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RESEARCH PAPER



Disease relevant modifications of the methylome and transcriptome by particulate matter (PM_{2.5}) from biomass combustion

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ABSTRACT

Exposure to particulate matter (PM) is recognized as a major health hazard, but molecular responses are still insufficiently described. We analyzed the epigenetic impact of ambient PM_{2.5} from biomass combustion on the methylome of primary human bronchial epithelial BEAS-2B cells using the Illumina HumanMethylation450 BeadChip. The transcriptome was determined by the Affymetrix HG-U133 Plus 2.0 Array. PM_{2.5} induced genome wide alterations of the DNA methylation pattern, including differentially methylated CpGs in the promoter region associated with CpG islands. Gene ontology analysis revealed that differentially methylated genes were significantly clustered in pathways associated with the extracellular matrix, cellular adhesion, function of GTPases, and responses to extracellular stimuli, or were involved in ion binding and shuttling. Differential methylations also affected tandem repeats. Additionally, 45 different miRNA CpG loci showed differential DNA methylation, most of them proximal to their promoter. These miRNAs are functionally relevant for lung cancer, inflammation, asthma, and other PM-associated diseases. Correlation of the methylome and transcriptome demonstrated a clear bias toward transcriptional activation by hypomethylation. Genes that exhibited both differential methylation and expression were functionally linked to cytokine and immune responses, cellular motility, angiogenesis, inflammation, wound healing, cell growth, differentiation and development, or responses to exogenous matter. Disease ontology of differentially methylated and expressed genes indicated their prominent role in lung cancer and their participation in dominant cancer related signaling pathways. Thus, in lung epithelial cells, PM_{2.5} alters the methylome of genes and noncoding transcripts or elements that might be relevant for PM- and lung-associated diseases.

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Introduction


Exposure to ambient particulate matter (PM) is recognized as a major health hazard, associated with acute respiratory infections, lung cancer, and chronic respiratory or cardiovascular diseases.^{1,2} Whereas industrial and traffic-related pollutants have been extensively investigated, the number of studies evaluating the impact of air pollution resulting from burning biomass is limited.



Worldwide, biomass fuel represents about 10% of direct human energy consumption and will remain the main source of energy for most of humanity.^{3–6} According to the World Health Organization, more than 3 billion people are affected by emissions from biomass fuels, claiming globally over 4 million deaths per year.⁷ Epidemiologic and exposure studies have associated wild land fires, agricultural burning, and residential wood combustion with a variety of adverse respiratory health effects, including asthma, chronic obstructive lung disease, and

acute lower respiratory tract infections, but also with cardiovascular effects and lung cancer.^{3,6} Thus, emissions from residential energy use have the largest impact on premature mortality worldwide.⁵ The use of biomass fuels and wood burning is still expected to rise due to high fossil energy costs, the desire to use renewable, CO₂-neutral energy sources, the rise in population, need for cropland, and climate changes.³ Even in developed countries, residential biomass combustion is a major source of particle emissions.³

Emissions from industrial or traffic related sources are essentially under legislation, but residential combustion of biomass fuels, agricultural burning, and forest fires are largely uncontrolled and may result in particle concentration up to 3 mg/m³ or even higher.^{3,8} Particles less than 2.5 μm (fine particles; PM_{2.5}), are considered to be particularly harmful, as they efficiently evade the mucociliary defense system and are deposited in the peripheral airways, where they can exert their

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pathophysiological mechanisms, linking exposure to ambient fine particles to diseases.⁹

Emissions from biomass fuels usually contain this harmful size range. Their chemical composition substantially depends on fuel composition and combustion characteristics and is different from industrial or traffic-related particles on which most health studies have focused.³ However, molecular studies have demonstrated that particulate matter from different sources may all induce oxidative stress and cellular inflammatory responses, potential mechanisms linking PM to respiratory or cardiovascular diseases and lung cancer.^{10–13}

Adaption to environmental changes occurs by epigenetic chromatin modifications resulting in durable alterations in gene expression.¹⁴ Indeed, for most of PM-associated diseases, epigenetic aberrations have been described.¹⁵ Correspondingly, changes in the DNA methylation pattern have been observed in the presence of ambient PM. Initially, studies determining methylation changes and its disease relevance have mainly focused on specific candidate target genes or repetitive elements in blood DNA, e.g., after exposure to black carbon, cigarette condensate, or traffic-related particles.^{16–18} Only lately, global methylation changes were determined upon high-occupational PM_{2.5} exposure.¹⁹ Despite the significant impact of PM from biomass combustion on health, comprehensive genome-wide methylation studies are still missing.

In our study, we generated profiles of the human methylome and transcriptome by microarray technologies in the presence of PM_{2.5} from biomass combustion using human airway epithelial cells (BEAS-2B) that represent the first barrier to inhaled extraneous particles. Exposure to PM_{2.5} from biomass combustion resulted in genome-wide changes of the methylome including repetitive elements and noncoding transcripts that may be relevant for lung cancer, inflammation, asthma, and other PM-associated diseases. Additionally, we filtered 152 differentially expressed genes, which were either hyper- or hypomethylated. From these genes, 66 were related to lung diseases, especially to lung cancer. Our results provide first insights on how changes in the methylation pattern of genes induced by chronic exposure to PM from biomass combustion may especially influence the development of various lung diseases.

Results

Characterization of PM_{2.5} from biomass combustion

PM_{2.5} was obtained from a biomass power plant. Mineralogical analysis revealed that the particles mainly consisted of crystalline arcanite (K₂SO₄) or amorphous components (for detailed composition see Table S1 and Dornhof et al.).²⁰

Methylome of PM_{2.5}-exposed BEAS-2B cells

To analyze whether PM affects the human methylome, human bronchial epithelial BEAS-2B cells were persistently exposed to PM_{2.5} from biomass combustion to accumulate DNA methylation changes. Cytotoxic effects were excluded by the absence of caspase-3/7 activity and DNA fragmentation (Fig. S1). Genome-wide DNA methylation was determined by using the Illumina Infinium HumanMethylation450 BeadChip (450K) Array. After 5 weeks, differential DNA methylation was

observed in PM_{2.5}-exposed BEAS-2B cells at 1.29% of the annotated sites (5501 CpGs) (Fig. 1A). The majority of alterations in DNA methylation were related to hypomethylation (decrease in $\Delta\beta > 0.02$; 4234 CpGs; Fig. 1B). Some individual differentially methylated CpGs were annotated to several different sites in relation to gene region or distance to CpG island (Fig. 1C/D).

CpG hypo- and hypermethylation events in PM_{2.5}-exposed BEAS-2B cells occurred frequently in the body of genes or unrelated to any gene region. Because methylation changes at CpG island-associated promoter sites have high relevance for gene expression,¹⁴ these sites were further investigated. The promoter region, including CpGs located in the 5'UTR, the TSS200, and the TSS1500, was hypomethylated at 35.45% (1501 CpGs) and hypermethylated at 28.02% (355 CpGs) of the differentially methylated sites (Fig. 1C/D). However, most of the hypo- and hypermethylation events, observed in the proximal promoters of PM_{2.5}-exposed cells occurred in promoters not associated with CpG islands (Figs. S2/S3). Only 9.6% (144 CpGs) of the hypomethylated sites and 15.21% (54 CpGs) of the hypermethylated sites in the promoter region were associated with CpG islands.

Additionally, the number of the differentially methylated CpG sites in the regions island, N-shelf, N-shore, open sea, S-shelf, and S-shore were compared with the expected number of CpG sites, assuming that methylation changes appear equally distributed in all regions. However, it became evident that CpG sites located in islands appeared less frequently (negative enrichment, $p < 0.05$) compared with a relatively high number of differentially methylated CpG sites (positive enrichment, $p < 0.05$) located in the shelf or open sea region (Table S2).

Chromosomal distribution of DNA methylation of PM_{2.5}-exposed BEAS-2B cells

Next, the chromosomal distribution of PM_{2.5} dependent DNA methylation was studied (Fig. 2, Table S3). DNA methylation changes were distributed on all analyzed chromosomes. Most methylation changes were detected on chromosome 1 ($\geq 10\%$ of either the hypo- or hypermethylated sites). In relation to the total amount of annotated CpGs per chromosome, hypomethylation was observed more frequently on chromosomes 1, 16, 17, and 22 (each $\geq 1.25\%$; enrichment tested by χ^2 -test, $p < 0.05$), whereas hypermethylation was enriched on chromosome 5 ($\geq 0.4\%$; $p < 0.05$). Moreover, differential methylation was observed in both G- and R-bands. The fraction of hypomethylated CpGs compared with hypermethylated CpGs was significantly higher in R-bands (2868 hypomethylated CpGs; 746 hypermethylated CpGs) than in G-bands, which stain positive for Giemsa (1353 hypomethylated CpGs; 507 hypermethylated CpGs; χ^2 -test, $p < 0.05$). R-bands are known for their high gene density.

Functional clustering of differentially methylated genes

Differently methylated genes were functionally clustered by a gene ontology analysis, using the Enrichr web application.²¹ Genes with hypomethylated CpGs were most significantly enriched in pathways associated with GTPase activity, regulatory signal transduction to growth factor stimuli and acidic chemicals, or coded for proteins involved in the architecture of

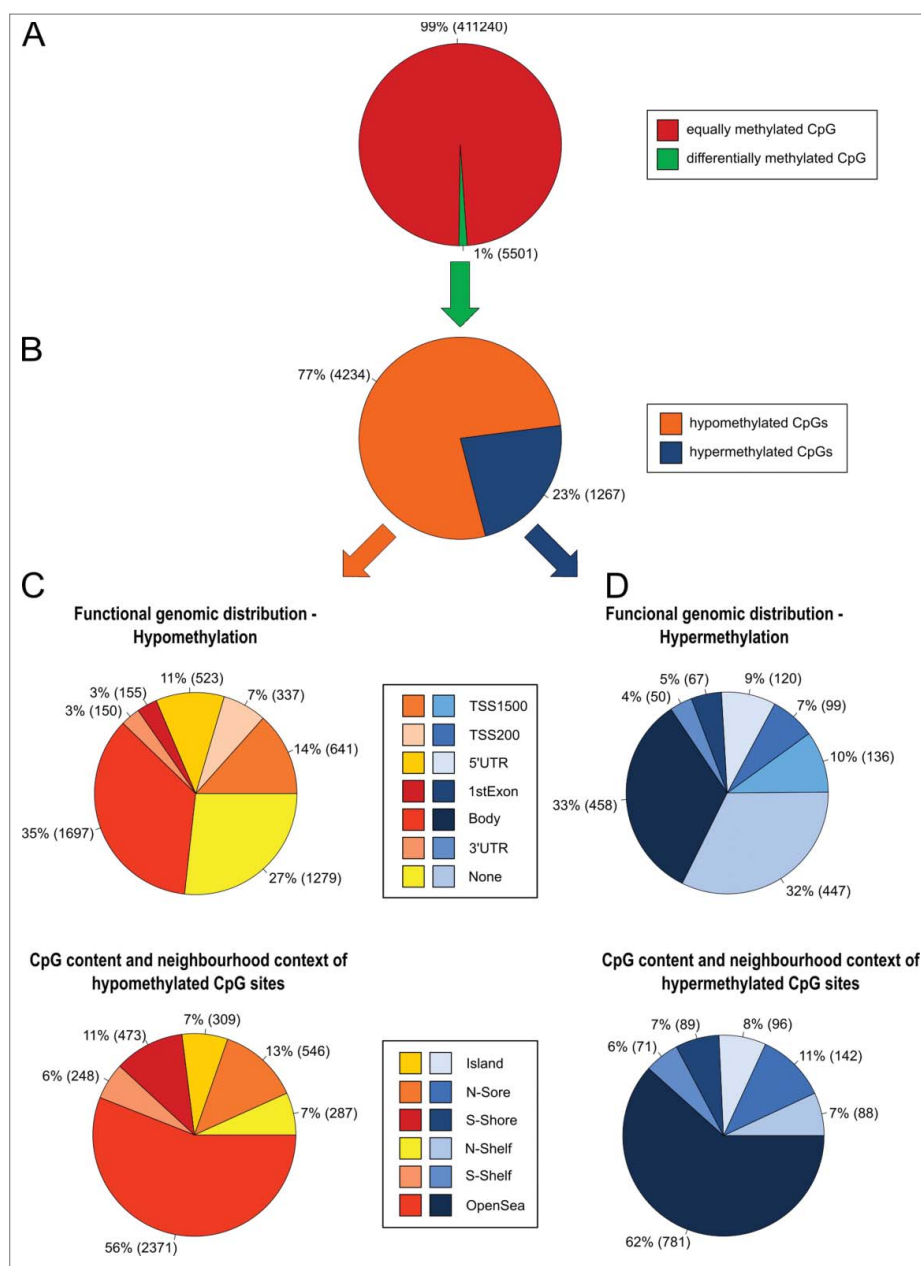


Figure 1. Methylation profile of BEAS-2B cells exposed to PM_{2.5} for 5 weeks. (A) Percentage of differentially methylated CpG sites in untreated BEAS-2B cells vs. PM_{2.5}-exposed cells (change in $\Delta\beta > 0.02$). (B) Percentage of hypermethylation and hypomethylation. (C) Functional genomic distribution of hypomethylated CpGs (upper pie chart) or in relation to CpG island regions (lower pie chart). (D) Functional genomic distribution of hypermethylated CpGs (upper pie chart) or in relation to CpG island regions (lower pie chart).

the extracellular matrix ($p < 1.0 \times 10^{-6}$; Fig. 3A). Genes with hypermethylated CpG sites were significantly enriched in pathways connected to cellular adhesion and pathways responsible for ion binding, transport, or channel activity ($p < 1.0 \times 10^{-5}$; Fig. 3B). The affected genes are listed in Tables S4/S5.

PM_{2.5} treatment affects tandem repeats and the miRNA-specific methylome

Differential methylation of repetitive DNA by environmental pollutants might have serious consequences for the structural integrity of the genome and on transcriptional activity, consequently affecting human health.²² Therefore, the methylation profile of these tandem repeats was also

analyzed. Interspersed repeats (long interspersed nuclear elements, LINEs; short interspersed nuclear elements, SINEs; long-terminal repeats, LTRs) satellite DNA (simple sequence repeats, SSRs; satellite) and low complexity regions (LCRs) were included in the study (Table 1). Reduced methylation of tandem repeats was prominent, but also some DNA loci were hypermethylated, indicating potentially impaired functionality of these sites.

Furthermore, the differential methylation of noncoding transcripts (miRNAs, miRs) was analyzed using positional data from the miRBase database (<http://mirbase.org>). Persistent exposure to PM_{2.5} significantly modified the methylation profile at 45 CpG loci annotated to 43 miRs. A large part of these CpGs were located proximal to the promoter and were

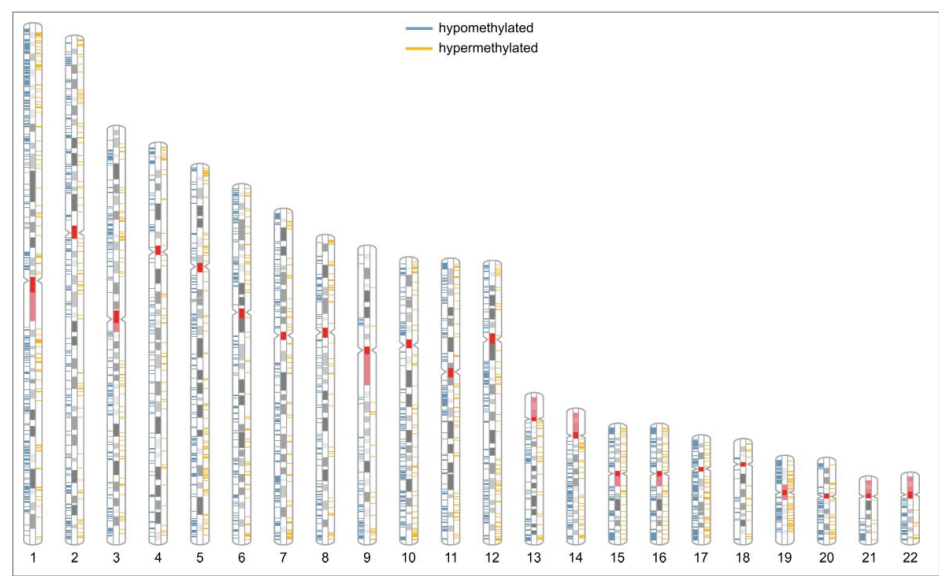


Figure 2. Ideogram of the human karyotype. R-bands are shown in white, G-bands in gray; the centromere and the coding region for rRNA are shown in red, regions of hypomethylation in blue, and regions of hypermethylation in yellow. The gender-specific XY-chromosomes were not further analyzed, because BEAS-2B cells originate from various individuals.

hypomethylated, indicating a potential mechanism for their transcriptional activation (Table 2).

The direct disease association of miR was examined by the human microRNA Disease Database v.2.0 (<http://cuilab.cn/>

hmdd). We found that miRs with a distinct methylation pattern were prominently involved in lung and PM-associated diseases, such as lung cancer and neoplasms, asthma, interstitial lung disease, or pulmonary embolism (Table 2). Moreover, miRs

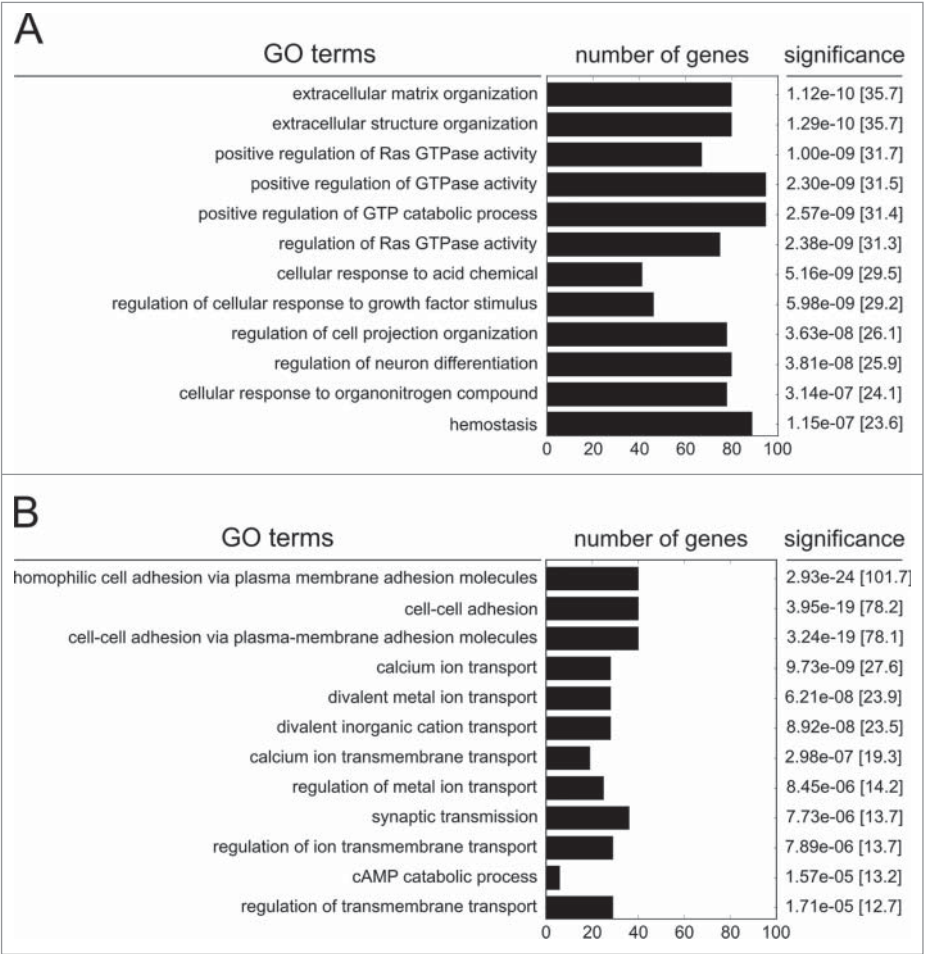


Figure 3. Gene ontology of hypomethylated genes (A) or hypermethylated genes (B) following persistent exposure to 100 µg/ml PM_{2.5} for 5 weeks. The 12 most significant functionally linked biologic processes, the number of clustered genes, the *p*-values and combined scores (in brackets) are shown.

Table 1. PM-dependent methylation changes of tandem repeats.

Tandem repeat	LINE	SINE	LTR	SSR	LCR	Satellite DNA
Hypomethylated repeats	202 (1.47%)	199 (1.35%)	92 (1.10%)	8 (0.39%)	3 (0.10%)	0 (0.00%)
Hypermethylated repeats	74 (0.54%)	54 (0.37%)	46 (0.55%)	3 (0.15%)	3 (0.10%)	4 (1.50%)
Analyzed repeats in total	13771	14793	8338	2043	2898	267

were linked to different types of cancer, regulation of inflammation and viral responses, which may also adversely affect lung physiology. A high proportion of miRs could be allocated to heart diseases. Several miRs contribute to the metabolic syndrome, and affect hypertension, diabetes, or lipid metabolism. All these diseases can be related to high ambient PM exposure.^{1,2,23}

Effect of gene methylation on gene transcription

To determine the biologic significance of differential DNA methylation, gene expression profiling of PM_{2.5}-exposed BEAS-2B cells was performed using the Human Genome U133 Plus 2.0 Array. Subsequent correlation of the transcriptome with changes in methylation resulted in 152 individual genes that show significant congruence (p -value threshold ≤ 0.05 ; Tables S6/S7). Mapping of expression vs. CpG methylation of these genes indicated a trend toward transcriptional activation by hypomethylation (Fig. 4). Accordingly, 78.42% of the hypomethylated CpGs were positioned on upregulated genes and located in the upper left quadrant. However, a correlation of hypermethylation and gene repression was not evident (lower right quadrant, 51.28% of hypermethylated CpGs). This trend did not change significantly, when only CpG island or promoter-associated alterations in the methylation pattern were included in the analysis (data not shown).

Gene ontology analysis using the Enrichr web application allocated genes that show both differential CpG methylation and transcription to functional properties (Fig. 5, Tables S8/S9). Functional enrichment of downregulated genes revealed that PM_{2.5} largely affected common pathways related to cytokine signaling, cell motility, differentiation, and development. Upregulated genes were largely involved in biologic responses to extracellular stimuli, e.g., chemotaxis, cell growth, wound healing, inflammation, angiogenesis, and responses to foreign particles or molecules.

PM_{2.5}-associated changes in the methylome and transcriptome are linked to human disease markers

Exposure to PM_{2.5} has been epidemiologically related to pulmonary and cardiovascular disease, metabolic disorders, and lung cancer.^{1,2,23} Therefore, a disease ontology approach was used to find a causal relationship between alterations in the methylome and transcriptome of PM_{2.5}-exposed BEAS-2B cells and diseases known to be exacerbated by PM. The 152 genes that showed both exposure-associated alterations in DNA methylation and gene expression were included in the analysis. Biocomputational profiling using the human disease methylation database (<http://bioinfo.hrbmu.edu.cn/diseasemeth>), the Dragon Database for Methylated Genes in Diseases (<http://cbrk.kaust.edu.sa/ddmgd/>), and the Human Gene Database

GeneCards® (<http://genecards.org>) revealed 66 molecular regulators mainly associated with lung diseases, in particular with lung cancer (Table 3). Extracted genes involved in lung independent diseases are listed in Table S9.

Next, the cellular and molecular relevance of the genes associated with lung cancer was investigated by a Medline search in more detail (data are included in Table 3, for references see Table S10). A significant number of these genes are upregulated in tumors and modulated by methylation. Some of these genes are implicated in tumor suppression and improved prognosis, whereas other genes were found to be largely involved in cellular transformation, proliferation and cell growth, tumor progression, metastasis, angiogenesis, inhibition of apoptosis, and therapeutic resistance. These tumor-promoting genes are prominently associated with Wnt, Akt, growth factor or hypoxia-related signaling pathways, epithelial-mesenchymal transition (EMT), and the circadian system. All of these pathways are central in promoting cellular survival and tumorigenesis.²⁴⁻³⁰

Discussion

This study demonstrates that long-term exposure to PM_{2.5} results in genome-wide alterations of the methylome and significantly alters gene expression. Predominantly, we observed hypomethylation of CpG sites, which is consistent with other studies, analyzing tandem repeats or individual candidate genes involved in cancer or cardiovascular disease upon exposure to polluted air.^{18,31} Extensive DNA hypomethylation can be induced by oxidative stress.³² Correspondingly, we detected increased intracellular levels of reactive oxygen species in chronically PM_{2.5}-exposed lung epithelial cells (Regina Dornhof, unpublished observation).

Although methylation changes were also found in the promoter region, only a minor part of those were located within CpG islands. The methylation pattern of CpG islands within the promoter region seems to be most critical for gene expression,¹⁴ but methylation outside this region may also be functionally relevant, because these CpG sites are evolutionarily conserved and not subject to negative selection. Accordingly, it was shown that changes in the methylation pattern of non-CpG island promoters have almost an identical impact on gene expression and disease relevance as changes in CpG island promoters.³³

PM-mediated variations in DNA methylation were distributed among most chromosomes, but accumulated at regions with high gene density. The fraction of hypomethylated to hypermethylated CpG sites was enriched in the transcriptionally competent R-bands compared with G-bands and hypomethylation was most prominent on the chromosomes 17 and 19, which contain the highest gene density in the genome. The observed intra-chromosomal clustering of DNA methylation changes may be a specific biologic response to PM_{2.5}. However,

Table 2. Disease relevance of miR with a PM-dependent, differential DNA methylation pattern.

miRNA	CpG locus	$\Delta\beta$ -value ^d	p-value	Lung cancer/ neoplasms	Asthma	Interstitial lung disease	Pulmonary embolism	Cancer/ neoplasms	Inflammation	Infection, viral response	Heart disease/ heart failure	Cardiomyopathy, muscular disorders	Lipid disorders	Atherosclerosis	Vascular disease	Hypertension	Diabetes	Stroke	Neurological disorders
miR-23a	TSS200/S_Shelf	-0.029	0.0008100	x				x	x	x	x	x			x				
miR-24-2	TSS1500/S_Shelf	-0.029	0.0008100	x				x	x	x	x	x			x				x
miR-27a	TSS1500/S_Shelf	-0.029	0.0008100	x				x		x	x	x			x				
miR-122	TSS1500/OpenSea	-0.077	0.0003100					x		x	x	x				x			x
miR-126	TSS1500/N_Shore	-0.051	0.0005800	x				x	x	x	x	x			x				x
miR-134	TSS1500/OpenSea	0.029	0.0004700	x			x	x		x	x	x			x				x
miR-299	Body/OpenSea	0.049	0.0000600					x			x								x
miR-323b	TSS1500/OpenSea	0.039	0.0002700					x			x								
miR-338	TSS200/N_Shelf	-0.131	0.0000010	x				x											x
	TSS200/N_Shelf	-0.102	0.0000300	x				x											x
miR-365a	TSS200/OpenSea	-0.072	0.0006900	x				x	x	x	x								
miR-382	TSS1500/OpenSea	0.029	0.0004700					x			x								
miR-425	TSS1500/N_Shore	-0.033	0.0002500					x		x	x								
miR-485	TSS1500/OpenSea	0.039	0.0002700	x				x											x
miR-570	TSS1500/OpenSea	-0.062	0.0000010					x											
miR-591	TSS200/OpenSea	-0.072	0.0006400					x											
miR-657	TSS1500/N_Shelf	-0.076	0.0007600					x											
	TSS1500/N_Shelf	-0.131	0.0000010					x											
miR-661	TSS1500/N_Shelf	-0.102	0.0000300					x											
	TSS200/S_Shore	-0.034	0.0004000					x											
	TSS200/S_Shore	-0.059	0.0004000					x											
miR-668	TSS200/S_Shore	-0.056	0.0003600					x											
miR-675	TSS200/OpenSea	0.039	0.0002700					x											
miR-1249	TSS200/Island	-0.057	0.0001300			x		x											
	TSS200/N_Shore	-0.062	0.0005800					x											
	TSS1500/N_Shore	-0.067	0.0000090					x											
	TSS1500/N_Shore	-0.062	0.0006700					x											
miRNA	CpG locus	$\Delta\beta$ -value ^d	p-value	miRNA	CpG-locus	$\Delta\beta$ -value ^d	p-value	miRNA	CpG-locus	$\Delta\beta$ -value ^d	p-value								
miR-380	TSS1500/OpenSea	0.049	0.0000600	miR-4512	TSS1500/S_Shore	-0.022	0.0000100												
miR-584	TSS1500/OpenSea	-0.052	0.0003300	miR-4686	TSS1500/S_Shore	-0.031	0.0000400												
miR-662	TSS1500/OpenSea	-0.047	0.0004200	miR-5091	TSS1500/OpenSea	0.062	0.0004800												
miR-1208	Body/OpenSea	-0.071	0.0000600	miR-6753	TSS1500/Island	0.053	0.0005300												
miR-1250	TSS200/N_Shelf	-0.047	0.0006000		TSS1500/OpenSea	-0.070	0.0000001												
miR-3065	TSS1500/N_Shelf	-0.088	0.0001800	miR-6775	TSS1500/OpenSea	-0.166	0.0000020												
	TSS1500/N_Shelf	-0.076	0.0007600	miR-6787	TSS1500/OpenSea	-0.042	0.0001300												
miR-3189	TSS1500/N_Shore	-0.035	0.0001500		TSS1500/N_Shore	-0.054	0.0000700												
miR-3195	TSS1500/Island	-0.050	0.0000300	miR-6887	TSS200/Island	-0.047	0.0002700												
miR-3912	TSS1500/N_Shore	0.026	0.0007200	miR-7107	TSS1500/N_Shelf	-0.069	0.0000900												
miR-4297	TSS1500/OpenSea	0.380	0.0008400		TSS200/Island	-0.021	0.0000500												
miR-4425	TSS1500/OpenSea	-0.027	0.0005200		TSS1500/Island	-0.032	0.0003900												
miR-4496	TSS1500/OpenSea	-0.030	0.0004600	miR-7846	TSS1500/N_Shore	-0.068	0.0001300												
miR-4496	TSS1500/OpenSea	-0.071	0.0000003																

^amiRNAs identified by the miRBase database (<http://mirbase.org>); ^bdisease association according to the human microRNA Disease Database v.2.0 (<http://cuilab.cn/hmdd>); ^cmiRNAs with known contribution to PM-associated diseases are listed in the upper part of Table 2, miRNAs with yet unidentified disease relevance are listed below; ^dnegative values: hypomethylated; positive values: hypermethylated

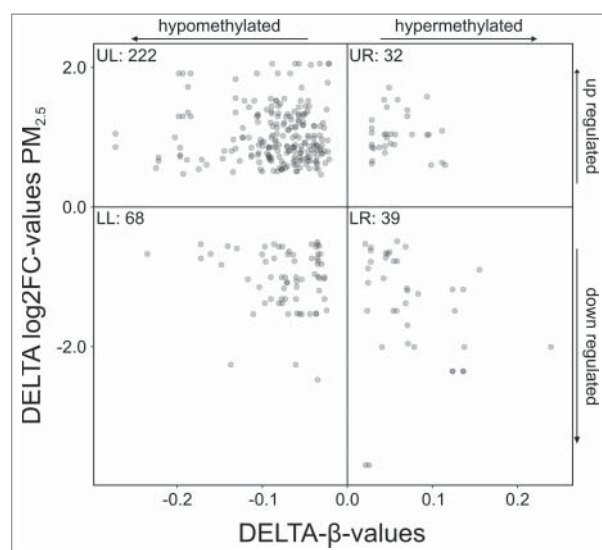


Figure 4. Mapping of gene expression vs. DNA methylation (p -value threshold ≤ 0.05). Each dot represents a single CpG, located on a gene that is both differentially methylated and expressed upon persistent exposure to 100 $\mu\text{g/ml}$ $\text{PM}_{2.5}$ for 5 weeks. Hypomethylated CpGs located on genes that cluster in the upper left quadrant were upregulated, whereas hypermethylated CpGs located on genes that were downregulated clustered in the lower right quadrant. The number of CpGs in each quadrant is indicated (UL, upper left quadrant; UR, upper right quadrant; LL, lower left quadrant; LR, lower right quadrant).

it may also demonstrate that changes in methylation is enriched at loci of high gene density or simply is a matter of technical bias by annotation of variable CpG sites to these regions.

Gene ontology enrichment analysis revealed that hypomethylated genes prominently affected pathways involved in G-protein signaling, cellular structure and extracellular interaction. Correspondingly, we observed PM-dependent cytoskeletal changes, such as the formation of stress fibers,²⁰ which might progress to epithelial barrier disruption in the respiratory tract, systemic spread of inhaled particles and lung-independent diseases. Hypermethylated genes were also associated with pathways involving cellular adhesion, indicating that these pathways are a dominant field of epigenetic regulation by PM. Loss of cellular adhesion by repressive hypermethylation might promote cancer cell progression, permeability of epithelial barriers, and metastasis. Furthermore, hypermethylation also clustered for pathways responsible for ion binding and shuttling, which might represent a biologic response to the high salt content in $\text{PM}_{2.5}$.

Hypomethylation was evident at repetitive element-associated DNA. Likewise, exposure to ambient air pollution or cigarette smoke induces hypomethylation of interspersed elements (LINE-1) or tandem repeats.^{18,31,34} Reduced methylation of interspersed repeats is associated with genomic instability, overexpression of oncogenes, and a higher risk of developing and dying from cancer.^{35,36} It has been proposed that hypomethylation of repetitive elements may also be used as a marker for many other human diseases, including metabolic disorders, lung injury, cardiovascular diseases, and associated risk factors.^{16,37-39} Thus, genome-wide hypomethylation of repetitive elements might represent a major mechanism to promote PM-associated diseases.

miRs posttranscriptionally affect gene expression and their deregulation may be involved in the pathogenesis of human disease.⁴⁰ Transcriptional activation of miRs can be regulated

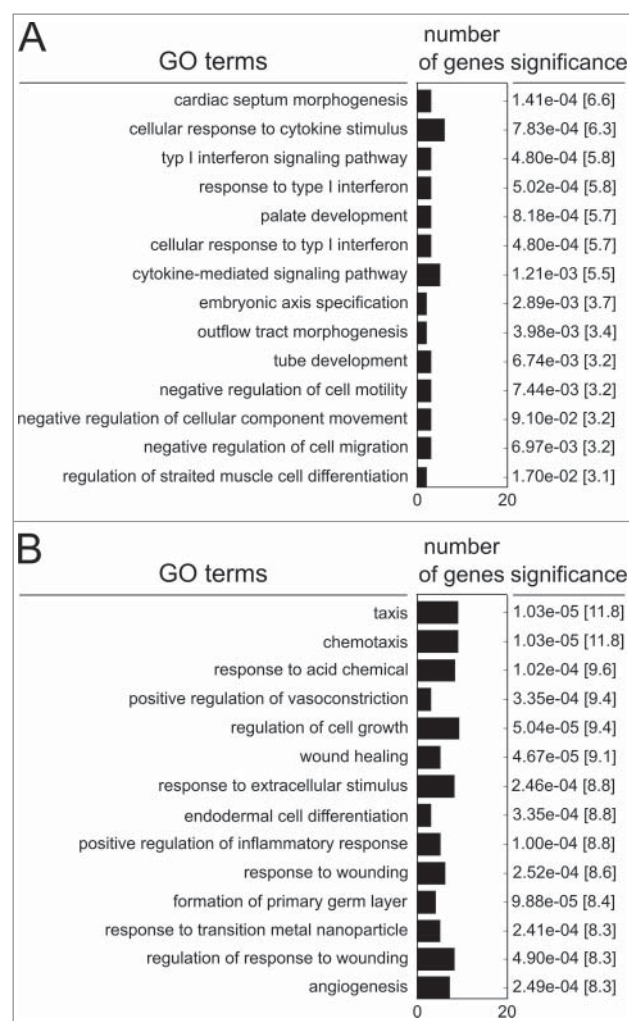


Figure 5. Functional enrichment of genes that show both differential methylation and transcription following persistent exposure to 100 $\mu\text{g/ml}$ $\text{PM}_{2.5}$ for 5 weeks identified by the Enrichr web server. The top 14 of the most significant functionally linked pathways, p -values, combined score, and number of clustered genes are indicated.

epigenetically⁴¹ and exposure to environmental pollutants or cigarette smoke may result in differential expression of miRs.^{42,43} We found methylation changes in some of these miR loci but also identified others for which relationship to PM exposure has not yet been reported (miRs listed in Table 2). Methylation changes were located near to the transcription start point suggesting functional relevance. A miR-specific disease database predicted a connection to PM-associated diseases. Almost all hits were related to the development or progression of cancer. miR-loci with a tumor suppressive allocation were frequently hypermethylated,⁴⁴⁻⁴⁸ whereas some oncogenic miR-loci were hypomethylated.⁴⁹⁻⁵⁵ However, the biologic functions of miRs are complex and most often tumor-type specific. Several miRs were also involved in the regulation of inflammation, response to pathogens, cardiovascular diseases, and associated risk factors, or various lung specific diseases (see Table 2). Some of these miRs have been described as useful biomarkers, such as for early detection of non-small cell lung cancer (NSCLC),⁵⁶ COPD,⁵⁷ or type 2 diabetes,⁵⁸ indicating that their dysregulation might represent an early event in disease development. Furthermore, dysregulation of some of these

Table 3. Differentially methylated and expressed genes and allocated lung-specific and lung-associated diseases.

Gene	Gene expression		DNA methylation portrait		Lung diseases ^d	Disease relevance																					
						Tumor						Tumor relevant	Cell death	Signaling													
			Hypomethylated	Hypomethylated at promoter	Hypermethylated	Hypermethylated at promoter	Lung cancer	Inflammation	Fibrosis	Tumor expression, activity	Candidate tumor suppressor	Poor prognosis	Malignance (stage/grade)	Proliferation/tumor growth, cell cycle	Tumor formation and progression	Motility (metastasis/invasion)	Angiogenesis	Treatment resistance	Endothelial to mesenchymal transition (EMT)	Adhesion, ECM, gap junction component	DNA damage response	Apoptosis, cell death	Epigenetically regulated (methylation)	Wnt-signaling	Akt-signaling	EGFR-signaling	HIF, hypoxia
log2FC	FC																										
AGPAT9 ^a	1.65	3.14	x	x		x					+/-	+/-		+/-								-	-				+
ANPEP ^a	1.44	2.71	x	x		x					+	+															
ANTXR2 ^a	0.80	1.74	x	x		x										+		x									
ARHGAP26 ^a	0.72	1.65	x			x					-		-	-	-			x				-					
ARID3A ^a	0.56	1.47	x			x					+	-															
ARNTL2 ^{a,b}	0.83	1.78	x	x		x						+	+	+	+	+											x
ASPH ^c	0.56	1.48	x	x		x								+	+												x
CD82 ^b	-0.50	0.71	x	x		x					-	+	*	-	-	-		+		+		-	-	-	-	-	
CDKN1A ^{b,c}	0.51	1.42	x	x		x					+/-	+/-	+	+/-	+	+			+	+/-		x	-	x	x	x	x
CHST11 ^a	0.82	1.77	x		x							+	+	+	+	+						x	x				
CLDN1 ^a	0.91	1.88	x			x					-	+				+/-		-	+			-	x		x	x	x
DCBLD2 ^a	1.33	2.52				x					+	+	+		+	+						-	+	+	+	+	x
DHRS3 ^a	-1.08	0.47	x			x						+	*	-	-					+		-		x			
DMBT1 ^b	0.60	1.52	x	x	x	x					-	+				+		x				-					
DUSP2 ^a	-1.49	0.36			x	x	x				-	+	*	+	-	-	-	-			+		-				x
DUSP5 ^a	0.79	1.73	x	x		x					x	-		-								-	+				
EGFR ^{b,c}	0.84	1.79	x			x					+		+	-*	+	+	+			+	+	+	+	+	+	+	x
EPHA2 ^b	0.61	1.53	x			x					+	+	+	+	+	+	+	+	+		-	+	+	x	x	x	x
ERRF1 ^a	1.37	2.58	x	x		x					-	+	+	*	-	-	-			+		-	-	-			x
FAM129A ^a	1.09	2.13	x			x					+													+/-			
FGR ^b	0.84	1.78	x	x		x								+	+	+				-				x			
FHOD3 ^a	1.04	2.06		x		x								+					x								
FLNB ^a	0.70	1.62	x			x								+	*	-	+	+	+		x						
FLVCR2 ^a	0.52	1.43	x			x																					
FOSL1 ^a	0.89	1.85	x	x		x					+/-	+	+	+		+	+				-		x	x	+		
G0S2 ^{a,c}	1.25	2.37	x	x		x					x				-					+		x					
GJB2 ^{b,c}	1.02	2.03	x	x		x					+/-	+	+	+	+	-			x			-					
HMCN1 ^a	-0.68	0.63	x			x					-								x								
KDM4B ^a	1.10	2.15	x			x					+			+	+	-*	-*										x
KRT6A ^b	1.48	2.79	x	x		x																					
LAPTM5 ^c	1.21	2.32	x		x	x					-	+	+	-						+		-		-			
LIFR ^b	-0.69	0.62		x	x	x					-	+	-		-	-						-	x	-		x	
LIMD1 ^{a,c}	0.71	1.64	x	x		x					-	x	+	+	*	-	-		+	+		-	-	-			
LPL ^{b,c}	-0.98	0.51		x		x						+											x				x
MAMDC2 ^b	-2.47	0.18	x			x													x			-					
MCC ^c	-2.26	0.21	x			x					-	+		-	-							-	-				
MEGF6 ^a	0.86	1.81	x		x	x											+										
MICAL2 ^a	1.00	2.00	x	x		x					+			+	+	+	+	+	+				x				
MSLN ^{b,c}	-0.53	0.69		x	x	x					+	+	+	+	+	+			x								
MXD1 ^a	0.94	1.91	x			x					+/-	x	+	-	-	-	+	+				-		x		x	
NPAS2 ^a	0.77	1.71	x	x		x						-		-						+						x	x
NT5E ^{a,b,c}	1.18	2.26	x			x						-*	+	+	+	+	+	+				-	+	+	+	x	
PI3 ^b	1.20	2.29	x	x		x	-					+	*	-	-	-	-					-	-				
PPP1R14A ^{a,b}	0.84	1.79		x	x	x					-	-			+							-					x
PTGS2 ^{b,c}	1.38	2.60	x	x		x	x				+	+	+	+	+	+	+	+	+			-	+	+	x	+	x
RAB27B ^a	0.99	1.98	x	x		x					+	+	+	+	+	+	+	+	+			+					
RNF145 ^a	-0.64	0.64	x			x																					
SEL1L3 ^a	0.75	1.68	x			x																					
SERINC2 ^a	0.94	1.92	x			x					+																
SERINC5 ^a	0.89	1.85	x			x																					
SH2B3 ^a	0.93	1.90	x	x		x					+			+/-	-						-						
SLC7A11 ^b	1.58	2.99	x	x		x					+	+	+	+	+	-*	+	+			+	+	-*	+	+	+	x
SLCO4A1 ^a	1.14	2.20	x	x		x																					
SMAD6 ^a	-0.56	0.68	x			x					+	+	+	-*	+				-		+	+	-				
SOX7 ^{a,b,c}	1.09	2.13	x			x					-	+	+	+	*	+	-			+		-	-				
SPOCK1 ^{a,b}	1.04	2.05		x		x					+	+	+	-*	+	+	-*		+			x	+	+		x	
SPP1 ^{b,c}	1.06	2.09	x	x		x		x				+	+	+	+	+	+	+	x				x	+	+	x	
SV2C ^a	-3.70	0.08		x	x	x																					
TCP11L2 ^a	0.65	1.57	x			x																					
TGFA ^b	2.04	4.10	x	x		x					+			+			+	+							+	x	x
TSPAN12 ^a	0.99	1.99	x	x		x						+		-									x				
TSPAN14 ^a	0.86	1.81	x	x		x						+	+					+									
UST ^a	-0.67	0.63	x			x									+												
ZC3HAV1L ^a	1.16	2.24	x	x		x										+											
ZEB1 ^a	0.63	1.55	x	x		x						+		-*	+	+	+	+	+	+	x	-*		+	+	x	x
ZNF395 ^a	0.82	1.76	x	x		x	+	+	-		+/-	+	+	+	*	-	-						-	-			x

^afor references see supplemental Table S8; ^aDiseaseMeth (<http://bioinfo.hrbmu.edu.cn/diseasemeth/>); ^bGeneCard® (<http://genecards.org/>); ^cDragon Database for Methylated Genes and Diseases (<http://cbr.kaust.edu.sa/ddmgd/>); ^daccording to data base search; +promoted/overexpressed; -suppressed/downregulated; *when absent or inhibited; x involved but not specified

miRs frequently correlates with outcome of cancer and is predictive for refractory therapy,^{59–61} development or relapse of disease before clinical manifestation,⁶² and poor prognosis.^{50,51,63} Additionally, it may lead to hypertension⁶⁴ and is also indicative of increased risk of death upon chronic heart disease⁶⁵ or post infarction.⁶⁶ However, further experiments are needed to prove that methylation changes affect miR and target gene expression, and thus represent a prominent mechanism for PM-associated diseases.

Hypomethylation of genes is associated with transcriptional activation.¹⁴ Accordingly, most genes that were hypomethylated also showed increased gene expression. In contrast, only less than half of the hypermethylated genes showed transcriptional repression. Because hypermethylation of CpG islands within promoter regions has a high impact on transcriptional repression,¹⁴ these genes were separately analyzed, but also showed no statistical bias for gene repression (data not shown). Controversially, increased gene body methylation or high methylation of shore and shelf regions, flanking demethylated CpG islands are associated with high gene expression, which might partly explain lack of correlation.^{67,68} Nevertheless, methylated CpG dinucleotides are considered to be mutational hot spots that cause genetic diseases and cancer through spontaneous deamination of 5-methylcytosine (5mC) and its transition to thymine.¹⁴

We functionally clustered differentially methylated genes according to their up- or downregulated expression. Interestingly, most of the selected genes were repeatedly identified by different GO terms with potential impact on PM-associated diseases (genes listed in Tables S8/S9 and referenced in Table S10). Some antiviral genes were downregulated, whereas other genes, encoding viral docking sites, were upregulated. This may facilitate respiratory infections, frequently associated to ambient PM or cigarette smoke exposure.^{69,70} Furthermore, genes that promote respiratory or cardiovascular diseases were primarily upregulated, whereas suppressors of these diseases were transcriptionally repressed. Encoded proteins are known to be critically involved in the pathogenesis of acute and chronic inflammation, asthma, COPD, atherosclerosis, or lung injury, but also often have pleiotropic functions and thus may additionally contribute to epithelial-mesenchymal transition, chronic inflammation and, consequently, to a pro-tumorigenic microenvironment.^{71,72} Other allocated genes may directly promote tumorigenesis by facilitating proliferation, invasiveness, metastasis, angiogenesis, immune evasion or resistance and are associated with poor patient survival when upregulated in tumor cells, tumor stroma, or surrounding tissue (Tables S8/S9). Transcriptional and functional activation by cigarette smoke or airborne particulate matter and regulation by methylation have been independently confirmed for some of these genes (Tables S9/S10). In contrast, tumor suppressive genes were transcriptionally repressed in the presence of PM (Tables S8/S10). Interestingly, some upregulated genes contradictorily showed hypermethylation whereas some repressed genes were hypomethylated (Tables S6–S9). This indicates that these genes may not be exclusively epigenetically regulated, but that additional, unknown mechanisms might predominate for their expression. However, most of these genes were repeatedly identified by multiple gene ontology terms with highest significance

highlighting their potential relevance. Because they represent central mediators of severe diseases, their reciprocal interaction and pathophysiological impact on adverse health effects by PM should be further examined.

In addition, the disease relevance of differentially methylated and expressed genes was confirmed by disease ontology. Some of the previously functionally clustered genes were allocated again and numerous additional genes were identified (Table 3). Genes, significant for cancer formation and progression were most highly enriched, indicating that exposure to PM may facilitate pro-tumorigenic conditions and transform lung epithelial cells to tumor cells. The cancer-associated molecular functions of these genes are referenced in Table S10. Again, tumor-promoting genes and genes associated with poor outcome in cancer patients were generally upregulated, whereas tumor-suppressive genes were often downregulated. However, the observed upregulation of some tumor-suppressive genes might represent an adaptive response to protect from cellular transformation. Independent studies have shown that many of these genes can be regulated by DNA methylation (see Table 3 and Table S10) and, interestingly, most of the allocated genes were associated with the same dominant pro-tumorigenic pathways, such as EGF/TGF- β -signaling, EMT including downregulation of E-cadherin, b-catenin activation, and response to hypoxia. Since these pathways are substantially linked to malignant progression, metastasis, angiogenesis, therapy resistance, and poor clinical outcome,^{25–29} it is crucial to further verify their activation and pathologic function during PM exposure.

In addition to cancer, dysregulation of some of these disease relevant genes may also exacerbate pulmonary inflammation, lung injury, severity of respiratory infections, and progression of COPD and ARDS (Table S10), but also lung-independent diseases, such as cardiovascular disease, hypertension, metabolic syndrome, neurologic disorders and chronic inflammation, or cancer in different tissue types (Table S11) and, thus, may contribute to other common pathological features symptomatic for PM exposure. However, here we used bronchial epithelial cells and therefore our discussion focuses on lung diseases. Whether PM_{2.5} induces similar biologic responses in different cell types needs further investigation.

Our results may open the door for preventive or therapeutic considerations, e.g., by using dietary compounds to reduce or reverse detrimental methylation changes when exposed to high PM_{2.5} concentrations. It has already been shown that folic acid can enhance methylation and that DNA methylation inhibitors can significantly reduce carcinogen-induced lung cancer development in animal models.^{73,74}

However, the presented data may have some limitations by the use of a single fraction of PM from biomass combustion. Particle composition and cellular responses can vary depending on the type of biomass and combustion conditions.⁷⁵ Additionally, the concentration of PM and duration of exposure may have a direct impact on biologic responses. The given concentration (100 $\mu\text{g/ml}$), which had no significant impact on cell viability upon prolonged treatment, was also used in other studies investigating biocombustion.¹¹ Moreover, our approach for efficient identification of putative target sites for methylation changes is additionally supported by the fact that ambient PM concentrations from biomass combustion may significantly

exceed industrial or traffic-related emissions,^{3,8} and that inhaled PM <2.5 μm may accumulate in the lung parenchyma.⁹ Consistently, health effects are especially evident in regions with recurrent and intense pollution, but rarely after a single and short exposure.¹ Nevertheless, further studies will be necessary for targeted validation of the most important top hits. We also acknowledge that the use of an immortalized cell line and cell culture conditions may not reflect *in vivo* processes. Moreover, both 5mC and 5-hydroxymethylcytosine (5hmC) are interrogated by the Illumina 450K array. The 5hmC modification, which is an intermediate of 5mC demethylation by TET enzymes,⁷⁶ may be responsible for some of the activation, correlated with higher promoter methylation. Finally, the Illumina 450K array does not completely cover the entire epigenome.

In conclusion, using BEAS-2B cells as a model, we could observe genome wide DNA methylation changes at CpG sites residing in disease relevant genes, repetitive elements, and miRs. Consequently, DNA methylation analysis should be included in future efforts to understand the interactions between environmental exposures and PM-associated diseases. By identifying putative targets and their allocation to selective prominent disease relevant pathways, there is now an urgent need to independently confirm the biologic effects of methylation changes on gene expression, to clarify the interplay of the affected mediators, and to demonstrate how they contribute to adverse health effects. This might finally result in the establishment of new sensitive and reliable diagnostic biomarkers and therapeutic targets for the prevention and/or treatment of PM-associated diseases.

Materials and methods

Preparation of PM_{2.5}

Preparation of PM_{2.5} has been described previously.²⁰ Briefly, bulk fly ash was collected from an electrostatic precipitator of a medium-scale biomass power plant with a nominal thermal output of 1.7 MW (Bürger Energie St. Peter eG, St. Peter, Schwarzwald, Germany), which exclusively combusts chips of soft wood (mainly spruce) derived from the local forests. The wood chips included debarked stem wood and branches with a minimum of 7 cm in diameter (merchantable wood) without leaves and twigs. The collected ash was subsequently size-fractionated by a cyclone with an aerodynamic cut-off diameter of 2.5 μm (Labor für Partikeltechnologie/Mechanische Verfahrenstechnik, Hochschule Konstanz, Technik, Wirtschaft und Gestaltung). The resultant size fraction (PM_{2.5}) was then used for all biologic assays.

Cell culture and PM_{2.5} treatment

Immortalized bronchial epithelial cells (BEAS-2B; LGC Standards GmbH, #ATCC-CRL-9609) were maintained in Dulbecco's Modified Eagle Medium/F12 with stable Glutamine (GE Healthcare, #E15-889) containing 5% fetal bovine serum (Life Technologies, #10270-106) 100 IU streptomycin and 100 IU penicillin (Sigma-Aldrich, #11074440001) at 37°C in a humidified incubator. BEAS-2B cells were plated at a density of

0.7×10^6 cells per 75 cm² cell culture flasks (Greiner bio-one, #658175) and were split twice a week after rinsing 2 times with phosphate buffered saline and subsequently using trypsin, 0.25% EDTA (Life Technologies, #25200056) for detachment. For the experiments, adherent cells were repeatedly treated with PM_{2.5} every first day after each passage. Particles were immersed in BEAS-2B growth medium at a concentration of 200 $\mu\text{g/ml}$ and sonicated for 20 min (SONOREX RK510H, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) immediately before dilution to the cells at a final concentration of 100 $\mu\text{g/ml}$ PM_{2.5}. The cells were maintained in the same PM_{2.5}-containing medium until the next passage. For the last passage, cells were seeded in 6-well plates and were exposed on the next day with 100 $\mu\text{g/ml}$ PM_{2.5} for a final time period of 48 h. Control cells were similarly treated in the absence of PM_{2.5}. After 5 weeks of exposure, cells were harvested for DNA (DNeasy Blood & Tissue Kit, Qiagen, #69504) and RNA (RNeasy® Plus Mini Kit, Qiagen, #74136) extraction following the manufacturer's instructions. To avoid RNA contamination, 400 μg RNase A (Carl Roth, #7156.1) were added during cellular lysis for genomic DNA isolation. For each condition (control and PM_{2.5}-treated cells) 3 independent replicates have been performed for DNA and for RNA isolation.

DNA methylation chip preparation and data analysis

The Illumina HumanMethylation450 BeadChip (450K) array was performed by AROS Applied Biotechnology A/S (Aarhus, Denmark) resulting in IDAT files containing the raw data. Analysis of Illumina 450K v1.2 data was performed with the R minfi package.⁷⁷ For the preprocessing of the raw data, the preprocessQuantile function was used. After calculation of β -values, 56,235 cross hybridizing probes were removed.⁷⁸ Differentially methylated positions were identified using the dmpFinder function and a False Discovery Rate (FDR) of less than 5% was obtained (q-value cutoff of 0.05). The differences of averaged β -values of the 4 replicates of each control and particular matter sample were defined with a cutoff for minimum difference of 0.02 (decrease in $\Delta\beta > 0.02$, hypomethylated; increase in $\Delta\beta > 0.02$, hypermethylated). Probes were annotated using the getAnnotation function implemented in the minfi package. Enrichment calculations (Table S2) were based on the χ^2 -test ($p < 0.05$).

Gene expression and chip preparation

Affymetrix HG-U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, #900467) were used to determine transcription levels of more than 54,000 transcripts and variants, representing over 20,000 genes. RNA was quality assessed (Agilent RNA 6,000 Nanokit, Agilent Technologies, #AGI 5067-1511; Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany) and quantified (NanoDrop ND-1,000 spectrophotometer, Thermo Fisher Scientific, Berlin, Germany). The RNA quality revealed values of RIN ≥ 9.9 for all samples. After reverse transcription of 300 ng total RNA (Invitrogen™ Ambion™ RETROscript™ Reverse Transcription Kit, Thermo Fisher Scientific, #AM1710) and generation of cRNA probes (3' IVT Plus Amplification Kit, Affymetrix, #902395), 50 $\mu\text{g/mL}$ of biotinylated and

fragmented cRNA was hybridized to HG-U133 Plus 2.0 arrays. After washing and fluorescence labeling (GeneChip Hybridization, Wash & Stain Kit, Affymetrix, #900720), arrays were scanned (Affymetrix GeneChip Scanner 3000, Affymetrix).

Gene expression analysis

Analysis of the raw expression data was performed using the *simpleaffy*⁷⁹ and the *affy* R packages⁸⁰ for uploading and RMA normalization of the data. The *lmFit*, *contrasts.fit*, as well as the *eBayes* functions of the *limma* package⁸¹ were used to preprocess the data, and, finally, the *topTable* function was applied to identify differentially expressed genes, with a false discovery rate defined by the Benjamini-Hochberg method of smaller than 0.05 and each *p*-value smaller than 0.05. Annotation data of probes were obtained from the supplier's homepage (<http://www.affymetrix.com/support/technical/byproduct.affx?product=hg-u133-plus>, file: "HG-u133_Plus_2.na35.annot.csv," creation_date = 2014-10-06). If several probe sets were assigned to the same LocusID either all sets were kept individually since variances between those values might be a result of alternative splicing of the related transcript (Fig. 4)⁸² or the average of the transcripts were calculated (Tables 3, S6, and S7) for a clearer presentation.

Data plotting

Mean β values of the control probes were plotted against the β values of the PM-treated samples of all differentially methylated CpG sites using the *ggplot2* package of R.⁸³

Transcription start site (TSS) positions were obtained from the UCSC website (<http://hgdownload.soe.ucsc.edu/goldenPath/hg19/database/>; file: "knownGene.txt.gz") taking into account the strandedness of annotated genes. Distances of all differentially methylated CpG positions to their nearest TSSs were calculated with the help of the *DistanceToNearest*-function of the "GenomicRanges" package,⁸⁴ and plotted against their corresponding Dbvalue.

The phenogram shown in the manuscript is a collage of 3 different phenograms (for hypomethylated CpGs, hypermethylated CpGs, and Giemsa bands). Each phenogram was generated using the web application *PhenoGram*.⁸⁵ The input data table for the Giemsa-stained cytobands was obtained from the *PhenoGram* website (*cytoBand-human.txt*, <http://visualization.ritchielab.psu.edu/phenograms/document>).

The names of all differentially methylated or differentially expressed genes were converted with the help of the web application *GeneCards* (<http://www.genecards.org/>) and the *db2db* database (<https://biobdnet-abcc.ncifcrf.gov/db/db2db.php>), and subsequently analyzed for enrichment using the default parameters of the *Enrichr* platform.²¹ Plotting of the tabular output of *Enrichr* was performed using Python's *matplotlib* module (<http://matplotlib.org/>).

Overlaps of all differentially methylated CpG positions with different kinds of repetitive elements ("LINE," "SINE," "LTR," "Simple_repeat," "Low_complexity," "Satellite") were counted using the *GenomicRanges* package in R.⁸⁴ The repetitive element positions were downloaded from the UCSC website (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/>, file: *rmsk*.

txt.gz). Overlaps with miRs were counted and incorporated with the input data from the miRBase website (<http://www.mirbase.org/ftp.shtml>, file: *hsa.gff3*; *gff-version 3 - miRBase v21*, build GRCh38, hg38). The CpG positions were converted from hg19 to hg38 by using the *liftOver* tool (<http://genome.ucsc.edu/cgi-bin/hgLiftOver>).

Circos plots were generated using the default settings of related web application (<http://mkweb.bcgsc.ca/tableviewer/>).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Authors contribution

Katharina Heßelbach provided biologic samples, was involved in data processing, their interpretation and assisted with manuscript preparation. Gwang-Jin Kim and Stefan Günther were responsible for bioinformatics data processing, statistical analysis and manuscript preparation. Stephan Flemming established the bioinformatics data processing and was involved in the interpretation of results. Thomas Häupl and Marc Bonin conducted the Affymetrix HG-U133 Plus 2.0 transcriptome analysis. Regina Dornhof provided biologic samples. Irmgard Merfort and Matjaz Humar were responsible for study design, sample preparation, data analysis, interpretation and presentation of results and manuscript preparation.

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References

1. Anderson JO, Thundiyil JG, Stolbach A. Clearing the air: a review of the effects of particulate matter air pollution on human health. *J Med Toxicol.* 2012;8:166-75. doi:10.1007/s13181-011-0203-1. PMID:22194192
2. Raaschou-Nielsen O, Andersen ZJ, Beelen R, Samoli E, Stafoggia M, Weinmayr G, Hoffmann B, Fischer P, Nieuwenhuijsen MJ, Brunekreef B, et al. Air pollution and lung cancer incidence in 17 European cohorts: prospective analyses from the European Study of Cohorts for Air Pollution Effects (ESCAPE). *Lancet Oncol.* 2013;14:813-22. doi:10.1016/S1470-2045(13)70279-1. PMID:23849838
3. Naeher LP, Brauer M, Lipsett M, Zelikoff JT, Simpson CD, Koenig JQ, Smith KR. Woodsmoke health effects: a review. *Inhal Toxicol.* 2007;19:67-106. doi:10.1080/08958370600985875. PMID:17127644
4. Programme UND. World energy assesment. New York:UNPD; 2004.
5. Lelieveld J, Evans JS, Fnais M, Giannadaki D, Pozzer A. The contribution of outdoor air pollution sources to premature mortality on a global scale. *Nature.* 2015;525:367-71. doi:10.1038/nature15371. PMID:26381985
6. Kim KH, Jahan SA, Kabir E. A review of diseases associated with household air pollution due to the use of biomass fuels. *J Hazard Mater.* 2011;192:425-31. doi:10.1016/j.jhazmat.2011.05.087. PMID:21705140
7. WHO. Household air pollution and health. WHO Media Centre; 2016. :Fact sheet N°292.
8. Rumchev K, Zhao Y, Spickett J. Health Risk Assessment of Indoor Air Quality, Socioeconomic and House Characteristics on Respiratory Health among Women and Children of Tirupur, South India. *Int J Environ Res Public Health.* 2017;14:E429. doi:10.3390/ijerph14040429. PMID:28420188
9. Valavanidis A, Fiotakis K, Vlachogianni T. Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcino-

- genic mechanisms. *J Environ Sci Health C Environ Carcinog Ecotoxico Rev.* 2008;26:339-62. doi:10.1080/10590500802494538. PMID:19034792
10. Maatta J, Luukkainen R, Husgafvel-Pursiainen K, Alenius H, Savolainen K. Comparison of hardwood and softwood dust-induced expression of cytokines and chemokines in mouse macrophage RAW 264.7 cells. *Toxicology.* 2006;218:13-21. doi:10.1016/j.tox.2005.09.001. PMID:16202497
11. Danielsen PH, Moller P, Jensen KA, Sharma AK, Wallin H, Bossi R, Autrup H, Molhave L, Ravanat JL, Briede JJ, et al. Oxidative stress, DNA damage, and inflammation induced by ambient air and wood smoke particulate matter in human A549 and THP-1 cell lines. *Chem Res Toxicol.* 2011;24:168-84. doi:10.1021/tx100407m. PMID:21235221
12. Ovrevik J, Refsnes M, Lag M, Brinchmann BC, Schwarze PE, Holme JA. Triggering Mechanisms and Inflammatory Effects of Combustion Exhaust Particles with Implication for Carcinogenesis. *Basic Clin Pharmacol Toxicol.* 2016. PMID:28001342.
13. Lodovici M, Bigagli E. Oxidative stress and air pollution exposure. *J Toxicol.* 2011;2011:487074. doi:10.1155/2011/487074. PMID:21860622
14. Li E, Zhang Y. DNA methylation in mammals. *Cold Spring Harb Perspect Biol.* 2014;6:a019133. doi:10.1101/cshperspect.a019133. PMID:24789823
15. Heerboth S, Lapinska K, Snyder N, Leary M, Rollinson S, Sarkar S. Use of epigenetic drugs in disease: an overview. *Genet Epigenet.* 2014;6:9-19. PMID:25512710.
16. Bellavia A, Urch B, Speck M, Brook RD, Scott JA, Albeti B, Behbod B, North M, Valeri L, Bertazzi PA, et al. DNA hypomethylation, ambient particulate matter, and increased blood pressure: findings from controlled human exposure experiments. *J Am Heart Assoc.* 2013;2:e000212. doi:10.1161/JAHA.113.000212. PMID:23782920
17. Bruniquel D, Schwartz RH. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat Immunol.* 2003;4:235-40. doi:10.1038/ni887. PMID:12548284
18. Guo L, Byun HM, Zhong J, Motta V, Barupal J, Zheng Y, Dou C, Zhang F, McCracken JP, Diaz A, et al. Effects of short-term exposure to inhalable particulate matter on DNA methylation of tandem repeats. *Environ Mol Mutagen.* 2014;55:322-35. doi:10.1002/em.21838. PMID:24436168
19. Panni T, Mehta AJ, Schwartz JD, Baccarelli AA, Just AC, Wolf K, Wahl S, Cyrus J, Kunze S, Strauch K, et al. Genome-Wide Analysis of DNA Methylation and Fine Particulate Matter Air Pollution in Three Study Populations: KORA F3, KORA F4, and the Normative Aging Study. *Environ Health Perspect.* 2016;124:983-90. doi:10.1289/ehp.1509966. PMID:26731791
20. Dornhof R, Maschowski C, Osipova A, Gieré R, Seidl M, Merfort I, Humar M. Stress fibers, autophagy and necrosis by persistent exposure to PM_{2.5} from biomass combustion. *PLoS One.* 2017;12(7):e0180291. doi:10.1371/journal.pone.0180291. PMID:28671960
21. Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, Koplev S, Jenkins SL, Jagodnik KM, Lachmann A, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* 2016;44:W90-7. doi:10.1093/nar/gkw377
22. Ross JP, Rand KN, Molloy PL. Hypomethylation of repeated DNA sequences in cancer. *Epigenomics.* 2010;2:245-69. doi:10.2217/epi.10.2. PMID:22121873
23. Coogan PF, White LF, Jerrett M, Brook RD, Su JG, Seto E, Burnett R, Palmer JR, Rosenberg L. Air pollution and incidence of hypertension and diabetes mellitus in black women living in Los Angeles. *Circulation.* 2012;125:767-72. doi:10.1161/CIRCULATIONAHA.111.052753. PMID:22219348
24. Xu CX, Jin H, Shin JY, Kim JE, Cho MH. Roles of protein kinase B/Akt in lung cancer. *Front Biosci (Elite Ed).* 2010;2:1472-84. PMID:20515818
25. Lindsey S, Langhans SA. Epidermal growth factor signaling in transformed cells. *Int Rev Cell Mol Biol.* 2015;314:1-41. PMID:25619714
26. Miyazono K, Ehata S, Koinuma D. Tumor-promoting functions of transforming growth factor-beta in progression of cancer. *Ups J Med Sci.* 2012;117:143-52. doi:10.3109/03009734.2011.638729. PMID:22111550
27. Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer Metastasis Rev.* 2009;28:151-66. doi:10.1007/s10555-008-9179-y. PMID:19153669
28. Tiwari N, Gheldof A, Tatari M, Christofori G. EMT as the ultimate survival mechanism of cancer cells. *Semin Cancer Biol.* 2012;22:194-207. doi:10.1016/j.semcancer.2012.02.013. PMID:22406545
29. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene.* 2010;29:625-34. doi:10.1038/onc.2009.441. PMID:19946328
30. Arellanes-Licea E, Caldelas I, De Ita-Perez D, Diaz-Munoz M. The circadian timing system: a recent addition in the physiological mechanisms underlying pathological and aging processes. *Aging Dis.* 2014;5:406-18. PMID:25489492
31. Chen R, Meng X, Zhao A, Wang C, Yang C, Li H, Cai J, Zhao Z, Kan H. DNA hypomethylation and its mediation in the effects of fine particulate air pollution on cardiovascular biomarkers: A randomized cross-over trial. *Environ Int.* 2016;94:614-9. doi:10.1016/j.envint.2016.06.026. PMID:27397927
32. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC. Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). *Nucleic Acids Res.* 2004;32:4100-8. doi:10.1093/nar/gkh739. PMID:15302911
33. Han H, Cortez CC, Yang X, Nichols PW, Jones PA, Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet.* 2011;20:4299-310. doi:10.1093/hmg/ddr356. PMID:21835883
34. Liu F, Killian JK, Yang M, Walker RL, Hong JA, Zhang M, Davis S, Zhang Y, Hussain M, Xi S, et al. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene.* 2010;29:3650-64. doi:10.1038/onc.2010.129. PMID:20440268
35. Zhu ZZ, Sparrow D, Hou L, Tarantini L, Bollati V, Litonjua AA, Zano-betti A, Vokonas P, Wright RO, Baccarelli A, et al. Repetitive element hypomethylation in blood leukocyte DNA and cancer incidence, prevalence, and mortality in elderly individuals: the Normative Aging Study. *Cancer Causes Control.* 2011;22:437-47. doi:10.1007/s10552-010-9715-2. PMID:21188491
36. Lamprecht B, Walter K, Kreher S, Kumar R, Hummel M, Lenze D, Kochert K, Bouhrel MA, Richter J, Soler E, et al. Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. *Nat Med.* 2010;16:571-9, 1p following 9. doi:10.1038/nm.2129. PMID:20436485
37. Baccarelli A, Wright R, Bollati V, Litonjua A, Zano-betti A, Tarantini L, Sparrow D, Vokonas P, Schwartz J. Ischemic heart disease and stroke in relation to blood DNA methylation. *Epidemiology.* 2010;21:819-28. doi:10.1097/EDE.0b013e3181f20457. PMID:20805753
38. Martin-Nunez GM, Rubio-Martin E, Cabrera-Mulero R, Rojo-Martinez G, Oliveira G, Valdes S, Soriguer F, Castano L, Morcillo S. Type 2 diabetes mellitus in relation to global LINE-1 DNA methylation in peripheral blood: a cohort study. *Epigenetics.* 2014;9:1322-8. doi:10.4161/15592294.2014.969617. PMID:25437047
39. Nزاباروشimana E, Prior S, Miousse IR, Pathak R, Allen AR, Latendresse J, Olsen RH, Raber J, Hauer-Jensen M, Nelson GA, et al. Combined exposure to protons and (56)Fe leads to overexpression of Il13 and reactivation of repetitive elements in the mouse lung. *Life Sci Space Res (Amst).* 2015;7:1-8. doi:10.1016/j.lssr.2015.08.001. PMID:26553631
40. Osman A. MicroRNAs in health and disease-basic science and clinical applications. *Clin Lab.* 2012;58:393-402. PMID:22783567
41. Xi S, Xu H, Shan J, Tao Y, Hong JA, Inchauste S, Zhang M, Kunst TF, Mercedes L, Schrupp DS. Cigarette smoke mediates epigenetic repression of miR-487b during pulmonary carcinogenesis. *J Clin Invest.* 2013;123:1241-61. doi:10.1172/JCI61271. PMID:23426183
42. Vrijens K, Bollati V, Nawrot TS. MicroRNAs as potential signatures of environmental exposure or effect: a systematic review. *Environ Health Perspect.* 2015;123:399-411. PMID:25616258
43. Rodosthenous RS, Coull BA, Lu Q, Vokonas PS, Schwartz JD, Baccarelli AA. Ambient particulate matter and microRNAs in extracellular

- vesicles: a pilot study of older individuals. Part Fibre Toxicol. 2016;13:13. doi:10.1186/s12989-016-0121-0. PMID:26956024
44. Sun CC, Li SJ, Li DJ. Hsa-miR-134 suppresses non-small cell lung cancer (NSCLC) development through down-regulation of CCND1. *Oncotarget*. 2016;7:35960-78. doi:10.18632/oncotarget.8482. PMID:27166267
 45. Wang C, Liu P, Wu H, Cui P, Li Y, Liu Y, Liu Z, Gou S. MicroRNA-323-3p inhibits cell invasion and metastasis in pancreatic ductal adenocarcinoma via direct suppression of SMAD2 and SMAD3. *Oncotarget*. 2016;7:14912-24. doi:10.18632/oncotarget.7482. PMID:26908446
 46. Zhou B, Song J, Han T, Huang M, Jiang H, Qiao H, Shi J, Wang Y. MiR-382 inhibits cell growth and invasion by targeting NR2F2 in colorectal cancer. *Mol Carcinog*. 2016;55(12):2260-67. <https://doi.org/10.1002/mc.22466>
 47. Mou X, Liu S. MiR-485 inhibits metastasis and EMT of lung adenocarcinoma by targeting Flot2. *Biochem Biophys Res Commun*. 2016;477:521-6. doi:10.1016/j.bbrc.2016.04.043. PMID:27262438
 48. Sun LL, Wang J, Zhao ZJ, Liu N, Wang AL, Ren HY, Yang F, Diao KX, Fu WN, Wan EH, et al. Suppressive role of miR-502-5p in breast cancer via downregulation of TRAF2. *Oncol Rep*. 2014;31:2085-92. PMID:24677135
 49. Li X, Liu X, Xu W, Zhou P, Gao P, Jiang S, Lobie PE, Zhu T. c-MYC-regulated miR-23a/24-2/27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting Sprouty2. *J Biol Chem*. 2013;288:18121-33. doi:10.1074/jbc.M113.478560. PMID:23649631
 50. Qu WQ, Liu L, Yu Z. Clinical value of microRNA-23a upregulation in non-small cell lung cancer. *Int J Clin Exp Med*. 2015;8:13598-603. PMID:26550300
 51. Zhao G, Liu L, Zhao T, Jin S, Jiang S, Cao S, Han J, Xin Y, Dong Q, Liu X, et al. Upregulation of miR-24 promotes cell proliferation by targeting NAIF1 in non-small cell lung cancer. *Tumour Biol*. 2015;36:3693-701. doi:10.1007/s13277-014-3008-4. PMID:25725584
 52. Cristobal I, Madoz-Gurpide J, Rojo F, Garcia-Foncillas J. Potential therapeutic value of miR-425-5p in metastatic colorectal cancer. *J Cell Mol Med*. 2016;20:2213-4. doi:10.1111/jcmm.12902. PMID:27396018
 53. Tong XD, Liu TQ, Wang GB, Zhang CL, Liu HX. MicroRNA-570 promotes lung carcinoma proliferation through targeting tumor suppressor KLF9. *Int J Clin Exp Pathol*. 2015;8:2829-34. PMID:26045791
 54. Zhang L, Yang L, Liu X, Chen W, Chang L, Chen L, Loera S, Chu P, Huang WC, Liu YR, et al. MicroRNA-657 promotes tumorigenesis in hepatocellular carcinoma by targeting transducin-like enhancer protein 1 through nuclear factor kappa B pathways. *Hepatology*. 2013;57:1919-30. doi:10.1002/hep.26162. PMID:23175432
 55. Wang J, Zhao YC, Lu YD, Ma CP. Integrated bioinformatics analyses identify dysregulated miRNAs in lung cancer. *Eur Rev Med Pharmacol Sci*. 2014;18:2270-4. PMID:25219825
 56. Zhu W, Zhou K, Zha Y, Chen D, He J, Ma H, Liu X, Le H, Zhang Y. Diagnostic Value of Serum miR-182, miR-183, miR-210, and miR-126 Levels in Patients with Early-Stage Non-Small Cell Lung Cancer. *PLoS One*. 2016;11:e0153046. doi:10.1371/journal.pone.0153046. PMID:27093275
 57. Wang M, Huang Y, Liang Z, Liu D, Lu Y, Dai Y, Feng G, Wang C. Plasma miRNAs might be promising biomarkers of chronic obstructive pulmonary disease. *Clin Respir J*. 2016;10:104-11. doi:10.1111/crj.12194. PMID:25102970
 58. Yan S, Wang T, Huang S, Di Y, Huang Y, Liu X, Luo Z, Han W, An B. Differential expression of microRNAs in plasma of patients with prediabetes and newly diagnosed type 2 diabetes. *Acta Diabetol*. 2016;53:693-702. doi:10.1007/s00592-016-0837-1. PMID:27039347
 59. Zheng D, Dai Y, Wang S, Xing X. MicroRNA-299-3p promotes the sensibility of lung cancer to doxorubicin through directly targeting ABCG1. *Int J Clin Exp Pathol*. 2015;8:10072-81. PMID:26617714
 60. Guo L, Liu Y, Bai Y, Sun Y, Xiao F, Guo Y. Gene expression profiling of drug-resistant small cell lung cancer cells by combining microRNA and cDNA expression analysis. *Eur J Cancer*. 2010;46:1692-702. doi:10.1016/j.ejca.2010.02.043. PMID:20371173
 61. Li J, Wang Y, Song Y, Fu Z, Yu W. miR-27a regulates cisplatin resistance and metastasis by targeting RKIP in human lung adenocarcinoma cells. *Mol Cancer*. 2014;13:193. doi:10.1186/1476-4598-13-193. PMID:25128483
 62. Philipone E, Yoon AJ, Wang S, Shen J, Ko YC, Sink JM, Rockafellow A, Shammay NA, Santella RM. MicroRNAs-208b-3p, 204-5p, 129-2-3p and 3065-5p as predictive markers of oral leukoplakia that progress to cancer. *Am J Cancer Res*. 2016;6:1537-46. PMID:27508095
 63. Chen SW, Wang TB, Tian YH, Zheng YG. Down-regulation of microRNA-126 and microRNA-133b acts as novel predictor biomarkers in progression and metastasis of non small cell lung cancer. *Int J Clin Exp Pathol*. 2015;8:14983-8. PMID:26823832
 64. Zou X, Wang J, Chen C, Jose PA, Zeng C. Secreted Monocyte miR-27a Causes Hypertension by Reducing Mas Receptor Expression and Function in the Artery. *J Am Soc Hypertens*. 2016;10Suppl 1:e3. doi:10.1016/j.jash.2016.06.010. PMID:27677133
 65. Qiang L, Hong L, Ningfu W, Huaihong C, Jing W. Expression of miR-126 and miR-508-5p in endothelial progenitor cells is associated with the prognosis of chronic heart failure patients. *Int J Cardiol*. 2013;168:2082-8. doi:10.1016/j.ijcard.2013.01.160. PMID:23465244
 66. Matsumoto S, Sakata Y, Nakatani D, Suna S, Mizuno H, Shimizu M, Usami M, Sasaki T, Sato H, Kawahara Y, et al. A subset of circulating microRNAs are predictive for cardiac death after discharge for acute myocardial infarction. *Biochem Biophys Res Commun*. 2012;427:280-4. doi:10.1016/j.bbrc.2012.09.039. PMID:22995291
 67. Edgar R, Tan PP, Portales-Casamar E, Pavlidis P. Meta-analysis of human methylomes reveals stably methylated sequences surrounding CpG islands associated with high gene expression. *Epigenetics Chromatin*. 2014;7:28. doi:10.1186/1756-8935-7-28. PMID:25493099
 68. Lou S, Lee HM, Qin H, Li JW, Gao Z, Liu X, Chan LL, Kl Lam V, So WY, Wang Y, et al. Whole-genome bisulfite sequencing of multiple individuals reveals complementary roles of promoter and gene body methylation in transcriptional regulation. *Genome Biol*. 2014;15:408. doi:10.1186/s13059-014-0408-0. PMID:25074712
 69. Logan J, Chen L, Gangell C, Sly PD, Fantino E, Liu K. Brief exposure to cigarette smoke impairs airway epithelial cell innate anti-viral defence. *Toxicol In Vitro*. 2014;28:1430-5. doi:10.1016/j.tiv.2014.07.012. PMID:25111775
 70. Kelly FJ, Fussell JC. Air pollution and airway disease. *Clin Exp Allergy*. 2011;41:1059-71. doi:10.1111/j.1365-2222.2011.03776.x. PMID:21623970
 71. Thierry JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009;139:871-90. doi:10.1016/j.cell.2009.11.007. PMID:19945376
 72. Vendramini-Costa DB, Carvalho JE. Molecular link mechanisms between inflammation and cancer. *Curr Pharm Des*. 2012;18:3831-52. doi:10.2174/138161212802083707. PMID:22632748
 73. Gonda TA, Kim YI, Salas MC, Gamble MV, Shibata W, Muthupalani S, Sohn KJ, Abrams JA, Fox JG, Wang TC, et al. Folic acid increases global DNA methylation and reduces inflammation to prevent Helicobacter-associated gastric cancer in mice. *Gastroenterology*. 2012;142:824-33 e7. PMID:22248660. doi:10.1053/j.gastro.2011.12.058
 74. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, Baylin SB. Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res*. 2003;63:7089-93. PMID:14612500
 75. Cassee FR, Heroux ME, Gerlofs-Nijland ME, Kelly FJ. Particulate matter beyond mass: recent health evidence on the role of fractions, chemical constituents and sources of emission. *Inhal Toxicol*. 2013;25:802-12. doi:10.3109/08958378.2013.850127. PMID:24304307
 76. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*. 2011;333:1300-3. doi:10.1126/science.1210597. PMID:21778364
 77. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*. 2014;30:1363-9. doi:10.1093/bioinformatics/btu049. PMID:24478339

78. Chen EY, Dobrinski KP, Brown KH, Clagg R, Edelman E, Ignatius MS, Chen JY, Brockmann J, Nielsen GP, Ramaswamy S, et al. Cross-species array comparative genomic hybridization identifies novel oncogenic events in zebrafish and human embryonal rhabdomyosarcoma. *PLoS Genet.* 2013;9:e1003727. doi:10.1371/journal.pgen.1003727. PMID:24009521
79. Wilson CL, Miller CJ. Simpleaffy: a BioConductor package for Affymetrix Quality Control and data analysis. *Bioinformatics.* 2005;21:3683-5. doi:10.1093/bioinformatics/bti605. PMID:16076888
80. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics.* 2004;20:307-15. PMID:14960456. doi:10.1093/bioinformatics/btg405
81. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43:e47. doi:10.1093/nar/gkv007. PMID:25605792
82. Stalteri MA, Harrison AP. Interpretation of multiple probe sets mapping to the same gene in Affymetrix GeneChips. *BMC Bioinformatics.* 2007;8:13. doi:10.1186/1471-2105-8-13. PMID:17224057
83. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer Science & Business Media; 2009.
84. Lawrence M, Huber W, Pages H, Aboyoun P, Carlson M, Gentleman R, Morgan MT, Carey VJ. Software for computing and annotating genomic ranges. *PLoS Comput Biol.* 2013;9:e1003118. doi:10.1371/journal.pcbi.1003118. PMID:23950696
85. Wolfe D, Dudek S, Ritchie MD, Pendergrass SA. Visualizing genomic information across chromosomes with PhenoGram. *BioData Min.* 2013;6:18. doi:10.1186/1756-0381-6-18. PMID:24131735