



PM_{2.5}-induced *ADRB2* hypermethylation contributed to cardiac dysfunction through cardiomyocytes apoptosis via PI3K/Akt pathway

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ABSTRACT

Background: Long-term exposure to fine particulate matter (PM_{2.5}) can causally contribute to progression of atherosclerosis, risk of ischemic heart disease and death, but the underlying mechanism is little known. Since DNA methylation impacts the process of heart disease, it might be useful in exploring potential mechanistic pathways linking PM_{2.5} exposure and heart disease.

Objectives: Here, we investigated the PM_{2.5}-induced *ADRB2* hypermethylation and the involving epigenetic mechanism of PM_{2.5}-induced cardiomyocytes apoptosis and cardiac dysfunction.

Methods and results: *In vitro*, PM_{2.5} markedly augmented cardiotoxicity including oxidative damage and apoptosis in cardiomyocytes AC16 as well as epigenetic alteration. DNA methylation profiling revealed a significant gene-*ADRB2* was involved in the cardiac relative GO and KEGG pathways. Methylation chip and Bisulfite Sequencing PCR (BSP) both identified the hypermethylation status of *ADRB2* which encodes β2-Adrenergic receptor (β2AR). Mechanistic study showed *ADRB2* hypermethylation-induced down-regulation of β2AR inhibited PI3K/Akt and then activated Bcl-2/BAX and p53 pathway in AC16. The transgenic cell lines showed over-expression of *ADRB2* weakened the PM_{2.5}-induced cardiomyocytes apoptosis in opposite way, but was augmented by PI3K inhibitor (LY294002). *In vivo*, echocardiography showed the heart contractile function was decreased after SD rats intratracheal instillation of PM_{2.5} for 30 days. The myocardial interstitial edema, myocardial gap expansion and myofibril disorder in PM_{2.5} treated group were observed in rats heart tissue. What's more, basal expression of β2AR and VEGFR2 decreased in heart tissue as the dosage of PM_{2.5} increasing, meanwhile PM_{2.5} markedly attenuated PI3K/Akt pathway followed by augmented Bcl-2/BAX and p53 pathway, thus caused a greater number of TUNEL positive cardiomyocytes resulted in cardiac dysfunction *in vivo*.

Conclusions: PM_{2.5} exposure could cause the myocardial *ADRB2* hypermethylation and activate the β2AR/PI3K/Akt pathway, resulted in PM_{2.5}-induced cardiomyocytes apoptosis and cardiac dysfunction. Our study suggested that the *ADRB2* demethylation or *ADRB2*/β2AR activation may serve as a potential pathway to prevent cardiac dysfunction induced by PM_{2.5} exposure.

1. Introduction

Fine particulate matter (PM_{2.5}), with an aerodynamic diameter < 2.5 μm, emerged as one of the most important air pollution. Long-term exposure to PM_{2.5} is associated with heart disease risk (Anderson et al., 2012). A large body of epidemiological studies had reported the association between PM_{2.5} exposure and the increased risk of heart diseases including coronary artery calcification (Kaufman et al., 2016), arrhythmias (Link et al., 2014), hypertension (Chan et al., 2015), myocardial infarction (MI), ischemic heart disease (IHD) (Wang

et al., 2015) and congestive heart failure (CHF) (Kim et al., 2017). Most recently, World Health Organization (WHO) reported that the account about 36% of the IHD deaths attributable to ambient air pollution globally (Landrigan et al., 2018). In 2010, American Heart Association (AHA) had published a scientific statement which finally concluded that PM_{2.5} exposure is deemed a modifiable factor that contributes to cardiovascular morbidity and mortality (Brook et al., 2010). And the IHD accounted for the largest relative (RR 1.18, 95% CI 1.14 to 1.23) and absolute risk for mortality per 10 μg/m³ elevation in PM_{2.5} (Pope 3rd et al., 2004). The public health burden caused by PM_{2.5} was bigger

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in the developing country like China, in which level of particles was often much higher than those monitored in the US and Western Europe (Richman, 1994). Although PM_{2.5} could cause cardiac adverse health effects, the disease progress underlying the association between PM_{2.5} and heart disease remains uncertain, and the exactly mechanism of PM_{2.5}-associated heart disease is not clear.

Evidences accumulated indicate that epigenetic modifications (mainly including DNA methylation, histone modifications, non-coding RNAs and chromatin remodeling) play key roles in the adverse biological outcomes and environmental-exposure-associated detrimental health impact (Sun et al., 2018). Among the epigenetic alterations, DNA methylation is one of the most important mechanism which is studied earliest and most completely (Moore et al., 2013). Due to the complex mechanism of PM_{2.5} exposure, DNA methylation study might be a meaningful epigenetic approach to investigate target gene and also can provide a clue of the mechanism. Previous studies demonstrated that DNA methylation impacts the process of heart disease (Huang, 2013; Lee et al., 2018). A growing body of evidences showed that several key gene promoter hypermethylation may trigger the initiation of heart diseases. It is reported that PM_{2.5} could cause pleiotropic effects on DNA methylation patterns, including both global DNA hypomethylation and gene-specific hypermethylation which may cause instability of chromosome and trigger the initiation of increased blood pressure (Bellavia et al., 2013), blood coagulation (Tarantini et al., 2013) and vascular constriction (Chen et al., 2016). Yet, until now, epigenetic/DNA methylation evidence in heart is very limited. Our previous study had identified the PM_{2.5}-caused cardiomyocytes apoptosis *in vitro* and cardiotoxicity including reducing heart rate and cardiac output as well as pericardial edema in zebrafish (*Danio rerio*) model (Duan et al., 2017; Yang et al., 2018). It is much meaningful to investigate the epigenetic mechanism of PM_{2.5}-induced apoptosis and other adverse effects on the heart.

As we knew, *ADRB2* encodes β_2 adrenergic receptors (β_2 AR) (A, P, et al., 2017), which is one of the G protein-coupled receptors (GPCRs) expressed in cardiomyocytes and their crucial role involved in heart physiology as they regulate chronotropic and inotropic responses of the sympathetic nervous system (Cannavo et al., 2013). Recently, Rorabaugh et al. found β_2 AR-GRK2-dependent signaling is an important mechanism for the treatment of IHD (Rorabaugh et al., 2017). And expression of *ADRB2* gene/ β_2 AR in the heart failure (HF) cardiomyocytes was significantly changed (Gong et al., 2017). It is well-documented that β_2 AR play a key role in cardiomyocytes apoptosis, which is an important cellular response and was reported as crucial mechanisms leading to the terminal point of cardiomyopathy (Steiner and Lang, 2017). Cardiomyocytes apoptosis also contributes to loss of contractility and implicates several kinds of heart disease involving HF, MI, arrhythmias, IHD and so on (Gill et al., 2002; Shiraishi et al., 2004). In clinical practice, inhibiting apoptosis emerged as one of the main goal of gene therapy for CHF to reduce the undesirable heart remodeling (Moe and Marin-Garcia, 2016). In previous study, Zheng et al. found that persistent activation of β_2 AR protected myocardium from apoptosis through a cell survival pathway involving PI3K and Akt (Zheng et al., 2005). But the β_2 AR knockout mice experienced increased myocyte apoptosis and greater mortality rates (Patterson et al., 2004). Yet, as far as we know, there is no report about PM_{2.5}-treated epigenetic change of *ADRB2* promoter in cardiomyocytes. Thus, it is much meaningful to clarify whether the apoptosis triggered by PM_{2.5} was through epigenetic mechanism triggered by *ADRB2* hypermethylation.

Here, in this study, the hypermethylation status of *ADRB2* which encodes β_2 AR was firstly identified by both methylation chip and Bisulfite Sequencing PCR (BSP) analysis. We therefore hypothesize that PM_{2.5} exposure could first lead to epigenetic alteration (including *ADRB2* hypermethylation), thus activate the PI3K/Akt signaling to trigger cardiomyocytes apoptosis and resulted in cardiac dysfunction. Cardiomyocytes AC16 cell line and Sprague Dawley (SD) rats were chosen as cell and animal model respectively. We designed a series of

experiment to explore the underling epigenetic mechanism of *ADRB2* hypermethylation-triggered cardiomyocytes apoptosis. Our findings reveal an important adaptive mechanism by which PM_{2.5} exposure induced cardiomyocytes apoptosis *in vivo* and *in vitro* through *ADRB2* hypermethylation mediated PI3K/Akt. Moreover, this study provided a potential mechanism of which PM_{2.5} caused cardiac dysfunction and increase the risk of heart disease. And it can identify specific interventions to prevent apoptosis and cardiac dysfunction.

2. Method

2.1. PM_{2.5} preparation

A particle collector with high-volume was used to consecutive collect PM_{2.5} samples in Fengtai District, Beijing, P.R. China in winter. PM_{2.5} samples were prepared under the way we reported before (Yang et al., 2018). PM_{2.5} samples were consecutive collected using a high-volume sampler particle collector (TH-1000CII, Wuhan Tianhong, China). The site for collecting PM_{2.5} was in university campus adjacent moderate road and commercial activities. PM_{2.5} samples were absorbed on quartz membrane. And then quartz membrane absorbing PM_{2.5} samples was clipped into some strips. Then we immersed the strips in a sonicator for water bath and sonicated them for 1 h. After we obtained the PM_{2.5} samples, we preserved the samples at temperature of -80°C for better conservation. Three 100 μL aliquots of PM_{2.5} samples was dropped on the small piece of filter paper (weighted prior to air dry) and air dry overnight as to calculate the average concentration of PM_{2.5} samples.

2.2. Cell culture

Shanghai Institutes for Biological Sciences (SIBS) provided the human cardiomyocytes AC16 cell line. The cardiomyocytes were growing in DMEM (Corning, USA) contained 10% fetal bovine serum (FBS) (GIBCO, Pittsburgh, PA, USA), 100 mg/mL streptomycin and 100 U/mL penicillin (KeyGEN, China), and kept in an incubator containing 95% air and 5% CO₂ at the temperature of 37°C . Cardiomyocytes AC16 were seeded in 6-well plates (96-well for CCK8 assay) at the density of 1×10^5 cells/mL. The cells grew adherently need 24 h, then AC16 cells were treated with the designed concentrations (25, 50, 100 $\mu\text{g}/\text{mL}$) of PM_{2.5} samples for 24 h in addition. We sonicated PM_{2.5} for 10 min samples before exposing cells while same volume of DMEM for control group. In the experiment treated with PI3K inhibitor, AC16 was pretreatment with 25 μM LY294002 (Selleck, USA) for 12 h before PM_{2.5} exposure.

2.3. Animal groups and PM_{2.5} intratracheal instillation

The Animal experiment were accord to the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals and was supervised by Capital Medical University's the Animal Experiments and Experimental Animal Welfare Committee (ethical review number: AEEI-2016-076), and Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) provided a total of 32 male SD rats, which meet specific pathogen free (SPF) standard and the rats' average weight is 200 ± 20 g. All rats were randomly divided into four groups ($n = 8$ per group): three experimental groups treated with PM_{2.5} (1.8, 5.4, 16.2 mg/kg body weight) by intratracheal instillation using a rat intubation pack coupled with laryngoscope (Hallowell EMC, USA) every 3 days for 30 days (in total 10 times), and one control group received equivalent volume of saline which used as dosing vehicle. Based on WHO air quality guidelines (Organization, 2007) and physiological parameters of SD rats, we convert the PM_{2.5} dose for intratracheal instillation. Dose conversion of PM_{2.5} was reported in our previous study (Zhang et al., 2017). Animal experiment design was presented in Fig. S1.

2.4. CCK8 measurement and LDH release detection

Cell Counting Kit (CCK8) was often chosen to detect cell viability or the cytotoxicity after cells was exposed to toxicant (Zhao et al., 2018). First AC16 were treated with PM_{2.5} at a series of dose (25, 50, and 100 µg/mL) for 24 h. Then cell viability was detected with the CCK8 assay (Tongren, Japan). Next we detect the lactate dehydrogenase (LDH) using LDH kit (KeyGEN, China). Usually, the intracellular LDH releasing to extracellular is one of the sensitive indicators of irreversible cell death as a result of membrane damage of cells (Yamamoto et al., 2014). The 100 µL cell culture supernatants were collected. At the absorbance of 440 nm, LDH measurement was conducted with a UV–visible spectrophotometer (Beckman DU-640B, USA).

2.5. ROS generation, lipid peroxidation and antioxidant activity measurement

The 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma, USA) was used to detect the ROS levels. Firstly, the cardiomyocytes were exposed to PM_{2.5} or DMEM (control medium) for 24 h. A volume of 2 mL working solution was added to the cell culture medium after the AC16 cells were wash 2 times using PBS. Then we put the cells into incubator for 30 min at 37 °C in dark. Then cardiomyocytes were washed again with PBS for two times. After trypsinization, the cells were resuspended in the PBS for next measure. We measured the fluorescent intensity of ROS generation using Flow cytometry (Becton-Dickson, USA). Each time, $> 1 \times 10^4$ cells are required to be collected for data analyzing. As an addition to the analysis of cytotoxicity and oxidative damage, we used another Malondialdehyde (MDA) detection kit (Dingguo, China) to detect the MDA content. In order to analyze the main enzymatic defense system, the superoxide dismutase (SOD) measurement kit and glutathione peroxidase GSH-Px detection kit were chosen to detect the activities of superoxide SOD and GSH-Px. After AC16 treated with DMEM (control group) and 50 µg/mL dose of PM_{2.5} for 24 h, then the cardiomyocytes were washed with PBS. Next the cell was lysed for 30 min by RIPA lysis buffer (KeyGEN, China). The buffer we used contains 1 mM Phenylmethanesulfonyl fluoride (PMSF) (KeyGEN, China). Then we centrifuged the lysates (12,000 rpm, 10 min) at 4 °C. Cell culture supernatant was obtained to detect the levels of MDA, SOD and GSH-Px. And the bicinchoninic acid (BCA) protein assay (Dingguo, China) was used to measure the concentration of the protein in the extract.

2.6. Determination of cell apoptosis

Apoptotic rate of cardiomyocytes AC16 was detected with the Annexin V-propidium iodide (PI) apoptosis measurement kit (Jiangcheng, China). Cardiomyocytes cell was treated with 50 µg/mL dose of PM_{2.5} for 24 h. DMEM treated group was treated as control. After the cells were washed twice with PBS, we centrifuged cells at 1200 rpm for 3 min and were resuspended in 500 µL binding buffer. Next 5 µL Annexin V-FITC and 5 µL PI were added and mixed. The cardiomyocytes were put into incubator at dark for 15 min at last. A Flow cytometer (Millipore, USA) was used to analysis apoptotic rate of AC16. In order to analysis, we analyzed $> 1 \times 10^4$ cells in each sample. About Annexin V-PI analysis, different cell populations were identified based on different labeling patterns. That is, The left upper quadrant represents cell fragments on the behave of FITC positive; the live cells were designed on the left lower quadrant because of FITC negative and PI negative; And the right upper quadrant represents late apoptotic cells on behalf of PI positive; And early apoptotic cells was designed as FITC positive and PI negative and located in the right lower quadrant.

2.7. Global DNA methylation quantification/DNA methylation chip and methylation profiling

The whole genomic DNA was isolated and bisulfite converted with Kit (Qiagen, Germany). We used Illumina HumanMethylation450K BeadChip (Illumina, USA) to perform DNA methylation profiling. Samples don't meet the purity standard were excluded from the data analysis. Methylation microarray profiling was performed to analyze 485,000 CpG sites methylation status covering 99% of the RefSeq gene. And the normalization method used in our study was SWAN, which is short for Subset-quantile Within Array Normalization. Next we used Illumina GenomeStudio software (version 2.0.4) to conduct normalization of the data. β value ranging from $\beta = 0$ (lowest methylation status) to $\beta = 1$ (highest methylation status) was used to identify the methylation status. We defined the differential methylation sites (DMSs) (absolute β values > 0.12 and p -value < 0.05). Then we did the GO functional enrichment and KEGG analyses based on differential methylation genes (DMGs), which were specific genes covering differential methylation sites (DMSs) were further performed.

2.8. Bisulfite sequencing PCR (BSP) detection

In order to verify the methylation status of ADRB2, we conduct the bisulfite sequencing PCR BSP assay which is a powerful tool in DNA methylation study. The genomic DNA of cardiomyocytes AC16 was bisulfite-treated under the manual instruction of EZ DNA Methylation-Gold™ Kit (ZYMO, USA) for the next BSP analysis. We cloned the purified products of PCR into pMD-18 T vector (Takara, China) for next sequencing after the promoter of ADRB2 sequence was amplified from the converted DNA by touch-down PCR. We sequenced 10 individual clones to recognize the methylated cytosine bases for each group. BSP primer sequences and PCR conditions for CpG islands were presented as dot chart.

2.9. Cell transfection for recombinant plasmid

The cells were cultured into the 6-well plates at a dose of 4×10^5 /mL, high expression ADRB2 (hADRB2) recombinant enhanced green fluorescent protein (EGFP) plasmid pEGFP-N1-hADRB2 and empty pEGFP-N1 plasmids (Likeli, Beijing, China) into human cardiomyocytes AC16, when the cell confluence was up to 80–95%. All the steps were conducted according to the instructions of Lipofectamine 3000 (Thermo Fisher Scientific, US), respectively. Transfection efficacy was verified by qRT-PCR 24 h later.

2.10. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The total RNA was extracted from cardiomyocytes AC16 with SV Total RNA Isolation System kit (Promega, USA) strictly based on manual provided by the manufacture. A RNA PCR kit (Takara, Japan) was performed for cDNA synthesis, qRT-PCR reaction was conducted using ABI PRISM 7500 Sequence Detection System (Thermo Fisher, USA). Results are expressed as increase or decrease fold relative to the expression of GAPDH. Table S4 list the test genes' primer sequences. We conducted the assay in three biological repeats and three duplicated repeats.

2.11. Western blotting analysis

The protein expression levels of β 2AR, PI3K, Akt, p-Akt, p53, Bcl-2, BAX were detected by western blot to analyze impact of PM_{2.5}, p-EGFP-N1-hADRB2, PI3K inhibitor-LY294002 on cardiomyocytes AC16. A kit for protein extraction (Jiancheng, China) was performed to extract the total protein in cardiomyocytes, and the BCA protein assay (Jiancheng, China) was used to measure the concentration of the total protein We

loads 40 µg lysate proteins into SDS-polyacrylamide 12% separation gels (gels). Then the PVDF membranes were immersed and blocked in BSA (5%) in Tris-buffered saline (TBS) for a period of 1 h, then PVDF membranes were incubated using primary antibody (#4247 for PI3k 110α, #2870s for Bcl-2, #5023s for BAX, #9272s for AKT, #4060s for p-AKT, CST; ab182136 for β2AR, abcam; NB200-103 for p53, NOVUS) at 4 °C overnight, washed three times using TBST (10 min each time), and incubated with anti-mouse or anti-rabbit Ig G secondary antibody (CST, USA) for 1 h at room temperature. TBST was used to wash the PVDF membranes for 3 times (10 min each time). At the final step, the ECL chemiluminescence reagent (Pierce, USA) was used to measure the antibody-bound proteins.

2.12. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

After intratracheal instillation of PM_{2.5} for 30 days, the SD rat was sacrificed. The prepared heart tissues were dehydrated with gradient alcohol after being embedded using conventional paraffin. Myocardial apoptosis was measured with TUNEL detection Kit (Roche, USA). We conducted the procedures strictly according to the manufacturer's instructions. We stimulated DAPI using ultra violet (UV) light (365 nm) in the same field of view under the fluorescence microscope (Olympus, X81, Japan), then under microscope the blue light was observed and the pictures were obtained. Next in order to stimulate TUNEL, we replaced UV light from 365 nm to 565 nm. Pictures were taken after green light appeared. We selected 5 non-overlapping pictures randomly from the heart specimen of SD rats. TUNEL positive cells rate/index was defined as apoptotic positive cells number/total cellular number.

2.13. Ultra-structural and histopathological evaluation of heart tissue

The heart specimen preparation of ultra-structural and histopathological evaluation was conducted immediately after the SD rat was sacrificed. And then we did physiological observation subsequently. The rats were sacrificed using anesthesia with an intraperitoneal administration of chloral hydrate (0.3 mL/100 g) (Beyotime, China) (10% mass fraction). Heart specimens were excised into small pieces after they were cut from heart tip. Heart specimen was fixed in 2.5% glutaraldehyde in PBS as to prepare the tissue for next ultra-structural evaluation, and then heart specimens were fixed in buffered osmium tetroxide (1%). At the same time, another piece of heart specimens were preserved in 4% paraformaldehyde (Beihua, China) in phosphate-buffered saline (PBS, pH 7.4) for next hematoxylin and eosin (HE) staining. Heart specimens were embedded in paraffin and cut into smaller sections. Then the small sections were stained using hematoxylin and eosin and then were evaluated histopathologically under a light microscope.

2.14. Immunohistochemical staining

Immunohistochemical staining was conducted as previously described (Duan et al., 2014). The expression levels of β2AR and VEGFR2 were measured immunohistochemically in the paraffin-embedded heart specimens and rehydration, as for antigen retrieval, the heart specimens were immersed in a citrate buffer solution (10 mM, pH 6.0). The heart specimens were treated for 5 min with 3% H₂O₂ in PBS and later washed using PBS in order to quench endogenous peroxidase activity. Then the specimens were blocked with 10% normal serum at 37 °C for 10 min. We incubated the specimen using an equivalent amount of normal IgG as negative control or primary antibody overnight at 4 °C. The heart samples were then stained with 3–3' diaminobenzidine substrate after treated with an avidin-biotin affinity system at room temperature for 30 min, and then counter-stained with hematoxylin. Under observation, a brown staining represents positive expression of β2AR and VEGFR2. Outcome was calculated as the total positive cell numbers from 50 continuous and random observation zones in each heart

specimens. Moreover, the pathologist was blinded to all the analyses of the slides.

2.15. Echocardiography

We performed echocardiography after the last PM_{2.5} treat prior to the SD rats was sacrificed. Rat was removed from our study if it did not meet the study inclusion criteria. For better operation, rats were lightly anesthetized with inhalation of 0.75–1% Isoflurane in 100% oxygen mixture through a nose cone. SD rats were set in a supine position. A high resolution imaging system (Vevo 2100 Imaging System, FUJIFILM VisualSonics Inc., USA) was used to obtain Two-dimensional (2D) guided M-mode echocardiography. Heart dimensions including left ventricular diastolic and systolic diameters (LVIDs, LVIDd), left ventricular posterior wall thickness and left ventricular posterior wall thickness (LVPW, LVAW) were get from echocardiograph M-mode tracings. Ejection fraction (EF), Stroke volume (SV) and Fractional shortening (FS) was obtained using defined calculations. Each parameter was measured in at least 3 cycles. Typically, we analyzed the data from 3 independent cardiac cycles. We calculated LV fractional shortening (FS%) as [(LV diastolic diameter–LV systolic diameter)/LV diastolic diameter] × 100 to represent the contractile function of heart. We conducted the experiment based on the American Society of Echocardiography guidelines.

2.16. Statistical analysis

All the assays were repeated more three times independently, result was expressed as mean ± S.D. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). SPSS 18.0 software (SPSS, USA) was conducted to do the statistical analysis. Statistical significance of differences in experiment with only one independent variable was assessed by two tailed unpaired Student's *t*-test. Differences in experiments with more than one independent variable were analyzed by One-way ANOVA. Differences were considered significant when *p* < 0.05.

3. Results

3.1. PM_{2.5} induced toxicity to cardiomyocytes including cell membrane damage, oxidative damage and apoptosis

To evaluate the cytotoxicity caused by PM_{2.5}, CCK8 measurement and LDH release detection were performed after exposing AC16 to PM_{2.5} for 24 h. Cell viability of AC16 after different dose of PM_{2.5} exposure showed no significant change (*p* > 0.05) with the concentration of 25 µg/mL. But as time passed, we can see cell viability decreased remarkably at 50 µg/mL and 100 µg/mL treated group contrast with no treating group. The cell viability in 50 µg/mL and 100 µg/mL group were significantly decreasing (*p* < 0.05) after PM_{2.5} exposure for 24 h. Result revealed PM_{2.5} caused cell death through a dose and time-dependent manner (Fig. 1A). 50 µg/mL PM_{2.5} was used for next assay including cell membrane damage, oxidative damage measurement, apoptosis detection and so on. As we known, LDH release showed the membrane damage. LDH release is strongly in accordance with the CCK8 results (Fig. 1B). In next measurement, compared with control, ROS levels and production of MDA in PM_{2.5} group were significant higher (Fig. 1D and Fig. 1E), followed by the decreasing in SOD and GSH-Px in cardiomyocytes AC16 (Fig. 1G and Fig. 1H). It is documented that antioxidant contained GSH-Px, SOD was involved in defense system to maintained the redox homeostasis, which is responsible for eliminating a wide range of oxidants, including ROS, MDA (Soeur et al., 2015). And moreover, significant higher apoptotic rate was detected in AC16 after PM_{2.5} exposure for 24 h (Fig. 1F and Fig. 1I). So our result indicated the PM_{2.5} induced serious cytotoxicity including cell death, cell membrane damage, oxidative damage and apoptosis in cardiomyocytes AC16.

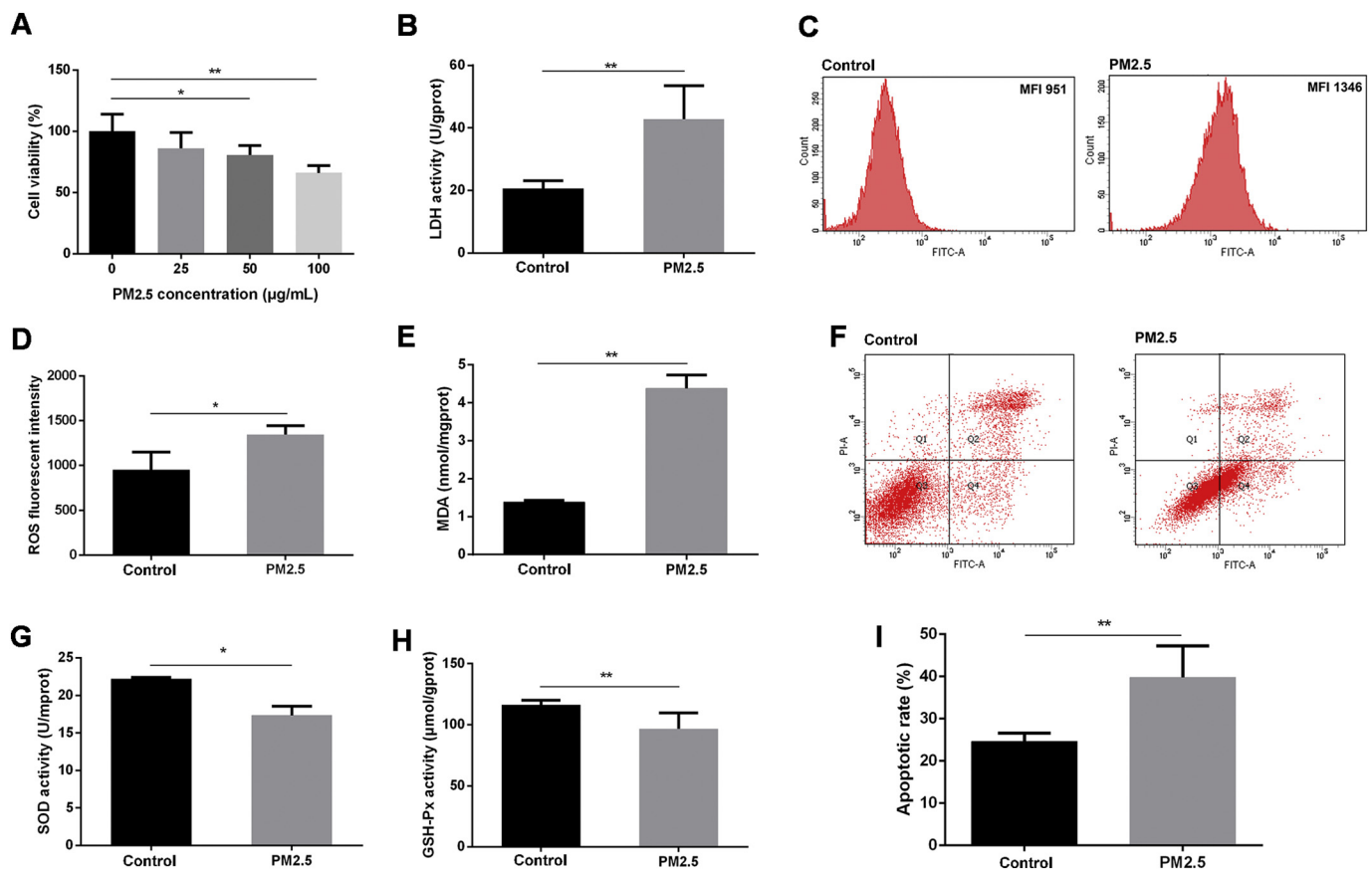


Fig. 1. PM_{2.5} induced cytotoxicity and apoptosis in cardiomyocytes AC16. **A**, cell viability was measured by CCK8 assay after AC16 was exposed to different concentration (0, 25, 50, 100 µg/mL) of PM_{2.5} for 24 h. **B**, LDH leakage was measured after AC16 was treated with PM_{2.5} (50 µg/mL) and DMEM for 24 h. **C** and **D**, ROS fluorescence intensity pictures (**C**) were got from flow cytometry, intracellular levels of ROS generation (**D**) was detected after AC16 was treated with PM_{2.5} (50 µg/mL) and DMEM for 24 h. **E**, MDA level increased obviously after AC16 was treated with PM_{2.5} compared with control. **G** and **H**, 24 h PM_{2.5} exposure induced the inhibition of SOD and GSH-Px in AC16. **F** and **I**, Apoptotic populations of cells (**F**) double stained with PI- and FITC-labeled Annexin V showed apoptotic rate (**I**) in AC16 was measured by flow cytometry after 24 h PM_{2.5} exposure. At least three independent experiments were performed for flow cytometric analysis. **p* < 0.05, ***p* < 0.01, Data are expressed as means ± S.D. from three independent experiments at least.

3.2. PM_{2.5} induced epigenetic alteration and DNA methylation profiling identified the key gene-ADRB2 in cardiomyocytes

To explore the epigenetic alteration in AC16 after PM_{2.5} exposure, we conducted methylation chip using Illumina HumanMethylation450K BeadChip. β difference > 0.12 or < -0.12 and *p*-value < 0.05 is the criteria we set in our study. A total of 13 differentially methylated CpGs were identified in PM_{2.5} groups compared with control (Fig. 2A). Among them, (69.23%) were hypermethylated and 4 (30.77%) were hypomethylated (Table S1). CpGs hyper- and hypomethylation events in PM_{2.5} exposed in human cardiomyocytes AC16 occurred frequently in the body of genes or unrelated to any gene region (Fig. 2B). Additionally, the expected number of CpG sites was compared with the number of the differentially methylated CpG sites in the regions including S-shore, S-shelf, open sea, N-shore, N-shelf, island. Totally 10 differently methylation genes (DMGs) were associated with the differentially methylated CpGs (DMCs) (Fig. 2C). The top DMGs methylation status is presented in Fig. 2D, which showed ADRB2 is hypermethylation. Go and KEGG analysis revealed the GO terms and important KEGG pathways which PM_{2.5} may trigger (Fig. 2E and Fig. 2F). Top go terms including beta2-adrenergic receptor activity, adrenergic receptor signaling pathway, positive regulation of apoptotic signaling pathway and so on (Table S2). What's more, among the top 10 pathways, there are four are cardiac/heart relative pathways including adrenergic signaling in cardiomyocytes, cGMP-PKG signaling pathway, calcium signaling pathway, cAMP signaling pathway. It is significant

and meaningful that a key gene-ADRB2 was involved in all the four cardiac/heart relative pathways (Table S3). Overall, we found PM_{2.5} induced epigenetic alteration and DNA methylation chip revealed a very significant hypermethylation gene-ADRB2. It is interesting and meaningful to investigate ADRB2 and the impact ADRB2 functioned on cardiomyocytes apoptosis in next experiment.

3.3. PM_{2.5} caused myocardial ADRB2 hypermethylation and thus led to down-expression of β 2AR

In order to verify the methylation status of ADRB2, we conducted Bisulfite sequencing PCR (BSP) assay which is a powerful tool in DNA methylation study (Fig. 3A). Significant hypermethylation status was observed (*p* < 0.01) (Fig. 3B). In the next qRT-PCR analysis, decreased mRNA expression of ADRB2 was observed as PM_{2.5} concentration decreased (Fig. 3C). Overall, methylation chip and BSP assay both identified the hypermethylation status. And qRT-PCR identified β 2AR down-regulated after ADRB2 hypermethylation. After treated with methylation inhibitor 5-aza (5 µM), which have no significant cellular toxicity to AC16 at concentration of 5 µM. Then we found the PM_{2.5} induced ADRB2 hypermethylation was inhibited (Fig. 3E), meanwhile single 5-aza treated group has no significant compared with the control. As we know, usually DNA methylation can regulate gene expression through epigenetic modification (Tao et al., 2018). So our results showed PM_{2.5} directly caused ADRB2 hypermethylation and thus led to down expression of β 2AR.

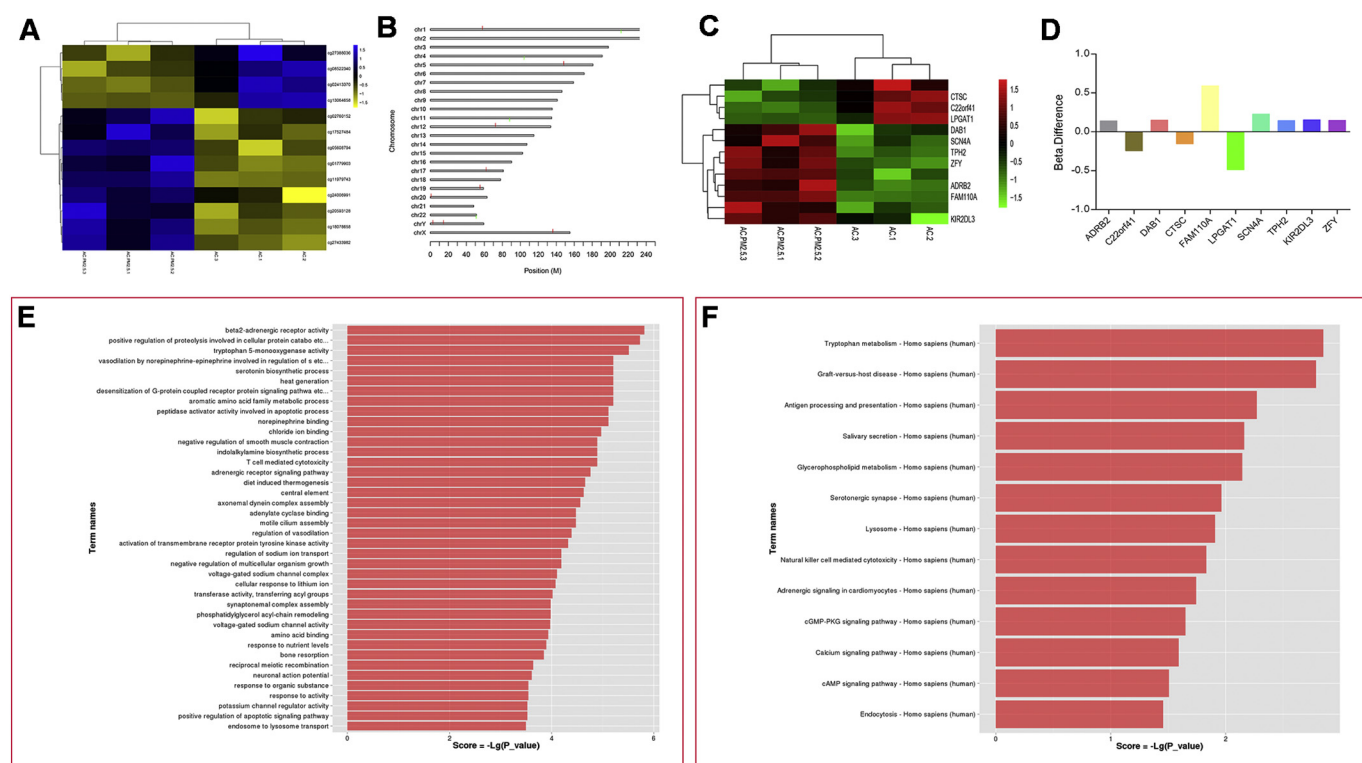


Fig. 2. Identification of DNA methylation alteration was conducted in AC16 after 24 h $PM_{2.5}$ exposure. **A**, Hierarchical cluster analysis and heat-map analysis to differential methylation status of DMCs. The heatmap revealed differential methylation level ranging between blue and yellow (hypomethylation to hypermethylation), respectively in the $PM_{2.5}$ -treated group, and control/untreated group in human cardiomyocytes AC16 cells line. The dendrograms at the top of the heatmaps was used to depict the clustering (based on z scores) of the samples. The names of different samples are indicated at the bottom of the heatmaps. "AC" represents control group and "AC $PM_{2.5}$ " represents $PM_{2.5}$ treated group. Colour bar at the left represents the colour scale reflecting methylation differences. **B**, The genome-wide distribution characteristics showed the location of 13 DMCs in the differential chromosomes. **C**, Hierarchical cluster analysis and heat-map analysis to differential methylation status of CpG sites relative DMGs. **D**, DNA methylation status of 10 DMGs. **E** and **F**, Bar graph of the top GO terms (**E**) and KEGG pathway analysis (**F**) based on DNA methylation profiling. The enriched terms were filtered with combined scores. The colour and size of the dots represent the $-\log_{10}$ (p-value) of each term. DMCs: Different methylation CpG sites; DMGs: Different methylaiton genes; Go: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. $PM_{2.5}$ induced ADRB2 hypermethylation/ β 2AR down expression mediated PI3K/Akt pathway trigger the cardiomyocytes apoptosis

PI3K/Akt pathway is the important mediator between β 2AR and the event of apoptosis. Further to understand the mechanism of ADRB2 hypermethylation/ β 2AR down expression induced apoptosis in cardiomyocytes AC16. After 24 h $PM_{2.5}$ exposure, Western blot results showed a decrease in expression of β 2AR, PI3K p-Akt/Akt at a concentration depend way (Fig. 3G, Fig. 3H and Fig. 3I). Meanwhile the expression of p53 in AC16 was obviously activated with the dose of $PM_{2.5}$ increasing (Fig. 3J). In our previous study, we found protein expression of Bcl-2 in cardiomyocytes AC16 decreased but BAX increased in an opposite way after 24 h $PM_{2.5}$ exposure (Yang et al., 2018). Taken together, Results showed that $PM_{2.5}$ induced ADRB2 hypermethylation/ β 2AR down expression mediated PI3K/Akt pathway further to trigger Bcl-2/BAX/p53 dependent Apoptosis.

3.5. Up-regulated ADRB2 impact on PI3K/AKT pathway and apoptosis in cardiomyocytes

To investigate the ADRB2/ β 2AR impact on PI3K/Akt pathway and apoptosis, high expression ADRB2 (hADRB2) recombinant enhanced green fluorescent protein (EGFP) plasmid pEGFP-N1-hADRB2 and empty pEGFP-N1 plasmids was used to do gene transient transfection in cardiomyocytes AC16 after $PM_{2.5}$ exposure. Fluorescence picture show our high expression cell model was successfully (Fig. 4A). Then we conducted qRT-PCR to verify the transfection efficacy of the

recombinant plasmids. The β 2AR expression of pEGFP-N1-hADRB2 group was 60-fold of the pEGFP-N1-empty group and the control (blank) (Fig. 4B). Western blot also showed the expression of β 2AR in pEGFP-N1-hADRB2 group was significant higher than pEGFP-con group and control (blank) (Fig. 4C). In next step, qRT-PCR was conduct to examine up-regulated ADRB2 impacted on the PI3K/AKT pathway. Compared with pEGFP-con group, relative mRNA expression in hADRB2 have no change, but PI3K/Bcl-2/BAX expression in $PM_{2.5}$ + pEGFP-hADRB2 group in significant increased compared with $PM_{2.5}$ + pEGFP-con. Results showed that high expression ADRB2 activated the PI3K/Akt and thus inhibited Bcl-2/BAX or p53 to decrease the apoptosis.

In apoptosis assay, we found that PI3K inhibitor LY294002 increased the apoptosis and high expression ADRB2 inhibited the apoptosis compared with control. Meanwhile, high expression ADRB2 decreased the $PM_{2.5}$ induced apoptosis and LY294002 increased the $PM_{2.5}$ induced apoptosis (Fig. 5A and Fig. 5B). Then western blot was conduct to examine up-regulated ADRB2 or inhibitor of PI3K impacted on the PI3K/Akt pathway (Fig. 5C and Fig. 5D). Compared with pEGFP-con group, relative protein expression in hADRB2 have no change, but PI3K/Bcl-2/BAX expression in $PM_{2.5}$ + pEGFP-hADRB2 group in significant increased compared with $PM_{2.5}$ + pEGFP-con. Results showed that high expression ADRB2 activated the PI3K/Akt and thus inhibited Bcl-2/BAX/p53 to decrease the apoptosis, but $PM_{2.5}$ and LY294002 triggered apoptosis in an opposite way.

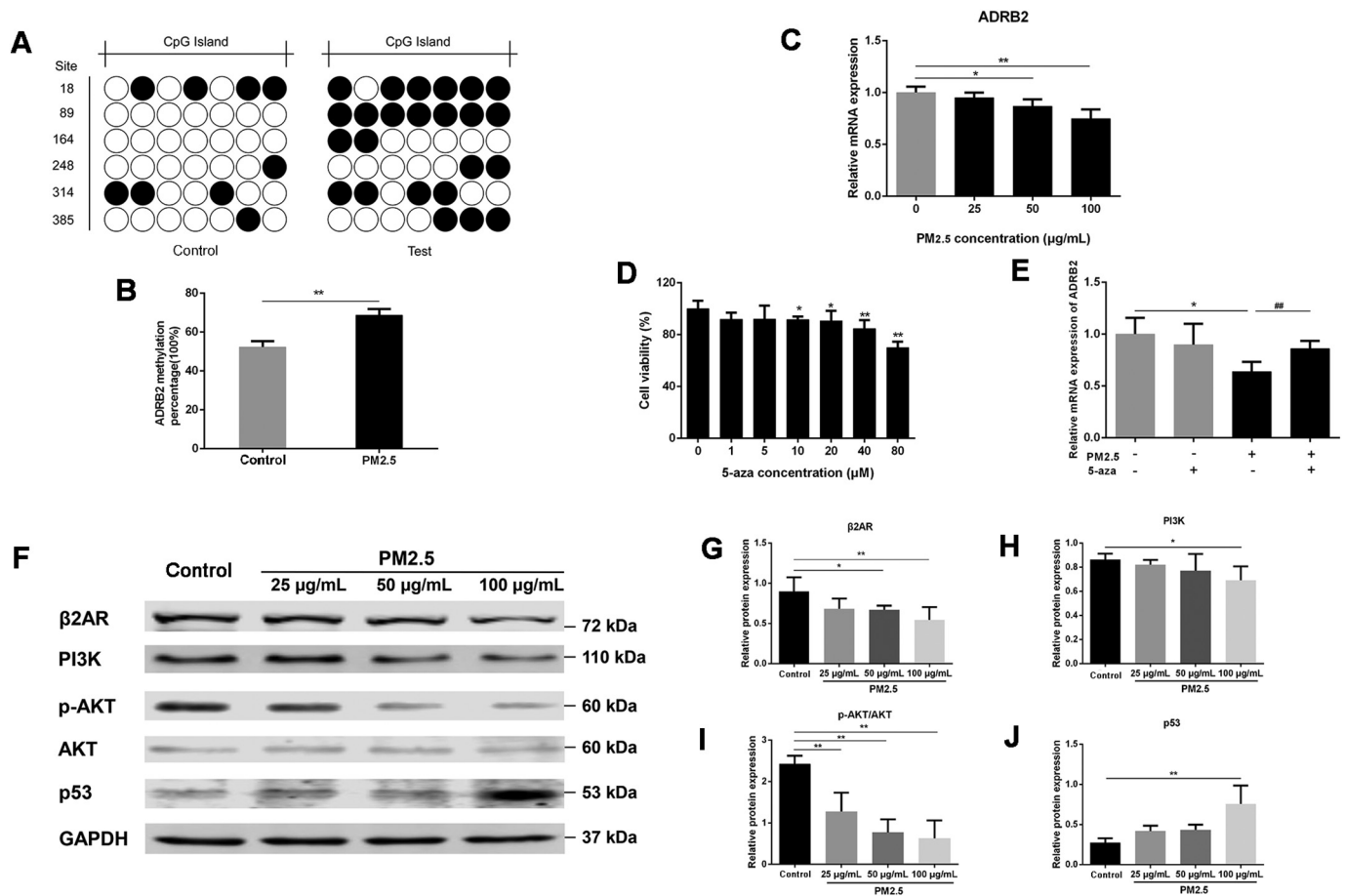


Fig. 3. *ADRB2* hypermethylation in $PM_{2.5}$ -trigger PI3K/Akt pathway. **A**, Methylation status of cytosine residues in *ADRB2* promoter (+18 to +385 bp) was detected after 24-h exposure to 50 μ g/mL of $PM_{2.5}$ or control/pure DMEM. **B**, BSP assay identified the hypermethylation status of *ADRB2* ($p < 0.01$). **C**, qRT-PCR analysis of *ADRB2* in AC16 after 24 h $PM_{2.5}$ exposure at different concentrations (0, 25, 50, 100 μ g/mL). **D**, Cell viability measurement after AC16 was treated with different concentration of gene methylation inhibitor 5-aza for 24 h, and 5 μ M with no significant cytotoxicity was used in next experiment. **E**, qRT-PCR analysis of *ADRB2* after $PM_{2.5}$ and/or 5-aza treated for 24-h. **F**, Western blot results showed a significant change in the protein expression of the $PM_{2.5}$ treated groups compared to control after 24 h exposure. **G**, Relative protein expression of β 2AR. **H**, Relative protein expression of PI3K. **I**, Relative protein expression of p-AKT/AKT. **J**, Relative protein expression of p53. * $p < 0.05$, ** $p < 0.01$, Data are expressed as means $\pm$ S.D. from three independent experiments. BSP: Bisulfite Sequencing PCR.

3.6. $PM_{2.5}$ exposure induced myocardial interstitial edema and ultra-structural changes in vivo

As shown in Fig. 6A, the histopathological examination was conducted to find out whether $PM_{2.5}$ exposure could induce pathological changes in heart tissues of SD rats. This suggested that there existed no significant toxicity to myocardium after rats exposure to control or low-dose, middle-dose $PM_{2.5}$, but there existed harmful effects to myocardium after exposure to high-dose $PM_{2.5}$. In other hand, heart ultra-structural damage measurement was performed. Under the observation of by TEM (magnification of 20,000 $\times$), myocardial gap expansion and myofibril disorder in middle-dose and high-dose treated group were significantly observed in rats heart tissue compared with control (Fig. S2), what's more, the mitochondrial damage including mitochondrial fission, mitochondrial swelling, mitochondrial crista disorder or vacuolization were observed. The observed adverse toxic effects were weaker in low-dose exposure group compared with the middle-dose and high-dose exposure group. Results suggested that $PM_{2.5}$ exposure can exhibited damage to mitochondria, cardiomyocytes and myocardium.

3.7. $PM_{2.5}$ exposure decreased cardiac function of SD rats

SD rats were exposed to high dose- $PM_{2.5}$ or normal saline through intratracheal instillation for 30 days. LVFS ($32.79 \pm 1.59\%$) (Fig. 6C), LVIDd (9.04 ± 0.62 cm) (Fig. 6E) and LVPW (1.21 ± 0.11 cm)

(Fig. 6G) and EF ($57.71 \pm 3.44\%$) (Fig. 6G) in $PM_{2.5}$ exposure group decreased significantly ($p < 0.05$), compared with LVFS ($26.68 \pm 0.49\%$), LVIDd (8.16 ± 0.59 cm), LVPW (0.94 ± 0.17 cm) and EF (59.77 ± 2.57) of control. But there was no statistically significant between control and $PM_{2.5}$ exposure in LVIDs (6.08 ± 0.65 cm) (Fig. 6F) and LVAW (1.51 ± 0.25 cm) (Fig. 6G), compared with LVIDs (6.56 ± 0.41) and LVAW (1.33 ± 0.26 cm) of control group. What's more, there is no significant difference in heart rate (HR) between $PM_{2.5}$ exposure and control (Fig. S2C). Stroke volume (SV) decreased significantly after $PM_{2.5}$ exposure compared with control (Fig. S2E), Cardiac output (CO) decreased significantly after $PM_{2.5}$ exposure compared with control (Fig. S2D). And there is a significant difference in left ventricle end-systolic volume (LV EDV) after SD rat exposed to high-dose $PM_{2.5}$ (Fig. S2A). Overall, those results demonstrated that SD rats exhibited decreased cardiac function after $PM_{2.5}$ intratracheal instillation. This may indicated that $PM_{2.5}$ -induced cardiomyocytes apoptosis lead to the loss of cardiomyocytes, thus injured contractile and decreased cardiac function.

3.8. β 2AR was down expression in heart tissue while apoptosis increased after $PM_{2.5}$ intratracheal instillation in vivo

In order to investigate the apoptosis in SD rat heart tissue, SD rat was treated with concentrated $PM_{2.5}$ intratracheal instillation for 30 days. TUNEL test was conducted (Fig. 7A) and TUNEL staining

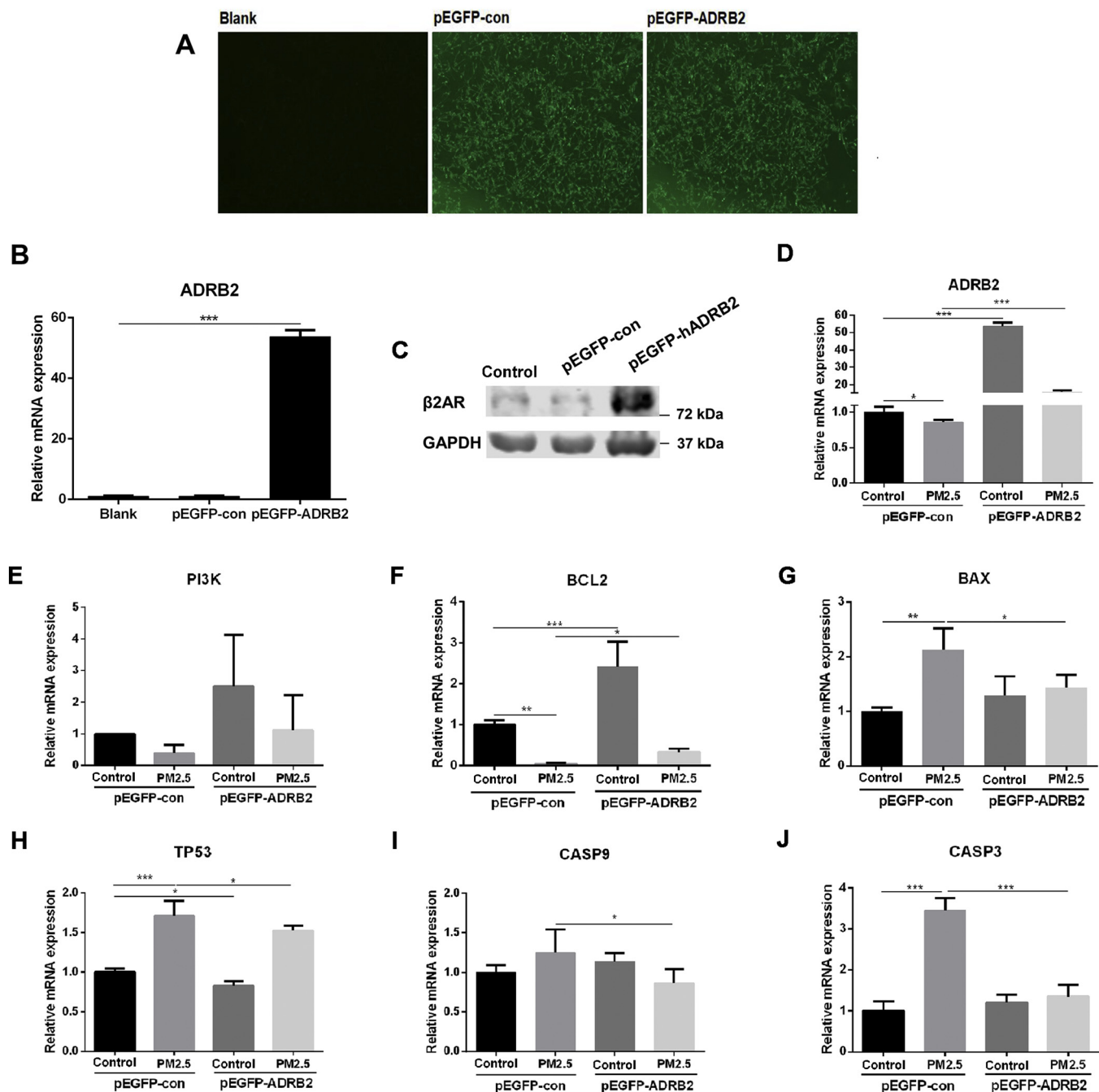


Fig. 4. pEGFP-hADRB2 transfection assay and qRT-PCR analysis after transfection in PM_{2.5}-treated AC16. **A**, Fluorescence picture showed the transfection efficiency in AC16 cells (magnification, ×10). pEGFP-con indicates cells transfected with empty pEGFP-N1; pEGFP-hADRB2 indicates cells transfected with ADRB2 over-expression pEGFP-N1 recombinant plasmid. pEGFP: enhanced green fluorescent protein plasmid. **B**, qRT-PCR analyzed the mRNA expression of ADRB2 after recombinant plasmid transfection. **C**, Western blot showed β2AR was significantly increased after AC16 was transfected with pEGFP-hADRB2 recombinant plasmid. **D**, **E**, **F**, **G**, **H**, **I** and **J**, qRT-PCR analysis for relative mRNA expression of ADRB2, PI3K, BCL2, PI3K, TP53 and CASP9 after recombinant plasmid transfection and/or PM_{2.5} exposure in AC16. **p* < 0.05, ***p* < 0.01 compared with control group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 compared with PM_{2.5} + pEGFP-con. Data are expressed as means ± S.D. from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results showed TUNEL positive cells in middle-dose and high-dose group was significant higher than the control (Fig. 7B). Results identified PM_{2.5} induced apoptosis in heart tissue. Meanwhile, immunohistochemical staining was conducted and we found β2AR expression was decreased while the PM_{2.5} dose increased (Fig. 7C and Fig. 7D). Meanwhile TUNEL detection showed that the apoptosis positive cells increased significantly while the dose of PM_{2.5} intratracheal instillation decreased *in vivo*. Overall, TUNEL result and

immunohistochemical staining revealed β2AR down expression in heart tissue while apoptosis increase after PM_{2.5} intratracheal instillation.

3.9. Down-expression of ADRB2 impacted the PI3K/Akt pathway after PM_{2.5} exposure *in vivo*

After intratracheal instillation of PM_{2.5} for 30 days, the western blot assay was conducted to detect the PI3K/Akt pathway in SD rat's heart

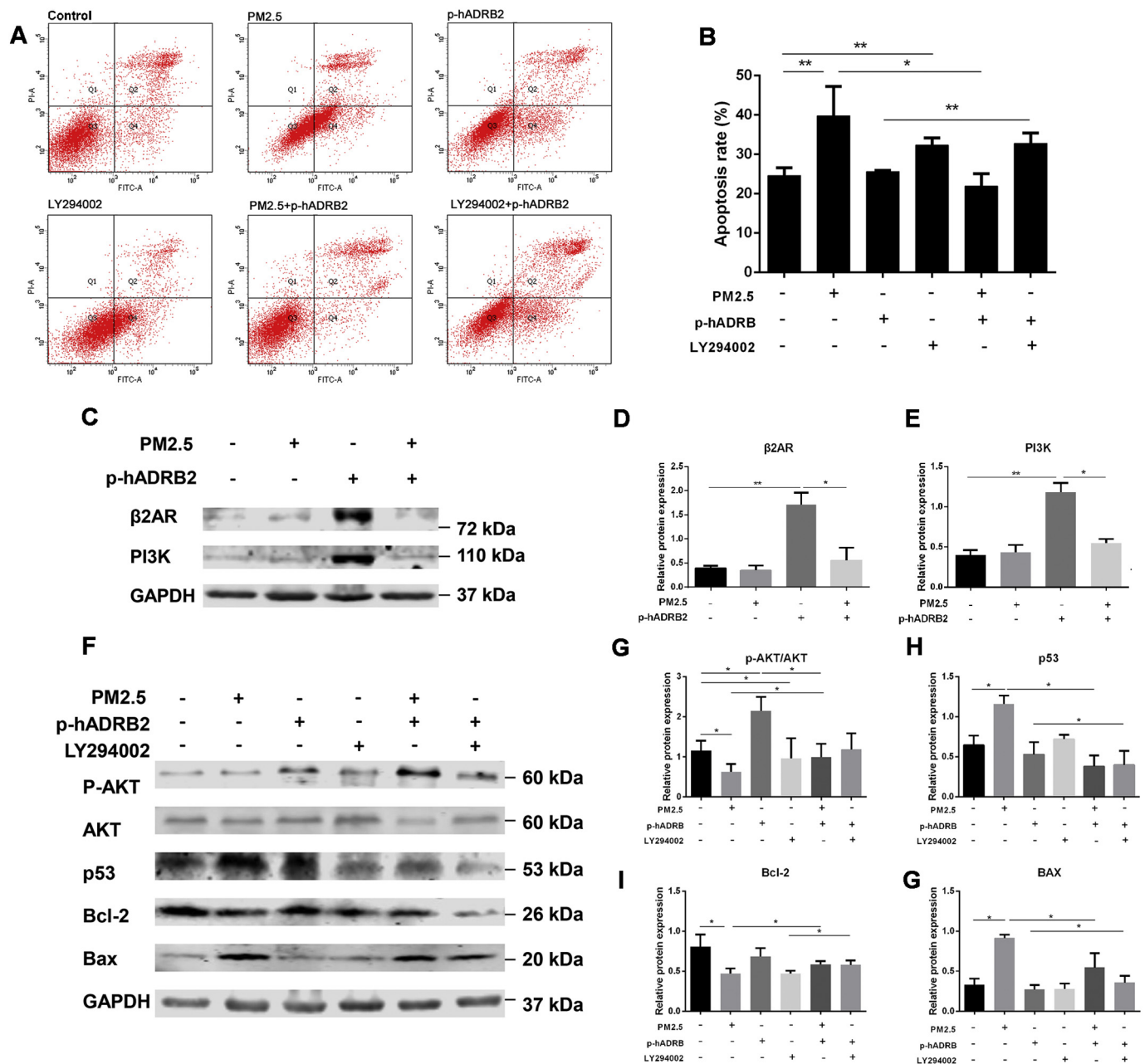


Fig. 5. Apoptosis and PI3K/Akt pathway relative protein expression measurement after AC16 was transfected with pEGFP-hADRB2 plasmid and/or treated with PM_{2.5} or PI3K inhibitor LY294002. **A**, Apoptotic populations of cells double stained with PI- and FITC-labeled Annexin V were measured by flow cytometry. **B**, The apoptotic rate of AC16 changed after AC16 was transfected with pEGFP-hADRB2 plasmid and/or treated with PM_{2.5} or PI3K inhibitor LY294002. **C**, Western blot results showed a significant change in the protein expression after AC16 was transfected with pEGFP-hADRB2 plasmid and/or treated with PM_{2.5}. **D** and **E**, Western blot results showed relative protein expression of β2AR and PI3K. **F**, Western blot results showed a significant change in the relative protein expression after AC16 was transfected with pEGFP-hADRB2 plasmid and/or treated with PM_{2.5} and/or LY294002. **G**, **H**, **I** and **J**, Western blot results showed the relative protein expression of p-Akt, Akt, p53, Bcl-2 and BAX. **p* < 0.05, ***p* < 0.01, Data are expressed as means ± S.D. from three independent experiments.

tissue (Fig. 7E). Result from western blot showed a decrease in expression of β2AR, PI3K, p-Akt/Akt, Bcl-2 at a concentration depend manner (Fig. 7F, Fig. 7G, Fig. 7H and Fig. 7J). But the expression of p53 and BAX in cardiomyocytes AC16 were obviously activated with the dose of PM_{2.5} increasing (Fig. 7F and Fig. 7G). Taken together, results showed that down expression ADRB2 impact Bcl-2/BAX and p53 dependent Apoptosis by regulating PI3K/Akt Pathway *in vivo*. The putative schematic representation of ADRB2 hypermethylation/β2AR down expression mediated PI3K/Akt pathway in PM_{2.5}-triggered trigger the apoptosis *in vivo* and *in vitro* was presented in Fig. 8.

4. Discussion

PM_{2.5} is deemed an important environmental factor that contribute to heart disease morbidity and mortality (Parker et al., 2018; Pope 3rd et al., 2006), but the potential mechanism is not clear. Previous studies indicated air pollution could cause DNA methylation and impact the progress of heart disease. Since the gene methylation represents a powerful epigenetic approach to investigate target gene and biological process, thus it might make the molecular mechanism of PM_{2.5} on heart disease more clearly. Here, our research explored the PM_{2.5} epigenetic effect on heart and firstly identified crucial role of ADRB2 hypermethylation as a modulator of cardiomyocytes apoptosis contributes

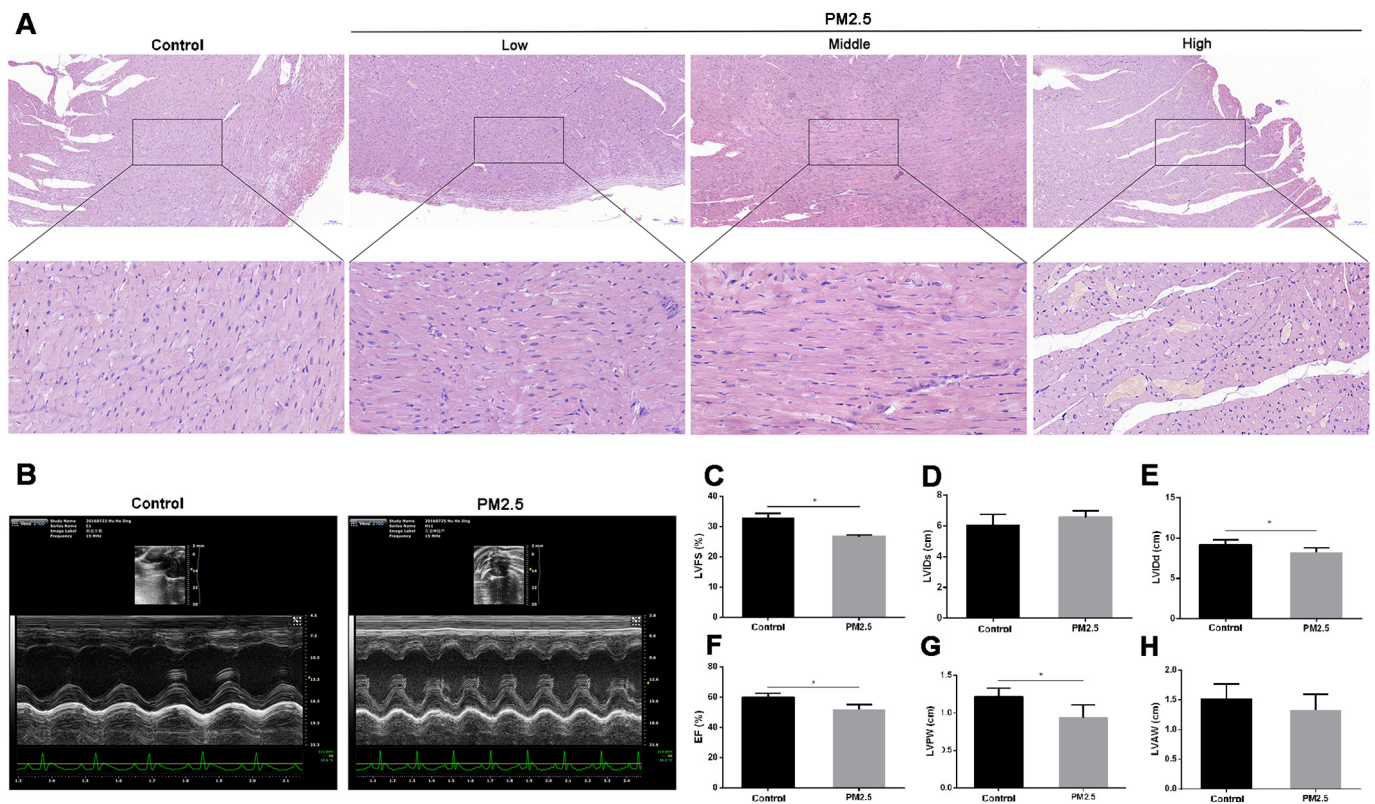


Fig. 6. Heart histopathological changes and cardiac dysfunction of SD rats after exposure to PM_{2.5}. **A**, Hematoxylin and eosin-stained abdominal heart: pathological changes on myocardial tissue. The blow panels are high magnification (40×) images corresponding to the upper panels (10×). **B**, Representative M-mode echocardiographic recordings are shown from control and high dose PM_{2.5}-intratracheal instillation SD rats after 30 days. **C**, Changes in LVFS in control and PM_{2.5} exposure rats. **D**, There was a statistically significant between control and PM_{2.5} exposure in LVIDs. **E**, Changes in LVIDd in control and PM_{2.5} exposure rats. **F**, There was a statistically significant between control and PM_{2.5} exposure in EF. **G**, Changes in LVPWd in control and PM_{2.5} exposure rats. **H**, Changes of LVAWd in control and PM_{2.5} exposure rats. Data are expressed as means ± S.D. **p* < 0.05, ***p* < 0.01. LVFS, left ventricular fractional shortening. LVIDs, left ventricular internal systolic diameters, LVIDd, left ventricular internal diastolic diameter. EF, ejection fraction. LVPWd, diastolic left ventricular posterior wall thickness. LVAWd, diastolic left ventricular anterior wall thickness.

to cardiac dysfunction via PI3K/Akt pathway.

Firstly, cytotoxicity indicators were measured in order to get closer insight in PM_{2.5}-induced cardiomyocytes toxic effects. After PM_{2.5} exposure, result showed PM_{2.5} induced cell viability decreased in a dose and time-dependent manner. The extracellular LDH releasing was significantly higher in PM_{2.5} treated group compared with control. As we known, the release of LDH could reflect the cellular membrane damage (Monnery et al., 2017) and LDH result was strongly in accordance with that of CCK8. Meanwhile, the ROS generation and production of MDA in PM_{2.5} group increased followed by the decreasing in GSH-Px and SOD compared with control. It is well-documented that antioxidant contained GSH-Px and SOD was involved in defense system to maintain the redox homeostasis, which is responsible for eliminating a wide range of oxidants, including ROS and MDA (Soeur et al., 2015). In previous study, oxidative damage emerged as a potential mechanism to explain the biologic response to PM_{2.5} (Brook et al., 2004; Pope 3rd et al., 2004). What's more, the significant higher apoptotic rate was detected after PM_{2.5} exposure compared with control (Fig. 1F). In consistent with our results, other reports revealed PM/PM_{2.5}-induced oxidative stress and apoptosis in other different cell types (Piao et al., 2018; Zhu et al., 2018). *In vivo* study, the amount of TUNEL positive cardiomyocytes increased significantly while the dose of PM_{2.5} for intratracheal instillation elevated (Fig. 7A), meanwhile, heart contractile function decreased and myocardial interstitial edema were also observed in high-dose PM_{2.5} group (Fig. 6).

Growing evidence demonstrated PM_{2.5} exposure is associated with global and specific gene methylation alteration (De Prins et al., 2013; Hou et al., 2011). In our study, a total of 13 DMCs and 10 DMGs were

identified after DNA methylation profiling (Fig. 2A and Table S1). The top DMGs methylation status is presented in Fig. 2D. Go and KEGG pathway analysis revealed the Go terms and KEGG pathways which PM_{2.5} might trigger (Fig. 2, Table S2 and Table S3). It is significant and meaningful that a key gene-*ADRB2* was involved in all the 4 cardiac/heart relative pathways. Overall, we found PM_{2.5}-induced global DNA methylation alteration and methylation chip revealed a significant hypermethylation gene-*ADRB2* (Beta. Difference = 0.14, *p* < 0.05). Next BSP assay again identified the significant hypermethylation status of *ADRB2* (*p* < 0.01). As we knew, investigation of PM/PM_{2.5} impact on specific gene methylation involved in cardiovascular disease is few, and the identified candidate genes including *F3*, *SERPINE1*, *ACE*, *EDN*, *NOS3*, *EDN1* et al. were just related to blood coagulation and vascular constriction (Chen et al., 2015; Tarantini et al., 2013; Wang et al., 2016). This is the first study focused on cardiac specific gene-*ADRB2* hypermethylation induced by PM_{2.5}. Next we observed decreased mRNA expression of *ADRB2* as PM_{2.5} concentration increased (Fig. 3C). Overall, DNA methylation chip and BSP both identified the significant hypermethylation status of *ADRB2*. And PM_{2.5}-induced *ADRB2* hypermethylation led to down-regulation of β₂AR thus may exert crucial role between PM_{2.5} and cardiac adverse effects.

Our previous study showed the main chemical components of PM_{2.5} used in this study as follow (Zhang et al., 2017): organic carbon (OC) and element carbon (EC) were the largest number of elements in PM_{2.5}, the average concentrations of OC and EC were 222.27 ± 35.55 and 47.97 ± 42.46 mg/g, respectively. In addition, the S, Si, K and Fe were the most abundant elements among the total of 28 inorganic elements of PM_{2.5}. And the heavy metals (including Pb, Mn, Cd, Cr and Ni) and

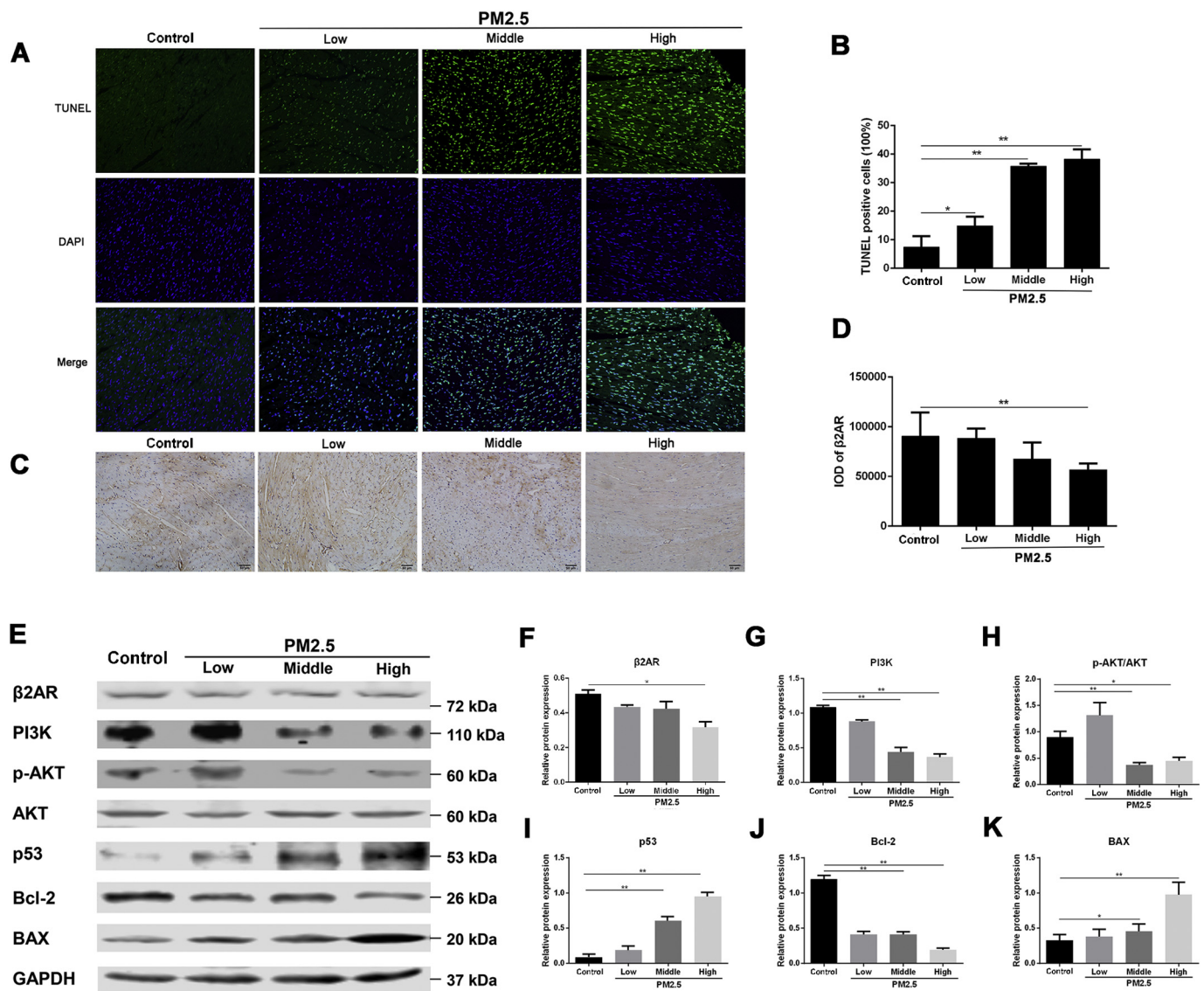


Fig. 7. PM_{2.5} induced myocardial apoptosis and triggered PI3K/Akt pathway *in vivo*. **A** and **B**, TUNEL fluorescence staining map ($\times 200$) (**A**) showed myocardial TUNEL positive apoptosis cells and myocardial tissue apoptotic indexes (**B**) of SD rats ($n = 6$) increased while the dose of PM_{2.5} elevated. **C** and **D**, Immunohistochemical staining (**C**) and bar graph (**D**) which indicating the mean staining intensity showed base *ADRB2* expression levels in heart tissues of SD rat after different concentration of PM_{2.5} exposure group ($n = 3$) ($\times 200$). **E**, Western blot results showed a significant change in the protein expression of SD rat heart tissue after different dose of PM_{2.5} treated compared to control. **F**, Relative protein expression of β 2AR. **G**, Relative protein expression of PI3K. **H**, Relative protein expression of p-AKT/AKT. **I**, Relative protein expression of p53. **J**, Relative protein expression of Bcl-2. **K**, Relative protein expression of BAX. * $P < 0.05$, ** $P < 0.01$ compared with control group; Data are expressed as means $\pm$ S.D. TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

nonmetallic elements (As) were also detected. As we all known, PM_{2.5} is one of the most complex air contaminants. Yet, until now, the lab evidence about the association between DNA methylation with PM/PM_{2.5}'s main chemical composition is limit. Epidemiology study revealed some evidence underling the main chemical constituent's toxic effect on DNA methylation. In the former study, Madrigano et al. found the increase in sulfate and black carbon (BC) exposures for 90 days were respectively associated with a decrease of 5-mC in LINE-1 and Alu, pointing to the influence of PM compositions (Madrigano et al., 2011). Dai et al. significantly observed 20 CpGs for Fe, 8 for Ni, and 1 for V, which may be relevant to relative pathways (Dai et al., 2017). Tarantini et al. found lower *NOS3* and *EDN1* methylation was related to PM and metal component exposure (Tarantini et al., 2013). Due to the complex components of PM_{2.5} and large number of genes methylation PM_{2.5} may trigger, more extensive work may be required in the future in order to clarify the main compositions of PM_{2.5}'s toxic effects/DNA methylation impact.

β 2AR subtypes are archetypal members of the GPCR superfamily and have much links to PI3K/Akt pathway (Bang and Choi, 2015). The stimulation of β 2AR plays a pivotal role in regulating myocardial function and morphology in the normal and failing heart. Previous study reported that β 2AR activates a Gi-G $\beta\gamma$ -PI3K-Akt signaling pathway. Therefore, it is well-known that PI3K consist of β 2AR-to-Gi pathway's intracellular messenger, which able to protect myocytes against Gs-mediated apoptosis via activation of the survival factor Akt. But PM_{2.5} impact on β 2AR mediated this pathway is not clear. Here, *in vitro* study, results showed the PM_{2.5} induced decreased expression of β 2AR, PI3K, p-AKT and Bcl-2 as PM_{2.5} concentration increasing, meanwhile p53 increased at an opposite way (Fig. 3). And in our previous study, we revealed PM_{2.5}-induced down-regulation of Bcl-2 and up-regulation of BAX (Yang et al., 2018). *In vivo*, immunohistochemical staining result reveled β 2AR down-expression in heart tissue while TUNEL positive cardiac myocytes increased after PM_{2.5} dose elevated by intratracheal instillation (Fig. 6A and Fig. 6B). Bcl-2 and BAX

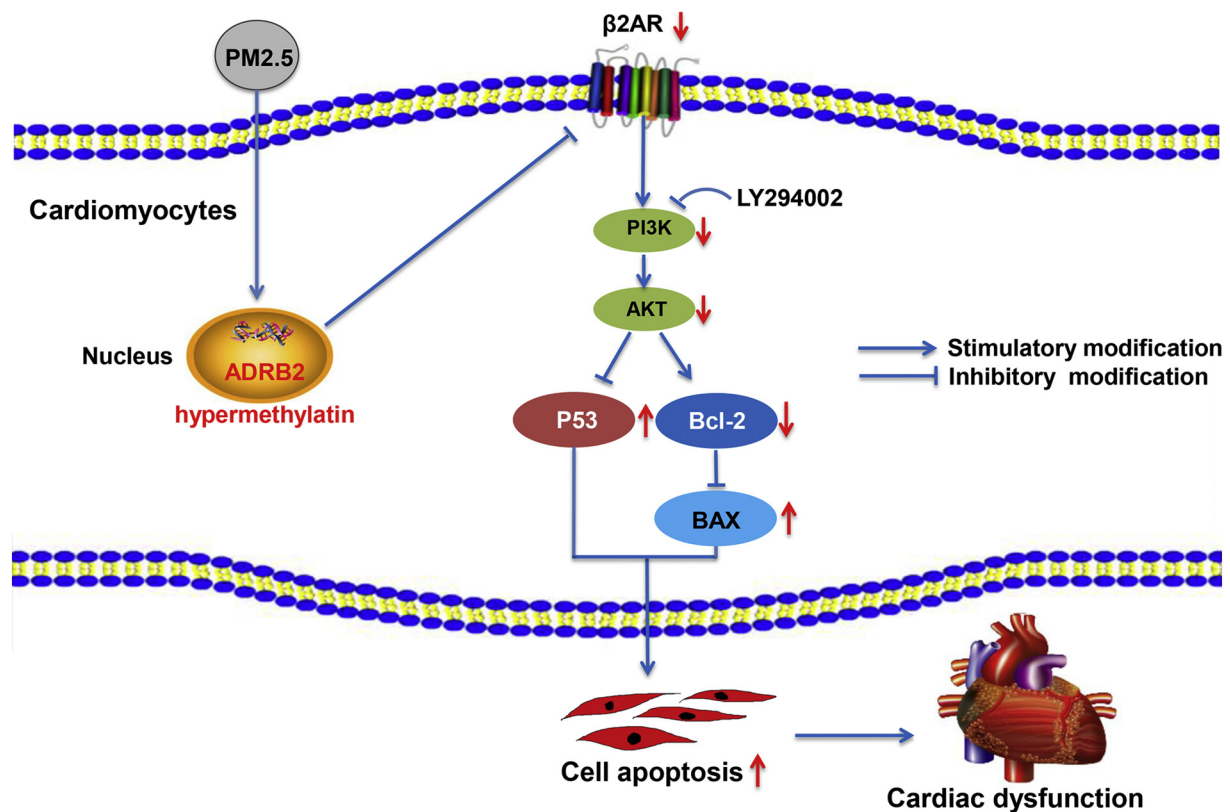


Fig. 8. Schematic illustration of the proposed molecular mechanism by which PM_{2.5} induce ADRB2 hypermethylation regulated cardiomyocytes apoptosis *in vitro* and *in vivo*. PM_{2.5} caused cytotoxicity including cell death, oxidative damage and so on. Meanwhile epigenetic alteration was occurred and PM_{2.5}-induced ADRB2 hypermethylation was ascertained. PM_{2.5}-induced ADRB2 hypermethylation induced down-regulation of β₂AR thus triggered apoptosis through the inhibition of the PI3K/Akt pathways followed by the up-regulation of p53 and Bcl-2/BAX. On the contrary, recombinant plasmid transfection showed overexpression of β₂AR can result in activation of PI3K/Akt and inhibition of Bcl-2/BAX and p53 thus weaken the PM_{2.5}-induced cardiomyocytes apoptosis, but was augmented by treatment with LY294002, a PI3K inhibitor. Therefore, PM_{2.5}-induced ADRB2 hypermethylation contributed to cardiac dysfunction through cardiomyocytes apoptosis via PI3K/Akt pathway.

proteins are the two important members of the Bcl-2 multi-gene family. BAX have a pro-apoptotic effect whereas Bcl-2 can prevent apoptosis (Adams and Cory, 1998). Usually, The Akt activation augments the expression of Bcl-2 anti-apoptotic gene through phosphorylation of cyclic AMP response binding protein (Pugazhenthil et al., 2000). Additionally, AKT is a master regulator involved in the transcriptional regulation of the anti-apoptotic protein Bcl-2 (Ghosh et al., 2013).

In our study, PM_{2.5} could induce inhibition of PI3K/Akt activated Bcl-2/BAX in similar way (Fig. 5 and Fig. 6). On the other hand, p53 regulates the transcription of genes associated with apoptosis (e.g., DR5, BAX, caspase-3 and so on) (Kawamoto et al., 1997). The activation of Akt could phosphorylate diverse downstream factors, including MDM2, p53. MDM2 comprises several conserved domains, which provide the structural basis for its functions. The N-terminal domain could bind to tumor suppressor protein p53 and inhibit the transcriptional activity of p53. MDM-2 could bind to p53 and make it ubiquitination for proteasomal degradation. Ubiquitination activity of MDM-2 can be enhanced when MDM-2 is phosphorylated by Akt at Ser-166/186 (Zhou et al., 2001). Thus our mechanistic study suggest that PM_{2.5}-induced ADRB2 hypermethylation could mediated PI3K/Akt pathway further trigger Bcl-2/BAX or p53 to augment cardiomyocytes apoptosis (Fig. 5 and Fig. 7).

To reaffirm the PM_{2.5}-induced ADRB2 hypermethylation impact on β₂AR-PI3K-Akt pathway, we do cell transfection using high-expression ADRB2 recombinant plasmid as a reversing verification method. Results showed that high expression of ADRB2 activated the PI3K/Akt and inhibited p53 and Bcl-2/BAX pathway. In apoptosis assay, activation of β₂AR/high expression ADRB2 protected cardiomyocytes from PM_{2.5}-

induced apoptosis, while PI3K inhibitor LY294002 augmented the apoptosis (Fig. 5A). In consistent with our result, Chesley et al. found that pre-treatment of cultured neonatal rat cardiomyocytes with β₂AR agonists can protected myocytes from apoptosis by the Gi dependent, PI3K-mediated mechanism (Chesley et al., 2000). Thus our study clearly demonstrated that PM_{2.5}-induce β₂AR down-expression play key role in cardiomyocytes apoptosis and cardiac dysfunction by regulating PI3K/Akt pathway *in vivo* and *in vitro*. *In vivo* study also identified cardiac function decreased and myocardial interstitial edema after PM_{2.5} exposure. As we knew, cardiomyocytes apoptosis participates in the myocardial loss thus lead to acute myocardial infarction and plays an important role on the process and the development of various heart diseases including HF, IHD and so on (Teringova and Tousek, 2017). Vascular endothelial growth factor prevents apoptosis and preserves contractile function (Friebs et al., 2006). On the contrary, we observed VEGFR2 expression in was significantly decreased as PM_{2.5} dose elevated in our study (Fig. S3). Hence, the β₂AR is essential for limiting PM_{2.5} induced cardiomyocytes apoptosis relative heart diseases.

At present, there was no credible alternative explanation about PM_{2.5}-induced heart disease exists since publication of the AHA scientific statement in 2010 (Pope 3rd et al., 2004). Our study strengthened the body of evidence and might provide one kind of treatment modalities on heart health outcomes. But our study has some limitations that must be acknowledged. Gene methylation chip revealed 10 novel genes and involving pathways, we study the function of the most significant and meaningful one, ADRB2. Other genes and pathways might also exert important role in the process involved in PM_{2.5} associated

heart disease. Related work is meaningful to perform the assays to exam the relation between other key genes/relative pathways and heart disease. We believe that despite this limitation, our study help provide a novel methylation mechanism to understand how PM_{2.5} exposure may exert epigenetic effects on heart.

5. Conclusions

In summary, this study was for the first time to provide both *in vitro* and *in vivo* evidence that PM_{2.5} exposure-induced *ADRB2* hypermethylation contributed to cardiac dysfunction through cardiomyocytes apoptosis via PI3K/Akt Pathway. Demethylation of *ADRB2* or activation of β 2AR in cardiomyocytes may be a novel molecular target for preventing PM_{2.5}-induced cardiac toxicity.

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Disclosures

The authors have declared that no conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.03.057>.

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