MicroRNA-760 Resists Ambient $PM_{2.5}$-induced Lung Injury Through Upregulating Heme-oxygenase 1 Expression

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Research

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Abstract

Background: $PM_{2.5}$ (particles matter smaller aerodynamic diameter of 2.5 μm) exposure, as one major environmental risk factor for the global burden of disease, is associated with high risks of respiratory diseases and lung cancer. Heme-oxygenase 1 (HMOX1) has been considered as one of the major molecular antioxidant defenses to mediate cytoprotective effects against diverse stressors, including $PM_{2.5}$-induced toxicity and SARS-CoV-2 infection; however, the regulatory mechanism of HMOX1 expression still needs to be elucidated. In this study, using $PM_{2.5}$ as a typical stressor, we explored whether microRNAs (miRNAs) might modulate HMOX1 expression in lung cells.

Results: Systematic bioinformatics analysis showed that seven miRNAs have the potential to target *HMOX1* gene. Among these, hsa-miR-760 was identified as a response miRNA to $PM_{2.5}$ exposure. More importantly, we revealed a “non-conventional” miRNA function in hsa-miR-760 upregulating HMOX1 expression, by targeting the coding region and interacting with YBX1 protein. In addition, we observed that exogenous hsa-miR-760 effectively elevated HMOX1 expression, reduced the reactive oxygen agents (ROS) levels, and then rescued the lung cells from $PM_{2.5}$-induced apoptosis.

Conclusion: Our results revealed that hsa-miR-760 might play an important role in protecting lung cells against $PM_{2.5}$-induced toxicity, by elevating HMOX1 expression, and offered new clues to elucidate the diverse function of miRNAs.

Background

Air pollution has become an urgent environmental and public health issue worldwide. Ambient air particulate matter is an important pollutant of urban atmosphere and has been linked to adverse health effects of the respiratory system [1]. The main sources of $PM_{2.5}$ (particles matter smaller aerodynamic diameter of 2.5 μm) are anthropogenic, with known contributors being power plants, heating, and vehicles. Unlike PM_{10}, $PM_{2.5}$ is sufficiently small in size to reach the alveoli of the lungs, thus exhibits a significant impact on human health [1-4]. According to the Global Burden of Disease report, airborne $PM_{2.5}$ exposure might contribute to 4.2 million deaths and 100 million disability-adjusted life-year losses, and serves as the fifth largest risk factor for death in the world [5]. Epidemiological studies have consistently shown that long-term exposure to high levels of $PM_{2.5}$ is associated with high risks of ischemic heart disease, stroke, lung cancer, and chronic obstructive pulmonary disease [6, 7].

Numerous evidences suggested that oxidative stress and inflammation play a critical role in $PM_{2.5}$ induced lung injury [5, 8-10]. Exposure to $PM_{2.5}$ has been reported to stimulate the release of various proinflammatory molecules in the lung. Furthermore, $PM_{2.5}$ may carry soluble organic components and transition metals on their surfaces that can prolong inflammation. $PM_{2.5}$ mediated oxidative stress may be initiated by diverse events, including higher concentrations of reactive oxygen species (ROS), soluble fractions and transition metals, and inflammatory activation [11, 12].

Enzymatic antioxidant defense has been considered as one of major molecular antioxidant defenses to eliminate ROS. The antioxidant enzymes are produced to restore cellular redox homeostasis in the presence of oxidative stress [13]. Heme oxygenase 1 (*HMOX1*) is a rate-limiting enzyme for heme metabolism and a key enzyme for endogenous carbon monoxide (CO) synthesis [14, 15]. In addition, *HMOX1* is one of the *Nrf2*-targeted genes, and has been reported to be involved in the protection of inflammation and apoptosis [16]. In most tissues/organs, except for the spleen, *HMOX1* is usually expressed at low levels; however, it is highly inducible in response to a variety of stimuli to protect cells against oxidative and inflammatory injury. Several lines of evidence have indicated that $PM_{2.5}$-induced oxidative stress might function as a signaling molecule to activate *HMOX1* expression [5, 13, 17, 18] ; however, the detailed mechanism remains unrevealed.

In the past two decades, non-coding RNAs have been identified as an essential epigenetic factor in modulating gene expression and therefore affecting biological functions. MicroRNAs (miRNAs) are a class of small non-coding RNAs with multiple function in diverse biological and pathological processes[19-22]. Due to their involvement in extensive biological functions, miRNAs are becoming clinical biomarkers for disease diagnosis and have been considered to have therapeutic potential in pre-clinical research [23]. MiRNAs typically suppress gene expression at post-transcriptional levels, but increasing evidences showed that specific miRNAs might increase the target gene expression [24-26]. Till now, lots of dysregulated miRNAs have been identified after $PM_{2.5}$ exposure [27-30]; however, it remains largely elusive about how specific miRNAs might contribute to *HMOX1* upregulation under $PM_{2.5}$ exposure.

In this study, we explored the hypothesis that $PM_{2.5}$ might modulate specific miRNA expression, which contributes to *HMOX1* upregulation to resist $PM_{2.5}$-induced lung injury. We observed a significant up-regulation of hsa-miR-760 in the lung cells upon $PM_{2.5}$ exposure, and manifested that hsa-miR-760 might upregulate *HMOX1* expression by binding to the coding region, i. e., an unconventional miRNA function. Further, we discovered that hsa-miR-760 up-regulates *HMOX1* by elevating the mRNA stability in a YBX1-dependent manner. We also found that upregulation of hsa-miR-760 has an anti-apoptosis effect in lung cells exposed to $PM_{2.5}$.

Results

Hsa-miR-760 was predicted to modulate *HMOX1* expression in response to $PM_{2.5}$ exposure

Firstly, we retrieved the top ten genes interacting with $PM_{2.5}$ exposure from the CTD database. All genes were reported to interact with $PM_{2.5}$ over 50 times, suggesting their pivotal roles in response to $PM_{2.5}$ exposure (**Fig. 1a**). Among these, *HMOX1* has been documented to induce anti-apoptotic effects after PM2.5 exposure *in vivo* and *in vitro*, but the underlying biological mechanism is not clear. Using *HMOX1* as the target gene, a total of seven miRNAs, including has-miR-485-5p, hsa-miR-760, hsa-miR-671-5p, hsa-miR-520a-5p, hsa-miR-525-5p, hsa-miR-3126-5p, and hsa-miR-3142, were predicted to bind to the *HMOX1*

transcript (**Fig. 1b; Table 1**). Among them, hsa-miR-760 showed high free energy to bind to both the 3'UTR and coding region of the *HMOX1* transcripts, indicating its strong regulatory roles in modulating *HMOX1* expression.

We then treated HBE cells with different concentrations of $PM_{2.5}$, and measured the expression changes of hsa-miR-760 and *HMOX1* at 24h after $PM_{2.5}$ exposure. As shown in **Fig. 1c,** $PM_{2.5}$ exposure significantly elevated hsa-miR-760 levels in HBE cells. Intriguingly, both RNA and protein levels of HMOX1 were observed to be upregulated in a dose-dependent fashion after $PM_{2.5}$ exposure (**Fig. 1d**), indicating a potential unconventional miRNA function of hsa-miR-760 in modulating *HMOX1* expression.

Hsa-miR-760 binds to the coding region of *HMOX1*

As mentioned above, hsa-miR-760 was predicted to potentially target the 3'UTR and coding region of the *HMOX1* transcript. We first constructed the reporter gene plasmids containing the core 3'UTR of *HMOX1*, and observed that exogenous hsa-miR-760 failed to affect the luciferase activities (**Fig. S1**), suggesting that hsa-miR-760 was not able to bind to *HMOX1* 3'UTR. Subsequently, we constructed three modified reporter gene plasmids (**Fig. 2a**), including HMOX1 CDS-WT (containing two predicted binding sites of hsa-miR-760), and two mutant luciferase plasmids HMOX1 CDS MUT-1 and HMOX1 CDS MUT-2 (destroying the 1st and 2nd binding sites of hsa-miR-760, respectively). **Fig. 2b** showed that exogenous hsa-miR-760 transfection significantly increased the luciferase signal produced by the HMOX1 CDS-WT and HMOX1 CDS-MUT2 plasmids in both HEK293T and HBE cells (all $P < 0.01$), but failed to elevate the luciferase signal produced by HMOX1 CDS-MUT1 plasmid as compared with the ones transfected with miRNA NC, proving that hsa-miR-760 was able to bind to the 2nd binding site, which with the free energy of −36.60 kcal/mol, in *HMOX1* coding region.

The direct interaction between hsa-miR-760 and its cognate target in the *HMOX1* coding region was then further validated by FREMSAs. As shown in **Fig. 3a(*lane 3*)**, hsa-miR-760 formed distinct complexes with the 2nd binding site in the *HMOX1* coding region. In competition assays, excess unlabeled miRNA oligonucleotides attenuated the miRNA:mRNA complexes (**Fig. 3a, *lane 5***), but excess unlabeled nonspecific oligonucleotides failed to show any inhibitory effects (**Fig. 3a, *lane 4***), exhibiting the sequence-specific interaction between hsa-miR-760 and its cognate binding site. In addition, the miRNA pull-down assays were conducted to test the miRNA:mRNA complex *in vivo*. As shown in **Fig. 3b**, the *HMOX1* transcript was significantly enriched ($P < 0.01$) in cells transfected with the 3'-biotinylated oligonucleotides of hsa-miR-760, compared with the ones transfected with miRNA NC.

Hsa-miR-760 upregulates HMOX1 levels in lung cells

Our findings described above revealed a direct interaction between has-miR-760 and *HMOX1* transcript. To measure the regulatory significance of hsa-miR-760 in endogenous *HMOX1* expression, were transfected different concentrations of hsa-miR-760 mimics and inhibitors into HBE and H226 cells. Together with the elevation of hsa-miR-760 levels (**Fig. S2**), both mRNA and protein levels of HMOX1 were

significantly increased in a dose-dependent fashion in HBE and H226 cells (all $P < 0.05$) (**Fig. 3c, 3d**), respectively. Further, the cells transfected with hsa-miR-760 inhibitors significantly decreased *HMOX1* expression, at both RNA and protein levels, compared with the ones transfected with inhibitor negative control (all $P < 0.05$) (**Fig. 3c, 3d**).

Knockdown of YBX1 attenuates the upregulatory effect of hsa-miR-760 on HMOX1 expression

Having observed that has-miR-760 upregulates HMOX1 production, we further investigated the underlying molecular mechanism via multiple functional experiments. The miRNA pull-down assays were conducted, and the proteins that could specifically bind to the biotinylated has-miR-760 were identified by mass spectrometry (MS) analysis. Among the proteins specifically enriched by biotinylated has-miR-760 (**Supplementary Table 2**), YBX1, a multifunctional RNA binding protein, was selected for further functional validation. Indeed, evidences obtained from both miRNA pull-down and RIP assays showed that YBX1 could bind to has-miR-760, directly (**Fig. 4a and 4b**). Intriguingly, as indicated in **Fig. 4c**, YBX1 could also specifically bind to *HMOX1* transcript.

To check the biological role of YBX1 in the interaction between hsa-miR-760 and *HMOX1* transcript, HBE cells were transfected with or without hsa-miR-760 mimics, together with either negative control siRNA or *YBX1* siRNA, and the RNA and protein levels of *HMOX1* gene were measured, respectively. As shown in **Fig. 4d**, treatment with siRNAs against *YBX1* significantly eliminated the hsa-miR-760 activity ($P< 0.01$). Interestingly, has-miR-760 mimics was also able to upregulate the YBX1 production ($P < 0.05$) (**Fig. 4e**), which further indicates the interaction between hsa-miR-760 and *YBX1*.

It was known that YBX1 is involved in RNA biology, such as translational repression, RNA stabilization, and mRNA splicing. Our results showed that exogenous hsa-miR-760 transfection significantly inhibited the *HMOX1* mRNA degradation, compared with the control group (**Fig. 4f**). However, once the cells were treated with siRNAs against *YBX1*, hsa-miR-760 failed to affect the stability of *HMOX1* transcripts **(Fig. 4g)**. All these evidences proved that YBX1 protein participates in the regulatory process of hsa-miR-760 on *HMOX1* expression by increasing its mRNA stability.

Knockdown of HMOX1 upregulates of ROS and apoptosis levels in HBE cells

To understand the antioxidant mechanism of HMOX1, the HBE cells were transfected with *HMOX1* siRNAs, and the ROS and apoptosis levels were measured. We observed that the knockdown of HMOX1 (**Fig. S3a**) led to a significant ROS accumulation (both $P < 0.05$) (**Fig. 5a)**. Moreover, the apoptosis levels were also significantly increased in the siRNA against *HMOX1* groups (all $P < 0.05$), compared to the control group (**Fig. 5b and 5c**). In line with this phenotype, the levels of BCl2/BAX, a molecular indicator of apoptosis, was decreased after the knockdown of HMOX1 **(Fig. 5d)**. These results proved that HMOX1 is an antioxidant factor by inhibiting ROS accumulation and apoptosis.

Exogenous hsa-miR-760 expression reduced apoptosis levels in HBE cells exposed to $PM_{2.5}$

We further investigated the upregulatory effects of has-miR-760 on *HMOX1* expression under $PM_{2.5}$ exposure. As shown in **Fig. S3b**, transfection with hsa-miR-760 mimics significantly increased mRNA and protein levels of HMOX1 in $PM_{2.5}$ exposure group. Moreover, transfection with hsa-miR-760 inhibitor was able to reverse the increase of HMOX1 expression by $PM_{2.5}$ exposure, at both mRNA and protein levels (**Fig. S3c**).

Though *HMOX1* was also upregulated upon $PM_{2.5}$ exposure (**Fig.1d and Fig.S3b**), HBE cells exhibited significant ROS accumulation and apoptosis (**Fig. 6 a, b, c,** and **d**), indicating the endogenous *HMOX1* upregulation failed to protect the cell against apoptosis. However, exogenous transfection of hsa-miR-760 effectively reduced the ROS levels, and then rescued the cells from $PM_{2.5}$-induced apoptosis (**Fig. 6 a, b, c,** and **d**).

Discussion

MicroRNAs are the members of small non-coding RNAs contains about 20-24 nucleotides, which act as important regulators in diverse pathogenesis processes. It has been reported that lots of miRNAs are dysregulated under $PM_{2.5}$ exposure, and these miRNAs might regulate the process of $PM_{2.5}$-induced toxicity. For example, $PM_{2.5}$ exposure significantly dysregulated the miR-331 and miR-146-3p expression, which contributing to human airway epithelial cells inflammation following $PM_{2.5}$ exposure [25, 32]. Multiple epidemiologic studies also observed the negative correlation between $PM_{2.5}$ exposure and the expression of miRNAs, such as miR-146a, miR-21, miR-222, miR-21-5p, and miR-187-3p [31, 32]. However, our understanding of the biological significance of miRNAs in response to $PM_{2.5}$ exposure is still the tip of the iceberg. In this study, we reported hsa-miR-760 as a new response miRNA to $PM_{2.5}$ exposure for the first time. More importantly, we revealed a “non-conventional” miRNA function in hsa-miR-760 modulating HMOX1 expression and then proved the exogenous hsa-miR-760 might serve as a potential drug to prevent $PM_{2.5}$-induced ROS accumulation and apoptosis in lung cells.

HMOX1 is a heme oxygenase whose transcription is prone to be triggered by a wide variety of stressors, including heme, hypoxia, and oxidative stress [32]. Moreover, it was reported that the HMOX1 pathway could be a target for the treatment and prevention of SARS-CoV-2 of 2019 (COVID-19) [33]. To reveal whether HMOX1 is epigenetically modulated in its response to $PM_{2.5}$ exposure, we first predicted seven miRNAs targeting HMOX1 transcript using four databases. Among them, hsa-miR-760 exhibited the highest free energy to bind to the 3’UTR and coding region of HMOX1. Hsa-miR-760 has been reported as a potential anti-tumor miRNA in various cancers [23, 34, 35]. For example, Yan et al. reported that hsa-miR-760 suppressed NSCLC cell proliferation, cell cycle, and migration, by regulating the ROS1 pathway [36]. In addition, hsa-miR-760 was reported to mediate cell apoptosis and inhibit the phosphorylation of ERK and JNK in Rheumatoid arthritis [37].

Intriguingly, in the cells exposed to $PM_{2.5}$, both hsa-miR-760 and HMOX1 were significantly elevated, indicating that hsa-miR-760 might act as a “non-conventional” miRNA, i.e., increasing but not suppressing

target gene expression. Subsequent functional experiments, including dual-luciferase reporter gene assay, FREMSA, and miRNA pull-down assay, identified that hsa-miR-760 was able to bind to the 2nd binding site, which with the free energy of –36.60 kcal/mol, in the HMOX1 coding region. Moreover, exogenous hsa-miR-760 transfection resulted in a strong elevation of endogenous HMOX1 expression under our experimental conditions, validating the direct regulatory role of hsa-miR-760 in HMOX1 production.

It's well-known that most miRNAs typically bind to 3'UTR of the target gene, leading to mRNA degradation or translation inhibition [20, 38]. More and more studies noticed that miRNAs could elevate target gene expression via binding to the 3'UTR or 5'UTR region [25, 39, 40]. The functional significance of miRNAs targeting the coding region remains elusive, though a few studies proposed that such miRNAs might be involved in translation inhibition [41, 42]. We provided solid evidences that hsa-miR-760 is able to upregulate HMOX1 expression by targeting the coding region, which supplies new clues to elucidate the functional significance of miRNAs.

Another highlight in this study is the identification of pivotal proteins involved in the interaction between miRNA and target mRNA. Usually, miRNAs are loaded into Argonaute (AGO) protein-containing complexes to exert canonical repression mechanisms [43]. In the present study, by using miRNA pull-down together with MS analysis and RIP assays, we found that hsa-miR-760 exerts unconventional regulatory function by interacting with YBX1, but not AGO family proteins. YBX1, a versatile RNA binding protein (RBP), has been implicated in multiple cellular processes, including transcription regulation, RNA stabilization, and translation process [44] [45, 46]. RBP interacting with miRNA is not unprecedented. For example, Eiring et al. reported that miR-328 might interact with hnRNP E2 [47]. To our knowledge, this is the first study to report the interaction between YBX1 and miRNA. In addition, we found that hsa-miR-760 mediated HMOX1 upregulation might partially attribute to the elevated HMOX1 mRNA stability.

HMOX1 is induced rapidly in response to a wide variety of stressors and it plays a key role in cellular protection [48]. Once large amounts of ROS were produced and released, cells will suffer from redox imbalance, which leads to series of phenomena such as DNA damage, destruction of cell membrane structure, and damage of mitochondria and other organelles, and finally induces cell apoptosis [49]. Multiple studies have indicated that $PM_{2.5}$-induced ROS may function as signaling molecules to induce *HMOX1* expression to protect cells against oxidative stress [5, 13, 17, 18]. In the current study, we also found that HMOX1 knockdown could upregulate ROS and apoptosis levels in cells, which consistent with the notion that HMOX1 plays an important role in cellular defense by inhibiting ROS accumulation. However, although HMOX1 was upregulated upon $PM_{2.5}$ exposure, HBE cells still exhibited ROS accumulation and apoptosis, under our experimental conditions. One plausible explanation is that the amounts of HMOX1 induced by $PM_{2.5}$ exposure fail to protect the cell against oxidative stress. Indeed, we observed that exogenous hsa-miR-760 transfection effectively elevated HMOX1 expression, reduced the ROS levels, and then rescued the cells from $PM_{2.5}$-induced apoptosis. Our study provided a new mechanism on the miRNA function under $PM_{2.5}$ exposure on lung injury.

Conclusions

In summary, our findings revealed that $PM_{2.5}$ exposure upregulated has-miR-760 levels, and then activated *HMOX1* expression that contributes to the protection against ROS accumulation and cell apoptosis. Therefore, has-miR-760 upregulating the HMOX1 pathway could prove to be preventive agents for $PM_{2.5}$ -induced lung injury. Most important of all, we revealed a “non-conventional” miRNA function, by which hsa-miR-760 upregulated *HMOX1* expression via the YBX1-dependent manner.

Methods

In silico analyses

Ten genes exhibiting the strongest interaction with $PM_{2.5}$ were achieved from the Comparative Toxicogenomic Database (CTD, http://ctdbase.org). The potential miRNAs targeting *HMOX1* gene were predicted using TargetScan (www.targetscan.org), miRTar (mirtar.mbc.nctu.edu.tw), Starbase (starbase.sysu.edu.cn/), DIANA (diana.imis.athena-innovation.gr/DianaTools/), respectively. RNAhybrid algorithm (bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) was used to predict the free energy of potential miRNA: mRNA duplexes.

Cell culture, transfection, and $PM_{2.5}$ treatment

Human lung cancer cell line H226 and embryo kidney cell line HEK293T were obtained from American Tissue Type Culture Collection (ATCC, Manassas, VA). Human lung normal epithelial cell line (HBE) is a gift from professor Wen Chen (Sun Yat-Sen University, Guangzhou). H226 was cultured in RPMI-1640 medium, HEK293T was cultured in DMEM medium, and HBE was cultured in MEM medium plus 10% FBS, respectively. The cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Multiple commercially obtained miRNA mimics (Ruibo, Shanghai, China), miRNA inhibitor (IDT, Coraville, IA), and siRNAs targeting *YBX1* and *HMOX1* (Ruibo, Guangzhou, China) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), while constructed plasmids were transfected into cells by Lipofectamine 2000 (Invitrogen).

Ambient $PM_{2.5}$ were collected from Shijiazhuang, China, and the details about the collection, sample extraction and components analyses were presented in our previous study [50]. $PM_{2.5}$ was dissolved in DMSO to produce 100mg/ml stock solutions, and then added to the cell culture at the final concentration of 25μg/ml, 50μg/ml, and 100μg/ml, respectively. HBE cells treated with an equal amount of 0.1% DMSO were used as solvent control.

Fluorescent-based RNA electrophoretic mobility shift assay (FREMSA)

All oligonucleotides and primers used in this study were purchased from Sangon Biotech (Shanghai, China). The dye-hsa-miR-760 oligonucleotide was labeled with IRDye® 800 dye on its 5’ end, while the

cognate *HMOX1* mRNA FREMSA was performed according to the protocol described in our previous study [51]. Briefly, Briefly, both miRNA and mRNA oligonucleotides were heated for 2 minutes at 95℃ to relax RNA secondary structures and immediately placed on ice. And then, 400 nM synthetic miRNA or/and 400nM cognate mRNA oligonucleotides were mixed with basic reaction buffer (1x Binding Buffer, 5% glycerol, 200 mM KCl, and 100 mM $MgCl_2$). All reaction mixtures were cultured at 25℃ for 20 min to form miRNA:mRNA duplex. The reaction mixtures were then separated on a 12% PAGE by electrophoresis at 4℃, and the resultant mobility shifts were scanned by Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoin, NE).

MicroRNA pull-down assays and MS analysis

The miRNA pull-down experiment was carried out as described in the previous study [52]. Briefly, a biotin molecule was covalently attached to the 3' end of the mature hsa-miR-760 or negative control strand. Modified biotin-miR-760 mimics and negative control were transfected into the HBE cells. Cells were harvested at 24h after transfection, and lysed using the lysis buffer that containing 3% IGEPAL ® CA-630 (Sigma-Aldrich, St. Louis, MO), protease inhibitor cocktail (Roche, Basel, Switzerland), and RNaseOUT (Thermo scientific, Tewksbury, MA). The cytoplasmic lysate was mixed with precoated beads (Life technologies) and incubated (with rotation) at 4 °C overnight to obtain miRNA-mRNA-protein complex. The harvested complex was then divided into two parts. One portion was used to isolate RNA using Trizol (Life Technologies, Carlsbad, CA), while the other portion was used to analyze proteins based on LC-MS/MS system by Applied Protein Technology (Shanghai, China).

Dual-luciferase reporter gene assay

Hsa-miR-760 was predicted to target both the 3'UTR and coding region of the *HMOX1* gene. The core *HMOX1* 3'-UTR that harboring the hsa-miR-760 response element was chemically synthesized and subcloned into the psiCHECK2 reporter vector, to detect the potential interactions between hsa-miR-760 and *HMOX1* 3'UTR. We further modified the psiCHECK2 vector to express the luciferase-HMOX1 fusion protein, by deleting a "T" nucleotide from the stop codon "TAA" of *Renilla* luciferase gene based on the change mutation method. Then the coding region of *HMOX1* that containing the response elements of hsa-miR-760 was subcloned into the modified psiCHECK2 vector. Specific mutations in the target sites matching to hsa-miR-760 seed sequence were introduced by PCR-based site-specific mutagenesis.

For the luciferase assays, HBE and 293T cells were seeded at 60% confluency in 96-well plates and co-transfected by constructed plasmids (100 ng/well) together with hsa-miR-760, or negative control (NC) mimics (100 nM) using lipo2000. Cells were harvested at 48h after transfection, and the luciferase activity was measured using the dual-luciferase kit (Promega, Madison, WI).

RNA isolation and quantification

All mRNA and miRNA primer sequences used in this study were listed in **Supplementary Table 2**. Both mRNAs and miRNAs were extracted from cells using TRIZOL, and cDNA was synthesized with a random

primer or specific miRNA or stem-loop reverse transcription primers, using Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific). Quantitative Real-Time PCR (qRT-PCR) was conducted using SYBR Green Kit (Qiagen) on the Light Cycler 480 Analyzer (Roche). All of the reactions, including the no-template controls, were run in triplicate. The miRNA levels were normalized to U6 snRNA, while mRNA levels were normalized to *GAPDH*.

mRNA degradation assay

This assay is designed to measure the decay kinetics of mRNAs. HBE cells were transfected with hsa-miR-760 mimics or NC mimics, incubated for 24h, and exposed to actinomycin D (final concentration: 3μg/ml). The cells were then harvested at 0, 4, 8, and 16 h after actinomycin D treatment, respectively, and total RNAs were prepared to test the relative *HMOX1* expression as mentioned above.

RNA immunoprecipitation (RIP)

RIP assay was conducted using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Burlington, MA), according to the manufacturer's protocols. Briefly, the cell lysate was incubated with antibodies against YBX1 and IgG control (Abcam, Cambridge, MA) overnight at 4 °C. The RNA-protein complex was recovered by Protein G Dynabeads and reverse cross-linked with proteinase K. Finally, the protein-binding RNAs were extracted using TRIZOL reagent and analyzed by qRT-PCR.

Western blot analysis

Total proteins were isolated from the cells using RIPA lysis buffer (Thermo Scientific). Antibodies against HMOX1 and GAPDH were purchased from Abcam, and antibodies against BCL2 and BAX were obtained from Cell Signal Technology (Danvers, MA). Western blotting assays were carried out using the Odyssey™ Western Blotting Kit (LI-COR Biosciences), and analyzed based on the Odyssey CLx Infrared Imaging System.

TUNEL Apoptosis Assays

Apoptosis was detected using the riboAPO™ One-Step TUNEL Apoptosis Kit (RiboBio, Guangzhou, China), according to the manufacturer's protocol. In brief, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with TdT Enzyme and TAM-dUTP Mixture protected from light, serially. The reaction was stopped by SSC, and the apoptotic cells stained by TUNEL were observed under a fluorescence microscope.

ROS Detection

Cells were treated with $PM_{2.5}$ as indicated and then exposed to membrane permeable fluorescent probe DCFH-DA (final concentration: 10 mM). After incubation for 20min at 37°C, the cells were harvested and resuspended in PBS. Fluoresce signaling was detected by Beckman Coulter (USA).

Statistical analyses

The quantitative variables are exhibited as mean ± standard deviation (SD). Statistical analysis was conducted using SPSS Statistics Version 21.0 (SPSS Inc., Chicago, IL). Student's t test was used to compare the differences in luciferase reporter gene assays, while one-way ANOVA on ranks test was used to detect the differences between subgroups for protein or RNA levels. Differences were regarded as statistically significant when $P < 0.05$.

Abbreviations

HMOX1, Heme-oxygenase 1; ROS, reactive oxygen species; CO, carbon monoxide; CTD, Comparative Toxicogenomic Database; FREMSA, Fluorescent-based RNA electrophoretic mobility shift assay; NC, Negative Control; qRT-PCR, quantitative real-time PCR; RIP, RNA immunoprecipitation; CDS, coding domain sequences; AGO, Argonaute; RBP, RNA binding protein.

Declarations

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Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Not applicable

Author contributions

Y.Z., and D.Y. proposed and organized the study; D.Y. designed the study; L.X., Q.Z., D.L., J.L., and W.M. predicted and validated the interaction between miRNA and *HMOX1* transcript; L.X., Y.J., and C.L. identified the YBX1; L.X. and K.Z. measured the ROS levels and cell apoptosis. D.Y. and L.X. wrote the manuscript, and W.C. and Y.Z. revised the manuscript. All authors reviewed the manuscript.

Consent for publication

All authors agree for the publication.

Competing interests

The authors declare no competing interests.

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Tables

Table 1. miRNAs potentially targeting *HMOX1* transcript

Gene symbol	Transcript	Targeting position	Region	miRNA symbol	Free energy (kcal/mol) *
HMOX1	NM_002133	1386–1407	3′ UTR	hsa-miR-485-5p	−18.70
HMOX1	**NM_002133**	**439–458**	**CDS**	**hsa-miR-760**	**−29.90**
HMOX1	**NM_002133**	**751–769**	**CDS**	**hsa-miR-760**	**−36.60**
HMOX1	**NM_002133**	**1203–1219**	**3′ UTR**	**hsa-miR-760**	**−28.50**
HMOX1	NM_002133	999–1020	3′ UTR	hsa-miR-671-5p	−17.50
HMOX1	NM_002133	1076–1096	3′ UTR	hsa-miR-520a-5p	−14.60
HMOX1	NM_002133	1076–1096	3′ UTR	hsa-miR-525-5p	−16.30
HMOX1	NM_002133	1068–1088	3′ UTR	hsa-miR-3126-5p	−14.60
HMOX1	NM_002133	978–999	3′ UTR	hsa-miR-3142	−16.40

* calculated by the RNAhybrid program (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid).

Figures

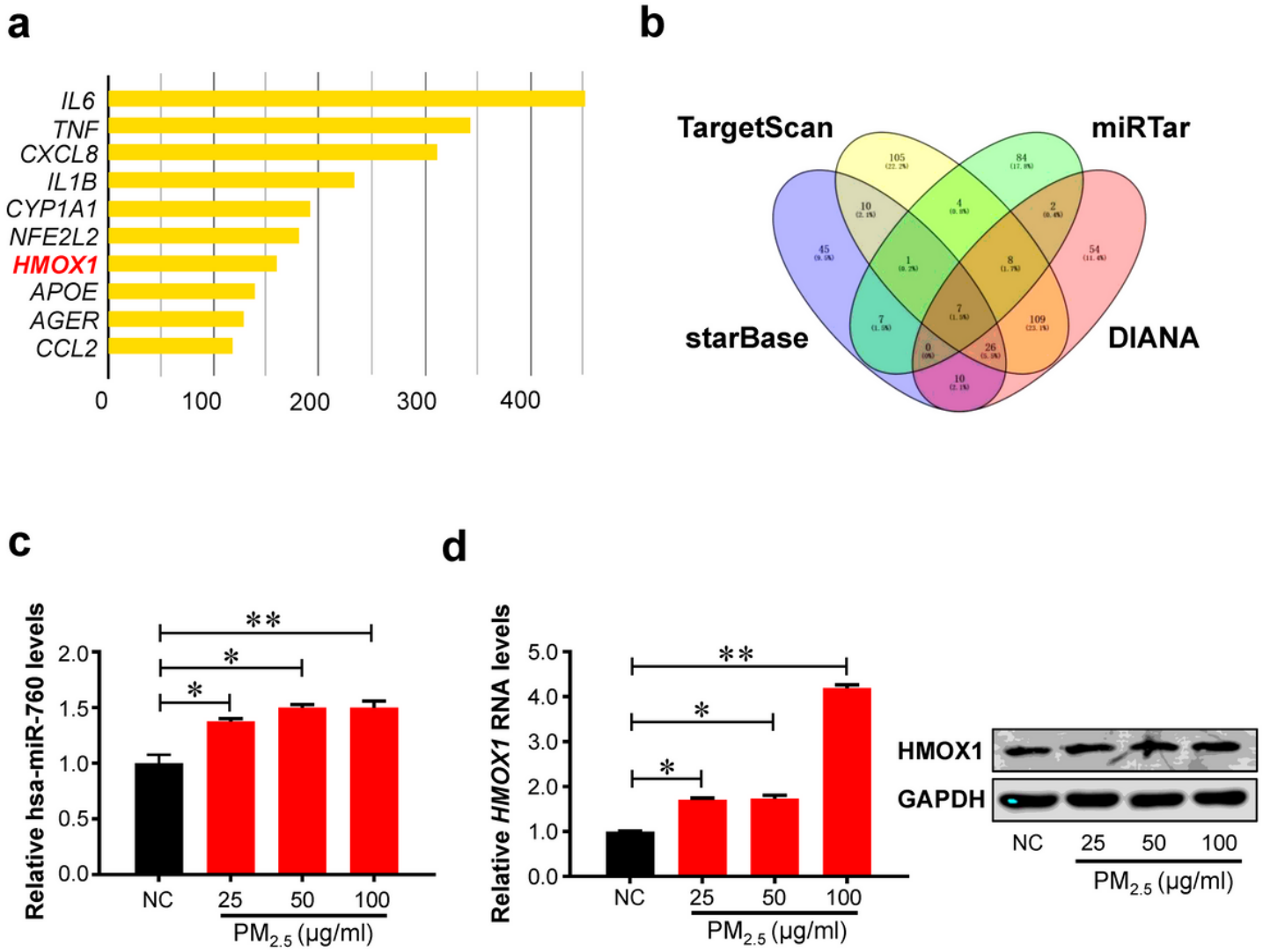

Figure 1

Selection of candidate genes and miRNAs response to PM2.5 exposure. (a) A total of 10 genes were recruited from the Comparative Toxicogenomic Database (CTD) database. (b) Venn diagram showing potential miRNAs targeting HMOX1 in four databases. (c) and (d) hsa-miR-760 levels, and HMOX1 mRNA and protein levels were significantly increased in HBE cells treated by different concentrations of PM2.5. *P < 0.05; **P < 0.01; NC, DMSO group.

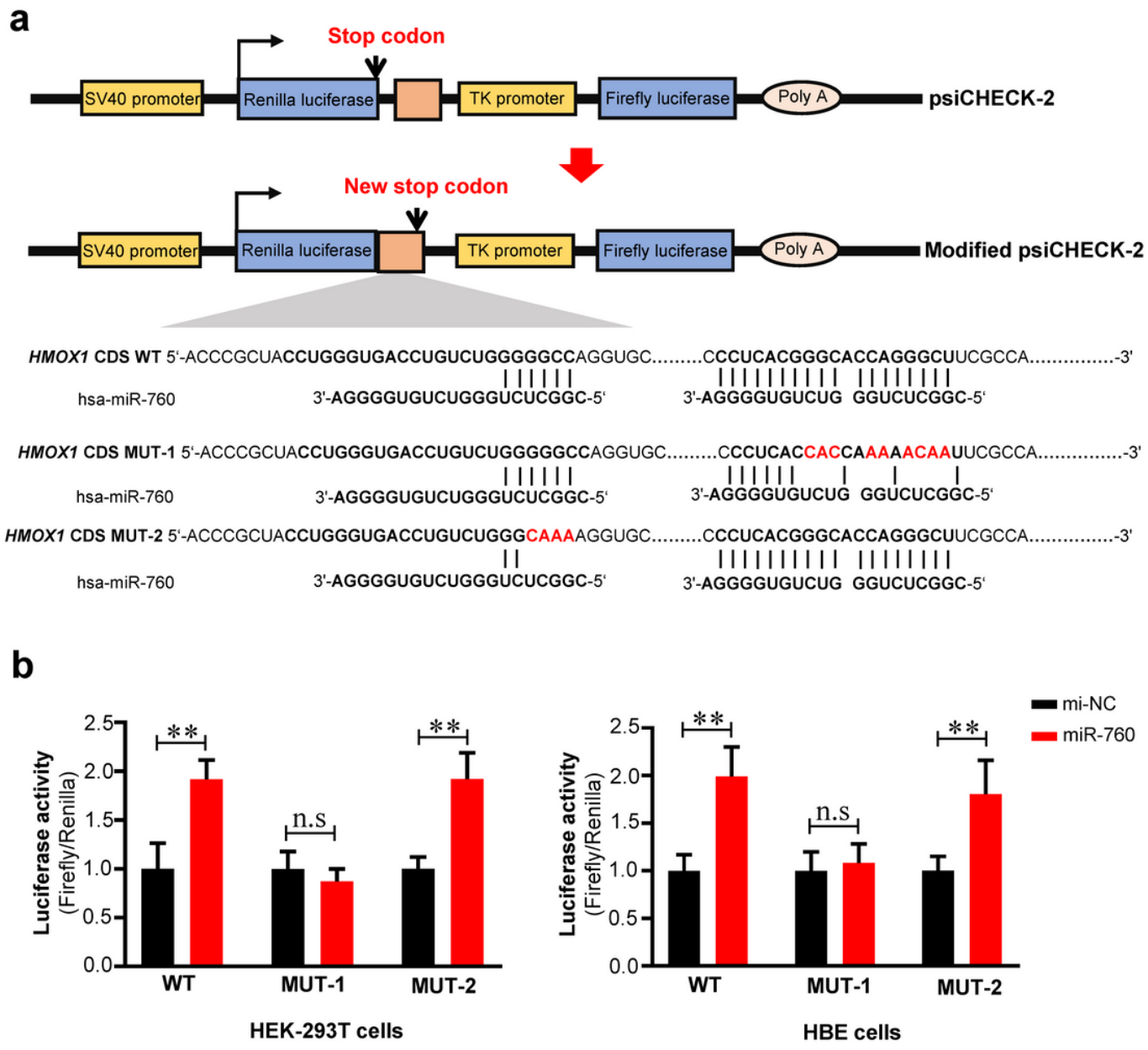

Figure 2

Hsa-miR-760 directly binds to the coding region of HMOX1. (a) The modified reporter gene plasmid and the potential miR-760 binding sites within the HMOX1 coding region. The solid vertical line indicates base pairing. HMOX1 coding sequences containing miRNA-recognition elements (MRE) were inserted into a modified psiCHECK2 reporter in which the stop codon of Renilla luciferase gene was deleted. (b) Reporter gene assays to investigate the regulatory effects of hsa-miR-760 on luciferase signal produced by HMOX1 coding region in HEK293 FT cells and HBE cells. **P < 0.01; n.s, no significant; mi-NC, miRNA mimic negative control.

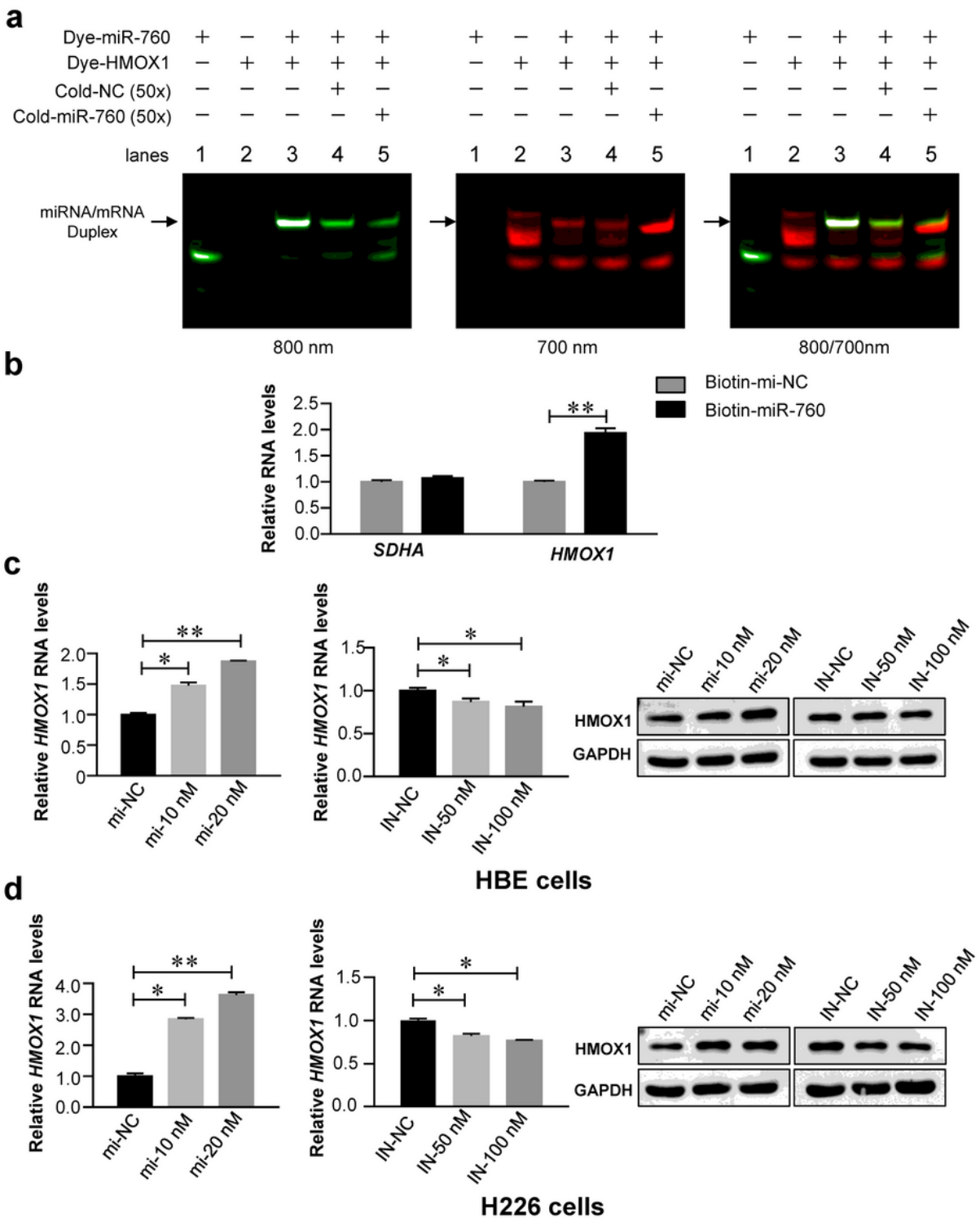

Figure 3

Hsa-miR-760 upregulates HMOX1 expression in lung cells. (a) FREMSA using 5'-dyed has-miR-760 or HMOX1 oligonucleotide, Lane 1 and 2 indicated the mobility of each type of oligonucleotide; lane 3 indicated the mobility status of the miRNA:mRNA complex; lane 4 and 5 revealed the mobility shift status of miRNA:mRNA complex in the presence of excess unlabeled nonspecific competitors or cold specific competitors (hsa-miR-760). Arrow indicates hsa-miR-760/HMOX1 complex. (b) The HMOX1 mRNA was

enriched by streptavidin pull-down for biotinylated has-miR-760. (c) and (d) Overexpression or knockdown of has-miR-760 affected the RNA (left panel) and protein (right panel) levels of HMOX1 in HBE and H226 cells, respectively. Results of mRNA represent means ± S.D. from three independent experiments. *P < 0.05, **P < 0.01; mi-NC, miRNA mimic negative control; IN-NC, miRNA inhibitor negative control.

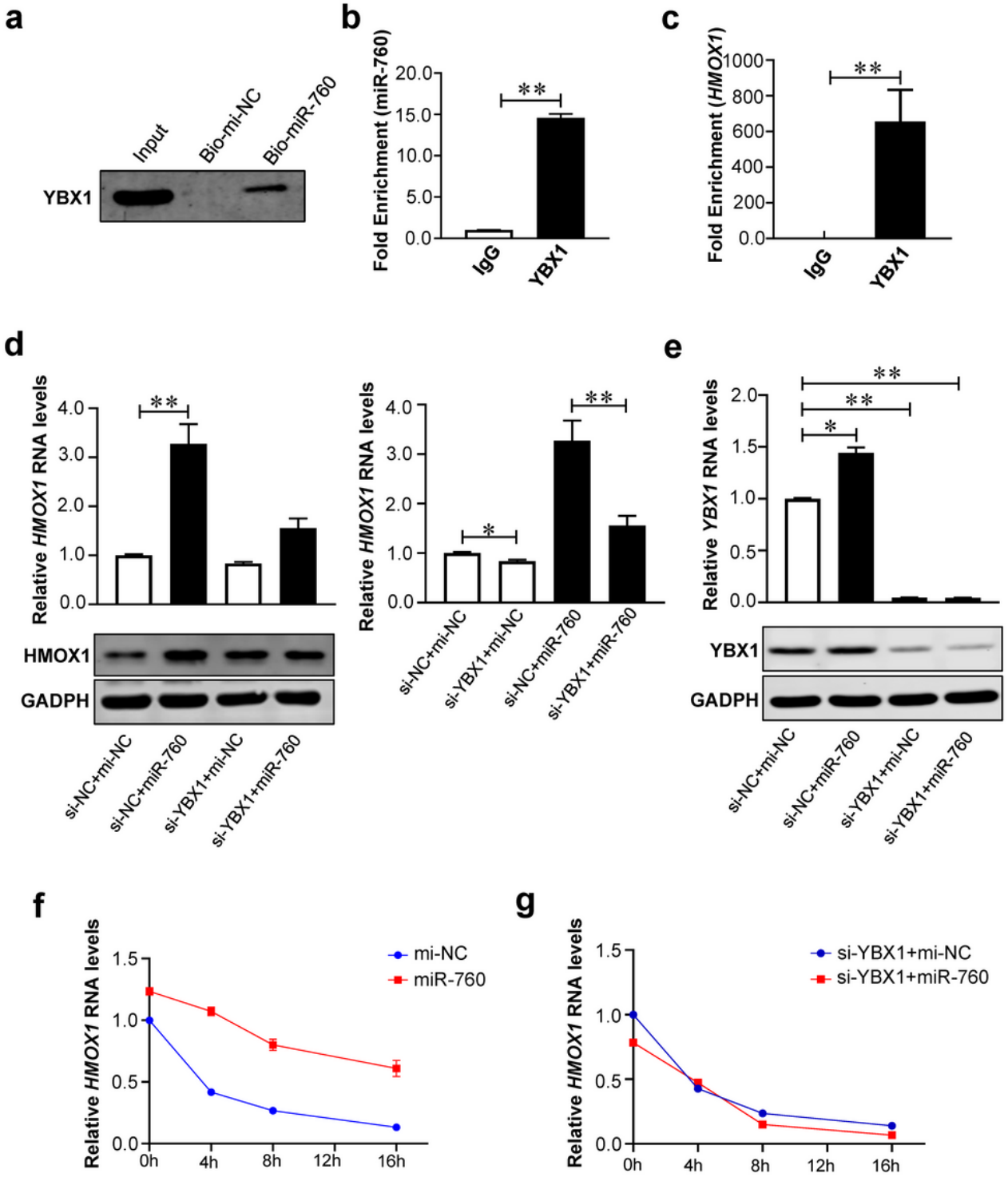

Figure 4

YBX1 is involved in hsa-miR-760-mediated upregulation of HMOX1 expression. (a) Confirmation of has-miR-760 binding to YBX1 using a biotin-based pull-down assay. Cell lysate was collected, and the RNA pull-down assay was performed using Bio-miR-760 or Bio-mi-NC (Biotin miRNA negative control). Associated proteins were pulled down with streptavidin beads, and bound levels of YBX1 were analyzed by western blotting. (b) and (c) RIP assays was performed in HBE cells using antibodies against YBX1. The enriched hsa-miR-760 and HMOX1 transcript were measured by qRT-PCR, respectively. (d) Knockdown of YBX1 attenuates the upregulatory effect of hsa-miR-760 on HMOX1 mRNA (upper) and protein (lower) expression. HBE cells were co-transfected with or without hsa-miR-760 mimics along with either siRNA negative control or YBX1 siRNA. (e) Exogenous hsa-miR-760 elevated YBX1 mRNA (upper) and protein (lower) levels. (f) Exogeneous hsa-miR-760 increases the stability of HMOX1 mRNA. HBE cells were transfected with hsa-miR-760 mimics or miRNA NC at the final concentration of 20 Nm, and then treated by actinomycin D for 4, 8, 12 and 16 h, respectively. The HMOX1 mRNA levels were measured by qRT-PCR. (g) Knockdown of YBX1 attenuated the effect of hsa-miR-760 on HMOX1 mRNA stability. HBE cells were co-transfected with YBX1 siRNA and has-miR-760 mimics or miRNA NC, and then the cells were treated by actinomycin D. *P < 0.05, **P < 0.01; mi-NC, miRNA mimic negative control; si-NC, siRNA negative control.

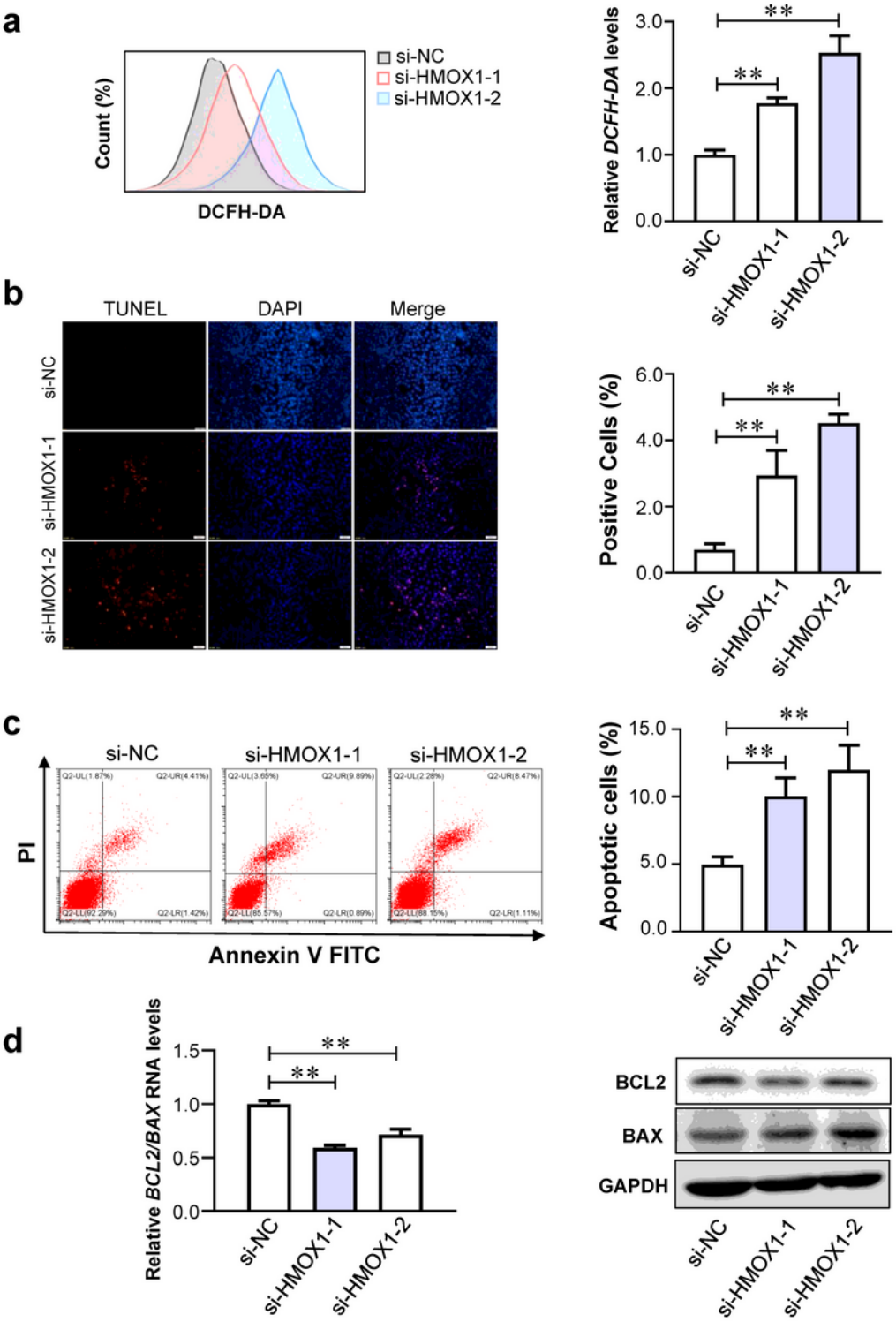

Figure 5

HMOX1 levels affected ROS accumulation and cell apoptosis in HBE cells. (a) ROS was determined in HBE cells with or without siRNA-mediated HMOX1 knockdown. Apoptosis level in HBE cells using TUNEL stanning (b) and flow cytometry (c) after transfection of HMOX1 siRNA or negative control. (d) BCl2/BAX mRNA (left) and protein (right) levels in HBE cells after transfection with HMOX1 siRNA or negative control. *P < 0.05, **P < 0.01; mi-NC, miRNA mimic negative control; si-NC, siRNA negative control.

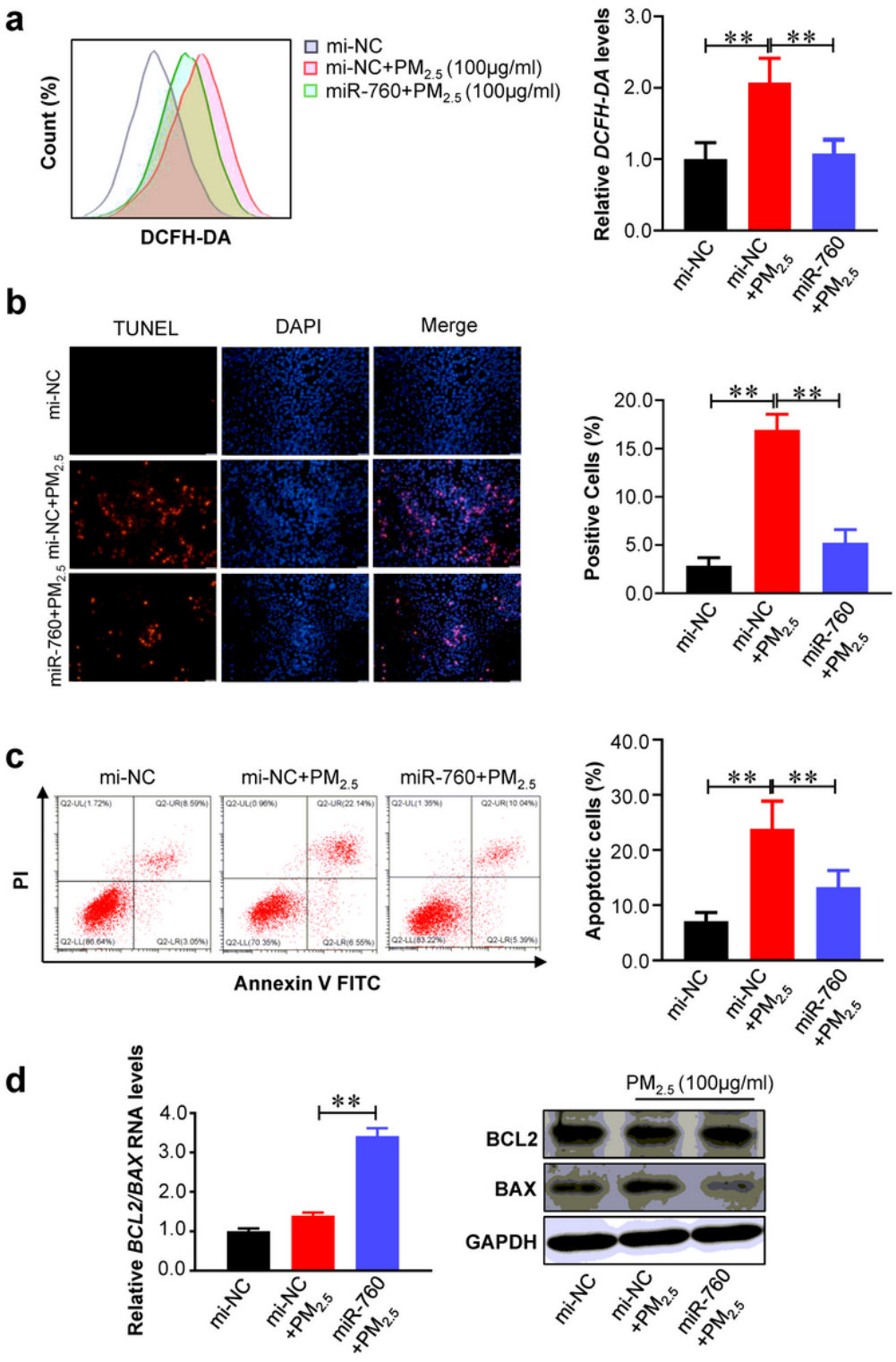

Figure 6

Exogenous hsa-miR-760 protected HBE cells against ROS accumulation and cell apoptosis induced by PM2.5 exposure. HBE cells were transfected with hsa-miR-760 mimics and negative control for 24 h and then cells were treated with PM2.5 (100μg/ml) for an additional 24 h. (a) ROS levels. Apoptosis levels assessed by TUNEL stanning (b) and flow cytometry (c). (d) BCl2/BAX mRNA (left) and protein (right) levels. *P < 0.05, **P < 0.01; mi-NC, miRNA mimic negative control.

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