Suppression of PNPase Alleviates Gestational Diabetes Mellitus-cardiovascular Injury through Up-regulating MicroRNA-26a and Down-regulating PTEN

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Research

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Abstract

**Objective:** Gestational diabetes mellitus (GDM) is often accompanied by cardiovascular injury (CI), while the specific pathology remains largely unknown. The purpose of this study was to investigate the role of Polynucleotide Phosphorylase (PNPase) in GDM-CI.

**Methods:** GDM-CI rats were modeled by giving high-glucose and high-saturated fat compound feed, and PNPase and miR-26a expression in rats was determined. Vascular smooth muscle cells (VSMCs) were isolated, and cells were transfected with si-PNPase, miR-26a mimic, or si-PNPase + miR-26a inhibitor. Cell proliferation, apoptosis and migration of VSMCs were measured by CCK-8 assay, flow cytometry, and scratch test. Dual-luciferase reporter gene assay was performed to verify the targeting relationship between miR-26a and PTEN. RT-qPCR was implemented to detect the expression levels of miR-26a and PTEN among cells in each group.

**Results:** GDM-CI increased PNPase expression and decreased miR-26a expression in cardiovascular tissues of GDM-CI rats. Silencing PNPase and miR-26a upregulation reduced VSMC apoptosis, and enhanced proliferation and migration abilities in GDM-CI. Treatment with miR-26a inhibitor reversed the alleviating effect of inhibiting PNPase expression on GDM-CI. There was a targeting relationship between miR-26a and PTEN, and miR-26a mimic inhibited the expression of PTEN. Suppressed PTEN was found to relieve the GDM-CI.

**Conclusion:** This study suggests that suppression of PNPase alleviates GDM-CI through up-regulating miR-26a and down-regulating PTEN.

1. Introduction

Gestational diabetes mellitus (GDM), as the most frequent manifestation is characterized by glucose intolerance-induced hyperglycemia that occurs or is primarily diagnosed in pregnancy [1]. GDM increases the risk for both maternal and fetal complications during pregnancy, and in even increases the risks of postpartum type 2 diabetes mellitus (T2DM) and cardiovascular injury (CI) [2]. Optimal glycemic control can evidently reduce the risk of GDM risk in a subsequent pregnancy, however its effect regarding the other long-term maternal metabolic complications has not been elucidated yet [3]. Additionally, the currently available therapeutic interventions for diabetes and to reduce inflammation could radically attenuate the subsequent risk of cardiovascular disease and further alleviate any debilitating long-term outcomes in GDM women [2]. Therefore, a definitive pathway is necessitated for the identification and treatment of women with GDM and complicated cardiovascular disease in the early postnatal period.

Polynucleotide Phosphorylase (PNPase) is located in the inner mitochondrial membrane that expands into the intermembrane space, which can essentially induce the transport of ribosomal RNA and transfer RNA based on their hairpin-loop structure [4, 5]. PNPase mutations in humans are genetically associated with severe ailments such as encephalomyopathy, hereditary hearing loss, chorioretinal defects, Leigh syndrome, axonal and auditory neuropathy, gut disturbances, and delayed myelination [6]. Research
supports the ability of PNPase to might regulate bioenergetics during T2DM [7]. However, the effect of PNPase on CI in GDM is still in infancy.

Accumulating evidence has shown the ability of manipulation of the PNPase expression levels may induce the delivery of anti-microRNA (miRNA) therapeutics to mitochondria in both the physiological and pathological conditions of T2DM [7]. Evidence supports the utilization of circulating miRNAs as diagnostic biomarkers to predict DM and its complications [8]. Several miRNAs are found to be overexpressed in the cardiovascular system, which is substantial in the occurrence and development of various cardiovascular diseases [9, 10]. Of which, the miR-26 family is reported to manipulate in cell behaviors via multiple mechanisms [11]. Moreover, Basak Icli et al. have identified the vital functionality of the miR-26 family in a wide range of cardiovascular repair mechanisms [12]. Essentially, miR-26a is dysregulated in cardiovascular diseases but it can regulate vascular smooth muscle cells (VSMCs) proliferation in neointimal hyperplasia [13]. Existing research elicits that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) could be a target gene of miR-26a [14, 15]. Recently, Grinder-Hansen et al. have supported that the PTEN variation is associated with insulin resistance and an increased T2DM risk [16]. In light of the aforementioned literature, we conducted this study to elucidate whether PNPase might regulate GDM-CI through miR-26a/PTEN axis, and provide a new theoretical basis for the treatment of GDM-CI.

2. Materials And Methods

2.1 Ethics statement

All the experimental procedures were in consistency with the guidelines issued by the National Institutes of Health and with approval of the Institutional Animal Care and Use Committee of Henan Provincial People's Hospital (HN-20190225). Adequate measures were taken to minimize the suffering of the included animals.

2.2 Establishment of GDM-CI rat models

The 12-week-old female rats (purchased from Beijing Union-Genius Pharmaceutical Technology Co. Ltd., the certificate number is SYXX (Beijing) 2020-0021) were randomly divided into two groups: the blank group (rats were instilled conventional feed) and the GDM-CI group (rats were instilled a high-glucose and high-saturated fat compound feed). The fasting blood glucose value of rats in the GDM-CI group was 150–250 mg/dl, which was considered to be diabetic, thus indicative of successful model establishment, while the fasting blood glucose value of the control rats was less than 100 mg/dl. The rats in the blank and GDM-CI groups were mated with the normal adult rats. The appearance of mating plugs was an indicator of successful mating, which was defined as the first day of successful pregnancy (10 rats per group).

2.3 Cell culture
After 21 days of rearing, the rats were euthanized. The heart tissues of rats were divided into small pieces, detached continuously with 0.1% trypsin for 30 minutes, treated at 37°C with 0.2% type II collagenase in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) for 10 hours, and then filtered using a nylon mesh (pore size: 70 mm, BD Falcon, Bedford, MA, USA). The separated cells were cultured using DMEM-containing 10% fetal bovine serum (FBS), and under saturated conditions of 37°C and 5% CO₂. The medium was replaced every 48 hours.

2.4 Immunofluorescence assay

The well-cultured cells were rinsed 3 times with preheated phosphate buffered saline (PBS), fixed using 4% paraformaldehyde for 15 minutes, then permeabilized by Triton-X-100 (2 mL/L) for 3 minutes, and incubated with the bovine serum albumin (BSA, 50 g/L) at room temperature for 30 minutes. Next, the cells were cultured with anti-a-SM-actin (1:100, ab124964, Abcam, Cambridge, MA, USA) at 4°C overnight, heated at 37°C for 30 minutes the following day and incubated at 37°C for 1 hour with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG H&L (1:1000, ab6785, Abcam) under conditions devoid of light. The cell nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) (Solarbio Science and Technology Co., Beijing, China), and then observed under a confocal microscope (Zeiss LSM 510 META, Carl Zeiss AG, Germany) to analyze the results.

2.5 Cell transfection and grouping

The cultured cells at the second and third passage were divided into the GDM-CI group and the blank group. The cell transfection was implemented in strict accordance with the provided instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Cells were transfected with the si-PNPase and si-negative control (NC) plasmids (siRNA/transfection reagent = 12.5 pmoles/µL) to establish the PNPase expression inhibition group (GDM-CI + si-PNPase group) and the NC group (GDM-CI + si-NC group). Cells were transfected with miR-26a mimic (Genecopoeia, Guangzhou, China) to establish the miR-26a overexpression group (GDM-CI + miR-26a mimic group) and the corresponding NC group (GDM-CI + mimic-NC group). Next, miR-26a inhibitor was utilized for treatment of cells in the GDM-CI + si-PNPase group to establish the GDM-CI + si-PNPase + miR-26a inhibitor group and the GDM-CI + si-PNPase + inhibitor-NC group.

2.6 Quantitative polymerase chain reaction (qPCR)

The total RNA content was separated using the TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Roche Light-Cycler480 real-time qPCR system was adopted to detect the transcription level. Next, qPCR was performed with the SYBR Green PCR Master Mix (total reaction volume, 20 µL). The $2^{-\Delta\Delta CT}$ method was adopted for subsequent quantification, and the expression level of the detected gene was normalized to U6 or glyceraldehyde phosphate dehydrogenase (GAPDH) to determine the relative quantification. The primer sequence is shown in Table 1.
Table 1
The primer sequence for genes

| Gene   | Sequence                                      |
|--------|-----------------------------------------------|
| miR-26a| F: 5’-CGTCCTTTAAGTAATCCAGGA-3’               |
|        | R: 5’-GCAGGGTCGAGGTATTC-3’                   |
| U6     | F: 5’-TGCGGGTGCTCGCTCGACG-3’                 |
|        | R: 5’-CCAGTCAGGGTCGAGGT-3’                   |
| PTEN   | F: 5’-TGGAAAGGGAGCCGAACTGTG-3’               |
|        | R: 5’-CATAGCGCCTCTGACTGGA-3’                 |
| GAPDH  | F: 5’-AGGTCGGTGAACGATTG-3’                   |
|        | R: 5’-TGTAGACCATGTTGAGGTC-3’                 |

Note: F, forward; R, reverse; miR, microRNA; PTEN, phosphatase and tensin homolog deleted on chromosome 10; GAPDH, glyceraldehyde phosphate dehydrogenase.

2.7 Cell counting kit (CCK)-8 assay

Cell suspension was prepared in conformity with the provided instructions of the CCK-8 reagent (Signalway Antibody, P002, College Park, USA), and the number of cells in each group was adjusted to $1 \times 10^5$ cells/well. Cells (100 µL per well) were seeded in a 96-well culture plate and incubated at 37°C with 5% CO$_2$. Each group was set with 3–5 duplicated wells. With 24, 48, and 72 hours of incubation, 100 µL of the CCK-8 solution was added to each well and then incubated for 90 minutes. A microplate reader (Infinite M200; Tecan Austria, GmbH, Grödig, Austria) was adopted to measure the absorbance value at wavelength of 450 nm to assess the cell proliferation activity.

2.8 Flow cytometry

Cell apoptosis was determined in strict accordance with the Annexin V-FITC Apoptosis Detection Kit (Abcam). Briefly, the cells were seeded onto 6-well plates at a concentration of $1.0 \times 10^5$ cells/well, after which the cells were incubated for 10 minutes with 5 µL of Annexin V-FITC and incubated for 15 minutes with 5 µL of propidium iodide at room temperature under conditions devoid of light. Cell apoptosis was measured using a FACScan flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

2.9 Western blot analysis

Cells collected from each group were rinsed with pre-cooled PBS, and the protein content was extracted by radioimmunoprecipitation assay lysis buffer (Thermo Scientific). The protein samples (50 µg each group) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotting onto polyvinylidene fluoride membranes, incubation at 4°C overnight with the primary antibody and subsequent incubation for 2 hours at room temperature with the horseradish peroxidase (HRP)-labeled
secondary antibody. The enhanced chemiluminescence kit (Thermo Scientific) was used to visualize the immune complexes. The included antibodies were as follows: PNPT1 (1:1000, ab157109), IgG H&L (HRP) (1:2000, ab205718) and β-actin (1:5000, ab6276) (all from Abcam).

2.10 Dual luciferase reporter gene assay

The PTEN 3'-untranslated region (UTR) containing the miR-26a wild-type (Wt) and mutant type (Mut) binding sites were amplified and cloned into the pGL3 vector (Promega Corporation, Madison, WI, USA) to construct pGL3-Wt-PTEN-3'-UTR and pGL3-Mut-PTEN-3'-UTR. The luciferase reporter vector was co-transfected with miR-26a mimic or the corresponding NC into the 293T cells (American Type culture collection, Manassas, VA, USA). The PRL-TK plasmid (Promega) was used as a control. After 48 hours, the Dual-Luciferase Reporter Assay System (Promega) was used to measure the two luciferase activities individually.

2.11 Hematoxylin and eosin (HE) staining and transmission electron microscope (TEM) observation

The pathological examination was performed according to defined laboratory standards. The heart tissues were preserved in 2.5% glutaraldehyde for 3 hours, rinsed with 0.1M PBS, embedded in 2% agarose gel, and then finally fixed in 4% osmium oxide solution for 1 hour. Subsequently, the tissues were rinsed under distilled water, stained with 0.5% uranyl acetate for 1 hour, and ultimately embedded in epoxy resin. The resin was polymerized at 60°C for 48 hours, after which the ultrathin sections were stained with 5% uranyl acetate and 2% lead citrate aqueous solution. The ultrastructure was observed under a TEM (JEOL JEM2100, Tokyo, Japan).

The heart sections were rinsed with PBS, deparaffinized with xylene, and hydrated with gradient ethanol. Then, the sections were stained using hematoxylin and eosin (Beyotime, Shanghai, China). The images were documented under a microscope (IX73, Olympus, Japan) in bright field mode.

2.12 Scratch test

The cells were seeded onto 6-well culture plates with 10% FBS-contained DMEM. Upon attaining 80% cell confluence, the fused monolayer membrane was scratched using the tip of a 200 µL pipette. Next, the cells were sustained in a stable and suitable environment for 72 hours. At each time point, a digital camera connected to a phase contrast microscope was used to document cell findings until the wound healed and closed. The wound gap area was measured by the IMAGE-PRO PLUS software.

2.13 Statistical analysis
All statistical data analyses were processed using the SPSS 22.0 software (IBM, Chicago, IL, USA) and GraphPad Prism 8.0. The measurement data were depicted as mean ± standard deviation. Firstly, a normality and variance homogeneity test was conducted and the data in compliance with the assumption of normality and homogeneity of variance were compared using the t-test (two groups) or one-way or two-way analysis of variance (ANOVA). The t test or Tukey's multiple comparisons test was employed as the post hoc test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 GDM-CI increases PNPase expression and decreases miR-26a expression in cardiovascular tissues

Initially, the HE staining and TEM observation (Fig. 1A) showed that the cells were locally vacuolated and the nuclear membrane was acutely dissolved in the cardiovascular tissues of GDM-CI rats. The detection of PNPase and miR-26a expression levels revealed an elevated PNPase and a reduced miR-26a expression level in the cardiovascular tissues of GDM-CI rats (Fig. 1B, C). Our results show that GDM-CI may induce an elevation in the PNPase expression and a decrease in the miR-26a expression of the cardiovascular tissues.

3.2 Identification of primary cultured rat VSMCs

α-SM-actin is a protein rich in VSMCs, which is principally expressed in contractile cells and is highly expressed in proliferating VSMCs. The cultured VSMCs at the second and third passage were stained by immunofluorescence assay and observed under a laser confocal microscope. The results revealed that α-SM-actin was localized in the cytoplasm as myofilaments along the longitudinal axis (Fig. 2). The cytoplasm emitted green fluorescence, and the nucleus was stained blue by DAPI. The positive cell rate was higher, indicating a higher purity of the cultured primary cells.

3.3 Inhibition of PNPase up-regulates miR-26a and alleviates GDM-CI

In order to validate if there was a relationship between PNPase and the miR-26a expression levels in the cardiovascular tissues, siRNA was adopted to inhibit the PNPase expression pattern in VSMCs, and performed western blot analysis and RT-qPCR to detect the PNPase and miR-26a expression levels in each group of cells. The results exhibited that the suppression of PNPase elevated the miR-26a expression pattern (Fig. 3A, B). The apoptosis, proliferation and migration of VSMCs in each group were detected by a combination of flow cytometry, CCK-8 assay and scratch test (Fig. 3C-E). The results revealed that the suppression of PNPase reduced VSMC apoptosis, and improved the proliferation and migration abilities in GDM-CI. The preceding results suggested that inhibiting the PNPase expression pattern can up-regulate the miR-26a expression pattern and alleviate GDM-CI consequent of a protective effect on VSMCs.
3.4 Overexpression of miR-26a can relieve GDM-CI

In an attempt to further explore the relationship between miR-26a expression and GDM-CI, VSMCs in GDM-CI were treated with overexpressed miR-26a, and the overexpression efficiency of miR-26a was verified by means of RT-qPCR (Fig. 4A). The degree of apoptosis, proliferation and migration of VSMCs in each group were determined by a combination of flow cytometry, CCK-8 assay and scratch test (Fig. 4C-D). The results revealed that the up-regulation of miR-26a inhibited VSMC apoptosis, and increased the proliferation and migration abilities in GDM-CI. Our results prove that the overexpression of miR-26a has a definitive alleviating effect on GDM-CI.

3.5 miR-26a targets PTEN

Starbase database analysis identified numerous target genes for miR-26a. Notably, PTEN expression plays a role in VSMC damage caused by hydrogen peroxide [17]. Database analysis predicted the presence of a binding site between miR-26a and PTEN (Fig. 5A). Next, by means of a dual luciferase reporter gene assay (Fig. 5B), it was ascertained that the overexpression of miR-26a reduced the luciferase activity of the PTEN-3'UTRwt reporter plasmid. Meanwhile, the PTEN expression pattern in VSMCs of each group was detected by RT-qPCR (Fig. 5C), which revealed that the elevation of miR-26a could down-regulate PTEN expression pattern in VSMCs of GDM-CI. The results validated that miR-26a has a targeted inhibitory effect on the PTEN expression.

3.6 PNPase up-regulates PTEN by inhibiting miR-26a expression to aggravate GDM-CI

In order to explore whether PNPase could up-regulate PTEN by inhibiting the miR-26a expression pattern, thereby exacerbating cardiovascular damage, we simultaneously inhibited the PNPase and miR-26a expression levels to establish GDM-CI + si-PNPase + miR-26a inhibitor group. PNPase expression in the VSMCs of each group was detected by western blot assay (Fig. 6A), and the expression levels of miR-26a and PTEN were determined by RT-qPCR (Fig. 6B, C). The results showed that the downregulation of miR-26a considerably reversed the inhibition of PNPase on PTEN, and the expression pattern of PTEN was significantly up-regulated. The experiments on apoptosis, proliferation and migration of VSMCs in each group demonstrated that the suppression of miR-26a reversed the effects of silencing PNPase on the apoptosis, proliferation and migration of VSMCs in GDM-CI (Fig. 6D-F). The preceding findings suggest that PNPase up-regulates the expression pattern of PTEN by inhibiting miR-26a, thereby exacerbating GDM-CI.

4. Discussion

GDM risk can be manipulated by various factors such as obesity, family history of T2DM, and ethnicity, principally these factors are also contradicted with an increased risk of cardiovascular disease [18]. Hence, it is trivial to identify the molecular mechanisms for the pathological processes of GDM-CI. In our
study, the findings revealed that inhibition of PNPass could up-regulate miR-26a and then down-regulate PTEN, thereby relieving GDM-CI.

During T2DM, PNPass has been identified as a constituent of the transfer mechanism of miRNAs into cardiac mitochondria [7]. Abnormal VSMC proliferation and migration are common pathogenesis in various cardiovascular diseases [19]. In our study, PNPass expression was identified to be elevated in cardiovascular tissues of GDM-CI rats, while the down-regulation of PNPass could reduce VSMC apoptosis, and enhance the proliferation and migration abilities. In consistency with our findings, elevated PNPass protein levels have been previously demonstrated in the cardiac tissues from both human and mouse models of T2DM [7]. Accumulating evidence supports the ability of up-regulated PNPass_{old−35} to induce the production of reactive oxygen species in the mitochondria to radically contribute to the expression of pro-inflammatory factors [20, 21]. Furthermore, Upneet K. Sokhi et al. have found that knockdown of PNPass_{old−35} causes evident gene expression alterations related to mitochondrial dysfunction and cholesterol biosynthesis, however the elevation of PNPass_{old−35} results in changes in cell-cycle associated functions [22]. Inhibition of hPNPass(old-35) may represent a novel target in the development of age-associated chronic diseases (PMID: 15978720). However, the role of PNPass in VSMCs has not been elucidated yet. The preceding literature elicits that the downregulation of PNPass plays a protective role in VSMCs and subsequently relieves injury.

Danielle L Shepherd et al. identified PNPass as a potential contributor to mitochondrial miRNA transfer, which could manipulate bioenergetics during T2DM [7]. Abnormal expression profiles of various miRNAs, such as miR-26a-5p, have been observed in women manifesting GDM [23]. In light of this, we speculated the presence of a correlation between PNPass and miR-26a in GDM-CI. Our findings suggested that miR-26a expression was considerably decreased in GDM-CI. Similarly, reduced levels of miR-26a have been identified in the serum and islets of high fat diet humans and db/db mouse models [24]. Overexpression of miR-26a could relieve GDM-CI, and the suppression of PNPass could up-regulate the expression of miR-26a to alleviate the impairment in GDM-CI. Additionally, an existing study identified the miR-26a expression to be down-regulated in the VSMCs of proliferative rat jugular vein, and the elevation of miR-26a could suppress VSMC proliferation and migration in response to platelet-derived growth factor-BB stimulation [13]. Moreover, Jiang et al. have stated that miR-26a overexpressing/transgenic mice exhibit reduced glucose level and fatty acid synthesis, with improved insulin sensitivity [25]. Interestingly, the upregulation of exoribonuclease (PNPass_{old−35}, PNPT1) could elevate the degree of DNA demethylation, which induces degradation of miR-26a [26]. The aforementioned data imply that the overexpression of miR-26a certainly has a definitive alleviating effect on GDM-CI.

Next, we explored the downstream process of miR-26a and its associated factors. Mechanistically, miR-26a could evidently suppress PTEN expression by binding with 3'-UTR both in vivo and in vitro [24]. In this study, we identified that the PTEN expression in GDM-CI + miR-26a mimic group was significantly down-regulated compared to the GDM-CI group. An existing study documented higher levels of PTEN in the GDM patients relative to the controls [27]. As verified by the online website prediction and detection of dual luciferase reporter gene assay, a relationship was exhibited between miR-26a and PTEN. The next
phase of our study focused at exploring whether PNPase up-regulated PTEN by inhibiting the miR-26a expression, thereby exacerbating cardiovascular damage. We inhibited miR-26a expression on the basis of suppressed PNPase. Our results suggested that PNPase up-regulated the expression of PTEN by inhibiting miR-26a to exacerbate GDM-CI. Essentially, a higher PTEN expression has been recognized as a potent therapeutic strategy for limiting disease development and elevating calorie expenditure so as to decrease the deposition of adipose tissue [28]. Wang et al. have found that PTEN overexpression could definitively impair VSMC proliferation and induce apoptosis, and PTEN overexpression serves as a promising intervention for restoring homeostatic heart function in coronary heart disease patients [29]. All these findings elicit that PNPase upregulates the PTEN expression by inhibiting miR-26a, thereby exacerbating CI.

In conclusion, our study demonstrates that PNPase increases the PTEN expression by down-regulating miR-26a expression, thereby aggravating GDM-CI. Nevertheless, the specific mechanism of action of PNPase on miR-26a/PTEN axis has not been identified yet, and further research is warranted for the verification of our results by in vivo experiments. We will focus on the specific mechanism of PNPase on miR-26a and validate its authenticity by in vivo experiments.

**Declarations**

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Not applicable.

**Conflict of Interests**

The authors declare that they have no conflicts of interest.

**Authors’ Contributions**

LL and HYW are the guarantor of integrity of the entire study; LL contributed to the study concepts, study design, definition of intellectual content, literature research and manuscript editing; HYW contributed to the data acquisition, data analysis, statistical analysis and manuscript preparation; XHC contributed to the data acquisition, data analysis, experimental studies and manuscript review. All authors read and approved the final manuscript.
Data Availability Statement

All the data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All the experimental procedures were in consistency with the guidelines issued by the National Institutes of Health and with approval of the Institutional Animal Care and Use Committee of Henan Provincial People's Hospital (HN-20190225). Adequate measures were taken to minimize the suffering of the included animals.

Consent for publication

Not applicable.

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**Figures**

![Figure 1](image)

**Figure 1**

An increase of PNPase expression and a decrease of miR-26a expression are found in cardiovascular tissues of GDM-CI rats. A. Observation of cardiovascular tissues in rats of the blank group and GDM-CI group by HE staining and transmission electron microscope. B. PNPase expression in cardiovascular tissues of rats in the blank group and GDM-CI group was detected by Western blot analysis. C. miR-26a expression in cardiovascular tissues of rats in the blank group and GDM-CI group was detected by RT-
qPCR. * P < 0.05. The measurement data were shown as mean ± standard deviation. The significance of the differences between two groups was determined by the t-test (panels B and C).

**Figure 2**

Identification of primary cultured rat vascular smooth muscle cells. The expression and localization of α-SM-actin (green) in VSMCs, and the nucleus is blue.

**Figure 3**

Inhibition of PNPase up-regulates miR-26a and alleviates GDM-CI. A. PNPase expression in VSMCs detected by Western blot analysis. B. miR-26a expression in VSMCs detected by RT-qPCR. C. VSMC apoptosis detected by flow cytometry. D. VSMC proliferation detected by CCK-8 assay. E. The wound healing ability of VSMCs was detected by scratch test. * P < 0.05. The measurement data were shown as mean ± standard deviation. The significance of the differences among multiple groups was determined by one-way ANOVA (panels A-C) or two-way ANOVA (panels D and E), followed by Tukey's multiple comparisons test.
Figure 4

Up-regulation of miR-26a relieves GDM-CI. A. miR-26a expression in VSMCs detected by RT-qPCR. B. VSMC apoptosis detected by flow cytometry. C. VSMC proliferation detected by CCK-8 assay. D. The wound healing ability of VSMCs was detected by scratch test. * P < 0.05. The measurement data were shown as mean ± standard deviation. The significance of the differences among multiple groups was determined by one-way ANOVA (panels A and B) or two-way ANOVA (panels C and D), followed by Tukey’s multiple comparisons test.
There is a binding site between miR-26a and PTEN. A. Starbase analyzed the binding sites of miR-26a and PTEN. B. The dual-luciferase assay verified the targeting relation between miR-26a and PTEN. C. PTEN expression in VSMCs was detected by RT-qPCR. * P < 0.05. The measurement data were shown as mean ± standard deviation. The significance of the differences among multiple groups was determined by one-way ANOVA (panel C) or two-way ANOVA (panel B), followed by Tukey’s multiple comparisons test.
Figure 6

Suppression of miR-26a reverses the effects of silencing PNPase on apoptosis, proliferation and migration of VSMCs in GDM-CI. A. PNPase expression in VSMCs was detected by Western blot analysis. B. miR-26a expression in VSMCs was detected by RT-qPCR. C. PTEN expression in VSMCs was detected by RT-qPCR. D. VSMC apoptosis was detected by ow cytometry. E. VSMC proliferation was detected by CCK-8 assay. F. The wound healing ability of VSMCs was detected by scratch test. * P < 0.05. The measurement data were shown as mean ± standard deviation. The significance of the differences among
multiple groups was determined by one-way ANOVA (panels A-D) or two-way ANOVA (panels E and F), followed by Tukey's multiple comparisons test.