Cellular response of lung fibroblasts and epithelial cells to particulate matter$_{10}$ treatment examined via comparative transcriptome analysis

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Abstract. Particulate matter (PM) can be categorized by particle size (PM_{10}, $PM_{2.5}$ and $PM_{1.0}$), which is an important factor affecting the biological response. Exposure to PM in the air (dust, smoke, dirt and biological contaminants) is clearly associated with lung disease (lung cancer, pneumonia and asthma). Although PM primarily affects lung epithelial cells, the specific response of related cell types to PM remains to be elucidated. The present study performed Gene Ontology (GO) analysis programs (Clustering GO and Database for Annotation, Visualization and Integrated Discovery) on differentially expressed genes in lung epithelial cells (WI-38 VA-13) and fibroblasts (WI-38) following treatment with PM_{10} and evaluated the cell-specific biological responses related to cell proliferation, apoptosis, adhesion and extracellular matrix production. The results suggested that short- or long-term exposure to PM may affect cell condition and may consequently be related to several human diseases, including lung cancer and cardiopulmonary disease.

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Abbreviations: PM_{10}, particulate matter$_{10}$; DAVID, Database for Annotation, Visualization and Integrated Discovery; ClueGO, Clustering Gene Ontology; DEGs, differentially expressed genes; ECM, extracellular matrix; DNMT, DNA methyltransferase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CCK-8, Cell Counting Kit-8; ER stress, endoplasmic reticulum stress

Key words: PM_{10}, fibroblasts, epithelial cells, RNA-sequencing

Introduction

Particulate matter (PM), also called particle pollution, is the suspension of liquid or solid particles in the air and includes inorganic and organic particles such as smoke, dust, pollen, biological contaminants, heavy metals and dirt (1-3). PM can be classified by the diameter of the particles, including PM_{10}, $PM_{2.5}$ and $PM_{1.0}$, and size is an important factor affecting the cellular response (3-5). Exposure to PM is linked to heart disease (cardiopulmonary disease, cardiac arrest and cardiovascular/hypertensive disease), lung disease (pneumonia, lung cancer, asthma and chronic obstructive lung disease) and neurodegeneration (1,6). The treatment of cells with PM is associated with both an inflammatory response and a pro-oxidative response (7). For example, PM (PM_{10} and $PM_{2.5}$) treatments induce Akt activation via phosphorylation at Ser473 and Thr308 in human lung epithelial cells (BEAS-2B) (8). Additionally, coal and silica dust treatments induce alterations in DNA methylation patterns via changes in DNA methyltransferase (DNMT) 1, DNMT3α, DNMT3β, methyl CpG binding protein 2 and methyl-CpG binding domain protein 2 expression (9,10). In addition, the treatment of A549 cells with $PM_{2.5}$ results in cytotoxicity and genotoxicity (3).

PM stimulates the immune system in the lungs and respiratory tract. Following inhalation, PM accumulates in air exchange regions of the lung, including the alveoli and stimulates defense mechanisms involving alveolar epithelial cells or macrophages (11). Lung tissue consists of various cell types, such as epithelium and fibroblasts, that maintain homeostasis and defense mechanisms in the lungs (12). Fibroblasts are responsible for maintaining the alveolar structure during repair and proliferation in injured regions (13). The lung epithelium provides an inert barrier for gas exchange and bulk airflow (14). Although PM exposure occurs in lung epithelial cells, the cell-specific responses of lung epithelial cells and fibroblasts to PM remain to be elucidated.

Thus, the present study performed an RNA-sequencing (RNA-seq) analysis on lung epithelial cells (WI-38 VA-13) and fibroblast cells (WI-38) following treatment with PM_{10} and used Gene Ontology (GO) analysis programs [Clustering GO (ClueGO) and Database for Annotation, Visualization and

Integrated Discovery (DAVID)] to identify the cell-specific responses to PM.

Materials and methods

PM_{10} sampling and components. The fine dust ERM®-CZ100 (PM_{10}) was purchased from Sigma-Aldrich (Merck KGaA). PM_{10} packaged in amber glass vials was used to prepare stock solutions in phosphate-buffered saline (PBS; 100 mg/ml) and experiments using the suspended PM_{10} composition were performed immediately. The PM_{10} used contained the following polyaromatic hydrocarbons (PAHs): i) Benzo(a)anthracene; ii) benzo(a)pyrene; iii) benzo(b)fluoranthene; iv) benzo(l) fluoranthene; v) benzo(k)fluoranthene; vi) dibenz(a,h)anthracene; vii) indeno(1,2,3-cd)pyrene; and viii) a combination of benzo(b)fluoranthene, benzo(k)fluoranthene and benzo(l) fluoranthene.

Cell culture and treatment. The normal human lung cell lines WI-38 and WI-38 VA-13 subclone 2RA (WI-38 VA-13) were purchased from the Korean Cell Line Bank (Korean Cell Line Research Foundation) and cultured in MEM (HyClone; Cytiva) and RPMI-1640 medium (Welgene, Inc.), respectively, supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere under 5% CO_2 at 37˚C. The WI-38 and WI-38 VA-13 cell lines were treated with PM_{10} (9 $\mu g/cm^2$) for 48 h.

Cell viability assay. A Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) was used to conduct the cell viability assays (15). WI38 and WI38 VA-13 cells were seeded in 6-well plates at $4x10^5$ cells/well and incubated for 24 h. Following PM_{10} treatment for 48 h, CCK-8 solution and RPMI-1640 medium with 10% FBS were added to each well and incubated under 5% CO_2 at 37˚C for 2 min or 5 min. The absorbance was measured using a microplate reader at 450 nm.

RNA isolation. Total RNA was isolated from WI38 and WI38 VA-13 cell lines using a Qiagen RNeasy Mini kit (74106; Qiagen, Inc.) according to the manufacturer's instructions. The concentration of the isolated RNA was confirmed through a SpectraMax QuickDrop Micro-Volume Spectrophotometer (Molecular Devices, LLC). The 260/280 ratio of the RNA used was >2.1.

RNA-seq and analysis. Using an Illumina TruSeq RNA Sample Preparation kit V2, purification and library construction were conducted with total RNA and Illumina HiSeq 2500 machines (Illumina, Inc.) were used for sequencing with a read length of 2x100 bases. FastQC v.0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of the paired-end reads. Cutadapt v.1.15 (https://cutadapt.readthedocs.io/en/v1.15/) and Sickle v.1.33 (https://github.com/najoshi/sickle/releases) were used to filter low-quality reads and adaptors. Cufflinks v.2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/) was used to calculate fragments per kilobase of transcripts per million mapped reads values. Cuffdiff (http://cole-trapnell-lab.github.io/cufflinks/) was used to select differentially expressed genes (DEGs; fold change >2). All GO analyses were performed using DAVID v.6.8 (https://david.ncifcrf.gov/) and ClueGO v.2.5.5 in Cytoscape v.3.7.1 (https://cytoscape.org/) (16).

Wound healing assay. WI38 and WI38 VA-13 cells (80% confluence) were seeded in 6-well plates at $4x10^5$ cells/well using MEM and RPMI-1640 medium, respectively, supplemented with 10% FBS and wounded by scratching with sterile plastic 10 μl micropipette tips after 24 h of PM_{10} treatment (17). Images were taken of the cells at 0 and 24 h after wounding using a CELENATM S Digital Imaging System (Logos Biosystems). Cell migration distance was measured in the images by using ImageJ software version 1.8 (National Institutes of Health).

Flow cytometric cell sorting analysis. After PM_{10} treatment for 48 h, WI38 and WI38 VA-13 cells were collected and incubated with the Muse Annexin V and Dead Cell Assay kit (cat. no. MCH100105; Luminex Corporation) for 20 min at room temperature in the dark. Following incubation, $5x10^4$ cells were analyzed using a Muse cell analyzer (Merck KGaA). The results were analyzed using Muse 1.5 analysis software (Merck KGaA).

Statistical analysis. The results were expressed as the means ± SDs (error bars) of three independent experiments. An unpaired Student's t-test was performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Research strategy for the biological effects of PM_{10} based on RNA-seq analysis in lung cell lines. To evaluate the biological effects of PM_{10} treatment in lung cells, two types of normal lung cell lines (WI-38, fibroblast cells and WI-38 VA-13, epithelial cells) were selected. Following PM_{10} treatment for 48 h, the total RNA was extracted and performed RNA-seq for a bioinformatic analysis (GO analysis). Cell-specific PM_{10} effects were evaluated and the biological relationship between the lung and PM predicted (Fig. 1). To assess the effect of PM_{10} on cell viability, CCK-8 and flow cytometric cell sorting analysis was performed following treatment with PM_{10} for 48 h. In Fig. 2A, although no significant effects on cell viability were observed as a result of PM_{10} treatment, slightly decreased cell growth was observed using bright field microscopy (Fig. 2B). Flow cytometric cell sorting analysis with Annexin V showed that the proportion of apoptotic cells in the PM_{10} treatment group was increased compared with that in the PBS group in both cell lines (Fig. 2C). This suggested that PM_{10} treatment may affect cell growth via induction of apoptosis. To verify whether PM_{10} treatment affected cell migration, wound healing analysis was performed following the treatment of two cell lines with PM_{10}. The rate of migration in VA-13 cells was decreased compared with that in the PBS treatment group. However, no difference in the migration rate in the WI-38 cell line was observed (Fig. 2D). Therefore, it was suggested that acute treatment with PM_{10} could induce cell apoptosis in the two cell lines, but inhibited the migration rate only in epithelial cells (VA-13), not in fibroblasts (WI-38).

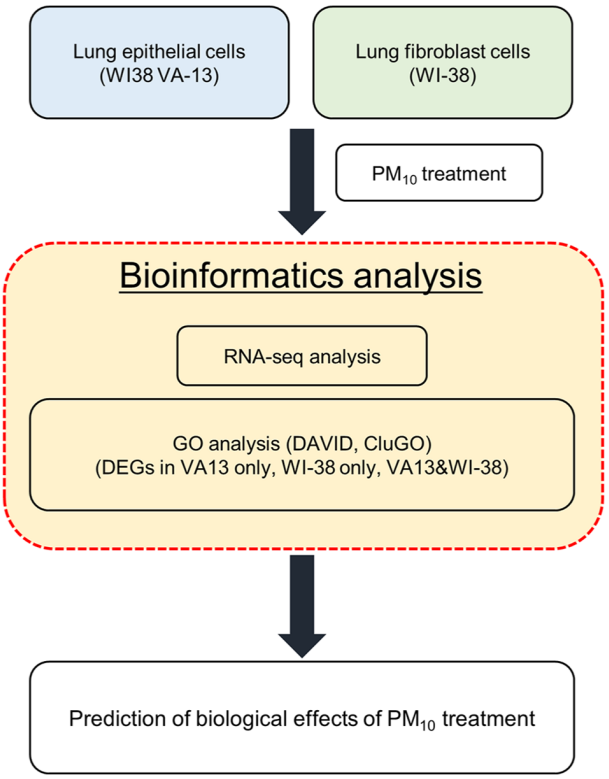

Figure 1. Research strategy for PM_{10} treatment of WI-38 or WI-38 VA-13 cells. PM, particulate matter; DAVID, Database for Annotation, Visualization and Integrated Discovery; GO, Gene Ontology; DEGs, differentially expressed genes; RNA-seq, RNA sequencing; ClueGO, Clustering Gene Ontology.

PM_{10} treatment affects the adhesion and extracellular matrix (ECM) of fibroblasts. RNA-seq analysis was used to observe the cell-specific DEGs in WI-38 and WI-38 VA-13 normal lung cell lines. Using 552 WI-38-specific DEGs, GO analysis was performed using the ClueGO plugin in Cytoscape (Fig. 3A). Fig. 3A shows that PM_{10} treatment was associated with 'apoptotic process', 'signal transduction', 'regulation of immune system process', 'structure morphogenesis' and 'positive regulation of metabolic process'. Additionally, heatmap analysis identified 189 downregulated genes and 363 upregulated genes following treatment with PM_{10} in WI-38 cell lines (Fig. 3B). The 189 downregulated genes in the GO term analysis were associated with migration-related terms ('melanocyte migration' and 'regulation of epithelial cell migration'), cell growth-related terms ('regulation of cell growth' and 'negative regulation of cell growth') and adhesion-related terms ('muscle contraction', 'cell-substrate adhesion' and 'extracellular matrix organization'). In addition, the 363 upregulated genes were associated with 'cell cycle arrest', the 'extrinsic apoptotic signaling pathway' and 'oxidation-reduction processes' (Fig. 3C). In conclusion, it was suggested that PM_{10} treatment affected cell growth ('extrinsic apoptotic signaling pathway', 'cell cycle arrest' and 'negative regulation of cell growth'), cell adhesion and ECM organization in fibroblasts.

PM_{10} induces ER stress in WI-38 VA-13 epithelial cells. Following PM_{10} treatment, 477 WI-38 VA-13-specific DEGs were identified using RNA-seq. For the WI-38 VA-13-specific DEGs, a GO analysis was performed using the ClueGO plugin in Cytoscape and associations with terms related to 'immune system process', 'cellular response to chemical stimulus', 'regulation of metabolic process' and 'biosynthetic process' were found (Fig. 4A). To analyze the 477 DEGs in greater detail, a heatmap analysis (246 downregulated genes and 231 upregulated genes) was performed (Fig. 4B). The 246 downregulated genes were clearly associated with 'negative regulation of apoptotic process', 'G2/M transition of mitotic cell cycle', 'cell-cell adhesion', 'cholesterol biosynthetic process via lathosterol' and 'cholesterol biosynthetic process via desmosterol'. In addition, the 231 upregulated genes were related to ER stress terms, including 'positive regulation of endoplasmic reticulum stress', 'PERK-mediated unfolded protein response' and 'negative regulation of endopeptidase activity' (Fig. 4C).

PM_{10} treatment is related to apoptotic processes in fibroblast and epithelial cell lines. In the analysis of DEGs, 107 genes were expressed in both the WI38 and WI-38 VA-13 cell lines following PM_{10} treatment. The GO analysis showed that the terms related to 'regulation of primary metabolic process' and 'cellular response' were enriched in genes affected by PM_{10} treatment (Fig. 5A). Additionally, the heatmap and GO analysis using DAVID indicated that DEGS in the two cell lines were associated with apoptosis processes, protein folding and signaling pathways ('p38 MAPK cascade' and 'negative regulation of protein kinase B signaling') following PM_{10} treatment (Fig. 5B and C). Thus, at the transcriptome level, PM_{10} treatment was associated with negative cell growth-related GO terms.

Discussion

GO analysis (DAVID and ClueGO) is a useful method for evaluating the total biological response of normal lung cell lines following PM_{10} treatment using cell-specific DEGs (18). The present study presented the cell-specific responses to PM_{10} observed in the cell lines WI-38 (lung fibroblast cells) and WI-38 VA-13 (lung epithelial cells) via transcriptome analysis using RNA-seq results. To select the PM_{10} concentration for the assessment of cellular response in normal lung cell lines, a high concentration of PM_{10} was selected in the present study. Chen *et al* (19) demonstrate that a concentration of 10 $\mu g/m^3$ PM_{10} is associated with lung cancer mortality (3.4~6% increase) in a cohort of 39,054 participants. From a cohort study conducted for 12 years, Quezada-Maldonado *et al* (20) report that a high concentration (10 $\mu g/cm^3$) of PM_{10} elicits an acute cellular response in lung tumors, changing the expression of 45 miRNAs for 72 h. At the *in vitro* level, cell culture is difficult to maintain in the long term following PM_{10} treatment. Thus, the present study decided on short-term cell culture following treatment with a high concentration of PM_{10} (9 $\mu g/cm^3$) for 48 h to determine the cellular effects of PM_{10} treatment. Hence, it demonstrated that continuous acute stimulation by PM_{10} had negative effects on cells and the loss of homeostasis in the cells may induce several types of disease in humans. However, future animal studies are needed to examine the long-term effects of PM_{10}.

Lung epithelial cells serve an important role in maintaining lung homeostasis and host defense mechanisms. In wound sites caused by toxins, PM and pathogens, several epithelial cell types spread and migrate as part of repair processes in the

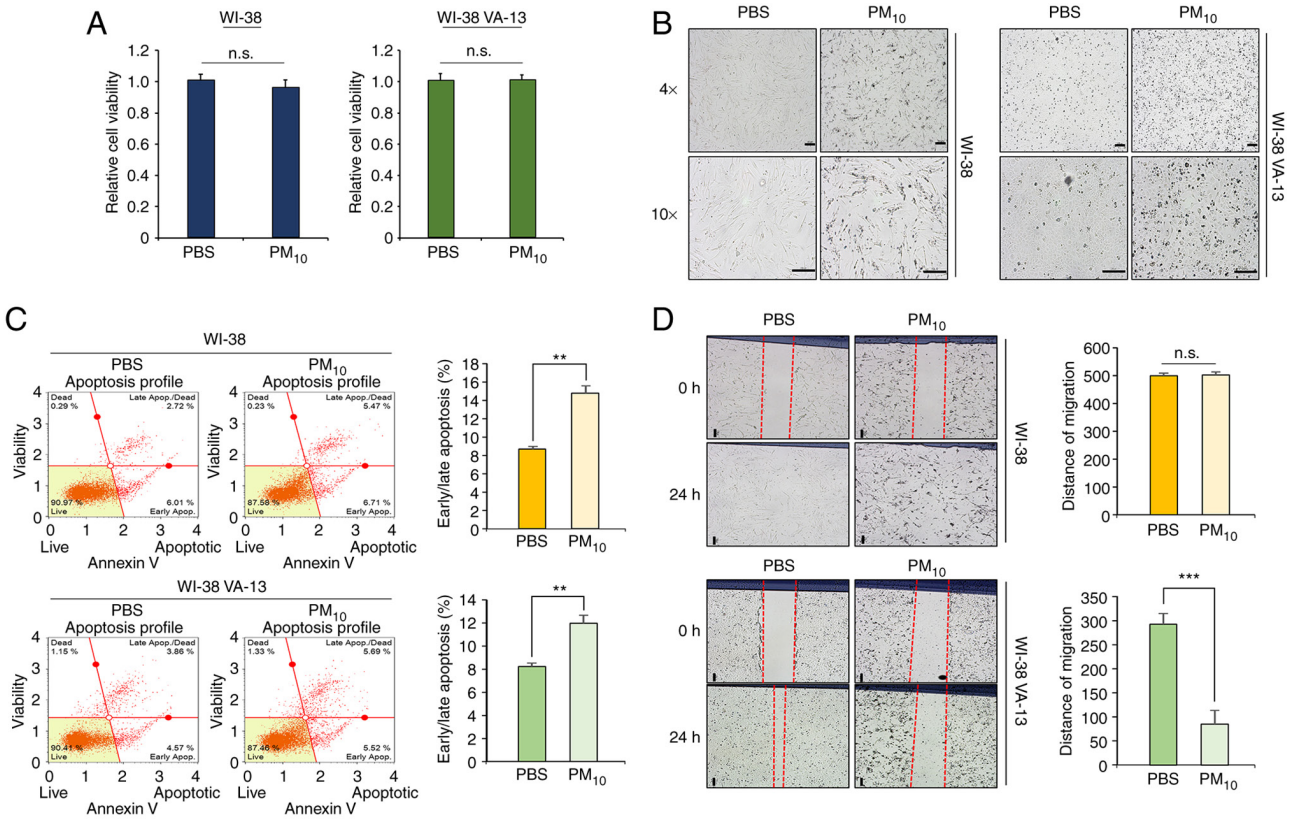

Figure 2. Cellular response to PM_{10} treatment of WI-38 or WI-38 VA-13 cells. (A) Cell viability assay following PM_{10} treatment for 48 h. Cell Counting Kit-8 solution was added to the culture medium and the cells were incubated for 5 min at 37˚C. Cell growth was measured using a microplate reader (450 nm). The mean ± SD of three independent experiments is shown. The P-values were calculated using Student's t-test. (B) Bright-field images following PM_{10} treatment. Scale bar, 200 μm. (C) Flow cytometric cell sorting analysis of Annexin V staining was performed following PM_{10} treatment. The lower right and upper right quadrants indicate early apoptosis and late apoptosis (left), followed by quantification of apoptosis (right). Mean ± SD of three independent experiments is shown. P-values were calculated using Student's t-test. **P<0.01. (D) Wound-healing assay. After 48 h of PM_{10} treatment, scratch assays of the WI-38 (above) and/or WI-38 VA-13 (below) cell lines were performed. After 24 h, wound closure was measured (right). The mean ± SD of three independent experiments is shown. The P-values were calculated using Student's t-test. ***P<0.001. PM, particulate matter; n.s., non-significant; PBS, phosphate-buffered saline.

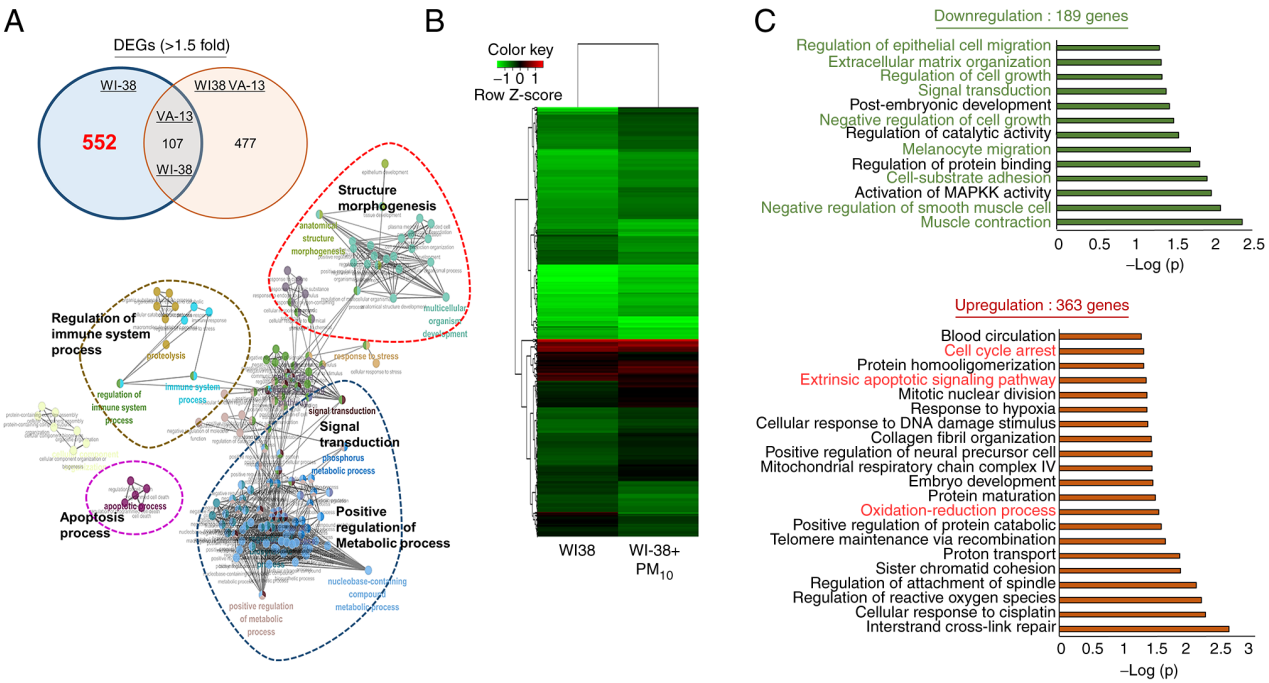

Figure 3. PM_{10} downregulates cell growth and adhesion terms in WI-38 cells. (A) Venn diagram of DEGs (above) and GO analysis (biological processes) of the DEGs following PM_{10} treatment in WI-38 grouped only by Clustering GO (below). (B) Heatmap of 552 WI-38-specific genes. (C) Database for Annotation, Visualization and Integrated Discovery-based GO analysis of RNA-sequencing results for 552 up- and downregulated genes. The enriched terms are shown. PM, particulate matter; DEGs, differentially expressed genes; GO, Gene Ontology.

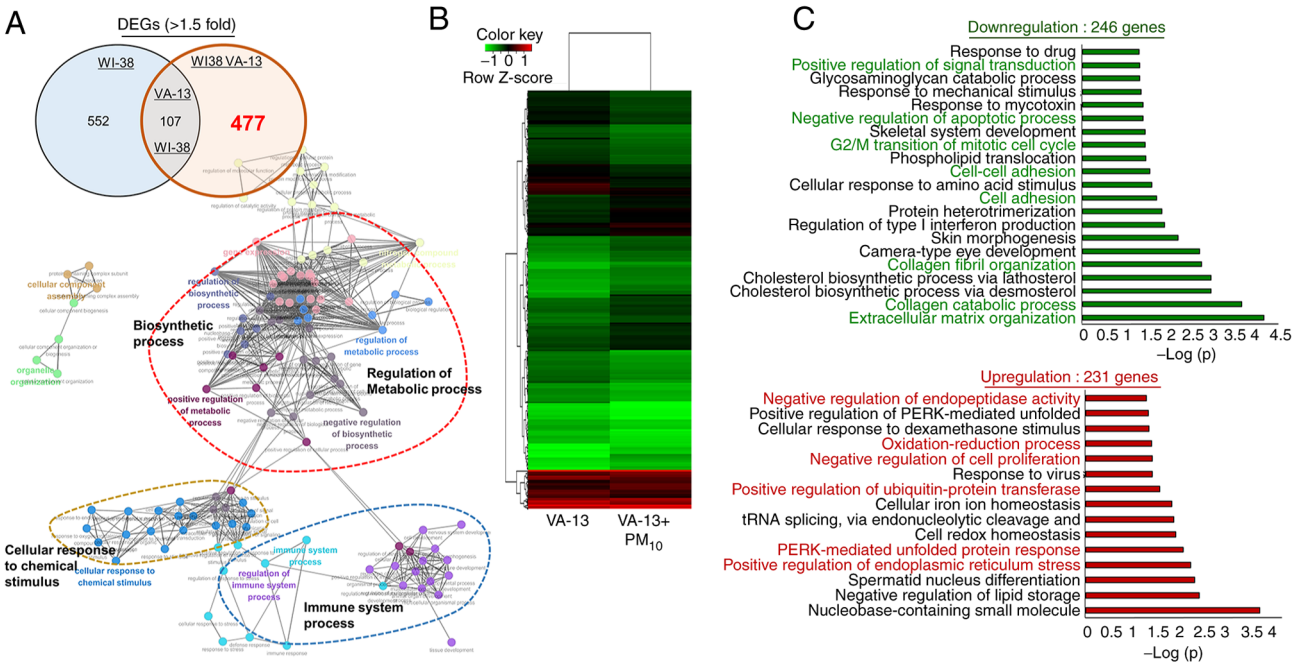

Figure 4. PM_{10} upregulates endoplasmic reticulum stress and apoptosis terms in WI-38 VA-13 cells. (A) Venn diagram of DEGs (above) and GO analysis (biological processes) of the DEGs following PM_{10} treatment in WI-38 VA-13 grouped only by Clustering GO (below). (B) Heatmap of 477 WI-38-specific genes. (C) Database for Annotation, Visualization and Integrated Discovery-based GO analysis of RNA-sequencing results for the 477 up- and downregulated genes. The enriched terms are shown. PM, particulate matter; DEGs, differentially expressed genes; GO, Gene Ontology.

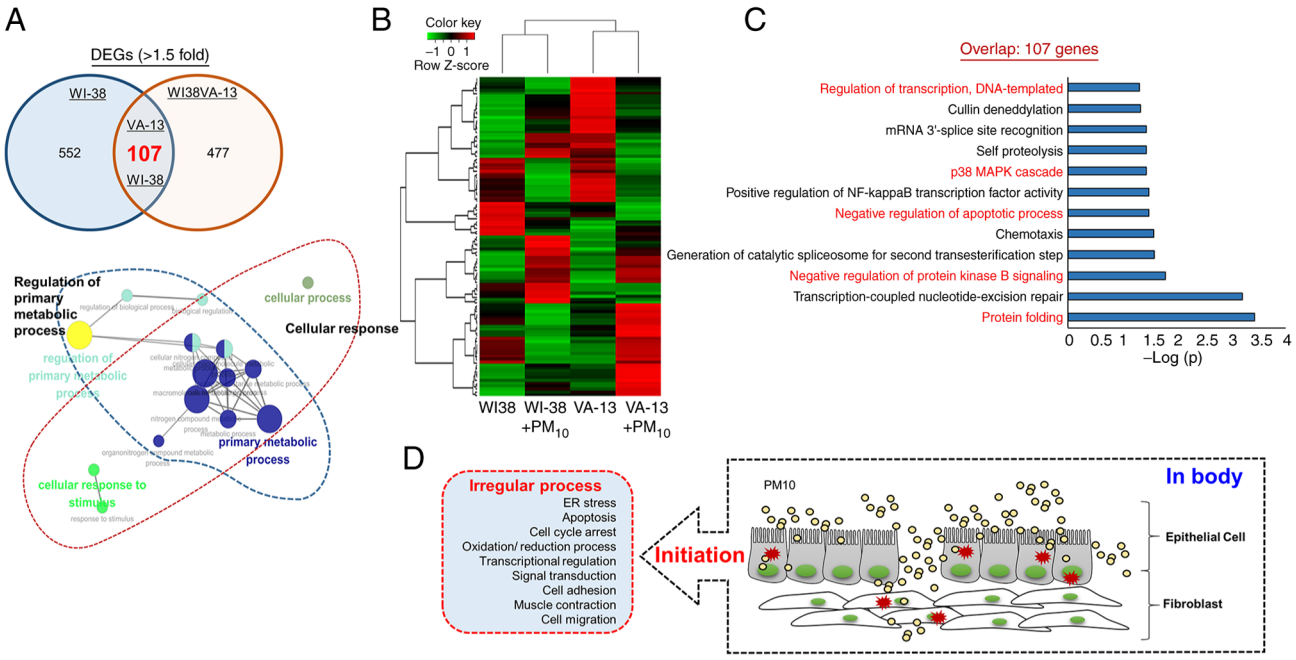

Figure 5. Overlapping DEGs following PM_{10} treatment in WI-38 and WI-38 VA-13 cells. (A) Venn diagram of DEGs (above) and GO analysis (biological processes) of the overlapping DEGs following PM_{10} treatment in WI-38 and WI-38 VA-13 grouped by Clustering GO (below). (B) Heatmap of 107 WI-38-specific genes. (C) Database for Annotation, Visualization and Integrated Discovery-based GO analysis of RNA-sequencing results for the 107 up- and downregulated genes. The enriched terms are shown. (D) Schematic summary of PM-induced biological responses. DEGs, differentially expressed genes; GO, Gene Ontology; PM, particulate matter; ER, endoplasmic reticulum.

lung (21,22). In wound healing analysis following PM_{10} treatment, it was clearly observed that PM_{10} treatment suppressed wound closure in epithelial cells (VA-13), implying that PM_{10} exposure may inhibit the wound healing process in the lung defense mechanisms. Additionally, in flow cytometric cell sorting analysis, the induction of apoptosis by PM_{10} treatment was associated with GO term results ('negative regulation of cell proliferation' and 'apoptosis'). Thus, PM_{10} treatment in epithelial cells may be a potential risk factor for several types of lung disease.

Lung fibroblasts produce ECM components, including elastin, type III collagen and proteoglycan, for lung structure and cell adhesion. The ECM is an important factor for physical support and normal organ function (23,24). Although the present study could not detect significant differences in cell growth and wound healing analysis following PM_{10} treatment, transcriptome analysis clearly revealed the enrichment of GO terms ('cell adhesion', 'cell growth' and 'extracellular matrix organization') suggesting that the primary functions of fibroblasts in the lung were slightly affected by exposure to PM_{10} and it was considered that continuous PM_{10} exposure may cause functional defects under normal conditions in lung fibroblasts.

Finally, in the DEGs of both cell types, PM_{10} induced changes related to apoptotic processes and metabolic signaling processes. Thus, the present study suggested that although brief exposure to PM_{10} was slightly reflected in phenotypic changes in cell processes including cell growth, various processes represented by functional GO terms (apoptotic GO terms and cell proliferation GO terms) were fully induced by PM_{10} treatment. In brief, longer exposure to PM_{10} may affect cell conditions and smaller particles ($PM_{2.5}$ or $PM_{1.0}$) could have stronger effects on lung epithelial or fibroblast cells.

In summary, RNA-seq analysis was performed on lung epithelial cells and fibroblast cells to identify cell-specific responses to PM_{10} treatment. GO analysis of DEGs showed that PM_{10} treatment was associated with terms related to cell apoptosis, immune system processes, cell cycle arrest and ER stress. Thus, the present study suggested that exposure to PM may affect cell conditions and long-term exposure to PM is likely to result in various disease processes (Fig. 5D).

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Availability of data and materials

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

Authors' contributions

Conception and design was conducted by DSK, MSL, YJP and HSC. TYR, JL, KK and SJL developed the methodology. Analysis and interpretation of data was conducted by KP, MYS and HSC. Manuscript writing and reviewing was performed by SJL, DSK, MSL and YJP. Study supervision was by DSK and HSC. All authors have read and approved the final manuscript. HSC and DSK confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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