

Article

MicroRNA miR-212-5p Regulates the MEK/ERK Signaling Pathway by Targeting A-Raf proto-oncogene serine/threonine-protein kinase (*ARAF*) to Regulate Cowshed PM_{2.5}-Induced NR8383 Apoptosis

Ke Sun ^{1,2}, Yize Sun ¹, Yunna Jia ¹, Xinran Duan ¹, Zhenhua Ma ¹, Xiqing Zhang ¹, Lixia Wang ³, Yanbin Zhu ², Yunhang Gao ^{1,*}, and Wangdui Basang ^{2,*},[†]

¹ College of Animal Science and Technology, Jilin Agricultural University, Changchun 130118, China; sun155198@163.com (K.S.)

² Institute of Animal Husbandry and Veterinary Medicine, Tibet Academy of Agricultural and Animal Husbandry Science, Lhasa 850009, China

³ Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun 130102, China

* Correspondence: gaoyunhang@163.com (Y.G.); bw0891@163.com (W.B.)

[†] These authors contributed equally to this work.

Abstract: **Objective:** To investigate the role of miR-212-5p-targeted *ARAF* during the apoptosis of rat alveolar macrophages induced by cowshed PM_{2.5}. **Methods:** miRNA and related target genes and pathways were predicted using the KEGG, TargetScan, and other prediction websites. NR8383 macrophages were treated with cowshed PM_{2.5} to establish an in vitro lung injury model in rats; meanwhile, for the assessment of cell viability, apoptosis, intracellular calcium ions, and mitochondrial membrane potential in NR8383 cells, RT-qPCR was used to detect the expression of miR-212-5p and the target gene *ARAF*. **Results:** The bioinformatic analyses showed that miR-212-5p and *ARAF* were involved in PM_{2.5}-associated cellular damage. Exposure to different concentrations (0 µg/mL, 60 µg/mL, 180 µg/mL, 300 µg/mL) with different durations (0 h, 12 h, 24 h, 48 h) of cowshed PM_{2.5} resulted in apoptosis, increased intracellular calcium ions, and decreased mitochondrial membrane potential. The miR-212-5p mimic group showed an up-regulation of Bax and cleaved Caspase 3 expression but decreased Bcl2 expression compared to the NC group, and overexpression of *ARAF* up-regulated the expression of p-MEK1/2 and p-ERK1/2 and simultaneously reversed the above phenomena. **Conclusions:** miR-212-5p targets *ARAF* to affect the cowshed PM_{2.5}-induced apoptosis through the MEK/ERK signaling pathway, providing a potential target for relevant farming industry and pathology studies.

Keywords: cowshed PM_{2.5}; miR-212-5p; mitochondrial damage; MEK/ERK signaling pathway; apoptosis



Citation: Sun, K.; Sun, Y.; Jia, Y.; Duan, X.; Ma, Z.; Zhang, X.; Wang, L.; Zhu, Y.; Gao, Y.; Basang, W. MicroRNA miR-212-5p Regulates the MEK/ERK Signaling Pathway by Targeting A-Raf proto-oncogene serine/threonine-protein kinase (*ARAF*) to Regulate Cowshed PM_{2.5}-Induced NR8383 Apoptosis. *Toxics* **2023**, *11*, 981. <https://doi.org/10.3390/toxics11120981>

Academic Editor: David R. Wallace

Received: 11 November 2023

Revised: 30 November 2023

Accepted: 30 November 2023

Published: 3 December 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

PM_{2.5} has become one of the most important disease-causing factors today, with the World Health Organization (WHO) reporting that approximately 7 million people die every year from fine particulate pollution [1], which causes respiratory diseases [2]. PM_{2.5} has a complex composition that includes biocides, metal ions, aromatic hydrocarbons, and other disease-causing factors [3]. They spread where the air speed is high and pose a serious threat to the respiratory systems of humans and animals. Therefore, research on the pathophysiological mechanisms underlying the action of PM_{2.5} can provide a relevant entry point for PM_{2.5} prevention and control. Studies have shown that PM_{2.5} exposure can cause a variety of respiratory diseases, such as inflammation of airways and lung macrophages, pulmonary fibrosis, etc. [4–6]. At the same time, it may cause pyroptosis and ferroptosis, which can lead to pulmonary toxicity [7], particularly in livestock housing due to the dense

and relatively enclosed animal population. The resulting fine particulate matter enters the lungs, causing loss of alveolar capillary membrane integrity, which in turn leads to lung destruction, fibrosing alveolitis, and lung tissue damage [8]. The immune system, especially macrophages, is a sensitive and powerful defense mechanism against harmful factors, including PM_{2.5} [9]. Normally, alveolar macrophages immediately remove PM_{2.5} through the phagocytosis–secretion–immunization network [10]. However, when persistent PM_{2.5} enters the respiratory system, macrophages in the lungs are hyper-activated, leading to an increase in acute damage [11]. Activated macrophages secrete pro-inflammatory cytokines (tumor necrosis factor alpha, interferons, and interleukins) and produce cytotoxic reactive oxygen species (ROS) [12,13], which in turn leads to apoptosis. Most of the PM_{2.5} in farm buildings comes from animal bedding, feed, and animal production activities, and may contain pathogens, leading to increased animal health problems, which will directly lead to a decline in economic efficiency. Thus, it becomes urgent to explore the virulence mechanism of PM_{2.5} in animal housing, which has great significance for the health and safety of animals and humans.

Mitochondrial apoptosis is the main pathway of endogenous apoptosis [14]. When it is stimulated by external stimuli (e.g., PM_{2.5}), it causes a change in membrane potential which in turn leads to a change in mitochondrial membrane permeability and promotes apoptosis through the release and activation of apoptosis-related proteins including Bcl-2, Bax, Cyt-c, caspase 3, and caspase 9 [15,16].

The MAPK signaling pathway, which consists of RAS, RAF, MEK, and ERK, is involved in a variety of cellular response processes such as apoptosis, cell proliferation, and other processes [17]. There are three isoforms of RAF (*ARAF*, *BRAF*, and *CRAF*) that are phosphorylated by MEK [18]. The RAF family also has two pseudokinases, including a kinase inhibitor of Ras 1/2 (*KSR1/2*), which generally acts as a bridge between MEK and RAF and plays a key role in the activation of RAF [19]. Members of the RAF family bind to Ras-GTP and lead to its homo- and heterodimerization and kinase activation [20], which constitutes the first step in the kinase cascade. RAF phosphorylates and activates mitogen-activated protein kinases (MEKs), which in turn phosphorylates and activates extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2) [21]. Activating mutations in members of the RAF, MEK, and ERK families have all been identified in cancer, with *BRAF* mutations being by far the most common [22]. *CRAF* mutations also occur in human cancers, but less frequently in comparison with the RAF, MEK, and ERK families [23]. Both *BRAF* and *CRAF* encode active kinases, and knockdown of these kinases significantly reduces ERK signaling [24]. Kinase-independent functions of *CRAF* are associated with a variety of cellular phenotypes, including the regulation of mitochondria-dependent apoptosis by blocking the MST2/HIPPO pathway [25]. However, little has been reported in relation to *ARAF*, particularly in relation to *ARAF*-mediated MEK/ERK signaling pathways affecting mitochondrial dysfunction and thus apoptosis.

MicroRNAs (miRNAs) are non-coding RNAs that consist of 21–23 nucleotides and endogenously regulate cell development, proliferation, differentiation, and death by binding to the 3′ untranslated region (3′UTR) of target messenger RNA to promote their degradation [26]. As an important component of epigenetics, microRNAs might be valuable biomarkers of environmental exposure [27]. Particulate matter-induced alterations in microRNAs suggest their regulatory importance in PM-related diseases. Studies have shown that airborne particulate matter may contribute to bronchial inflammation and affect immune regulation in lung injury [28,29]. The expression of miRNAs is altered after PM_{2.5} exposure, and miR-4726-5p expression is elevated in response to PM_{2.5} stimulation and affects the development of coronary heart disease [30]. Exposure of H9C2 cells to PM_{2.5} results in the upregulation of miR-421 expression and downregulation of SIRT3 expression, triggering mitochondrial damage and consequent cardiovascular diseases [31]. Previous studies have shown that miR-212-5p is associated with the development of a variety of diseases, including ischemic brain injury [32], fat accumulation [33], nasopharyngeal carcinoma, and various cancers such as breast cancer [34]. However, there are limited reports

on the mechanism of miR-212-5p's regulation of apoptosis in lung injury under cowshed PM_{2.5} stimulation. Thus, we proposed a scientific hypothesis that miR-212-5p-targeted *ARAF* plays an important role in the mechanism of rat alveolar macrophage apoptosis induced by cowshed PM_{2.5} through the MEK/ERK signaling pathway.

In this study, using the *in vivo* rat infection model constructed by our group, we found that miR-212-5p was differentially expressed based on transcriptome data analysis [35], and *ARAF* was also predicted as its target gene through bioinformatic analyses. We investigated the mechanism of miR-212-5p-targeted *ARAF* in PM_{2.5}-induced apoptosis of rat alveolar macrophages *in vitro*, aiming to provide gene therapy targets and a scientific basis for PM_{2.5}-induced apoptosis triggering respiratory diseases at the level of miRNA regulation.

2. Materials and Methods

2.1. Chemicals and Reagents

DMSO (C₂H₆O₅, CAS: 67–68–5) was obtained from Sigma-Aldrich, Merck, Germany, while anti-MEK1/2 antibody (9G3) (sc-81504), anti-ERK1/2 antibody (C-9) (sc-514302), anti-Bax antibody (sc-7480), and anti-caspase 3 antibody (sc-56053) were obtained from Santa Cruz Biotechnology, USA. Anti-p-MEK1/2 (S217/221) antibody (9154T) was obtained from CST, USA, while anti-p-ERK1/2 antibody (AP0472) and anti-*ARAF* antibody (A16346) were obtained from ABclonal, China. Anti-Bcl-2 antibody (26593-1-AP), anti-cytochrome C antibody (66264-1-Ig), anti-GAPDH antibody (60004-1-Ig), anti-β-actin antibody (20536-1-AP), and secondary goat anti-rabbit and rabbit anti-mouse antibodies were purchased from Proteintech Group (Wuhan, China). Anti-caspase 9 antibody (WL03421) was obtained from Wanleibio, China.

2.2. PM_{2.5} Sample Collection

PM_{2.5} was collected using a multi-level flow particulate sampler (2030, Laoying Haina Photovoltaic Environmental Protection Group Co., Qingdao, China) from a dairy cattle farm in Changchun, Jilin Province [36–38]. The sampler was located at the center of the cow farm, 1.5 m above the ground, with a sampling flow rate of 100 L/min that operated continuously for 24 h. After collection, ultra-pure water without endotoxin was used for elution, which was then filtered through six layers of sterile gauze and freeze-dried using a vacuum lyophilizer. The final PM_{2.5} was made into a master batch of 1×10^4 µg/mL and preserved. Ultrasound was used to shake it well before each use to prevent it from caking into larger particles.

2.3. Cell Culture

Rat alveolar macrophage cell line (NR8383) and human embryonic kidney cell line (HEK-293T) were purchased from Shanghai Cell Bank, China, and cultured in DMEM medium (GIBCO, CA, USA) with 10% FBS (fetal bovine serum, GIBCO, CA, USA) and 1% penicillin–streptomycin (C0222, Beyotime Biotechnology, Shanghai, China). The cells were grown within a humidified 5% CO₂ atmosphere at 37 °C.

2.4. CCK8 Cell Viability Assay

We use the CCK-8 kit to detect cell viability (C0037, Beyotime Biotechnology, China). NR8383 cells were inoculated into a 96-well plates at a density of 5×10^3 cells per well. PM_{2.5} exposure treatments were performed in the cowshed using concentrations of 0, 60, 180, and 300 µg/mL, with 5 biological replicates for each concentration. A 10 µL volume of the CCK-8 working solution was added to each well and incubated for 1 h, then the absorbance value at 450 nm was detected using a Microplate Reader (Thermo Fisher, Waltham, MA USA). Cell viability was measured according to the manufacturer's instructions.

2.5. Plasmid Construction and Cell Transfection

We transfected miR-212-5p mimics and miR-212-5p inhibitor into NR8383 cells to construct miR-212-5p overexpression and silencing cell lines, respectively. miRNA NC and

miRNA inhibitor NC were the corresponding negative controls. For the overexpression of *ARAF*, we linked the complete CDS sequence of the *ARAF* gene to the pcDNA3.1 plasmid to construct the *ARAF* overexpression vector, with the blank pcDNA3.1 plasmid as the negative control. The miR-212-5p mimics, miR-212-5p inhibitor, miRNA NC, miRNA inhibitor NC, and pcDNA3.1 were purchased from GenePharma (Suzhou China). NR8383 cells were seeded into 6-well plates and cultured until about 60% confluence. The Lipofectamine 3000 Kit (Thermo Fisher, USA) was used to transfect 50 nM oligonucleotides or 2 µg plasmids into the NR8383 cells.

2.6. Determination of Calcium Ion Concentration

We used the Fluo-4 AM calcium fluorescent probe method to determine intracellular calcium levels according to the manufacturer's protocol (S1061S, Beyotime Biotechnology, China). NR8383 cells were first inoculated into cell culture plates, the medium was aspirated after reaching a density of 10^6 , and the cells were washed with PBS. Then, 500 µL of Fluo-4 working solution was added for staining and incubated at 37 °C for 30 min in the dark, after which, the cells were examined using a fluorescence microscope, and then the data were analyzed using Image J software 1.8.0.

2.7. Determination of Reactive Oxygen Species (ROS) Level

The intracellular ROS level was determined using a fluorescent probe: dichlorodihydro-fluorescein diacetate (DCFH-DA). The cells were first inoculated in cell culture plates overnight at a density of 1×10^5 /mL according to the manufacturer's protocol (S0033S, Beyotime Biotechnology, China). The cells were then observed for their growth status, the cell culture medium was aspirated, and 1 mL of diluted prepared DCFH-DA working solution was added to each well. The cells were then incubated in a humidified 5% CO₂ atmosphere at 37 °C for 20 min. The cells were washed three times with phosphate-buffered saline (PBS) and then collected, and their luminescence intensity was measured using a fluorescent microscope (Olympus, Tokyo, Japan). The experiment was repeated three times for each sample. Finally, the analysis was performed using Image Pro Plus software 6.0 (Media Contronetics, Rockville, MD, USA).

2.8. JC-1 Detection of Mitochondrial Membrane Potential

We used the Enhanced Mitochondrial Membrane Potential Assay Kit (JC-1) to determine the mitochondrial membrane potential ($\Delta\psi$ m) (C2003S, Beyotime Biotechnology, China). We suspended 5×10^5 cells in the cell culture solution, added a 500 µL JC-1 staining working solution, and incubated them at 37 °C for 20 min in a cell culture incubator. After the incubation was completed, the cells were collected, and the JC-1 dyeing buffer was washed twice. After being re-suspended, a fluorescent microplate reader (Spark, Tecan Group Ltd., Männedorf, Switzerland) and fluorescent microscope (Olympus, Tokyo, Japan) were used to measure the staining.

2.9. Real-Time Quantitative PCR

Total RNA was extracted from cells using a column total RNA extraction kit (B511321, Sangon Biotech, Shanghai, China) and reverse transcription into cDNA was performed using a PrimeScript™ RT kit (RR047A, TaKaRa, Dalian, China). The primers for miR-212-5p, *ARAF*, U6, and GAPDH listed in Table 1 were obtained from Sangon Biotech, Shanghai, China. RT-qPCR was performed according to the manufacturer's protocol for the RT-qPCR kit (RR820A, TaKaRa, Dalian, China). The relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method.

Table 1. The primers for qRT-PCR.

Gene	Primer Sequence (5'-3')
<i>ARAF</i>	Forward: CAGTGAGGTACAGCTGTTGAAG Reverse: GCCACCTTGAGCACTTCA
<i>GAPDH</i>	Forward: CCTGCACCACCAACTGCTTA Reverse: CATCACGCCACAGCTTCCA
U6	Forward: CTCGCTTCGGCAGCACA Reverse: AACGCTTCACGAATTTGCGT
miR-212-5p	Forward: CGCGACCTTGGCTCTAGACTG Reverse: AGTGCAGGGTCCGAGGTATT

2.10. Dual-Luciferase Reporter Assay

The putative binding sites for miR-212-5p and *ARAF* were predicted using the online software TargetScan7.2 (TargetScanHuman 7.2). The wild-type *ARAF* (*ARAF*-WT) and mutant *ARAF* (*ARAF*-MUT) sequences were amplified, and their binding sites to rat miR-212-5p were cloned into the pmir-GLO dual-luciferase reporter vector. The constructed *ARAF* vector was co-transfected with NC or miR-212-5p in HEK-293T cells since the transfection efficiency of HEK-293T is higher than that of NR8383 cells. After 48 h of transfection, the luciferase activity was measured and normalized using a Dual-Luciferase Reporter Kit (DL101, Vazyme, Nanjing, China).

2.11. Flow Cytometry

The apoptosis analysis was performed using the Annexin V-FITC/PI kit (556547, BD Pharmingen™, Franklin Lakes, NJ, USA). NR8383 cells were collected after 72 h of transfection (1×10^6 cells/time), washed with cold PBS, suspended in 1 mL of $1 \times$ Binding Buffer, centrifuged at 300 rpm for 10 min, and the supernatant was discarded. The cells were resuspended in 1 mL of $1 \times$ Binding Buffer to a density of 1×10^6 cells/mL. A 100 μ L volume of cells suspension (1×10^5 cells) was incubated with 5 μ L of Annexin V-FITC and 5 μ L of PI for 15 min at room temperature in a dark environment. A 500 μ L volume of PBS was added and gently mixed. The apoptotic cells were finally examined using a BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA).

2.12. Western Blotting

Briefly, we lysed the cultured cells using RIPA lysis solution containing 1% PMSF which were then centrifuged and the supernatant was taken after 30–40 min at 4 °C. The extracted protein concentrations were determined using the BCA protein quantification method and then SDS-PAGE gel electrophoresis was performed, the proteins were transferred to a PVDF membrane and then blocked using a blocking solution (5% skimmed milk powder). Then, the membranes were incubated overnight at 4 °C with the corresponding primary antibody and an HRP-conjugated secondary antibody (anti-mouse/rabbit antibody) for 1 h at room temperature and finally ECL luminescent solution was added to detect the protein bands using an Amersham Imager 680 (GE Healthcare, Sunnyvale, Chicago, Illinois, USA). The image analysis was performed using Image J.

2.13. Statistical Analysis

The experiments were repeated at least three times, and the values are shown as the mean $\pm$ SE of the three measurements. GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA, USA) was used to plot the experimental results. Student's *t*-tests were used to evaluate the data. Relative gene expression data were calculated using the $2^{-\Delta\Delta CT}$ method. Differences between the groups were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple comparison tests. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. *PM_{2.5} Causes Apoptosis in Rat NR8383 Cells*

We used the Cell Counting Kit-8 (CCK-8) to detect the cell viability of rat NR8383 cells stimulated with the cowshed PM_{2.5} at different concentrations (0, 60, 120, 180, 240, and 300 µg/mL) for 12 h. The results showed that the cell viability decreased with increasing concentrations of PM_{2.5} (Figure 1A). Based on these results, we chose 60, 180, and 300 µg/mL as the low, medium, and high concentrations for subsequent use. The cell viability reached 70% after the 180 µg/mL stimulation, which was chosen as the single stimulus amount to detect the cell viability at different time points after cowshed PM_{2.5} stimulation (0, 12, 24, and 48 h) (Figure 1B). The results showed that cell viability decreased in a time-dependent manner. We have also examined the expression of apoptosis-related proteins, and found that Bcl-2 levels decreased, but the expression of Bax and caspase 3 increased. PM_{2.5} also decreased cell viability and induced apoptosis with increasing the concentration and time (Figure 1C,D). Subsequently, we examined apoptosis using flow cytometry and showed that PM_{2.5} caused apoptosis in a time- and concentration-dependent manner (Figure 1E,F). The results suggest that PM_{2.5} reduces cell viability, leading to apoptosis.

3.2. *PM_{2.5} Increases Calcium Ions and ROS to Trigger Mitochondrial Damage*

We used a fluoroprobe to detect calcium ion levels in NR8383 cells after stimulation with different concentrations of the cowshed PM_{2.5} and found that the calcium ion concentration increased in a cowshed PM_{2.5} concentration-dependent manner (Figure 2A). Similarly, the results of the luciferase labeling experiment showed that after PM_{2.5} stimulation, the ROS level increased and was proportional to the PM_{2.5} concentration (Figure 2B). Using the fluorescent probe JC-1, it was found that the green fluorescence gradually intensified and the red fluorescence decreased after PM_{2.5} treatment compared to the control, indicating that the PM_{2.5} treatment resulted in an increase in monomer and a decrease in polymer levels, resulting in a decrease in mitochondrial membrane potential, which was again corroborated by the luciferase labeling experiment (Figure 2C,D). We then examined the Cyt-c and caspase 9 protein levels and found that the expression of both proteins also increased with the cowshed PM_{2.5} treatment (Figure 2E). These results suggest that the cowshed PM_{2.5} can promote intracellular calcium and ROS production, reduce the mitochondrial membrane potential, and increase the expression of related proteins, thereby triggering mitochondrial damage.

3.3. *miR-212-5p Expression Increases under the Cowshed PM_{2.5} Stimulation*

miR-212-5p was found to be differentially expressed from the transcriptomic data (Table S1) and, given its multiple biological functions, we conjectured that it might have some role in cowshed PM_{2.5} stimulation. Therefore, we examined miR-212-5p expression after stimulating NR8383 cells with concentrations of the cowshed PM_{2.5} for different durations, and the results showed that miR-212-5p was up-regulated in a time- and cowshed PM_{2.5} concentration-dependent manner (Figure 3A,B).

3.4. *ARAF Is a Potential Target of miR-212-5p and Its Expression Is Down-Regulated in the Cowshed PM_{2.5}-Stimulated NR8383 Cells*

ARAF was predicted to be an miR-212-5p target gene using TargetScan, miRDB, and other websites. At the same time, we found that miR-212-5p may bind to the 3'UTR region of ARAF (Figure 3C). Subsequently, we verified by RT-qPCR and Western blot that ARAF expression was down-regulated after cowshed PM_{2.5} stimulation (Figure 3D–G). Next, we transfected miR-212-5p mimics and inhibitors to verify the transfection efficiency, and the results showed successful overexpression and knockdown of miR-212-5p (Figure 3H).

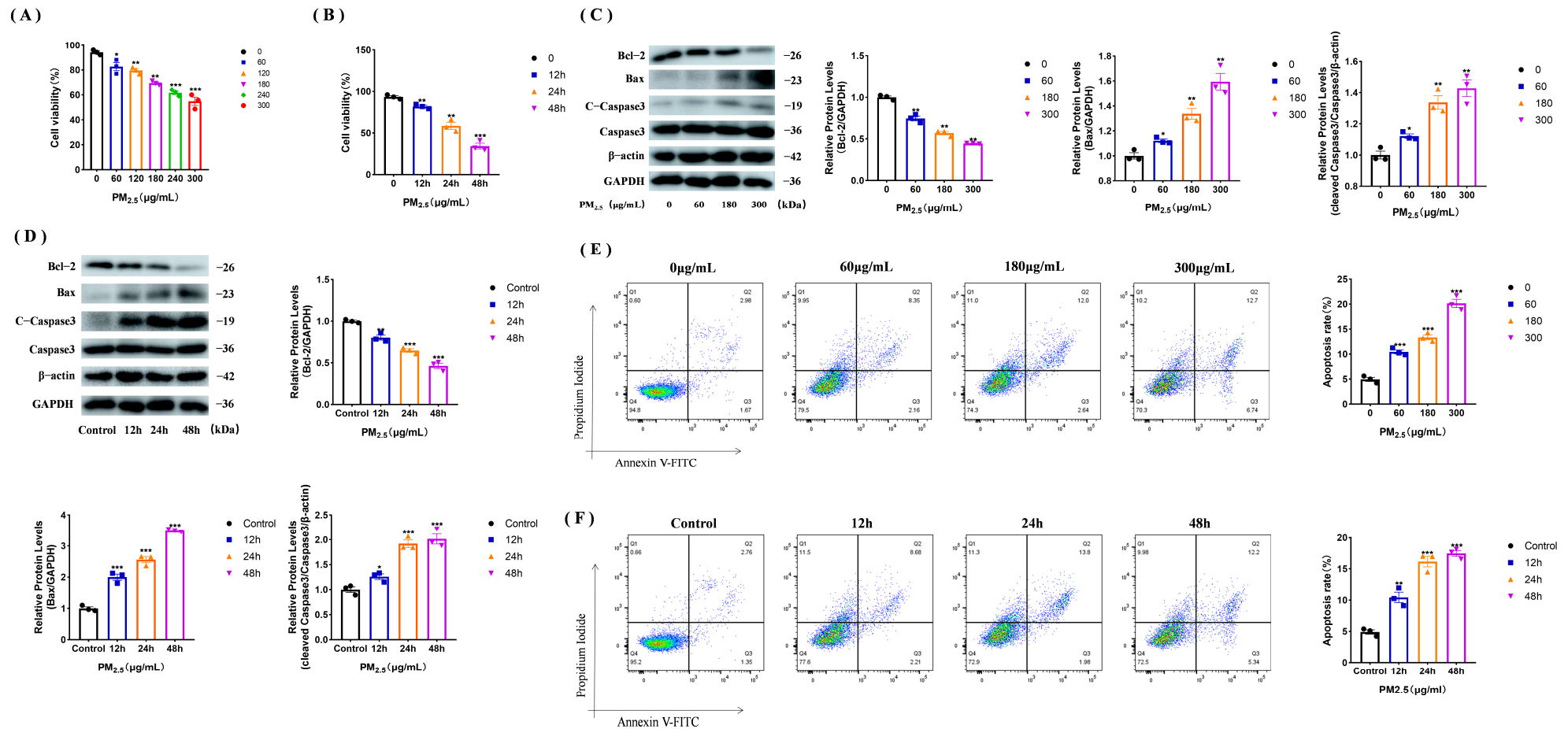


Figure 1. The cowshed PM_{2.5} reduces NR8383 cell viability and promotes apoptosis. (A) CCK8 assay for cell viability of the cells stimulated with cowshed PM_{2.5} (0, 60, 120, 180, 240 and 300 µg/mL) for 12 h assayed. (B) Cells were stimulated using 180 µg/mL of the cowshed PM_{2.5} for 0, 12, 24, and 48 h and then cell viability was assayed using the CCK8 assay. (C,D) Expression of apoptosis-related proteins after stimulation of NR8383 cells at different concentrations of the cowshed PM_{2.5} (C) at different time points (D). (E,F) Apoptosis analysis by flow cytometry using Annexin V-FITC and PI staining of NR8383 cells after stimulation with the cowshed PM_{2.5} at different concentrations (E) at different time points (F). All groups were analyzed in comparison to 0 µg/mL or control group. Each experiment was repeated at least three times, and values are shown as the mean ± SE of the three measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

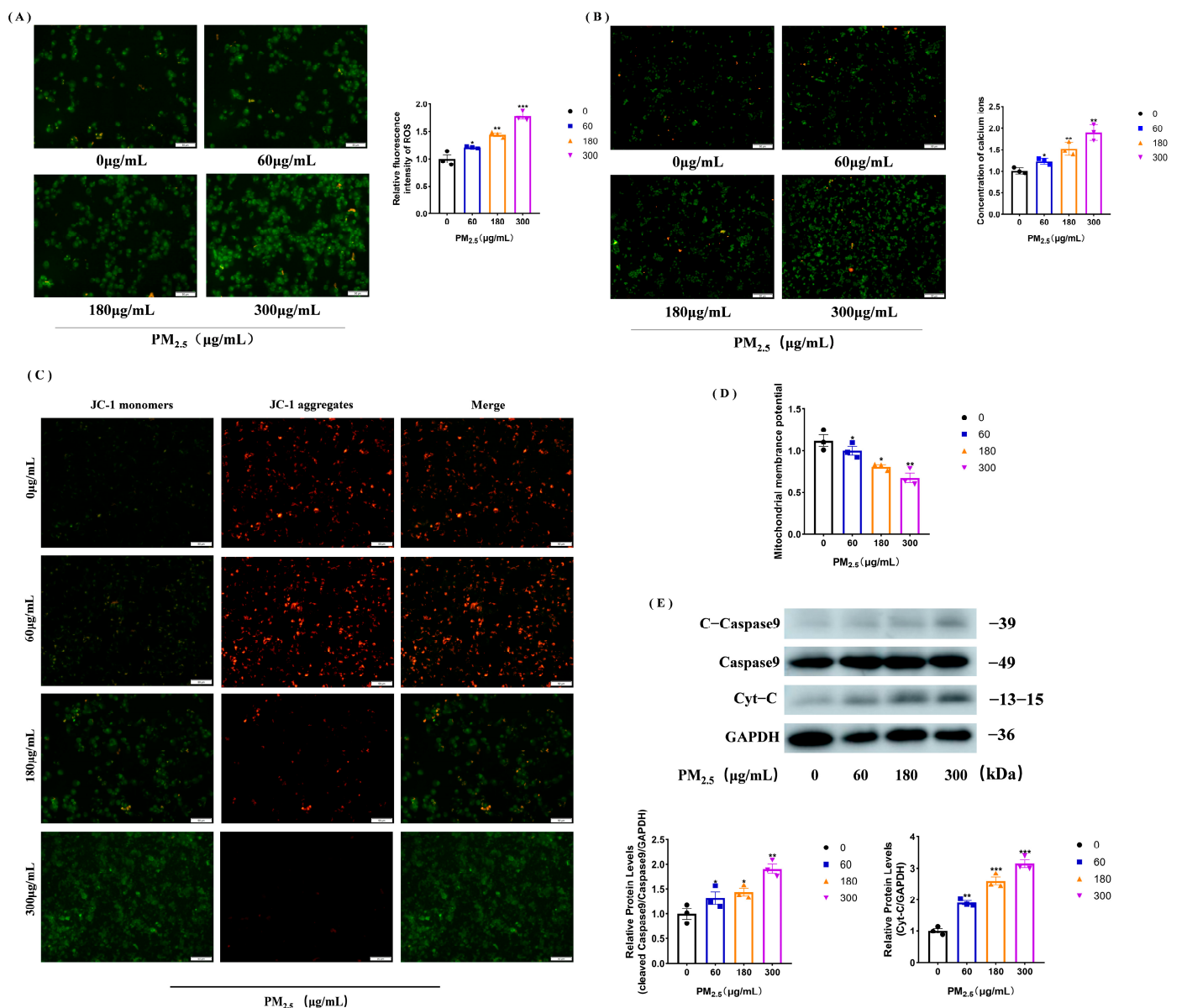


Figure 2. The cowshed PM_{2.5} increases calcium ion and ROS levels and thus triggers mitochondrial damage. (A) Changes in calcium ion concentration using fluorescence microscopy after the addition of Fluo-4 AM fluorescent probe to cells stimulated with different concentrations of the cowshed PM_{2.5}. (B) Changes in ROS were observed using fluorescence microscopy after the addition of DCFH-DA fluorescent probe to cells stimulated with different concentrations of the cowshed PM_{2.5}. (C,D) Cells stimulated with different concentrations of the cowshed PM_{2.5} were incubated with the JC-1 fluorescent probe and fluorescence microscopy was used to observe the changes in mitochondrial membrane potential (C). Simultaneous detection of changes in membrane potential using a fluorescent microplate reader (D). (E) Mitochondrial damage-associated protein assay was performed on cells after the treatment. All groups were analyzed in comparison to 0 µg/mL. Each experiment was repeated at least three times, and values are shown as the mean ± SE of the three measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

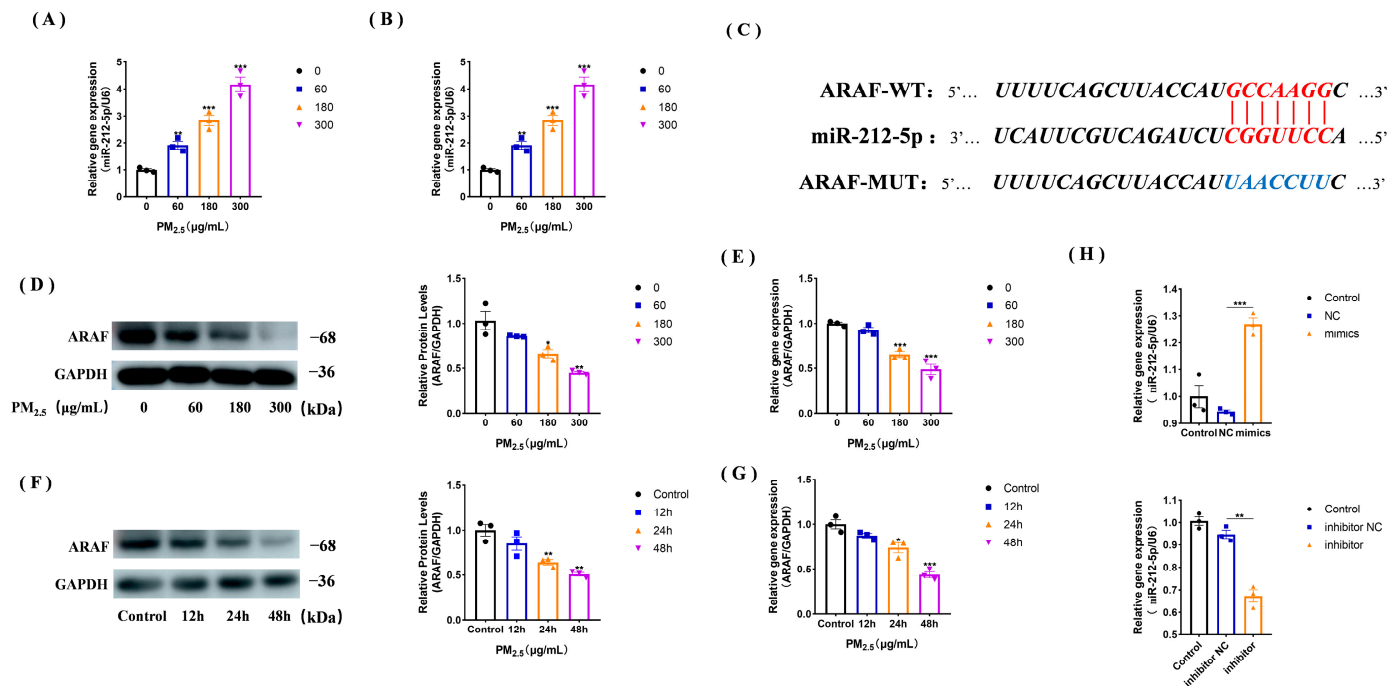


Figure 3. miR-212-5p expression increases after PM_{2.5} stimulation and its binds to ARAF. (A,B) qPCR validation of miR-212-5p expression changes after the cowshed PM_{2.5} cells stimulation at different concentrations (A) and different durations (B). (C) The prediction website identified a possible binding relationship between miR-212-5p and ARAF. (D,E) ARAF expression at the protein (D) and mRNA (E) levels after cells were stimulated with different concentrations of the cowshed PM_{2.5}. (F,G) The expression of ARAF decreased at the protein (F) and mRNA (G) levels after cells were stimulated with the cowshed PM_{2.5} for different durations. (H) Expression of miR-212-5p after transfection with mimics and inhibitors. All groups were analyzed in comparison to 0 ng/μL or control group. Each experiment was repeated at least three times, and values are shown as the mean ± SE of the three measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. miR-212-5p Targets ARAF, Promoting Mitochondrial Injury and Apoptosis after Cowshed PM_{2.5} Stimulation

To demonstrate that ARAF is a true target of miR-212-5p, the 3'UTR of the rat ARAF gene was introduced into the pmir-GLO reporter plasmid, and subsequent experiments using a dual-luciferase reporter assay revealed a reduction in dual-luciferase activity by miR-212-5p compared to the control, while ARAF-MUT showed no significant changes (Figure 4A). To demonstrate the regulatory relationship between miR-212-5p and ARAF, cells transfected with NC, mimics, inhibitor NC, and inhibitor were assayed for ARAF expression by Western blotting and RT-qPCR (Figure 4B,C). The results showed that ARAF expression was reduced upon overexpression of miR-212-5p, and the two were negatively correlated. To verify the role of miR-212-5p in cells, after transfection of the mimics, inhibitor, and their negative controls, the mitochondrial membrane potential and apoptosis-related proteins were analyzed after cowshed PM_{2.5} stimulation (Figure 4D–G). The results showed that after overexpression of miR-212-5p, the mitochondrial membrane potential was decreased, and the expression of Cyt-c, caspase 9, caspase 3, and Bax increased while the expression of Bcl-2 decreased. The opposite results were achieved with the addition of the inhibitor. Flow cytometry also confirmed the above conclusions (Figure 4H). In conclusion, we can say that overexpression of miR-212-5p leads to mitochondrial damage and promotes apoptosis.

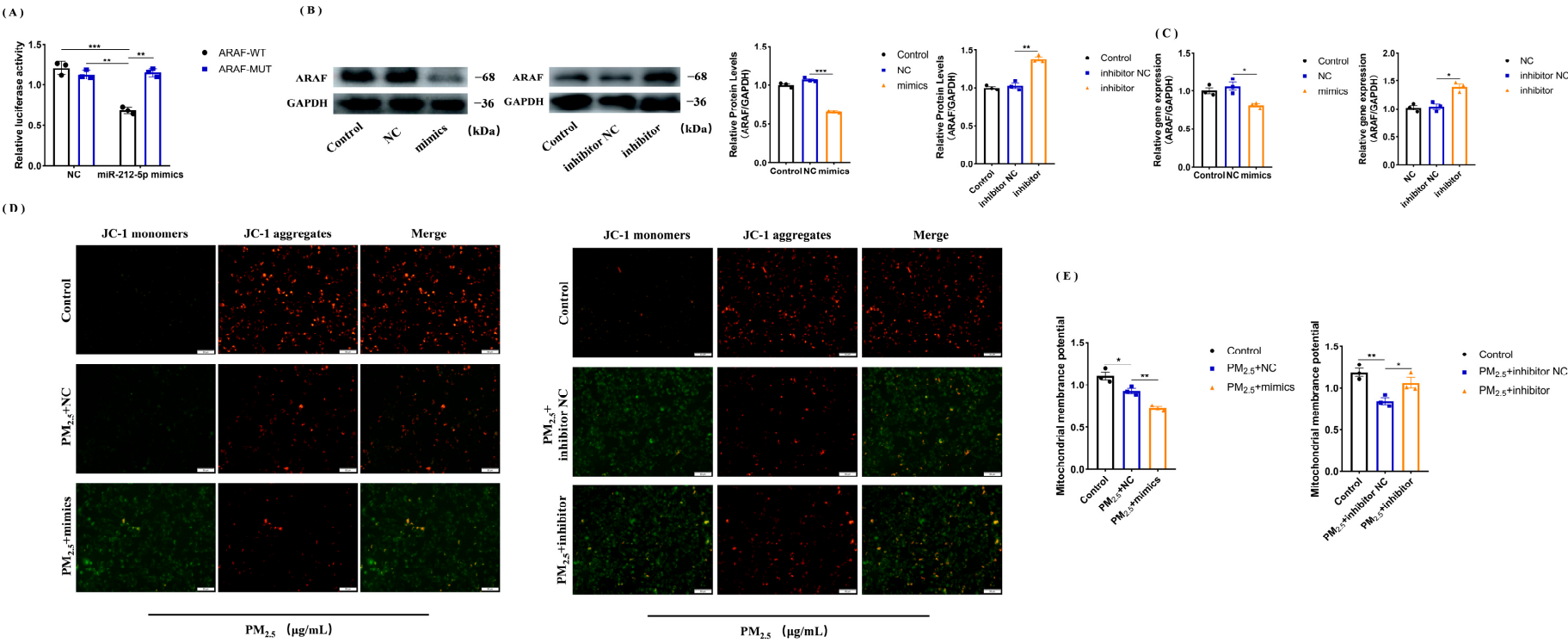


Figure 4. Cont.

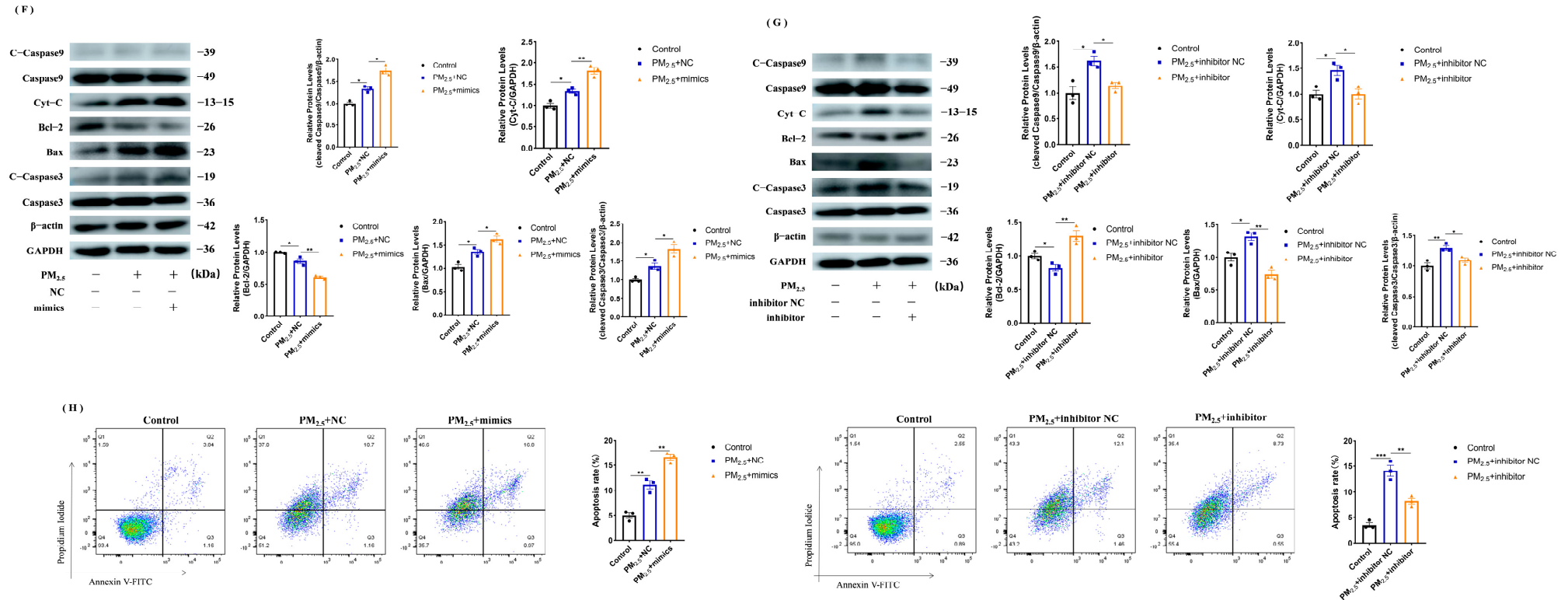


Figure 4. miR-212-5p binds to *ARAF* and promotes mitochondrial damage and apoptosis in response to PM_{2.5} stimulation. **(A)** Dual-luciferase reporter assay verified that miR-212-5p can target and bind to *ARAF*. **(B,C)** Western blotting **(B)** and qPCR **(C)** verified that miR-212-5p and *ARAF* are both negatively correlated. **(D)** Detection of mitochondrial membrane potential using the JC-1 probe. **(E)** Detection of mitochondrial membrane potential using a fluorescent microplate reader. **(F,G)** Changes in mitochondrial damage and apoptosis-related protein expression after transfection with miR-212-5p mimics **(F)** and inhibitor **(G)** in the cowshed PM_{2.5}-stimulated cells. **(H)** Apoptosis rates after transfection with mimics or inhibitor were detected using flow cytometry. All groups were analyzed in comparison with the control or NC groups. Each experiment was repeated at least three times, and values are shown as the mean $\pm$ SE of the three measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6. Activation of MEK/ERK Signaling Pathway after Overexpression of ARAF Inhibits the Cowshed PM_{2.5}-Induced Apoptosis

Previously, it was verified that miR-212-5p could promote apoptosis, but whether it was related to the MEK/ERK pathway is not known. We found that p-MEK1/2 and p-ERK1/2 expression was upregulated after transfection with the miR-212-5p inhibitor (Figure 5A). To verify the specific apoptotic mechanisms within the cells, we constructed pcDNA3.1-ARAF and transfected it into cells, and found that p-MEK1/2 and p-ERK1/2 expression was up-regulated in the presence of the cowshed PM_{2.5} (Figure 5B,C), indicating that the MEK/ERK signaling pathway was activated. We then examined the apoptotic proteins and mitochondria-associated proteins and found that as the expression of ARAF increased, Bcl-2 expression was up-regulated and Bax and caspase 3 expression was down-regulated, which was similarly confirmed by flow cytometry (Figure 5D,E). Similarly, after overexpression of ARAF, the mitochondrial membrane potential, ROS level, and calcium profile were all reversed (Figure 5F–H). The results suggest that overexpression of ARAF can inhibit the onset of apoptosis by activating the MEK/ERK signaling pathway.

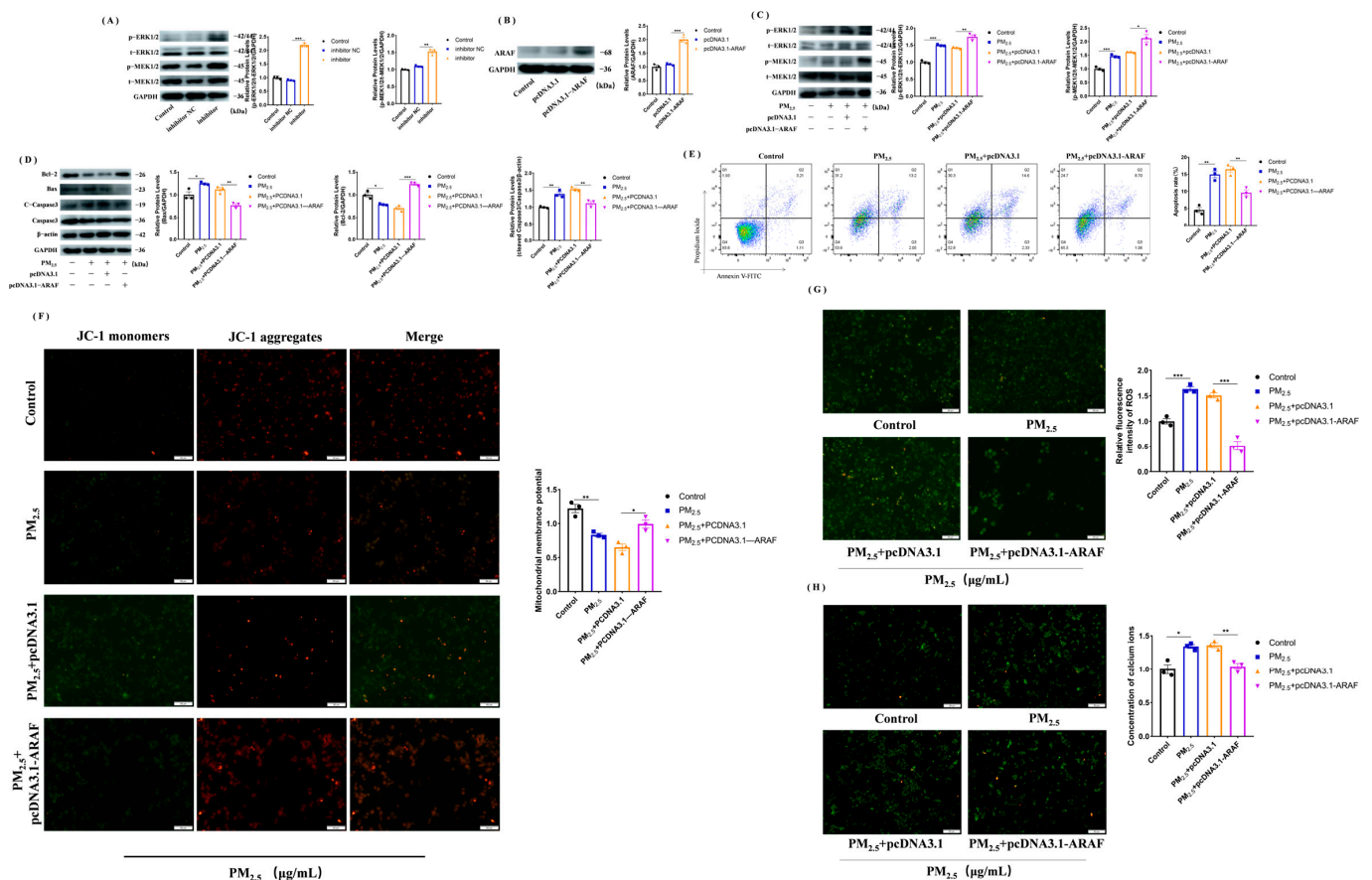


Figure 5. Overexpression of ARAF activates the MEK/ERK signaling pathway to affect apoptosis. (A) Changes in p-MEK1/2 and p-ERK1/2 protein expression after transfection with miR-212-5p inhibitor. (B) Western blotting verified the successful construction of pcDNA3.1-ARAF. (C) Changes in p-MEK1/2 and p-ERK1/2 protein expression after transfection of pcDNA3.1-ARAF in response to the cowshed PM_{2.5} stimulation. (D) Detection of associated proteins after overexpression of ARAF. (E) Detection of apoptosis rate after overexpression of ARAF using flow cytometry. (F–H) Mitochondrial membrane potential (F), ROS (G), and calcium (H) changes after overexpression of ARAF. All groups were analyzed in comparison with control or pcDNA3.1 empty vector groups. Each experiment was repeated at least three times, and values are shown as the mean $\pm$ SE of the three measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

To date, the number of deaths per year from airborne particulate matter has increased dramatically, while the more complex air environment at the farms poses a serious threat to human and animal health. The literature suggests that particulate matter emissions are higher in livestock and poultry housing, particularly in cowsheds, than in the general area [39,40]. This complex environment increases the expression of pro-inflammatory factors, which ultimately contribute to a variety of diseases, including non-small cell lung cancer (NSCLC) and cerebral atherosclerosis, which is consistent with our hypothesis [41,42]. This has a direct impact on the economic performance of farms and has a deterrent effect on economic development. Compared with other farms, cattle farms derive their economic benefits from meat and milk production, which occupy a large share of the market, and the monitoring of the health of the animals themselves should be taken into account. Therefore, it is particularly important to explore the specific intracellular mechanisms of cowshed PM_{2.5} regulation.

Macrophages are the first barrier against invaders. When a bacterium or virus enters the organism, macrophages quickly recognize the foreign invader. They form a small pocket in their outer cell membrane to encapsulate the invader or to digest and destroy them. At the same time, macrophages will call on T-cells and B-cells to work together to fight the infection. Previously, our group constructed an in vivo rat infection model and performed transcriptome sequencing. In this study, we constructed an in vitro injury model by treating rat alveolar macrophages with cowshed PM_{2.5}, with the aim of discovering a new mechanism of lung injury by cowshed PM_{2.5}. The PM_{2.5} treatment reduced the expression of *ARAF* in NR8383 cells while inducing alterations in the miRNA profile of rat lungs, consistent with the previous reports [43]. Notably, among the group of dysregulated miRNAs, miR-212-5p increased in response to bovine housing PM_{2.5} and promoted apoptosis in NR8383 cells through the downregulation of *ARAF* expression.

The RAF kinase family has been reported to be involved in the process of tumor progression, especially BRAF and CRAF, and their mutations lead to a number of outcomes, such as tumor metastasis and apoptosis [44]. However, mutations in *ARAF* have been rarely reported, which is why the aim of this study was to discuss the specific role and regulatory mechanism that *ARAF* plays in the cowshed PM_{2.5}-induced apoptosis.

A large number of miRNAs can regulate various physiological activities in organisms. The literature shows that there are a variety of miRNAs that are abnormally expressed in response to airborne particulate exposure, while PM_{2.5} has a more serious harmful effect on organisms [45]. This has become strong epiphenomenal evidence in this article to understand the process of apoptosis caused by cowshed PM_{2.5}. It is undeniable that miR-212-5p regulates multiple target genes, which have the same or different functions [32,46,47], all of which suggest that miR-212-5p has complex biological functions depending on the target genes and cellular context. Some articles have reported that miR-212-5p can regulate some physiological activities, such as cell proliferation and epithelial mesenchymal transition [48,49], but the regulation of apoptosis after cowshed PM_{2.5} stimulation by miR-212-5p has not been reported.

In our experiments, we concluded that the expression of miR-212-5p was altered in response to the cowshed PM_{2.5} stimulation. Interestingly, we found from RT-qPCR that the expression of miR-212-5p was significantly increased after 24 h of the cowshed PM_{2.5} stimulation, which corresponded to a significant increase in apoptosis 24 and 48 h after the cowshed PM_{2.5} exposure. It is reasonable to suspect that the change in miR-212-5p expression may have occurred in the pre-apoptotic phase and that the change in miR-212-5p expression led to an increase in the rate of apoptosis. Thus, miR-212-5p may regulate many physiological functions in cells, including apoptosis. In this article, we mainly discussed apoptosis levels at the cellular level in rats after stimulation with cowsheds PM_{2.5}. In the next step, we will focus on cells from other species, such as human macrophages or epithelial cells, to further explore whether this mechanism is feasible, which will have significant implications for the health and safety of breeders. In addition, it is unknown

whether there were other related results in the programmed death mode, and it is necessary to explore the toxic mechanism of the cowsheds PM_{2.5} and will also be the focus of our next research.

In the last section, we first verified that the miR-212-5p inhibitor may activate the MEK/ERK signaling pathway, and secondly, we verified that the overexpression of *ARAF* also activates the MEK/ERK signaling pathway when stimulated by the cowshed PM_{2.5}. However, it is worth noting that, compared with the control group, the cowshed PM_{2.5} group also had elevated expression of p-MEK1/2 and p-ERK1/2 proteins, which may be due to the fact that when the cells were stimulated by the cowshed PM_{2.5} and apoptosis occurred, it triggered the feedback regulation, which led to the elevation of the expression of p-MEK1/2 and p-ERK1/2. Moreover, due to the complexity of the pro-apoptotic network of the cowshed PM_{2.5}, it is possible that other pathways are also playing an important role in influencing the results. However, the overexpression group had a higher expression of p-MEK1/2 and p-ERK1/2, which had more obvious effects on pathway activation.

In this article, we first found that cell viability decreased after cowshed PM_{2.5} stimulation in a time- and concentration-dependent manner, which is consistent with previous studies [50]. It has been suggested in the literature that intracellular reactive oxygen species and calcium ions may contribute to lung injury [51,52]. We then confirmed our suspicions experimentally and found that intracellular reactive oxygen species and calcium ion levels increased significantly after the cowshed PM_{2.5} stimulation, which might be one of the reasons for the induction of apoptosis. As the mitochondrial apoptotic pathway is one of the main pathways through which apoptosis occurs [53], the accumulation of reactive oxygen species led us to associate it with the mitochondrial apoptotic pathway [54], as evidenced by the decrease in mitochondrial membrane potential and proteins such as cytochrome C, which is also consistent with previous experiments. Subsequently, a change in expression of miR-212-5p was found, and its target gene *ARAF* was confirmed using a dual-luciferase assay. The KEGG pathway database and the construction of overexpression vectors revealed that the MEK/ERK signaling pathway was specifically activated and led to the expression of anti-apoptotic proteins that inhibited the expression of pro-apoptotic proteins. In conclusion, our new findings suggest that the miR-212-5p/*ARAF*/MEK-ERK axis is involved in the cowshed PM_{2.5}-induced apoptosis in the alveolar macrophage cell line (NR8383).

5. Conclusions

In conclusion, our study showed that in the cowshed PM_{2.5}-induced apoptosis in NR8383 cells, the resistance to apoptosis occurs through overexpression of *ARAF* and activation of the MEK/ERK signaling pathway. These effects could be regulated by miR-212-5p. Therefore, we found that the cowshed PM_{2.5} affects mitochondrial damage in NR8383 cells through the miR-212-5p/*ARAF*/MEK-ERK axis and thus promotes apoptosis. miR-212-5p is upregulated as a pro-apoptotic regulator in the cowshed PM_{2.5}-treated NR8383 cells and inhibits the protective effect of *ARAF* on cells. Our results demonstrate a novel mechanism of the miR-212-5p/*ARAF*/MEK-ERK axis in the cowshed PM_{2.5}-induced apoptosis, providing a new perspective for the study of the cowshed PM_{2.5}-induced lung toxicity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxics11120981/s1>, Table S1: Differentially expressed miRNAs.

Author Contributions: All authors contributed to the study conception and design. K.S.: conceptualization, methodology, software, writing—original draft. Y.S. and Y.J.: methodology. X.D. and Z.M.: sample collection. X.Z. and L.W.: writing—review and editing. Y.Z.: writing—review and editing. Y.G. and W.B.: writing—review and editing, project administration. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Tibet Autonomous Region Science and Technology Project (XZ202101ZD0002N-04), Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDA28080400), and China Agriculture Research System of MOF and MARA (CARS-37).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

References

- Chen, X.W.; Deng, T.; Huo, T.T.; Dong, F.Q.; Deng, J.J. Mir-140-5p/tlr4 /nf-kappa b signaling pathway: Crucial role in inflammatory response in 16hbe cells induced by dust fall pm2.5. *Ecotoxicol. Environ. Saf.* **2021**, *208*, 111414. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kangas, T.; Gadeyne, S.; Lefebvre, W.; Vanpoucke, C.; Rodriguez-Loureiro, L. Are air quality perception and pm2.5 exposure differently associated with cardiovascular and respiratory disease mortality in brussels? Findings from a census-based study. *Environ. Res.* **2023**, *219*, 115180. [\[CrossRef\]](#) [\[PubMed\]](#)
- Qi, Z.H.; Zhang, Y.H.; Chen, Z.F.; Yang, C.; Song, Y.Y.; Liao, X.L.; Li, W.Q.; Tsang, S.Y.; Liu, G.G.; Cai, Z.W. Chemical identity and cardiovascular toxicity of hydrophobic organic components in pm2.5. *Ecotoxicol. Environ. Saf.* **2020**, *201*, 110827. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ren, J.Y.; Li, X.; Zhu, S.Q.; Yin, B.W.; Guo, Z.H.; Cui, Q.Q.; Song, J.S.; Pei, H.T.; Ma, Y.X. Sesamin ameliorates fine particulate matter (pm_{2.5})-induced lung injury via suppression of apoptosis and autophagy in rats br. *J. Agric. Food Chem.* **2022**, *70*, 9489–9498. [\[CrossRef\]](#) [\[PubMed\]](#)
- Fan, X.; Gao, Y.; Hua, C.; Peng, L.; Ci, X. Daphnetin ameliorates pm2.5-induced airway inflammation by inhibiting nlrp3 inflammasome-mediated pyroptosis in cs-exposed mice. *Biomed. Pharmacother.* **2023**, *165*, 115047. [\[CrossRef\]](#)
- Hsieh, C.-C.; Yu, S.-H.; Kuo, H.-C.; Cheng, K.-W.; Hsu, C.-C.; Lin, Y.-P.; Khumsupan, D.; Lin, S.-P.; Angkawijaya, A.E.; Cheng, K.-C. Alleviation of pm_{2.5}-induced alveolar macrophage inflammation using extract of fermented chenopodium formosanum koidz sprouts via regulation of nf- κ b pathway. *J. Ethnopharmacol.* **2024**, *318*, 116980. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wang, Y.; Duan, H.; Zhang, J.; Wang, Q.; Peng, T.; Ye, X.; Cheng, Z.; Li, X. Yap1 protects against pm2.5-induced lung toxicity by suppressing pyroptosis and ferroptosis. *Ecotoxicol. Environ. Saf.* **2023**, *253*, 114708. [\[CrossRef\]](#)
- Wu, B.; Qin, L.W.; Wang, M.; Zhou, T.; Dong, Y.X.; Chai, T.J. The composition of microbial aerosols, pm2.5, and pm10 in a duck house in shandong province, china. *Poult. Sci.* **2019**, *98*, 5913–5924. [\[CrossRef\]](#)
- Liu, H.L.; Nie, H.P.; Lai, W.Q.; Shi, Y.; Liu, X.; Li, K.; Tian, L.; Xi, Z.G.; Lin, B.C. Different exposure modes of pm2.5 induces bronchial asthma and fibrosis in male rats through macrophage activation and immune imbalance induced by tpe2 methylation. *Ecotoxicol. Environ. Saf.* **2022**, *247*, 114200. [\[CrossRef\]](#)
- Hamad, S.H.; Schauer, J.J.; Antkiewicz, D.S.; Shafer, M.M.; Kadhim, A.K.H. Ros production and gene expression in alveolar macrophages exposed to pm2.5 from baghdad, iraq: Seasonal trends and impact of chemical composition. *Sci. Total Environ.* **2016**, *543*, 739–745. [\[CrossRef\]](#)
- Yang, B.; Guo, J.; Xiao, C.L. Effect of pm2.5 environmental pollution on rat lung. *Environ. Sci. Pollut. Res.* **2018**, *25*, 36136–36146. [\[CrossRef\]](#)
- Jiang, X.; Han, Y.Q.; Qiu, X.H.; Chai, Q.Q.; Zhang, H.X.Y.; Chen, X.; Cheng, Z.; Wang, Y.W.; Fan, Y.F.; Xue, T.; et al. Organic components of personal pm2.5 exposure associated with inflammation: Evidence from an untargeted exposomic approach. *Environ. Sci. Technol.* **2021**, *55*, 10589–10596. [\[CrossRef\]](#) [\[PubMed\]](#)
- Xie, J.L.; Li, S.H.; Ma, X.N.; Li, R.Q.; Zhang, H.R.; Li, J.W.; Yan, X.X. Mir-217-5p inhibits smog (pm2.5)-induced inflammation and oxidative stress response of mouse lung tissues and macrophages through targeting stat1. *Aging* **2022**, *14*, 6796–6808. [\[CrossRef\]](#) [\[PubMed\]](#)
- Huang, J.; Zhang, Y.; Cheng, A.; Wang, M.; Liu, M.; Zhu, D.; Chen, S.; Zhao, X.; Yang, Q.; Wu, Y.; et al. Duck circovirus genotype 2 orf3 protein induces apoptosis through the mitochondrial pathway. *Poult. Sci.* **2023**, *102*, 102533. [\[CrossRef\]](#) [\[PubMed\]](#)
- Han, J.W.; Park, H.J. Perfluorooctanoic acid induces cell death in tm3 cells via the er stress-mitochondrial apoptosis pathway. *Reprod. Toxicol.* **2023**, *118*, 108383. [\[CrossRef\]](#) [\[PubMed\]](#)
- Song, M.; Zhang, J.; Huo, S.M.; Zhang, X.L.; Cui, Y.L.; Li, Y.F. Mitophagy alleviates aif-mediated spleen apoptosis induced by alcl3 through parkin stabilization in mice. *Food Chem. Toxicol.* **2023**, *176*, 113762. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zheng, G.Z.; Zhang, Q.H.; Chang, B.; Xie, P.; Liao, H.; Du, S.X.; Li, X.D. Dioscin induces osteosarcoma cell apoptosis by upregulating ros-mediated p38 mapk signaling. *Drug Dev. Res.* **2023**, *84*, 25–35. [\[CrossRef\]](#)
- Zhang, L.L.; Chen, L.; Qi, M.; Yu, F.C.; Ni, X.T.; Hong, H.Z.; Xu, H.T.; Xu, S.W. Glyphosate induces autophagy in hepatic l8824 cell line through no-mediated activation of ras/raf/mek/erk signaling pathway and energy metabolism disorders. *Fish Shellfish. Immunol.* **2023**, *137*, 108772. [\[CrossRef\]](#)
- Song, Y.L.; Bi, Z.F.; Liu, Y.; Qin, F.R.; Wei, Y.Q.; Wei, X.W. Targeting ras-raf-mek-erk signaling pathway in human cancer: Current status in clinical trials. *Genes Dis.* **2023**, *10*, 76–88. [\[CrossRef\]](#)
- Wu, C.Y.; Zhu, G.Y.; Qiu, F.; Ren, F.L.; Lin, B.B.; Zhang, D.Y.; Yang, Q.Y.; Huang, C.L. Plx8394, a raf inhibitor, inhibits enterovirus 71 replication by blocking raf/mek/erk signaling. *Virol. Sin.* **2023**, *38*, 276–284. [\[CrossRef\]](#)

21. Santarpia, L.; Lippman, S.M.; El-Naggar, A.K. Targeting the mapk-ras-raf signaling pathway in cancer therapy. *Expert Opin. Ther. Targets* **2012**, *16*, 103–119. [CrossRef] [PubMed]
22. Hendrikse, C.S.E.; Theelen, P.M.M.; van der Ploeg, P.; Westgeest, H.M.; Boere, I.A.; Thijs, A.M.J.; Ottevanger, P.B.; van de Stolpe, A.; Lambrechts, S.; Bekkers, R.L.M.; et al. The potential of ras/raf/mek/erk (mapk) signaling pathway inhibitors in ovarian cancer: A systematic review and meta-analysis. *Gynecol. Oncol.* **2023**, *171*, 83–94. [CrossRef]
23. An, S.; Yang, Y.; Ward, R.; Liu, Y.; Guo, X.X.; Xu, T.R. A-raf: A new star of the family of raf kinases. *Crit. Rev. Biochem. Mol. Biol.* **2015**, *50*, 520–531. [CrossRef]
24. Yang, S.F.; Liu, G.H. Targeting the ras/raf/mek/erk pathway in hepatocellular carcinoma. *Oncol. Lett.* **2017**, *13*, 1041–1047. [CrossRef] [PubMed]
25. O'Neill, E.; Kolch, W. Taming the hippo—Raf-1 controls apoptosis by suppressing mst2/hippo. *Cell Cycle* **2005**, *4*, 365–367. [CrossRef] [PubMed]
26. Wang, J.-C.; Huang, Y.; Zhang, R.-X.; Han, Z.-J.; Zhou, L.-L.; Sun, N.; Dong, W.-Y.; Zhuang, G.-S. Mir-338–3p inhibits autophagy in a rat model of allergic rhinitis after pm2.5 exposure through akt/mtor signaling by targeting ube2q1. *Biochem. Biophys. Res. Commun.* **2021**, *554*, 1–6. [CrossRef]
27. Zhou, T.Y.; Zhong, Y.J.; Hu, Y.; Sun, C.; Wang, Y.X.; Wang, G.F. Pm2.5 downregulates mir-194-3p and accelerates apoptosis in cigarette-inflamed bronchial epithelium by targeting death-associated protein kinase i. *Int. J. Chronic Obstr. Pulm. Dis.* **2018**, *13*, 2339–2349. [CrossRef] [PubMed]
28. Park, S.; Kim, M.; Park, M.; Jin, Y.; Lee, S.J.; Lee, H. Specific upregulation of extracellular mir-6238 in particulate matter-induced acute lung injury and its immunomodulation. *J. Hazard. Mater.* **2023**, *445*, 130466. [CrossRef]
29. Wang, J.; Zhu, M.C.; Song, J.; Zeng, Y.Y.; Xia, S.J.; Chen, C.C.; Jin, M.L.; Song, Y.L. The circular rna circctxnd1 promoted ambient particulate matter-induced inflammation in human bronchial epithelial cells by regulating mir-892a/cox-2 axis. *Chemosphere* **2022**, *286*, 131614. [CrossRef]
30. Guo, J.H.; Xie, X.X.; Wu, J.Y.; Yang, L.; Ruan, Q.S.; Xu, X.Y.; Wei, D.H.; Wen, Y.Y.; Wang, T.G.; Hu, Y.D.; et al. Association between fine particulate matter and coronary heart disease: A mirna microarray analysis. *Environ. Pollut.* **2022**, *313*, 120163. [CrossRef]
31. Chen, S.; Chen, W.; Li, Z.; Yue, J.; Yung, K.K.L.; Li, R. Regulation of pm2.5 on mitochondrial damage in h9c2 cells through mir-421/sirt3 pathway and protective effect of mir-421 inhibitor and resveratrol. *J. Environ. Sci.* **2024**, *138*, 288–300. [CrossRef]
32. Li, S.H.; Qu, X.; Qin, Z.X.; Gao, J.G.; Li, J.P.; Liu, J.L. Lncfos/mir-212-5p/casp7 axis-regulated mir-212-5p protects the brain against ischemic damage. *Mol. Neurobiol.* **2023**, *60*, 2767–2785. [CrossRef]
33. Guo, Y.J.; Yu, J.J.; Wang, C.X.; Li, K.; Liu, B.; Du, Y.; Xiao, F.; Chen, S.H.; Guo, F.F. Mir-212-5p suppresses lipid accumulation by targeting fas and scd1. *J. Mol. Endocrinol.* **2017**, *59*, 205–217. [CrossRef] [PubMed]
34. Ding, L.; Xie, Z.B. Circwhsc1 regulates malignancy and glycolysis by the mir-212-5p/akt3 pathway in triple-negative breast cancer. *Exp. Mol. Pathol.* **2021**, *123*, 104704. [CrossRef] [PubMed]
35. Jia, Y.N. Study on Mir-212-5p Targeting Lamc2 and Lama3 to Regulate Cell Apoptosis Induced by pm2.5 in Cowhouse. Jilin Agricultural University, 2023. Available online: https://kns.cnki.net/kcms2/article/abstract?v=jxCH2R2OgonxEmvdodzdIVK9LATq7Mza1UktlqVaU1AvnKYQ2z39D6RQiEvgwtaAZXTFx6Esnf5Sz9Zmj2hFqStlnt_Rx1mR-fda7mM6MOh49II2zSiPK_ph3f-Xkaa5leFDTXjizTxLsoZ8l32fMw==&uniplatform=NZKPT&language=CHS (accessed on 1 May 2023).
36. Ma, Z.H. Effect of Guanylate Binding Protein 2 on Rat Alveolar Macrophage Injury Induced by pm2.5 in Cowshe. Jilin Agricultural University, 2022. Available online: https://kns.cnki.net/kcms2/article/abstract?v=Eo9-C_M6tLlJ7OAgj-LWX2D4jNPAYLybL6bCSgzvzdnVIZUtv6jIYYb_p4S-yu4tj-B6k4P7spk2keVIGTwrEgz7KtpV-fqYOXzwtlt1d2J7vO0t6NZF1Jo9iuQfrJV7Mf-iKh0dHV96w46skDIA==&uniplatform=NZKPT&language=CHS (accessed on 1 May 2022).
37. Zhang, X.Q.; Ma, Z.H. Characteristics of bioaerosols in animal barns: Size distribution, bacterial community, environmental factors, antibiotic resistance genes, and health risks. Jilin Agricultural University: Changchun, China, 2023, *submitted*.
38. Ma, Z.H.; Du, X.H. Overexpression of rgs2 ameliorates cowshed pm2.5-induced alveolar macrophage damage by suppressing the gq/11-ca2+ pathway. Jilin Agricultural University: Changchun, China, 2023, *submitted*.
39. Pan, S.S.; Qiu, Y.T.; Li, M.; Yang, Z.Q.; Liang, D.P. Recent developments in the determination of pm2.5 chemical composition. *Bull. Environ. Contam. Toxicol.* **2022**, *108*, 819–823. [CrossRef] [PubMed]
40. Roman, M.; Roman, K.; Roman, M. Spatial variation in particulate emission resulting from animal farming in poland. *Agriculture* **2021**, *11*, 168. [CrossRef]
41. Liang, S.; Ning, R.H.; Zhang, J.Y.; Liu, J.Y.; Zhang, J.; Shen, H.Q.; Chen, R.; Duan, J.C.; Sun, Z.W. Mir-939-5p suppresses pm2.5-induced endothelial injury via targeting hif-1 alpha in haecs. *Nanotoxicology* **2021**, *15*, 706–720. [CrossRef]
42. Yao, H.M.; Zhao, X.X.; Wang, L.L.; Ren, Y. Atorvastatin ameliorated pm2.5-induced atherosclerosis in rats. *Arch. Environ. Occup. Health* **2023**, *78*, 267–272. [CrossRef]
43. Ma, W.L.; Xu, L.; Sun, X.Y.; Qi, Y.; Chen, S.; Li, D.C.; Jin, Y.; Chen, N.N.; Zhu, X.X.; Luo, J.; et al. Using a human bronchial epithelial cell-based malignant transformation model to explore the function of hsa-mir-200 family in the progress of pm2.5-induced lung cancer development. *Environ. Pollut.* **2023**, *319*, 120981. [CrossRef]
44. Khan, P.S.; Rajesh, P.; Rajendra, P.; Chaskar, M.G.; Rohidas, A.; Jaiprakash, S. Recent advances in b-raf inhibitors as anticancer agents. *Bioorg. Chem.* **2022**, *120*, 105597. [CrossRef]
45. Xu, D.; Lin, W.P.; Gao, J.; Jiang, Y.; Li, L.B.; Gao, F. Pm2.5 exposure and health risk assessment using remote sensing data and gis. *Int. J. Environ. Res. Public Health* **2022**, *19*, 6154. [CrossRef]

46. Chong, Y.L.; Zhu, C.R.; Hu, W.; Jiang, C.R.; Liang, W.B.; Zhu, Z.J. Mir-212-5p suppresses glioma development via targeting sumo2. *Biochem. Genet.* **2023**, *61*, 35–47. [[CrossRef](#)] [[PubMed](#)]
47. Zhou, H.Y.; Zhang, N.N. Mir-212-5p inhibits nasopharyngeal carcinoma progression by targeting mettl3. *Open Med.* **2022**, *17*, 1241–1251. [[CrossRef](#)] [[PubMed](#)]
48. Peng, N.X.; Zhang, Z.J.; Wang, Y.M.; Yang, M.L.; Fan, J.Q.; Wang, Q.J.; Deng, L.; Chen, D.; Cai, Y.F.; Li, Q.H.; et al. Down-regulated linc00115 inhibits prostate cancer cell proliferation and invasion via targeting mir-212-5p/fzd5/wnt/beta-catenin axis. *J. Cell. Mol. Med.* **2021**, *25*, 10627–10637. [[CrossRef](#)] [[PubMed](#)]
49. Zhao, Z.Y.; Zhang, G.M.; Yang, J.; Lu, R.; Hu, H.J. Dleu2 modulates proliferation, migration and invasion of platelet-derived growth factor-bb (pdgf-bb)-induced vascular smooth muscle cells (vsmcs) via mir-212-5p/ywhaz axis. *Cell Cycle* **2022**, *21*, 2013–2026. [[CrossRef](#)] [[PubMed](#)]
50. Shan, H.; Li, X.H.; Ouyang, C.; Ke, H.Y.; Yu, X.L.; Tan, J.F.; Chen, J.H.; Wang, C.P.; Zhang, L.P.; Tang, Y.F.; et al. Salidroside prevents pm2.5-induced beas-2b cell apoptosis via sirt1-dependent regulation of ros and mitochondrial function. *Ecotoxicol. Environ. Saf.* **2022**, *231*, 113170. [[CrossRef](#)] [[PubMed](#)]
51. Liu, H.; Wang, R.; OuYang, H.J.; Wang, Y.; Wu, J.; Li, M.Y.; Hu, Y.; Yao, Y.Y.; Liu, Y.H.; Ji, Y.L. Cadmium induced mouse spermatogonia apoptosis via mitochondrial calcium overload mediated by ip3r-mcu signal pathway. *Toxicology* **2023**, *486*, 153448. [[CrossRef](#)] [[PubMed](#)]
52. Wang, C.Y.; Meng, X.Z.; Meng, M.L.; Shi, M.; Sun, W.P.; Li, X.J.; Zhang, X.; Liu, R.H.; Fu, Y.; Song, L.Y. Oxidative stress activates the trpm2-ca2+-nlrp3 axis to promote pm2.5-induced lung injury of mice. *Biomed. Pharmacother.* **2020**, *130*, 110481. [[CrossRef](#)]
53. Flores-Romero, H.; Dadsena, S.; Garcia-Saez, A.J. Mitochondrial pores at the crossroad between cell death and inflammatory signaling. *Mol. Cell* **2023**, *83*, 843–856. [[CrossRef](#)]
54. Wang, C.; Shang, H.; Zhang, S.; Wang, X.; Liu, D.; Shen, M.; Li, N.; Jiang, Y.; Wei, K.; Zhu, R. Hexavalent chromium disrupts the skin barrier by targeting ros-mediated mitochondrial pathway apoptosis in keratinocytes. *Chem. Biol. Interact.* **2023**, *379*, 110523. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.