciferase reporter gene assay. As a result, we found that PDGF-BB could trigger a decrease of miR-29a-5p in a time- and dose-dependent manner ($P < 0.05$). Overexpression of miR-29a-5p could curb the effect of PDGF-BB on the proliferation and migration of VSMCs while upregulating the expression of markers of differentiation ($P < 0.05$). In addition, the expression of GSAP was also affected by the negative regulation of miR-29a-5p, while the restoration of GSAP eliminated the effect of miR-29a-5p on the VSMCs partially ($P < 0.05$). Moreover, vascular calcification models were also established in the CKD rats, suggesting that the inhibition of GSAP could prevent PTH-induced vascular calcification in CKD rats. In conclusion, miR-29a-5p could inhibit the PDGF-BB-induced proliferation, migration and phenotypic transition of VSMCs via targeting GSAP. Thus, miR-29a-5p/GSAP might be a potential target for the treatment of vascular calcification.

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Introduction

Chronic kidney disease (CKD) has now become one of the diseases severely threatening public health all around the world (1). In China, a cross-sectional epidemic study has shown that the prevalence of CKD in Chinese adults has risen to more than 10%, almost 100 million Chinese, and about 1% of CKD patients would progress into end-stage renal disease, requiring renal replacement therapy (2). Therefore, a high mortality rate and the relevant causes of CKD have become clinical problems. Thus, elucidating the pathogenesis of CKD is of great significance for developing substitute therapy.

Recent studies have shown that the phenotypic transition of vascular smooth muscle cells (VSMC) may have a critical role in the progression of CKD (3). In response to the external stimuli, VSMC experiences a transition from the contractive phenotype to the synthesis and secretion phenotype, while the morphology of VSMC also changes from the elongated spindle to the short spindle with a pseudopod, during which the function of synthesis and secretion is enhanced, while the contraction is curbed. Biomolecular markers of VSMC, including α-SMA and SM22-α, decrease, while the constituents of extracellular matrix, including collagen and proteoglycan, increase with the enhancement in the secretion of some proteases (4). Increased secretion and activity of matrix metalloproteinase accelerates the degradation and collapse of extracellular matrix to induce the osteogenic/chondroid phenotypic changes of VSMCs, eventually inducing atherosclerosis and vascular calcification (5), while the rupture of atherosclerotic plaque could further promote the pathological

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progress of vascular calcification of CKD (6).

Vascular calcification is one of the pathological features of the ectopic deposition of calcium phosphate crystal in the cardiovascular tissue, which could weaken the elasticity and hemodynamic force, thus being a risk factor for cardiovascular disease and relevant death in CKD patients (7-8). Apoptosis of VSMC is a key link in the calcification of tunica media vasorum. CKD always accompanies hyperphosphatemia. VSMC, under the hypophosphite status, may experience abnormal apoptosis due to mitochondrial dysfunction, and the resulting decrease in the quantity of VSMCs could reduce the synthesis and secretion of extracellular matrix, attenuation of fibrous membranes, thereby forming the necrotic core and calcified crystal, which could be released into the cytosol, finally altering the vascular wall structure and promoting the vascular calcification (9). As such, preventing vascular calcification and the resulting cardiovascular diseases is of great significance for decreasing the mortality rate of CKD patients. However, the exact mechanism of CKD to trigger vascular calcification remains unknown.

MicroRNAs (miRNAs), as a group of non-coding RNA in the length of 22 nt, are involved in the growth, development, tumorigenesis and development and progression of cardiovascular diseases by regulating the expression of various genes (10). Existing data have shown that miRNAs are involved in the phenotypic transition and dysfunction of VSMCs (11). For instance, VSMCs, after the treatment of PDGF-BB, presented the significant downregulation of miR-124; upregulation of miR-124 could induce increases in the expression of the markers of VSMCs differentiation, including α-SMA and SM-22α and decreases in the proliferation and migration of VSMCs (12).

The miR-29 family has been shown to be able to alter fibrosis by regulating the expression of extracellular matrix, including the collagens. It has been found that miR-29a, as a multi-target regulator, is involved in the proliferation, apoptosis and invasion of cells (13). Previous literatures have reported the functions of miR-29a-5p in cancer. MiR-29a-5p, by targeting DHRS4, could modulate the proliferation, invasion and migration of glioma (14). Nevertheless, the exact role of miR-29a-5p in the diffusion, migration and invasion of VSMCs remains unclear. Therefore, this study investigated the effect of miR-29a-5p on the phenotypic transition, proliferation and migration of VSMCs and attempted to elucidate the mechanism of how miR-29a-5p works in the phenotypic transition of VSMCs.

Materials and Methods

Cell culture

Human VSMCs provided by American Type Culture Collection (ATCC, Rockville, MD, USA) were maintained in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum at 37°C and 5% CO_2. Cells were treated with PDGF-BB in varying concentrations (0, 10, 20 and 40 ng/mL) and for different time periods (0, 6, 12 and 24 h) to induce the excessive proliferation and migration and phenotypic transition of VSMCs.

Animal experiment

Eight-week-old, male Sprague Dawley rats were divided randomly into 4 groups: Control group (CTL group, n = 10), CKD group (n = 15), CKD + CINA group (n = 10), CKD+AAV-29a group (n = 10) and CKD + DAPT group (n = 15). According to the previous study, rats in the CKD group would be further induced by the hypophosphite diet (2.0%) following the 5/6 nephrectomy to establish the CKD models. After the *in vivo* experiments, rats were anesthetized by peritoneal injection of pentobarbital sodium (150 mg/kg).

Real-time quantitative PCR

TRIzol reagent (TaKaRa, Japan) was used to separate the total RNA, and PCR was performed by using the SYBR Premix Ex Taq and 7300 Real-time PCR system (Applied System). All results were normalized to GAPDH.

Detection of proliferation and migration of cell

Cell proliferation was measured by the BrdU method. In brief, VSMCs were inoculated into a 35 mm culture dish containing a slide and then incubated with BrdU solution (Beyotime, Shanghai, China) for 4 h. Subsequently, cells were fixed in paraformaldehyde for 10 min and then incubated with the anti-BrdU antibody for 2 h at room temperature. Cells were then stained in DAPI buffer (Beyotime, Shanghai, China). Following several washes in PBS, five visions were selected randomly under the fluorescent microscope (Olympus, Tokyo, Japan) to calculate the average quantity of BrdU-positive cells.

Cell migration was evaluated by the Transwell system. Specifically, 600 μL RPMI-1640 medium containing 10% FBS was added into the lower chambers, while in the upper chambers, 100 μL serum-free RPMI-1640 medium containing 10^{15} cells was added. Following 12 hours of culture at 37°C, cells that remained in the upper chambers were removed by a cotton swab, while those in the lower chambers were fixed in 4% paraformaldehyde for 10 min and stained in 0.5% crystal violet. Eventually, 5 visions were selected from each membrane randomly to calculate the average quantity of migrated cells.

Immunoblots

VSMCs were collected and lysed in 100 μL RIPA buffer (Beyotime, Shanghai, China). Total protein was also extracted to determine the concentration of proteins by using the Bradford method. Proteins in the same amount were isolated via the SDS-PAGE and then transferred onto the PVDF membrane. Thereafter, the membrane was soaked in 5% non-fat milk for 1 h at room temperature, and proteins on the membrane were incubated with the primary antibodies at 4°C overnight. Later, the resulting immunoblots were further incubated with the secondary antibodies at room temperature for 1 h. Final immunoblots were then visualized by incubating with the mixture of enhanced chemiluminescent A and B on Amersham Imager 600 and subjected to the analysis on Image J.

Statistical analysis

Biological repeats were conducted by using 3 parallel samples for all *in vitro* experiments, and for each sample, 3 technical repeats were also carried out. SPSS 23.0 software was used for statistical analysis. All measurement data were expressed in the form of mean ± standard deviation (SD). The difference in the data between the two groups was validated via *t*-test, while a one-way analy-

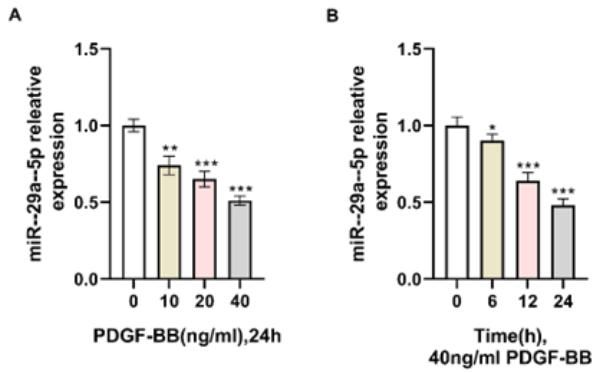

Figure 1. Effect of PDGF on the expression of miR-29a-5p in VSMCs. (a) Following 24 hours of treatment of PDGF in varying concentrations for VSMCs, the expression of miR-29a-5p is quantified by qRT-PCR. (b) Following the different time periods of treatment by 40 ng/mL PDGF for VSMCs, the expression of miR-29a-5p is quantified by qRT-PCR. *, ** and *** for $P < 0.05$, 0.01 and 0.001 when compared to the 0 ng/mL group or 0 h group.

sis of variance was performed for the comparison among groups. $P < 0.05$ suggested that the difference had statistical significance.

Results

miR-29a-5p is down-regulated by PDGF-BB

PDGF-BB could induce the proliferation and phenotypic transition of VSMCs (5), so we established the PDGF-BB-induced VSMC dysfunction models to investigate the expression profile of miR-29a-5p in VSMC. In this work, VSMCs were treated with PDGF-BB in varying concentrations (0, 10, 20 and 40 ng/mL), and we noted that the expression of miR-29a-5p was down-regulated as the concentration of PDGF-BB increased (Figure 1A). Moreover, following the treatment of 40 ng/mL PDGF for 0, 6, 12 and 24 h, we found that the expression of miR-29a-5p decreased in a time-dependent manner, attaining the lowest expression at 24 h (Figure 1B). Thus, 40 ng/mL and 24 h were selected for the following experiment for the treatment of VSMCs.

miR-29a-5p inhibits the phenotypic transition and improves the PDGF-BB-induced proliferation and migration

To investigate the role of miR-29a-5p in VSMC, we transfected the miR-29a-5p mimic into the VSMCs and the transfection efficiency was validated by qRT-PCR (Figure 2A). BrdU assay also showed that PDGF-BB enhanced the proliferation of VSMCs significantly, while the overexpression of miR-29a-5p also inhibited the proliferation of VSMCs (Figure 2B). Results of flow cytometry also demonstrated that miR-29a-5p overexpression reversed the PDGF-BB-induced cell cycle arrest in the G2/M phase (Figure 2C). Results of the Transwell assay also indicated that PDGF-BB enhanced the migration, while the overexpression of miR-29a-5p blocked the migration of VSMCs (Figure 2d). Furthermore, results of Western blot indicated that the treatment of PDGF-BB inhibited the protein expression of α-SMA, SM22α, SMMHC and Calponin, while the overexpression of miR-29a-5p could reverse the regulatory effect of PDGF-BB on these markers, thereby blocking the differentiation of VSMCs (Figure 2E).

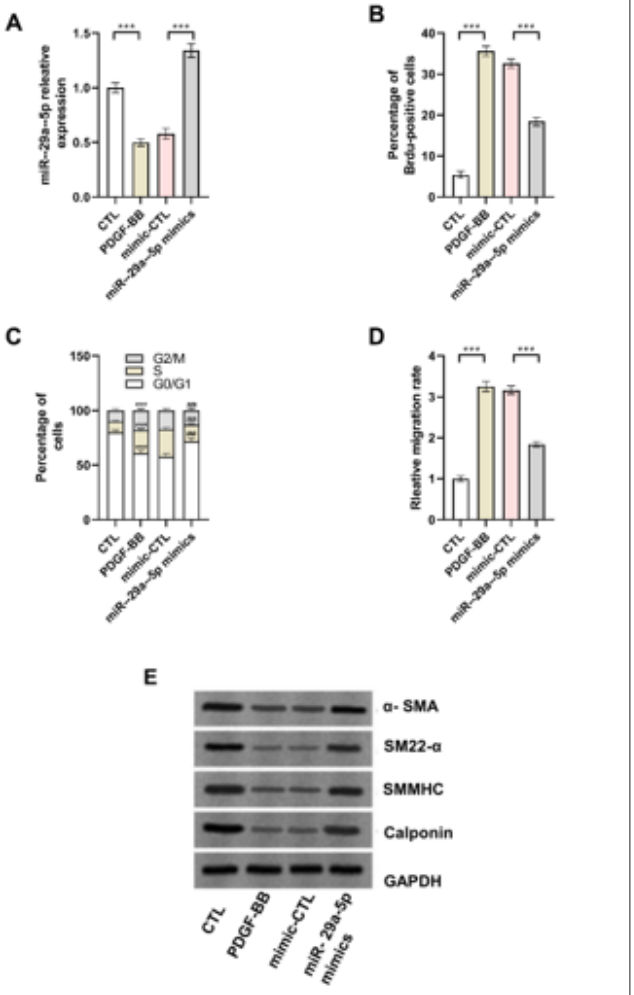

Figure 2. Effect of miR- 29a-5p on VSMCs. (A) Transfection efficiency of miR-29a-5p mimic is determined by qRT-PCR. (B) The proliferation of VSMCs is determined by the BrdU assay. (C) The cycle distribution of VSMCs is measured by flow cytometry. (D) Migration of VSMCs is detected by Transwell assay. (E) Protein expression of VSMCs differential markers, including α-SMA, SM22α, SMMHC and Calponin. *** $P < 0.001$. In Part C, *** $P < 0.001$ *vs.* the CTL group, ### $P < 0.001$ *vs.* the mimic-CTL group.

Overall, overexpression of miR-29a-5p could suppress the PDGF-BB-induced transition of VSMCs from the contractive phenotype to the synthetic phenotype and prevent the excessive proliferation and migration of VSMCs.

Prediction of the downstream mechanism of miR-29a-5p

To further clarify the potential mechanism of how miR-29a-5p modulates the phenotype of VSMCs, we predicted the downstream target of miR-29a-5p by using the TargetScan database, and as a result, GSAP might be a potential target of miR-29a-5p (Figure 3A). Therefore, wild-type (WT) and mutant (MUT) GSAP 3'UTR luciferase reporter gene vectors were constructed according to the binding sites predicted by the TargetScan database (Figure 3B). Then, the dual luciferase reporter gene assay also demonstrated that transfection of miR-29a-5p mimic could reduce the activity of the luciferase of the WT GSAP reporter gene but showed no effect on that of the MUT reporter gene (Figure 3C). Besides, overexpression of miR-29a-5p resulted in the significant downregulation of mRNA and protein of GSAP in VSMCs (Figure 3D). Taken together, GSAP expression is directly regulated by

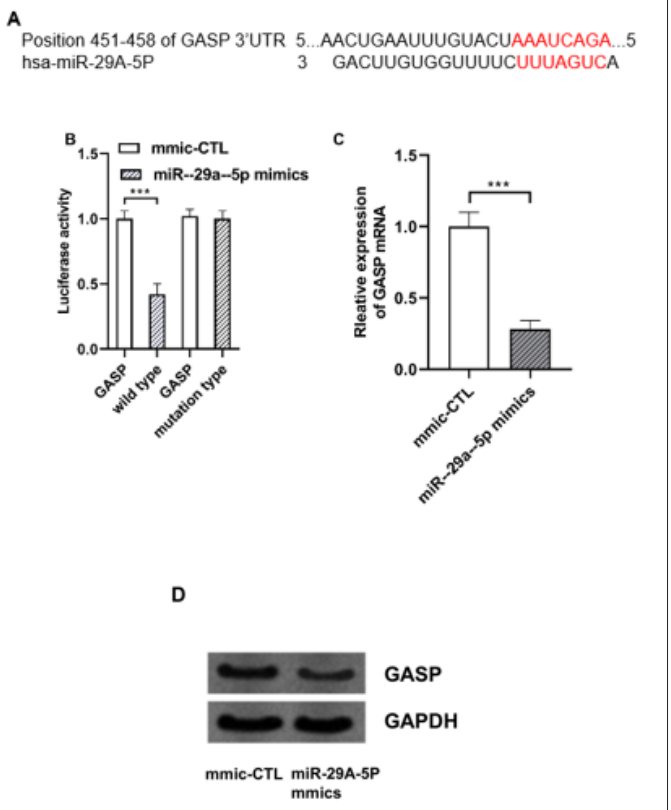

Figure 3. GSAP expression in VSMCs is inhibited by miR- 29a-5p. (A) The binding sequence between miR-29a-5p and GSAP was predicted by the TargetScan database. (B) The binding site between miR-29a-5p and GSAP was validated by a dual luciferase reporter gene assay. (C) The mRNA expression of GSAP in VSMCs by qRT-PCR after the transfection of miR-29a-5p mimic. (D) The protein expression of GSAP in VSMCs by qRT-PCR after the transfection of miR-29a-5p mimic. *** $P < 0.001$.

miR-29a-5p in VSMCs.

Expression of GSAP eliminates the effect of miR-29a-5p on VSMCs

To further testify the hypothesis, we transfected the VSMCs with miR-29a-5p mimic and GAPS-overexpressing plasmid before the treatment of PDGF-BB. The expression of miR-29a-5p and GSAP was also quantified by qRT-PCR and Western blot to confirm the transfection efficiency (Figure 4A). Overexpression of miR-29a-5p could reduce PDGF-BB-induced proliferation of VSMCs and advance the progression of cell cycle and migration, and the inhibitory effect of miR-29a-5p overexpression could be totally abolished by GSAP overexpression, while the promoting effect of PDGF-BB on VSMCs was partially abolished by GSAP overexpression (Figure 4B-D). In addition, in the miR-29a-5p mimic + GSAP group, the expression of α-SMA, SM22α, SMMHC and Calponin was up-regulated evidently. E) Western blotting determines the protein expression of VSMCs differential markers, including α-SMA, SM22α, SMMHC and Calponin (Figure 4E).

Inhibiting the GSAP pathway mitigates the PTH-induced vascular calcification in CKD rats

Parathyroid hormone has been regarded as a key constituent of nephropathy-induced cardiovascular calcification. Thus, we constructed the VC models on CKD rats on a hyperphosphatemia diet and received the 5/6 nephrectomy. In this study, we attempted to explore whether inhibiting

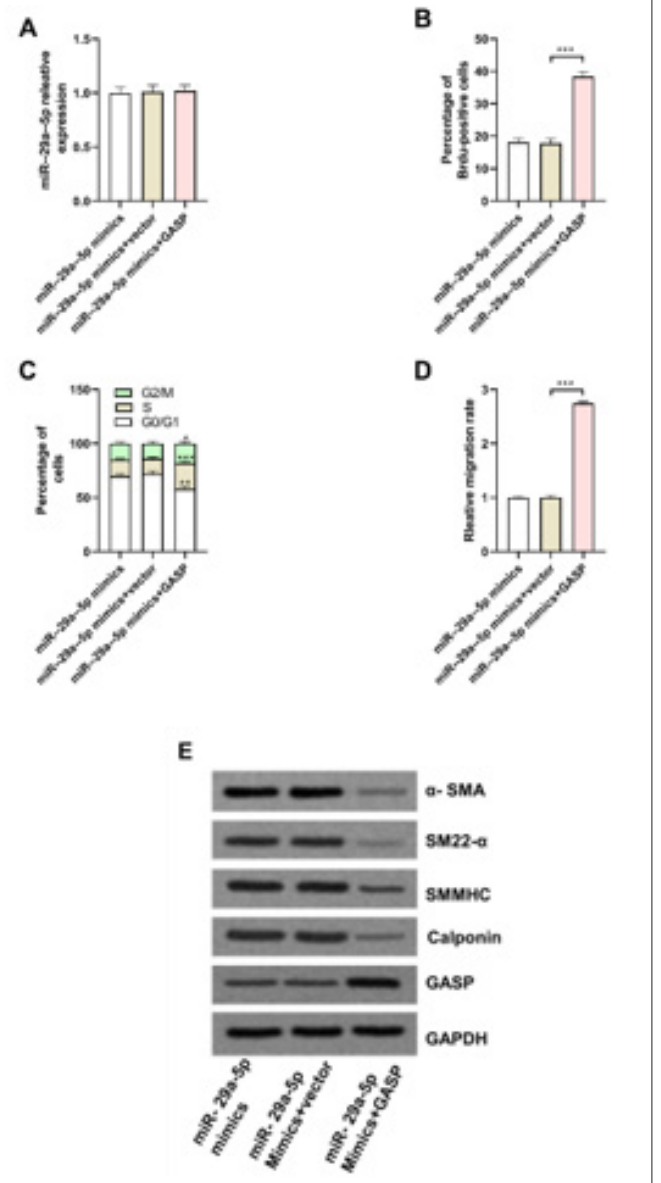

Figure 4. GSAP eliminates the effect of miR-29a-5p on VSMCs. (A) miR-29a-5p expression is determined by qRT-PCR after co-transfection. (B) The proliferation of VSMCs is determined by the BrdU assay. (C) The cycle distribution of VSMCs is measured by flow cytometry. (D) Migration of VSMCs is detected by Transwell assay. (E) Western blotting determines the protein expression of VSMCs differential markers, including α-SMA, SM22α, SMMHC and Calponin. *** for $P < 0.001$. Vector for pcDNA. *, ** and *** for $P < 0.05$, 0.01 and 0.001 when compared to the miR-29a-5p mimic + vector group.

the GSAP pathway could delay vascular calcification in CKD rats, thereby clarifying the effect of AAV-29a and DAPT on vascular calcification in CKD rats. The microscopic observation indicated that AAV-miR-29a and DAPT could reduce the deposition of calcium and phosphate bodies (Figure 5A). In comparison with the CTL group, rats in the CKD group presented a significant enlargement of the calcified lesion in the aortic valve, in spite of the shrinkage after the treatment of AAV-29a and DAPT (Figure 5B). In conclusion, inhibiting the GSAP pathway could delay vascular calcification in CKD rats.

Discussion

Current analysis of the global burden of disease indicates that the major diseases in China have shifted from in-

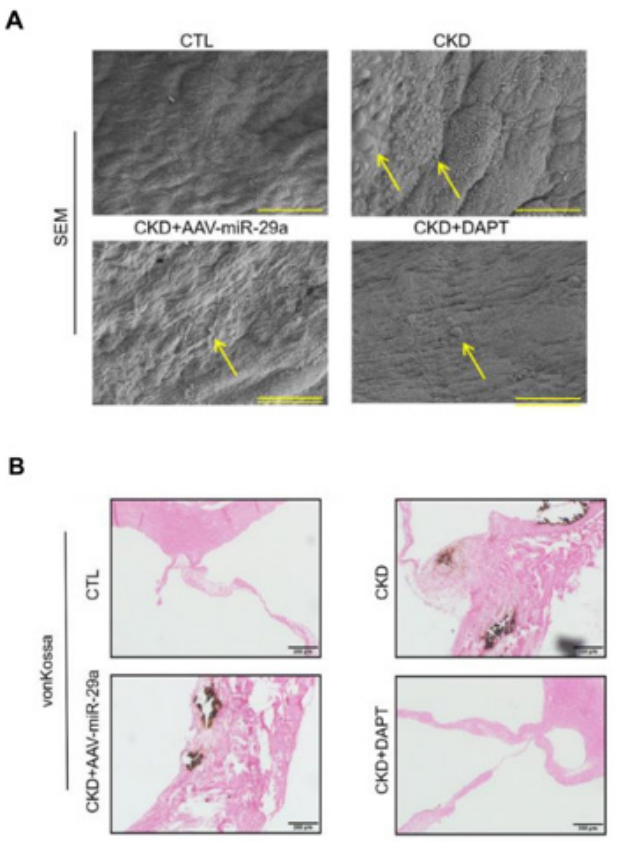

Figure 5. Inhibiting the miR-29a-5p/GSAP pathway could mitigate the PTH-induced vascular calcification in CKD rats. (A) Aortic valves under the scanning electron microscope. (B) Von Kossa staining for the aortic valve in different groups.

fectious diseases to non-infectious diseases, while chronic nephropathy has now become a great threat to the health of citizens (15). The phenotype of VSMCs is variable: In normal, mature vessel wall, VSMC is in contractive type; when it comes to the vascular endothelial injury or *in vitro* culture of VSMCs, VSMCs experience a rapid phenotypic transition, during which cells synthesize and secrete a large amount of extracellular matrix, thereby gaining the ability of migration and proliferation. The phenotypic transition of VSCMs could further induce vascular remodeling, which has been considered as a root cause of the development and progression of vascular lesions, including vascular calcification. As such, research on the molecular mechanism of how the phenotype of VSMCs is regulated is of great significance for the prophylaxis and treatment of chronic nephropathy (16).

MiR-29a, as a kind of conservative miRNA, is involved in the modulation of post-transcription programs in different biological events. For example, miR-29a could inhibit the translation of multiple extracellular matrix proteins, while the consumption of miR-29a results in the fibrosis of tissues (17). According to the previous study, Tokumaru Y *et al*. reported that the poor expression of miR-29a relates to the invasion and a low survival rate of gastric cancer (18). However, Park and his colleague found that any increase in the circulation level of miR-29a could further deteriorate leukemia, suggesting that miRNA may have different roles in the progression of cancer (19). In addition, miR-29a-5p is upregulated in the urine and serum of patients with Stage 1-4 chronic nephropathy (20). A further functional study demonstrates the effect of miR-29a-5p on the calcification of the aortic valve in PTH-induced EndMT CKD rats (21). Encouraged by the findings above, we chose miR-29a-5p as the subject of this study. In this study, we confirmed that miR-29a-5p was down-regulated by PDGF-BB, and the overexpression of miR-29a-5p could inhibit the PDGF-BB-induced transition of VSMCs from the contractive type to the synthetic type and prevent the excessive proliferation and migration of VSMCs. Thus, the findings of this study emphasize the value of miR-29a-5p in the phenotypic transition of VSMCs, but the underlying mechanism remains unclear.

GSAP, a newly found protein, is generated from the Caspase-3-dependent lysis of a precursor, interacts with the key component of the γ-secretase compound and acts as a rate-limiting enzyme in the generation of amyloid beta. Recent evidence has shown that GSAP is increased in the postmortem brain of Alzheimer's disease patients, and pharmaceutical or genetic inhibition could ameliorate the AD-like amyloid phenotype in transgenic models of the mouse (22). GSAP, encoded by the gene located in chromosome 7q11.23, is generated from the proteolysis of larger proteins. According to the available data, the knockout of the GSAP gene decreases the load of Aβ plaque in AD mouse models (23). However, the role of GSAP in vascular calcification is scarcely reported. Prediction of TragetScan and miRanda and our findings suggested that GSAP is the target of miR-29a-5p. In addition, qRT-PCR and Western blotting results demonstrated the negative correlation between the expression of miR-29a-5p and GSAP. Following that, we explored the correlation between miR-29a-5p and GSAP. Dual luciferase reporter gene assay indicated that miR-29a-5p could modulate the activity of 3'-UTR of GSAP directly. The results above, taken together, showed that GSAP may play a key role in VSCM as the direct target of miR-29a-5p. Further evidence has shown that the expression of GSAP eliminated the effect of miR-29a-5p on VSMCs. In addition, VC models were constructed on the hypophosphite-fed rats with the nephropathy induced by 5/6 nephrectomy, and we noted that inhibiting the GSAP pathway could suppress the PTH-induced vascular calcification in CKD rats. The results above provided a novel insight into the potential mechanism of miR-29a-5p in inhibiting the progression of CKD, in which GSAP may be involved directly. Nevertheless, the specific role and mechanism of GSAP to promote vascular calcification should be further investigated in future work.

In conclusion, miR-29a-5p could inhibit the PDGF-BB-induced proliferation, migration and phenotypic transition of VSMCs by targeting GSAP directly. Moreover, GSAP, as an adverse index for CKD patients, is of great value and, therefore, could be a therapeutic target in the future.

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