N6-methyladenosine-mediated Nrf2 Regulates the Defense Mechanism Against PM2.5-induced Pulmonary Fibrosis

Ding Ji
Hebei Medical University First Affiliated Hospital

Chenxi Hu
Hebei Medical University First Affiliated Hospital

Jie Ning
Hebei Medical University

Xiaoling Ying
Sun Yat-sen University First Affiliated Hospital

Haiqing Zhang
Sun Yat-sen University First Affiliated Hospital

Bohan Zhang
Hebei Medical University

Bixia Liu
Sun Yat-sen University First Affiliated Hospital

Qingping Liu
Hebei Medical University

Rong Zhang (rongzhang@hebmu.edu.cn)
Hebei Medical University

Weidong Ji
Sun Yat-sen University First Affiliated Hospital

Research

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Abstract

**Background:** It has been reported that particulate matter with an aerodynamic diameter of < 2.5 µm (PM2.5) could induce epithelial–mesenchymal transition (EMT)- and extracellular matrix (ECM)-related pulmonary fibrosis (PF). The transcription factor Nrf2 alleviated PM2.5-induced PF by antagonizing oxidative stress. The N6-methyladenosine (m6A) modifications play a significant role in the stress response. However, the effect of m6A modification on the mechanisms of Nrf2-mediated defense against PM2.5-induced PF remain unknown. Here, we investigated the role and the underlying molecular mechanisms of m6A methylation of Nrf2 mRNA in PM2.5-induced PF.

**Results:** Male C57BL/6 mice were exposed to filtered air (FA), unfiltered air (UA) and concentrated air (CA) for 16 weeks. 16HBE cells were treated with 0, 50, or 100 µg/mL PM2.5 for 24 h. Our data showed that chronic PM2.5 exposure could induce fibrosis in lung and increase Nrf2 signals. In Nrf2 deficient cells, α-SMA expression was significantly upregulated whereas E-cadherin decreased compared with WT cells after PM2.5 treatment which implied the aggravated fibrosis. m6A methyltransferase METTL3 was upregulated after PM2.5 treatment. m6A-methylated RNA immunoprecipitation (MeRIP) and qRT-PCR results showed that METTL3 improved the m6A modification of Nrf2 mRNA in PM2.5-exposed 16HBE cells. MeRIP-Seq and single-base T3 ligase-based PCR results showed that the m6A-modified sites of Nrf2 mRNA were 1317, 1376, and 935 in lung of mice after PM2.5 exposure. RIP results suggested that the m6A binding proteins YTHDF1/IGF2BP1 promoted Nrf2 translation by binding to Nrf2 mRNA m6A residues.

**Conclusions:** Our results revealed the mechanism by which m6A regulated the activities of the Nrf2-mediated signaling pathway against PM2.5-induced PF.

**Background**

Particulate matter with an aerodynamic diameter of < 2.5 µm (PM2.5) is involved in the pathogenesis of various respiratory diseases because of the toxicity of these particles, which originate from various sources [1, 2]. Harmful environmental factors can reportedly induce pulmonary fibrosis (PF) [3], which is associated with epithelial–mesenchymal transition (EMT) and extracellular matrix (ECM) [4, 5]. As an important pathway of stromal cell production and excess ECM deposition, EMT can promote scar tissue formation in the lungs, eventually leading to the development of PF [6].

PM2.5 causes lung fibrosis of mice by activating NLRP3 inflammasome [7]. PM2.5 increases ROS production, therefore, aggravates the pulmonary inflammatory response in mice [8, 9]. Nuclear factor erythroid-2-related factor 2 (Nrf2) signals regulate reduction-oxidation balance and play important roles during the development of PF [10]. A previous study found that Trametes orientalis polysaccharide (TOP-2) could alleviate PM2.5-induced lung injury in mice by activating the Nrf2/HMOX-1 pathway and inhibiting the NLRP3 inflammasome [11]. Nrf2 attenuates the EMT and PF development processes by regulating the expression of the transcription factor Snail in bleomycin-induced PF [5, 12]. Therefore, it is hypothesized that Nrf2 might regulate the development of EMT and PF induced by PM2.5.
Environmental pollutants can alter global N$^6$-methyladenosine (m$^6$A) levels and the expression of RNA methylation modulator genes, thereby inducing injury [13]. Our previous study found that m$^6$A-regulated miRNA-126 processing activated the PI3K-AKT-mTOR pathway and exacerbated PF in rats after nanoscale carbon black particle inhalation [14]. It has been reported that m$^6$A-regulated miR-873-5p can mediate the Keap1-Nrf2 pathway to resist colistin-induced oxidative stress [15]. Furthermore, m$^6$A-mediated nuclear receptor PPaR$\alpha$ mRNA stability to regulate lipid metabolism during oxidative stress, which was induced by circadian clock change in mice liver [16]. In our previous study, m$^6$A was concentrated in 3′-untranslated region (UTR) in Nis-treated 16HBE cells [17]. Zhao et al. found Nrf2 mRNA m$^6$A methylation in prepubertal testicular tissue of rats exposed to di-(2-ethylhexyl) phthalate (DEHP) [18], suggesting the presence of potential m$^6$A methylation sites in the 3′-UTR of Nrf2 mRNA. However, the m$^6$A methylation levels and sites of Nrf2 mRNA, as well as its regulation mechanism on PM2.5-induced PF, is still unknown.

In this study, we established PM2.5 exposure models in mice and 16HBE cells. Our data identified that altered m$^6$A modification of Nrf2 mRNA could affect the development of PF induced by PM2.5. Furthermore, we determined the exact Nrf2 mRNA m$^6$A sites (1317, 1376, and 935) which enhanced Nrf2 translation by binding the m$^6$A binding proteins, such as YTH domain-containing proteins (YTHDFs) and insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs). The m$^6$A-mediated Nrf2 signaling pathway might be used as a potential index to prevent PM2.5-induced PF.

Methods

**PM2.5 collection and extraction**

Airborne PM2.5 was collected on a Teflon® filter by a high-volume air sampler (Thermo Scientific, Franklin, MA, USA) operating at a ventilation frequency of 18-20 h for 6 h per day, and the filter membranes were replaced every 3 days. The retrieved filter membranes were divided into small pieces, placed in a 50-mL centrifuge tube, ultrasonicated in iced water for 30 min, and subsequently shaken for 20 min three times each. The resulting PM2.5 extracts were passed along sterile 40-μm nylon filters (Corning Life Sciences, Corning, NY, USA), freeze-dried, packed in tinfoil, and preserved at −80 °C.

**Animals and PM2.5 exposure**

Six-week-old male C57BL/6 mice were obtained from Vital River Laboratory (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China). Standard food and water were provided with all the mice, which were housed under a 12 h light/dark cycle and standard conditions set to 50% humidity at 25-26 °C. The mouse body weights were recorded. Twenty-four mice were randomly divided into three groups after 1 week of acclimatization. The mice were exposed to concentrated air (CA), unfiltered air (UA), and filtered air (FA) PM2.5 according to our previously reported procedures [19]. The FA and UA mice were housed in the chambers with or without high efficiency particulate air filters, respectively [20]. CA mice were housed in the chamber with the concentrated PM2.5 by the PM2.5 concentration enrichment system (Beijing Huironghe Technology Co., Ltd, Beijing, China). All the mice were exposed for 6 h per day (from 9:00 to 15:00), 7 days
per week for 16 weeks and housed in an individually ventilated cage (IVC) during the remaining time. The mice were then sacrificed, and their lungs were separated and weighed. All experimental protocols were approved in advance by the Committee of the Ethics Animal Experiments of Hebei Medical University (IACUC-Hebmu-20170163).

**Cell culture and treatment**

The human bronchial epithelial cell line (16HBE) was gifted by Dr. D. C. Gruenert (University of California, San Francisco, CA, USA). The cells were cultured in basic Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a water-saturated atmosphere under 5% CO₂ at 37 °C. All the mycoplasma contamination tests conducted using a polymerase chain reaction (PCR)-based universal mycoplasma detection kit were negative. The medium was replenished every day. A 0.05% solution of pancreatin ethylenediaminetetraacetic acid (EDTA; Gibco) was used to digest the cells when they completely covered the bottom of the culture dish.

The cells were transferred to 6-well plates with DMEM and starved for 12 h when the cells covered 80–90% of the board. The cells were then treated with 0, 50, or 100 µg/mL PM2.5 for 24 h.

**Histopathological analysis and immunohistochemical (IHC) staining**

Lungs were fixed with 4% paraformaldehyde (3 mice per group). The paraffin-embedded lung tissues were cut into 5-µm continuous sections then stained with hematoxylin and eosin (H&E). According to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA), Masson’s trichrome staining were performed to evaluate the distribution of fibrous collagen in lung.

IHC was used to detect the Nrf2, HMOX-1, METTL3 expression in lung tissue. The details were in additional files 1.

**Determination of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) concentrations in mouse lung tissues by high-performance liquid chromatography (HPLC)**

The concentrations of MDA, SOD, and GSH in lung tissues of mice were determined using HPLC (Shimadzu Corporation, Kyoto, Japan). Lung tissues (0.5 mg) were homogenized on ice and then centrifuged for 15 minutes at 4 °C, 20,000 rpm, and the resulting supernatants were diluted with a mobile phase (including 1 mM EDTA) mixed at a 1:1 (v/v) ratio, centrifuged again for 2 min, and then instantly analyzed using HPLC.

**Measurement of ROS concentrations**

The intracellular ROS concentration in lung of mice was detected using 2,7-dichlorofluorescein diacetate (DCFH-190 DA) labeling (Beyotime Biotechnology, Jiangsu, China) according to our previously study [21]. ROS levels were evaluated by the average fluorescence intensity using flow cytometry (FACS) analysis (AccuriTM C6 flow cytometer, Becton Dickinson, Franklin Lakes, NJ, USA). The results were
expressed as the mean ± standard deviation (SD). Six mice in each group were used to measure the ROS levels in lungs.

**Western blot**

The lysates of lung tissues or 16HBE cells were denatured by boiling for 10 min. The samples were loaded onto a polyacrylamide gel (PAG) containing 8-10% sodium dodecyl sulfate and were separated using electrophoresis (SDS-PAGE) for approximately 1 h, and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with anti-METTL3 (Proteintech, 1:1,000), anti-Nrf2 (Bioworld, 1:1,000), anti-ALKBH5 (Sigma-Aldrich, 1:1,000), anti-α-SMA (Cell Signaling Technology, Danvers, MA, USA, 1:1,000), anti-HMOX-1 (Proteintech, 1:1,000), anti-Vimentin (Santa Cruz Biotechnology, Dallas, TX, USA, 1:1,000), anti-YTHDF1 (Proteintech, 1:1,000), anti-YTHDF2 (Proteintech, 1:1,000), anti-YTHDF3 (Proteintech, 1:1,000), anti-IGF2BP1 (Cell Signaling Technology, 1:1,000), anti-IGF2BP2 (Abcam, 1:1,000), anti-IGF2BP3 (Abcam, 1:1,000), or anti-GAPDH (Cell Signaling Technology, 1:1,000) at −4 °C for 13-14 h. The incubated membranes were washed four times with Tris-buffered saline containing 0.1% Tween™ 20 and incubated with anti-rabbit IgG (Cell Signaling Technology, 1:5,000) or anti-mouse IgG (Cell Signaling Technology, 1:5,000) for 1 h at 37 °C. The target protein pieces were illuminated using chemiluminescent substrates and a chemiluminescent imaging system (Tanon, Beijing, China). The levels of protein were quantified using a Gel-Pro® Analyzer 3.0. The source of used antibodies are shown in additional files 2.

**Analysis of mRNA concentrations by quantitative real-time PCR (qRT-PCR)**

The total RNA was extracted from the lung tissues and cells with TRIzol™ (Life Invitrogen, Waltham, MA, USA) based on the manufacturer’s protocols. The HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) was used for the reverse transcription of the complementary DNA (cDNA). qRT-PCR was conducted using a Step-One Fast Real-time PCR System (Applied Biosystems, Waltham, MA, USA) using the HiScript® II One-Step qRT-PCR SYBR Green Kit (Vazyme). The qPCR primer sequences are shown in additional files 3.

**Stabilization of cell lines using lentiviral transduction**

The stable cell lines were generated according to our previously described methods [22]. Briefly, LentiCRISPR V2 and Lenti PLEX-MCS vector were used for METTL3 knockdown or overexpression, respectively. Lentivirus vector, the packaging vector PAX2, and VSVG were cotransfected into 293T cells to prepare lentivirus. Cells were transduced using the formentioned lentiviruses with 8 μg/mL polybrene (Sigma, St. Louis, MO, USA) and selected with 1 μg/mL puromycin for 1 week. Then, the 16HBE V2 METTL3 knockdown (KO-M3) and its control (V2) cells as well as METTL3-overexpressed knockdown (KO-M3 PLEX-M3) and its control (KO-M3 PLEX) cells were constructed. The source of recombinant DNAs are shown in additional files 2.

**m⁶A-methylated RNA immunoprecipitation (MeRIP)**
The MeRIP assay was performed according to a previously described protocol [23]. Briefly, RNA was extracted by TRIZol™ according to the manufacturer's instructions. The RNA was then fragmented using an RNA fragmentation kit (Ambion, Austin, TX, USA). Pierce M Protein A/G Magnetic Beads (Thermo Scientific) were incubated with the m^6^A antibody (Synaptic Systems, Goettingen, Germany) at 4 °C for 3 h. The mixture of antibody-coupled magnetic beads, RIP buffer, and fragmented RNA was incubated at 4 °C overnight, and m^6^A nucleotide solution (Sigma-Aldrich) was used to elute the immunoprecipitated RNA for qRT-PCR analysis.

**L-azidohomoalanine labeling of synthesized proteins**

The synthesized proteins were labeled with L-azidohomoalanine (AHA) according to a previously described method [24], with minor modifications. Briefly, cells were incubated in a methionine-free medium for 30 min and then incubated in 50 µM AHA. The protein from cell lysates (500 µg) was used for click reactions according to Click-iT™ Protein Reaction Buffer Kit's protocols (Invitrogen). The total proteins obtained from the click reactions were precipitated with methanol and dissolved in buffer (50 mM Tris, 0.01% SDS). The biotin-labeled proteins were incubated with 50 µL streptavidin-conjugated magnetic beads (Dynabeads® M-280 Streptavidin, Invitrogen) at room temperature for 5 h and washed with PBS and 0.5% SDS five times. The immunoprecipitated proteins were analyzed using SDS-PAGE and western blotting with Nrf2 antibodies.

**Single-base m^6^A validation using ligase-based PCR**

A refined single-base T3 ligase-based method was described previously [25]. Briefly, the designed DNA probes L and R matched the flanking sequences of the m^6^A site (additional files 4). Probes L and R were modified with phosphate at the 5' terminal and two ribonucleotides at the 3'-end codon. The 20 nm probes L and R, 1 × T3 ligation buffer (New England Biological Laboratory [NEB], Ipswich, MA, USA), and 300 ng total RNA were mixed and incubated at 85 °C for 3 min, then at 35 °C for 10 min. T3 DNA ligase (NEB) was added to the final volume of 10 µL.

The mixture was incubated at room temperature for 10 min and then immediately chilled on ice. 1 µL ligation product was amplified by PCR for 24 cycles. The samples were loaded on 2-3% agarose gels and detected by electrophoresis.

**Small interfering RNA (siRNA) transfection**

siRNAs against the Nrf2, YTHDFs, and IGF2BPs were synthesized by Sangon Biotech (Shanghai, China). The sequences of siRNA oligonucleotides are listed in additional files 5. 16HBE cells (5 × 10^5/mL) were temporarily transfected with siRNA using Lipofectamine® RNAiMAX transfection reagent (Invitrogen) based on the manufacturer's instructions. The knockdown effects were analyzed using western blotting.

**RIP**

The CDS regions of IGF2BP1 were synthesized and cloned into pcDNA3 FLAG 2AB vector by the HindIII and EcoRI restriction sites. The 2AB-FLAG-IGF2BP1 and 2AB-FLAG-YTHDF1 plasmids were transfected into 10^7...
cells using Lipofectamine® 3000 for 48 h. Then, the cell lysate was immunoprecipitated with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Magnetic beads coated with 5 µg antibodies against IgG (Millipore) or FLAG® (Sigma Aldrich) were incubated with prefrozen cell lysates overnight at 4 °C. The magnetic bead-bound complexes were washed with RIP washing buffer five times. The precipitated RNA was extracted by a purified phenol–chloroform solution and analyzed using qPCR. The cloning primers of 2AB-IGF2BP2-CDS and 2AB-IGF2BP3-CDS are shown in additional files 3.

**Statistical analysis**

All the results are expressed as the mean ± SD. Statistical analysis was performed using SPSS 24.0 software (SPSS®, IBM®, USA). Unpaired two-tailed Student's *t*-tests were used to compare the differences between two groups. One-way analysis of variance (ANOVA) was used to compare the differences among three or more groups with Dunnett’s or the least significant difference (LSD) post-hoc test. P < 0.05 represented a statistically significant difference.

**Results**

**PM2.5 concentration and physical characteristics**

The mice were exposed to PM2.5 from December 1, 2017 to March 25, 2018. During the exposure period, the PM2.5 concentrations were an average of 0, 86.78, or 671.87 µg/m³ in the FA, UA, or CA chambers, respectively. Our results showed that 91.22% and 99.49% of the particles in the CA and UA chambers were PM2.5, respectively (Table 1). Meanwhile, no particles were observed in the FA chamber. Besides the PM, no statistical significance was observed for the other air components, such as the SO₂, CO, NO₂, or O₃ in different chambers.

**PM2.5 induced PF and collagen deposition in lung tissues of mice and 16HBE cells**

In CA and UA mice, the histopathological results showed that the alveolar walls and bronchiole structure were disordered compared to FA mice (Fig. 1A). Meanwhile, the alveolar walls were thickened, the alveolar septa were narrowed, and the bronchial epithelial cells had proliferated in both CA and UA mice. Numerous inflammatory cells infiltrated into interstitium. Macrophages, monocytes, lymphocytes, and fibrous nodules were found in the lungs of the CA and UA mice. Specifically, damaged alveolar structures, thickened diaphragms, fibroblast formation, and increased collagen fibers were found in the CA and UA mice. The results of Masson's trichrome staining indicated positive staining representing metagenetic fibrosis in the lungs of mice after PM2.5 exposure. Compared with the FA mice, positive staining areas were significant increase in CA and UA mice (P < 0.01) (Fig. 1A).

Western blotting showed that α-SMA expression was 1.41-fold and 2.28-fold increases in UA and CA mice compared with FA mice, respectively (P < 0.05) (Fig. 1B, C). The representative bands of vimentin, α-SMA, and E-cadherin in 16HBE cells are shown in Figure 1D. α-SMA expression was significantly increased in 100 µg/mL PM2.5 group compared with the control (Fig. 1E). Vimentin expression was increased (Fig. 1F), whereas E-cadherin expression was decreased (Fig. 1G) in a dose dependent manner (P < 0.01).
Nrf2 defends against oxidative stress in lung and 16HBE cells after PM2.5 treatment

**Oxidative stress in lungs of mice**

ROS levels in the lungs of CA mice increased by 2.38-fold and 1.95-fold compared with FA and UA mice, respectively (P < 0.05) (Fig. 2A). Our data showed that the MDA levels increased by 1.28-fold in CA mice (P < 0.05) but slightly increased in UA mice (P > 0.05) compared with the FA mice (Fig. 2B). Compared with the FA mice, the SOD levels decreased by 1.32- and 1.66-fold in UA and CA mice, respectively (P < 0.01) (Fig. 2C). The GSH concentration decreased by 1.35-fold in CA mice compared with the FA mice (P < 0.05) (Fig. 2D). All above data demonstrated elevated oxidative stress in lung of mice after PM2.5 exposure.

**Changes of Nrf2-related proteins in lung of mice and 16HBE cells after PM2.5 exposure**

IHC staining was performed for Nrf2 and HMOX-1 expression in lung tissues. In the FA group, Nrf2 and HMOX-1 were expressed in the pulmonary vascular and airway smooth muscle cells, while no obvious positive staining was observed in other areas. After PM2.5 exposure, Nrf2 and HMOX-1 expressed in the fibroblast cytoplasm, except for above areas (Fig. 2E-G). As shown in Fig. 2H, the Nrf2 signal-related proteins expressed in mice lung tissues were detected by western blot. Nrf2 expression significantly increased by 1.15-fold and 1.8-fold in UA and CA groups compared with FA group, respectively (P < 0.05) (Fig. 2I). HMOX-1 expression significantly increased by 1.28-fold and 1.68-fold in UA or CA mice compared with the FA mice, respectively (P < 0.05) (Fig. 2J). No significant difference was observed in Cul3 and Keap-1 protein expression (P > 0.05) (Fig. 2K, L). Nrf2 expression increased significantly in 100 μg/mL PM2.5-treated 16HBE cells compared with the control (P < 0.05) (Fig. 2M, N). HMOX-1 protein levels significantly increased in 100 μg/mL PM2.5-treated 16HBE cells compared with the control (P < 0.01) (Fig. 2O). Cul3 and Keap-1 expression showed no significant difference between groups (P > 0.05) (Fig. 2P, Q).

16HBE siRNA-Nrf2 cells was constructed and our results found Nrf2 deficiency decreased the expression of HMOX-1 and E-cadherin and increased the expression of vimentin and α-SMA in 16HBE cells after PM2.5 treatment, compared with the 16HBE siRNA-control cells (additional file 6 Fig. S1)

**METTL3-mediated m\(^6\)A modification promotes Nrf2 translation in mice and 16HBE cells after PM2.5 treatment**

**METTL3 expression was upregulated in vivo and in vitro after PM2.5 treatment**

The levels of METTL3 protein in lung of mice were detected by IHC staining (Fig. 3A, B). The results of qRT-PCR showed that METTL3 mRNA expression was significantly upregulated by 1.58-fold and 1.11-fold in CA and UA groups compared with FA group, respectively (P < 0.05) (Fig. 3C). Western blotting showed that the METTL3 expression increased by 1.55-fold and 1.09-fold in CA and UA groups compared with FA group, respectively (P < 0.05) (Fig. 3D, E). However, METTL14, ALKBH5, and FTO mRNA expression was not significantly different among the different groups (Fig. 3F-H). In 16HBE cells, western blotting suggested that METTL3 expression increased by 1.66-fold in 100 μg/mL PM2.5 group compared with 0 μg/mL PM2.5 group (Fig. 3I, J).
The effects of METTL3 on oxidative stress in 16HBE cells after PM2.5 treatment

The deficient and over expressive METTL3 16HBE cells were established (Fig. 3K, L). In V2 cells, the expression of METTL3, Nrf2, and HMOX-1 increased in 100 µg/mL PM2.5 group compared with 0 µg/mL PM2.5 group (P < 0.05). In 100 µg/mL PM2.5 treatment group, METTL3, Nrf2, and HMOX-1 expression was significantly decreased in KO-M3 cells compared with V2 cells (P < 0.01) (Fig. 3M-P). However, in KO-M3 cells, there were no significant differences in METTL3, Nrf2, and HMOX-1 expression among the different PM2.5 groups (P > 0.05) (Fig. 3M-P).

In V2 cells, α-SMA levels were significantly increased in 50, 100 µg/mL PM2.5 group compared with 0 µg/mL PM2.5 group, respectively (P < 0.001). In KO-M3 cells, however, α-SMA levels were only significantly upregulated in 100 µg/mL PM2.5 group compared with 0 µg/mL PM2.5 group (P < 0.01) (Fig. 3Q). α-SMA expression was significantly increased in KO-M3 cells after 0, 50, or 100 µg/mL PM2.5 treatment compared with their counterpart V2 cells, respectively (P < 0.01). The rescued experiments were proceeded, and the results showed that METLL3 could affect Nrf2 signals and α-SMA in 16HBE cells after PM2.5 treatment (additional files 6 Fig. S2).

\( m^6A \) modification mediated Nrf2 mRNA translation

The \( m^6A \) modification data were extracted from the GEO datasets and \( m^6A \) motifs within the Nrf2 CDS at a single-nucleotide resolution by analyzing the data from the \( m^6A \) iCLIP (miCLIP)-Seq (additional files 6 Fig. S3). Based on the sequence, we designed three pairs of primers to detect the \( m^6A \) modification (Fig. 4A). \( m^6A \) MeRIP-qRT-PCR confirmed that \( m^6A \)-modified Nrf2 expression was significantly decreased in KO-M3 cells compared with V2 cells (Primer 2, Primer 3) (P < 0.01) (Fig. 4B). Furthermore, \( m^6A \)-modified Nrf2 expression was significantly increased in 100 µg/mL PM2.5-treated 16HBE cells compared with 0 µg/mL PM2.5-treated 16HBE cells (Primer 2, Primer 3) (P < 0.01) (Fig. 4C).

qPCR showed no significant differences of Nrf2 expression in 50 and 100 µg/mL PM2.5-treated 16HBE cells compared with 0 µg/mL PM2.5-treated 16HBE cells (P > 0.05) (Fig. 4D). Our data suggested that expression of Nrf2 protein (Fig. 2N), but not that of the corresponding mRNA, was upregulated in the PM2.5-exposed 16HBE cells. To support this result, we next monitored the expression of the Nrf2 synthesized in KO-M3 and V2 cells by incorporating CLICK chemistry and AHA. The V2 and KO-M3 cells were labeled with AHA, and the resulting AHA-labeled synthesized proteins were biotinylated and affinity-purified with streptavidin. Although the V2 cells robustly synthesized Nrf2 protein, protein synthesis was almost completely blocked in KO-M3 cells (Fig. 4E, F).

Single-base \( m^6A \) detection identified exact Nrf2 mRNA \( m^6A \) sites

It has previously been reported that Nrf2 has five \( m^6A \) sites (808, 935, 1317, 1333, and 1376). \( m^6A \) motifs within the CDS of Nrf2 are shown from the date of MeRIP-Seq in FA and CA mice. The results showed that \( m^6A \) enrichment increased by 1.41-fold in CA mice compared with FA mice (P < 0.01) (Fig. 4A, additional files 6 Fig. S3). To further determine the exact Nrf2 mRNA \( m^6A \) sites related to PM2.5 exposure, we designed...
probes L and R targeting the Nrf2 CDS and used the T3 ligase to concatenate both probes on templates, which could be subsequently amplified by PCR. Furthermore, we designed probes against a nonmethylated Nrf2 A site as a control. Previous studies have suggested that the T3 ligase is highly selective between the m^6A and A sites. Our results showed that the ligation efficiency was significantly decreased at the m^6A sites compared with the nonmethylated A sites. Furthermore, the difference between the ligation efficiencies was magnified by PCR. Therefore, the concentration of PCR products could be used to evaluate the ligation efficiency m^6A and indicate the methylation level of each site [26]. Our results showed that only three sites (1317, 1376, and 935) were methylated in PM2.5-exposed 16HBE cells but not the other two sites (1333 and 808) (Fig. 4G). Furthermore, the methylation could promote Nrf2 mRNA translation after PM2.5 exposure (Fig. 4H).

**YTHDF1/IGF2BP1 preferentially recognizes Nrf2-mRNA residues and promotes Nrf2 translation**

The results showed that Nrf2 expression was reduced significantly in the 16HBE siRNA-YTHDF1 and 16HBE siRNA-IGF2BP1 cells compared with 16HBE siRNA control cells (P < 0.05) (Fig. 5A, B), but not in 16HBE siRNA-YTHDF2, 16HBE siRNA-YTHDF3, 16HBE siRNA-IGF2BP2, and 16HBE siRNA-IGF2BP3 cells (P > 0.05) (additional files 6 Fig. S4). Based on the results obtained for exact Nrf2 mRNA m^6A sites (1317, 1376, and 935), we chose primers 2 and 3 to test whether the YTHDF1 and IGF2BP1 proteins could bind to the Nrf2 mRNA m^6A residues. The RIP-qPCR results showed that the Flag-tagged YTHDF1/IGF2BP1 selectively bound to Nrf2 mRNA. The effect of immunoprecipitation with an anti-FLAG antibody in RIP experiment were shown in additional files 6 Fig. S5. Nrf2 mRNA expression increased by 9-fold in 16HBE Flag-YTHDF1 cells (Primer 2), 8-fold in 16HBE Flag-YTHDF1 cells (Primer 3), 61-fold in 16HBE Flag-IGF2BP1 cells (Primer 2), and 64-fold in 16HBE Flag-IGF2BP1 cells compared with 16HBE Flag cells (Primer 3) (P < 0.01) (Fig. 5C, D). Moreover, METTL3 knockdown significantly decreased the interaction between YTHDF1/IGF2BP1 and Nrf2 mRNA (P < 0.01) (Fig. 5E, F). Meanwhile, PM2.5 treatment significantly increased the interaction between YTHDF1/IGF2BP1 and Nrf2 mRNA (P < 0.01) (Fig. 5G, H).

**Discussion**

In this study, the exposures duration was 6 h/day for 16 weeks. Therefore, the average PM2.5 concentration during this period was equal to 21.67 μg/m^3 in UA chamber and 168 μg/m^3 in CA chamber for an average of 24 h average, which was lower than or similar to the levels in Shijiazhuang from December 1st, 2017 to March 25th, 2018, respectively. After short-term PM2.5 (271.6 ± 84.8 μg/m^3 for 1 day or 138.2 ± 34.3 μg/m^3 for 5 days) exposure, the broken alveolar walls enlarged spaces, and the interstitium filled with inflammatory exudates were observed in lungs of mice [27]. In our previous studies, carbon black nanoparticles (CBNPs), a major airborne particle, could induce alveolar macrophage accumulation and inflammatory cell infiltration and increase the incidence of PF in rat lung tissues [14, 28]. Here, we observed thickened alveolar walls, narrowed alveolar septa, infiltrated inflammatory cells, and metagenetic fibrosis in lungs of UA and CA mice. Increased vimentin and decreased E-cadherin expression are hallmarks of EMT that usually contribute to fibrosis [5, 29]. Collagen is also an extracellular component, and its abnormal accumulation is a key feature of PF [30]. Increased α-SMA expression whereas decreased E-cadherin were
observed in lung of mice after PM2.5 treatment [31]. In the present study, vimentin and α-SMA expression increased whereas E-cadherin expression decreased in lungs and 16HBE cells after PM2.5 exposure. These results indicated the occurrence of fibrosis and increased EMT activity in lung of mice after PM2.5 exposure.

It has already been shown that PM2.5-induced oxidative stress is mainly caused by an imbalance between ROS production and antioxidant defense activity [32, 33]. Excess ROS lead to oxidative stress, induce tissue damage and then promote fibrosis related with the antiapoptotic fibroblast formation in idiopathic pulmonary fibrosis (IPF) [34]. In the present study, significantly increased ROS levels were observed in lung of mice. Furthermore, PM2.5 exposure increased the MDA concentration in lung of mice. In contrast, we observed significant decreases of SOD activities and GSH levels after PM2.5 exposure. Our results indicated that the PM2.5 could increase the concentrations of superoxide radicals, hydrogen peroxide, and MDA, whereas downregulate the SOD activity and decrease the GSH levels to aggravate oxidative stress [35].

Nrf2 could alleviate oxidative stress, increase cellular proliferation and migration, and decrease apoptosis, which may modulate MMP9, TGFβ1, and fibronectin expression [36]. Nrf2 is the primary transcription factor regulating HMOX1 mRNA expression [37]. In bleomycin-treated PF mice, Nrf2 expression increased after exposure for 7 or 14 days and decreased after 28 days [5, 38]. In previous studies, increased Nrf2 protein was found in the lungs of mice [36] and prefrontal cortex of rats [39] after PM2.5 exposure. In the present study, Nrf2 expression was increased in the lungs of the mice exposed to PM2.5 for 8 or 16 weeks, possibly because continuous PM2.5 exposure might induce persistent oxidative stress and then increase Nrf2 expression (additional files 6 Fig. S6 for 8-week mice experiment). HMOX-1 is an enzyme showing strong anti-inflammatory and antioxidative properties. It catalyzes the degradation of the proinflammatory free heme and produces anti-inflammatory compounds, such as bilirubin and carbon monoxide, which was selected as a marker for inducing the cellular protective mechanism [40]. In a previous study, both of HMOX-1 expression and Nrf2 translocation increased in a cell alveolar model treated with diesel exhaust particulate matter [41]. In a traumatic brain injury (TBI) mouse model, Dong et al. found that both of Nrf2 mRNA and protein expression increased. Moreover, downstream HMOX-1 was upregulated at the transcription and translation levels. In Nrf2-KO C57BL/6 mice, Nrf2 deletion weakened HMOX-1 expression [42]. Zhang et al. also found more severe PF and lower HMOX-1 and NQO1 expression in bleomycin-treated Nrf2-/- mice compared with WT mice [5]. Oh et al. found that sulforaphane promoted the expression of Nrf2 and its downstream HMOX-1, which attenuated hepatic fibrosis [43]. Furthermore, Nrf2 expression in prefrontal cortices of rats increased significantly after PM2.5 exposure for 12 weeks [39]. In our study, Nrf2 and HMOX-1 expression increased in mice and 16HBE cells after PM2.5 exposure. However, the Nrf2-related proteins Cul3 and Keap-1 were no significant differences in lung of mice and 16HBE cells after PM2.5 treatment. These findings suggest that Nrf2, but not Cul3 or Keap-1, plays a major role in the PM2.5-induced PF model. Similarly, we found that HOMX-1 expression was regulated by Nrf2. Furthermore, increased vimentin levels were observed in lungs of mice and 16HBE after PM2.5 treatment. After Nrf2 deletion, vimentin expression increased whereas HMOX-1 expression decreased compared with the WT cells after PM2.5 exposure. Therefore, we demonstrated that Nrf2 could promote the antioxidative enzyme HMOX-1 and inhibit vimentin expression induced by PM2.5. α-SMA expression was significantly
upregulated whereas E-cadherin levels were decreased in 16HBE siRNA-Nrf2 cells after PM2.5 treatment compared with that of 16HBE siRNA control cells. These data suggested that Nrf2 deficiency could aggravate PM2.5-induced PF by decreasing HMOX-1 and E-cadherin expression as well as increasing α-SMA and vimentin expression.

m^6^A could regulate RNA metabolism, including pre-mRNA splicing, mRNA nuclear export, mRNA stability, mRNA translation [44]. In a previous study, METTL3 and METTL14 expression was observed increases in mice after PM2.5 exposure (271.6 ± 84.8 µg/m^3^) but not found significant changes of FTO and ALKBH5 expression [27]. Similarly, in the present study, the levels of METTL3 transcription and protein were increased in lungs of mice and 16HBE cells after PM2.5 exposure but not about METTL14, ALKBH5 or FTO expression. Additionally, Wang et al. found that METTL3 might regulate Keap-1/Nrf2 pathway in colistin-induced oxidative stress by interacting with DGCR8 [15]. In our study, Nrf2, and HMOX-1 expression was significantly decreased in KO-M3 cells compared with V2 cells after PM2.5 exposure. In KO-M3 cells, α-SMA levels were significantly upregulated compared with in V2 cells after PM2.5 treatment. Rescued METTL3 expression could increase the Nrf2 expression and decrease α-SMA levels. These data indicated that METTL3 suppression might impair the ability of the Nrf2 pathway to defend against PM2.5-induced oxidative stress and increase fibrosis.

In the present study, we found the expression of Nrf2 protein but not mRNA was upregulated in the PM2.5-exposed 16HBE cells, which indicated that the m^6^A modification selectively mediated Nrf2 mRNA translation, but not its transcription. Our results indicated PM2.5-induced METTL3 expression promoted YTHDF1/IGF2BP1 to bind on Nrf2 mRNA through m^6^A modification and then induced Nrf2 translation. Overall, these data suggest that Nrf2 protein expression could be posttranscriptionally regulated by m^6^A modifications during PM2.5 exposure.

Indeed, distinct m^6^A sites within the same transcript are regulated by different modifying enzymes and may exert different biological functions [45, 46]. Multiple Nrf2 mRNA m^6^A loci have been found in various datasets. In the present study, five potential Nrf2 mRNA m^6^A methylation sites (808, 935, 1317, 1333, and 1376) were screened based on the analysis of the MeT-DB and GEO database (GSM1828594, GSM1828596). Recently, Zhao et al. reported that demethylase FTO mediated three Nrf2 mRNA m^6^A sites (75, 714, and 1164), which might decrease Nrf2 protein expression and then inhibit Nrf2-mediated antioxidant pathway in DEHP-induced prepubertal testicular injury [18]. In the present study, the exact Nrf2 mRNA m^6^A sites (1317, 1376, and 935) promoted Nrf2 translation in lung of mice after PM2.5 exposure, thereby facilitated Nrf2 antioxidant signaling pathway.

**Conclusions**

PM 2.5 could cause fibrosis of lung and Nrf2 antioxidant signaling pathway plays a critical role. Methyltransferase METTL3 deposits m^6^A on Nrf2 mRNA, which is upregulated by PM2.5 exposure. m^6^A binding proteins YTHDF1/IGF2BP1 facilitate Nrf2 translation by identifying the Nrf2 mRNA m^6^A sites (Fig. 6). Overall, our results revealed the mechanism by which m^6^A regulated the activity of the Nrf2-mediated
signaling pathway against PM2.5-induced PF, thereby broadened the knowledge of PF diagnosis and treatment.

**Abbreviations**

ARE, antioxidant response element; CA, concentrated air; CDS, coding sequence; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FA, filtered air; GSH, glutathione; MDA, malondialdehyde; m6A, N6-methyladenosine; PF, pulmonary fibrosis; PM2.5, particulate matter with an aerodynamic diameter of < 2.5 µm; ROS, reactive oxygen species; SOD, superoxide dismutase; UA, unfiltered air.

**Declarations**

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**Authors’ contributions**

DJ: Investigation, Data Curation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing. CH: Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing. JN: Investigation, Resources, Writing - Review & Editing. XY: Investigation, Resources. HZ: Investigation, Resources. BZ: Investigation. BL: Investigation. QL: Resources. RZ: Conceived the project and critically revised the manuscript, Funding acquisition. WJ: Conceived the project and critically revised the manuscript, Funding acquisition. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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**Availability of data and materials**

All the data that support the findings of this study are available on request from the corresponding author.

**Ethics approval and consent to participate**
The animal care and use protocol was reviewed and approved by the Laboratory Animal Ethical and Welfare Committee, Hebei Medical University, Shijiazhuang, China (Approval No. IACUC-Hebm-20170163).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Tables

Table 1. Particulate-matter size distributions (%) in different chambers.

| Size (µm) | FA | Percentage (%) | Mean ± SD | UA | Percentage (%) | Mean ± SD | CA | Percentage (%) |
|----------|----------------|----------------|----------|----------------|----------|----------|----------------|
| <0.523 | - | - | 15.35±0.95 | 4.0 | 390.83±8.30 | 3.03 |
| 0.523–1 | - | - | 265.03±2.35 | 60.13 | 6895.78±203.56 | 75.56 |
| 1–1.5 | - | - | 95.32±3.01 | 15.08 | 1898.56±20.38 | 16.44 |
| 1.5–2.0 | - | - | 30.56±4.50 | 8.05 | 235.06±3.98 | 3.69 |
| 2–2.5 | - | - | 17.45±3.50 | 3.96 | 65.56±3.26 | 0.77 |
| 2.5–5 | - | - | 30.03±2.56 | 6.36 | 39.89±2.03 | 0.42 |
| 5–10 | - | - | 9.89±4.05 | 2.03 | 1.98±1.56 | 0.09 |
| >10 | - | - | 1.30±0.22 | 0.39 | 0 | 0.00 |

Figures
Figure 1

Fibrosis in mouse pulmonary and 16HBE cells. (A) Changes in lung-tissue histopathology including H&E and Masson's trichrome staining in FA, UA, and CA mice. Scale bar represents 50µm with the outer box and 20µm with the inner box. In H&E, red arrow indicates thickened interstitial, green arrow indicates inflammatory cell infiltration, and yellow arrow indicates tracheal wall. The quantification data of Masson's trichrome staining showed that lung fibrosis deteriorated in UA and CA mice; n=3, *P < 0.05, **P < 0.01 and ***P < 0.001 compared with FA mice, respectively. (B-C) Representative protein changes in α-SMA
expressions in mouse lung tissues \( n = 3 \), \( *P < 0.05 \) compared with FA mice. (D) Representative protein changes in (E) \( \alpha \)-SMA, (F) Vimentin, and (G) E-cadherin expressions in 16HBE cells \( n = 5 \), \( *P < 0.05 \) and \( **P < 0.01 \) compared with nontreated control 16HBE cells.

**Figure 2**

Nrf2-related oxidative stress in PM2.5-exposed mouse-lung tissues and 16HBE cells. (A) PM2.5-induced ROS production in lung tissues of mice treated with CA, UA, or FA; \( n = 6 \), \( **P < 0.01 \) compared with FA mice; \( ###P < 0.01 \) compared with UA mice. (B–D) Concentrations of MDA, SOD, and GSH in lung tissues of CA, UA,
FA, and mice, respectively; n = 3, *P < 0.05 and **P < 0.01 compared with FA mice. (E) Representative images of immunohistochemically stained Nrf2- and HMOX-1-positive cells in mouse-lung tissues. Scale bar represents 50 µm. (F–G) The quantification data of immunohistochemistry showed that Nrf2 and HMOX-1 expressions were significantly upregulated in lung tissues of CA mice. n = 3. **P < 0.01 and ***P < 0.001 compared with FA mice, respectively. (H–L) Representative protein changes in Nrf2, HMOX-1, Cul3, and Keap-1 protein levels in lung tissues of mice exposed to FA, UA, and CA; n = 3, *P < 0.05 and **P < 0.01 compared with FA group, respectively. (M–Q) Representative protein changes in Nrf2, Cul3, Keap-1, and HMOX-1 protein levels in 16HBE cells treated with 0, 50, and 100 µg/mL of PM2.5; n = 5, *P < 0.05 compared with nontreated control 16HBE cells.
Figure 3

m6A mediated Nrf2-signaling pathway in mice and 16HBE cells. (A) Representative images of immunohistochemically stained METTL3-positive cells in mouse-lung tissues. Scale bar represents 50 µm. (B) The quantification data of immunohistochemistry showed that METTL3 expression upregulated significantly in the lung tissues of CA mice. n = 3. **P < 0.01 and ***P < 0.001 compared with FA group. (C) Expressions of METTL3 mRNA in lung tissues of FA, UA, and CA mice; n = 3, *P < 0.05 compared with FA group. (D–E) Representative protein bands and changes in METTL3 expression in lung tissues of FA, UA,
and CA mice; n = 3, *P < 0.05 compared with FA group. (F–H) Expressions of METTL14, ALKBH5, and FTO mRNA in lung tissues of FA, UA, and CA mice; n = 3. (I–J) Representative protein bands and changes in METTL3 expression in PM2.5-exposed 16HBE cells; n = 5, *P < 0.05 compared with nontreated control 16HBE cells. (K–L) Representative protein changes in METTL3 expressions in 16HBE V2, KO-M3, KO-M3 PLEX, and KO-M3 PLEX-M3 cells; n = 5, *P < 0.05 compared with 16HBE V2 cells; ###P < 0.01 compared with KO-M3 PLEX cells. (M–Q) Representative protein changes in METTL3, Nrf2, HMOX-1 and α-SMA expressions in 16HBE V2 cells and 16HBE KO-METTL3 cells; n=5, *P < 0.05 and **P < 0.01 compared with nontreated 16HBE V2 cells; ###P < 0.001 compared with 16HBE V2 cells treated with 100µg/mL of PM2.5; &&P < 0.01 compared with nontreated 16HBE KO-METTL3 cells.
Figure 4

Mechanism of m6A-mediated Nrf2 expression and single-base m6A detection identifying exact Nrf2-mRNA m6A sites. (A) Potential Nrf2 m6A modification sites. (B) m6A modification of Nrf2 expression in 16HBE KO-M3 cells; n = 5, **P < 0.01 (Primer 2) and #P < 0.05 (Primer 3) compared with 16HBE V2 cells. (C) m6A modification of Nrf2 expression in PM2.5-exposed 16HBE cells; n = 5, *P < 0.05 (Primer 2) and ##P < 0.01 (Primer 3) compared with nontreated control 16HBE cells. (D) Nrf2-mRNA expressions in PM2.5-exposed 16HBE cells; n = 5. (E–F) Affinity purification of biotinylated L-azidohomoalanine (AHA)-labeled proteins
acutely synthesized in 16HBE V2 and KO-M3 cells; n = 5, *P < 0.05 compared with 16HBE V2 cells. (G) Validation of 5 sites in 16HBE V2 and KO-M3 cells. Three sites (1317, 1376, and 935) were confirmed as m6A sites, whereas other sites (1333 and 808) were nonmodified and treated as control sites. (H) Validation of m6A sites (1317, 1376, and 935) in PM2.5-exposed 16HBE cells.

Figure 5

YTHDF1/IGF2BP1 preferentially recognizes Nrf2-mRNA m6A residues and promotes Nrf2 translation. (A) Representative protein changes in YTHDF1 and Nrf2 expressions in 16HBE siRNA-control and 16HBE siRNA-
YTHDF1 cells; n = 5, *P < 0.05 and **P < 0.01 compared with 16HBE siRNA-control cells, respectively. (B) Representative protein changes in IGF2BP1 and Nrf2 expressions in 16HBE siRNA-control and 16HBE siRNA-IGF2BP1 cells; n = 5, *P < 0.05 and **P < 0.01 compared with 16HBE siRNA-control cells, respectively. (C) RIP analysis of YTHDF1 protein binding in 16HBE cells; n = 5, **P < 0.01 compared with 16HBE Flag cells (Primer 2), ##P < 0.01 compared with 16HBE Flag cells (Primer 3). (D) RIP analysis of IGF2BP1 protein binding in 16HBE cells, n = 5, **P < 0.01 compared with 16HBE Flag cells (Primer 2), ##P < 0.01 compared with 16HBE Flag cells (Primer 3). (E) RIP analysis of YTHDF1 protein binding to Nrf2 mRNA in METTL3 knockdown and control 16HBE cells; n = 5, **P < 0.01 compared with 16HBE V2 Flag-YTHDF1 cells (Primer 2), ##P < 0.01 compared with 16HBE V2 Flag-YTHDF1 cells (Primer 3). (F) RIP analysis of IGF2BP1 protein binding to Nrf2 mRNA in 16HBE V2 and KO-M3 cells; n = 5, **P < 0.01 compared with 16HBE V2 Flag-IGF2BP1 cells (Primer 2), ##P < 0.01 compared with 16HBE V2 Flag-IGF2BP1 cells (Primer 3). (G) RIP analysis of YTHDF1 protein binding to Nrf2 mRNA in PM2.5-exposed 16HBE Flag-YTHDF1 cells; n=5, **P < 0.01 compared with nontreated 16HBE Flag-YTHDF1 cells (Primer 2); ##P < 0.01 compared with nontreated 16HBE Flag-YTHDF1 cells (Primer 3). (H) RIP analysis of IGF2BP1 protein binding to Nrf2 mRNA in PM2.5-exposed 16HBE Flag-IGF2BP1 cells; n=5, **P < 0.01 compared with nontreated 16HBE Flag-IGF2BP1 cells (Primer 2); ##P < 0.01 compared with nontreated 16HBE Flag-IGF2BP1 cells (Primer 3).

Figure 6

m6A-mediated Nrf2 signals regulates the defense mechanism against PF during PM2.5 exposure.

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