




Cite this: *Toxicol. Res.*, 2017, **6**, 432

Coal combustion related fine particulate matter (PM_{2.5}) induces toxicity in *Caenorhabditis elegans* by dysregulating microRNA expression†

Qiuli Wu, Xiaoxiao Han, Di Wang, Fang Zhao and Dayong Wang *

We employed an *in vivo* assay system of *Caenorhabditis elegans* to determine if and which microRNAs (miRNAs) were dysregulated upon exposure to coal combustion related fine particulate matter (PM_{2.5}) by profiling the miRNAs using SOLiD sequencing. From this, expression of 25 miRNAs was discovered to become dysregulated by exposure to PM_{2.5}. Using the corresponding *C. elegans* deletion mutants, 5 miRNAs (*mir-231*, *mir-232*, *mir-230*, *mir-251* and *mir-35*) were found to be involved in the control of PM_{2.5} toxicity. Furthermore, mutation of *mir-231* or *mir-232* induced a resistance to PM_{2.5} toxicity, whereas mutation of *mir-230*, *mir-251*, or *mir-35* induced a susceptibility to PM_{2.5} toxicity. SMK-1, an ortholog of the mammalian SMEK protein, was identified as a molecular target for *mir-231* in the regulation of PM_{2.5} toxicity. In addition, the genes of *sod-3*, *sod-4* and *ctl-3*, which are necessary for protection against oxidative stress, were determined to be important downstream targets of *smk-1* in the regulation of PM_{2.5} toxicity. The triggering of this *mir-231*-SMK-1-SOD-3/SOD-4/CTL-3 signaling pathway may be a critical molecular basis for the role of oxidative stress in the induction of coal combustion related PM_{2.5} toxicity.

Received 12th April 2017,

Accepted 21st April 2017

DOI: 10.1039/c7tx00107j

rsc.li/toxicology-research

Introduction

Epidemiological studies have suggested the statistical associations between fine particulate matter (PM) emission and human health.^{1,2} For example, an elevation in PM air pollution was associated with the increased risk of all-cause, cardio-pulmonary, and lung cancer mortality.¹ PM less than 2.5 μm in aerodynamic size (PM_{2.5}) is responsible for air pollution caused by coal combustion to a great degree. Coal combustion related PM_{2.5} has been suggested to be closely associated with lung dysfunction, cancer, and induction of oxidative stress.^{3–5} The cytotoxicity assay further indicated that exposure to coal combustion related PM_{2.5} could result in a decrease in cell viability, increase in global DNA methylation, and oxidative DNA damage in vascular endothelial cells.⁶

Nematode *Caenorhabditis elegans*, an important model animal, can be maintained in the laboratory, where it is grown on agar plates or liquid cultures with *Escherichia coli* OP50 as the food source.⁷ Numerous basic physiological processes, stress responses, and molecular regulation mechanisms have

been proven to be conserved between *C. elegans* and human beings or mammals.⁸ *C. elegans* has been employed as an important alternative model animal for toxicological study, and it can provide an assay system for asking *in vivo* questions.^{9–11} Recently, *C. elegans* has been successfully used for the toxicity assessment and the toxicological study of PM_{2.5}, including the coal combustion related PM_{2.5}.^{12–16} Exposure to coal combustion related PM_{2.5} can damage the functions of both primary targeted organs, such as intestine, and secondary targeted organs, such as neurons, and reproductive organs in nematodes.^{13,15}

MicroRNAs (miRNAs) are a large class of short noncoding RNAs found in various organisms, which usually act to post-transcriptionally inhibit the gene expression *via* sequence-specific base pairing with target mRNAs.^{17,18} Previous studies have suggested that the miRNAs could participate in the regulation of toxicity formation of certain environmental toxicants, such as engineered nanomaterials of graphene oxide and multi-walled carbon nanotubes.^{19–23} However, the role of miRNAs in the toxicity formation of coal combustion related PM_{2.5} is still largely unclear in nematodes. In this study, we employed the *in vivo* assay system of *C. elegans* to identify the dysregulated miRNA profiling induced by coal combustion related PM_{2.5} using the SOLiD sequencing technique. We further investigated the role of miRNAs in the regulation of the toxicity of coal combustion related PM_{2.5} in nematodes.

Key Laboratory of Environmental Medicine Engineering in Ministry of Education, Medical School, Southeast University, Nanjing 210009, China.

E-mail: dayongw@seu.edu.cn

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7tx00107j

Moreover, we focused on one of the miRNAs, *mir-231*, to examine the underlying molecular mechanism for its role in regulating the toxicity of coal combustion related PM_{2.5}. Our results will highlight the crucial role of miRNAs in the induction of coal combustion related PM_{2.5} toxicity in organisms.

Materials and methods

Sample collection

Coal combustion related PM_{2.5} was acquired from a site of coal production in the Datong, Shanxi province, China. The PM_{2.5} samples were first collected on glass fiber membranes, and then organic compounds within the samples were removed. Our previously published report contains the detailed protocols by which the PM_{2.5} was prepared for experimental analysis, as well as an analysis of its chemical components.¹³ Briefly, these chemical components included at least 2 mg m⁻³ of Fe, Pb and Zn, and less than 1 mg m⁻³ of As, Cd, Cr, Cu and Ni.¹³ In addition, there were also 14 types of polycyclic aromatic hydrocarbons (PAHs) identified, where most were present in the PM_{2.5} at concentrations of at least 1 ng m⁻³.¹³

C. elegans maintenance

Several of the *C. elegans* strains used in this study were obtained from *Caenorhabditis* Genetics Center. The nematodes were maintained at 20 °C in plates containing nematode growth medium (NGM) and *Escherichia coli* OP50 as previously described.⁷ The L1-larvae were kept synchronized by lysing gravid nematodes with a bleaching mixture of 0.45 mol L⁻¹ NaOH and 2% HOCl.²⁴

Exposure

Previously published research has shown that long term exposure of nematodes to more than 1 mg L⁻¹ of PM_{2.5} results in significant decreases in locomotion and brood size, and an increase in reactive oxygen species (ROS) production in the intestines.¹³ In this study, 1 mg L⁻¹ of PM_{2.5} was dissolved in K-medium (32 mmol L⁻¹ KCl, 5 mmol L⁻¹ NaCl) and used as the working concentration in experiments. Prolonged exposure to PM_{2.5} from L1-larvae to young adults (approximately 2.5 days) was performed in the liquid K medium in sterile tissue culture plate wells in the presence of OP50 food at 20 °C. The control nematodes were grown in the liquid K medium in sterile tissue culture plate wells in the presence of OP50 food at 20 °C without the addition of PM_{2.5}. Toxicity was assessed based on the endpoints of locomotion and intestinal ROS production.

Intestinal ROS production

The endpoint of intestinal ROS production was used to reflect the functional state of a primary targeted organ, the intestine, in nematodes.²⁵ Intestinal ROS production was analyzed as described previously.^{26,27} Following the incubation in PM_{2.5}, the exposed nematodes were transferred into sterile tissue culture plate wells and incubated in 1 μmol L⁻¹ CM-H₂DCFDA

in the dark for 3 h. The nematodes were then evaluated by microscopy after mounting them on 2% agar pads and then examining the relative fluorescence intensity of the intestine under a laser scanning confocal microscope. Relative fluorescence intensity of the intestine was semi-quantified using relative fluorescence units (RFU). For each treatment, 30 nematodes were evaluated in 3 replicates.

Locomotion behavior

Nematode locomotion is an indicative parameter of the functional state of the motor neurons.²⁸ In this study, locomotion was assessed based on examination of head thrash and body bend under a dissecting microscope by visual inspection as previously published.^{29,30} The head thrash and body bend were defined as directional changes in bending at the mid-body and pharynx posterior bulb, respectively, along the y axis when the nematode is moving along the x axis. For each treatment, 20 nematodes were assessed in 6 replicates.

Small RNA extraction, SOLiD sequencing, and bioinformatics analysis

Extraction of small RNAs and SOLiD sequencing were executed as previously described.³¹ Briefly, the control and PM_{2.5} (1 mg L⁻¹) treated nematodes were lysed and small RNAs were extracted using a mirVana™ miRNA isolation kit (Ambion) based on the manufacturer's protocol. These small RNAs were then reverse transcribed into a double-stranded cDNA library, on which adaptor ligation was then performed. Importantly, this library was created to be compatible with the high-throughput sequencing system used, the Applied Biosystems SOLiD™ system. SOLiD sequencing results were collected as nucleotide sequences and their coverage. The resulting sequences were compared against the Genbank (<http://www.ncbi.nlm.nih.gov/Genbank>) and miRNAbase (<http://www.mirbase.org>) databases, and the registered miRNAs were analyzed using all the collected sequences.

A total of three independent biological replicates were performed. The average expression of each of the miRNAs was compared between nematodes exposed to PM_{2.5} and the control. Dysregulation of miRNA expression was detected in PM_{2.5}-exposed nematodes by DESeq, which is an R package capable of estimating variance and testing for differential expression. The expression of each miRNA was denoted as up- or down-regulated, where a 2-fold, statistically significant ($P < 0.05$) change was the cutoff; the results were then normalized and displayed as a scatter diagram. The predicted target genes of dysregulated miRNAs that were the most notably affected by exposure to PM_{2.5} were then classified based on their gene ontology and the KEGG pathways using the bioinformatics tools at <http://www.geneontology.org> and <http://www.pantherdb.org>, respectively.

Reverse-transcription and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from the nematodes using Trizol (Invitrogen, UK) according to the manufacturer's protocol. The

extracted RNA was used as a template for the synthesis of cDNA. The relative expression levels of the RNA were measured by real-time PCR (RT-PCR) in an ABI 7500 with Evagreen (Biotium, USA). Three biological replicates were performed. The expression of each miRNA is presented as the relative expression ratio between each miRNA and *F35C11.9*, which encodes a small nuclear RNA U6. Quantification of each gene target was expressed as the relative expression ratio between the gene of interest and the reference gene *tba-1*, which encodes a tubulin protein. The sequences of the primers used for the RT-PCR of the miRNAs are presented in Tables S1 and S2,† while those used to amplify genes are in Table S3.†

RNA interference (RNAi)

To generate nematodes with specific knock-down, RNAi was used through the *E. coli* strain HT115, carrying double-stranded RNA corresponding to each target, and was fed to the nematodes. Primer sequences for RNAi carrying the *E. coli* strain construction are presented in Table S4.† First, RNAi carrying *E. coli* strains were incubated overnight at 37 °C and then were transferred to NGM plates. L1 larvae were then added to these wells and incubated for 2 days at 20 °C until the nematodes became gravid. The resulting eggs were incubated at 20 °C until the offspring became young adults and were then used for the subsequent assays.

Statistical analysis

Results in this article are all presented as mean \pm standard deviation (SD). Cohorts were analyzed for statistically significant

differences by ANOVA using SPSS 12.0 (SPSS Inc., Chicago, USA), where $P < 0.05$ and $P < 0.01$ were considered significant.

Results

SOLiD sequencing

To generate miRNA expression profiles so that comparisons could be made between control and PM_{2.5} (1 mg L⁻¹) exposed nematodes, SOLiD sequencing was performed. It was found that the miRNA sequences ranged in size from 19–25 nucleotides, and the most frequent lengths were 20 and 23 nucleotides, which are the lengths of mature miRNAs according to the miRNA database (Fig. 1a). In addition, all of the detected miRNAs mapped across all chromosomes, including both the autosomal and the X chromosomes (Fig. 1b). The coverage of the miRNAs was visualized by a scatter diagram, and both up- and down-regulated miRNAs were found following exposure to PM_{2.5} (Fig. 1c). Overall, this data suggest that RNAomics sequencing is a feasible method to identify the miRNAs with potential roles in regulating the *in vivo* response to coal combustion related PM_{2.5} toxicity.

Identification of miRNAs dysregulated by exposure to coal combustion related PM_{2.5}

To identify miRNAs with changes in expression triggered by exposure to PM_{2.5}, fold changes between control and PM_{2.5} exposed miRNAs were calculated, where a 2.0-fold change cutoff was considered statistically significant. To assign annotations to the resulting genes of interest, the miRNA sequences

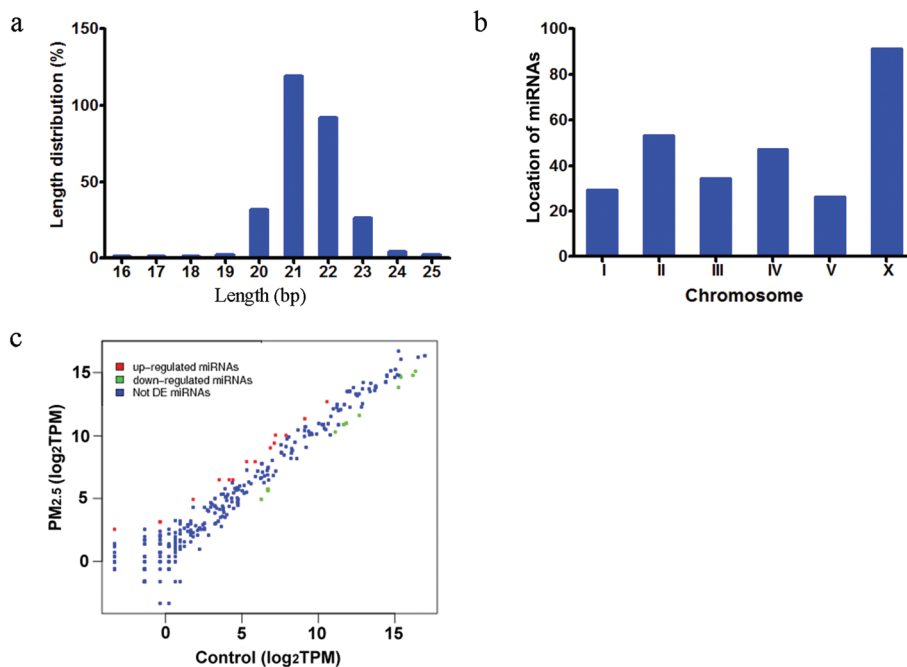


Fig. 1 Basic SOLiD sequencing results. (a) Length distribution of miRNAs. (b) Chromosome distribution of miRNAs. (c) Scatter diagram of miRNAs. DE miRNAs: differentially expressed miRNAs. TPM: transcripts per million. The concentration of coal combustion related PM_{2.5} was 1 mg L⁻¹. Prolonged exposure was performed from L1-larvae to young adults at 20 °C in the presence of food.

identified were compared against the Genbank and miRbase databases. Twenty-five miRNAs were found to be differentially expressed in nematodes exposed to PM_{2.5} compared to the control (Fig. 2 and Table S5†). The molecular signature of the 25 miRNAs that were found to be dysregulated following exposure to PM_{2.5} was displayed through hierarchical clustering (Fig. 2a). Of these 25 miRNAs, 15 were up-regulated, *i.e.* *mir-43*, *mir-62*, *mir-67*, *mir-79*, *mir-124*, *mir-229*, *mir-231*, *mir-232*, *mir-251*, *mir-392*, *mir-792*, *mir-797*, *mir-799*, *mir-1020* and *mir-481* (Fig. 2b and Table S5†). Moreover, the remaining 10 were downregulated, consisting of *mir-35*, *mir-37*, *mir-38*, *mir-*

40, *mir-49*, *mir-61*, *mir-83*, *mir-230*, *mir-234* and *mir-784* (Fig. 2b and Table S5†).

To confirm the SOLiD sequencing results, changes in the expression levels of several miRNAs that were dysregulated upon prolonged exposure to PM_{2.5} (1 mg L⁻¹) were measured by qRT-PCR. Compared to the control, the miRNAs *mir-62*, *mir-231*, *mir-232*, and *mir-251* were found to be significantly up-regulated, while *mir-35*, *mir-83*, *mir-230*, and *mir-234* were significantly down-regulated (Fig. 2c). Therefore, the alterations in expression for these candidate miRNAs were similar by both SOLiD sequencing and qRT-PCR.

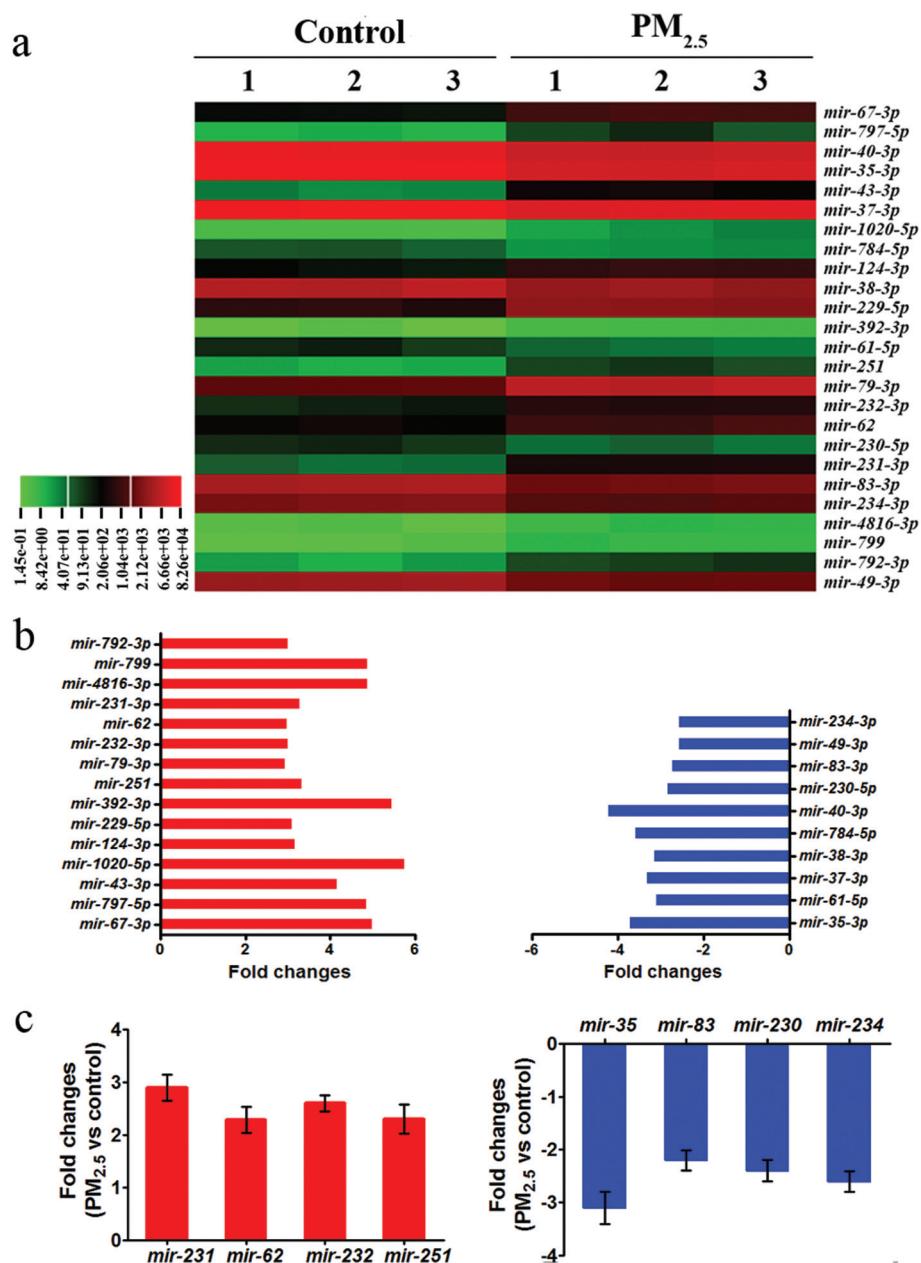


Fig. 2 Identification of miRNAs involved in the control of coal combustion related PM_{2.5} toxicity. (a) Hierarchical clustering assay of miRNAs expression. (b) Fold changes of miRNAs in coal combustion related PM_{2.5} exposed nematodes. (c) Expression pattern of mature miRNAs detected by real-time PCR after coal combustion related PM_{2.5} exposure. The concentration of coal combustion related PM_{2.5} was 1 mg L⁻¹. Prolonged exposure was performed from L1-larvae to young adults at 20 °C in the presence of food. Bars represent mean ± SD.

Gene ontology and signaling pathways of the predicted gene targets of the miRNAs dysregulated in response to coal combustion related PM_{2.5}

To characterize the functions of the miRNAs that were dysregulated in response to PM_{2.5} in the nematodes, the genes targeted by these miRNAs were predicted using the TargetScan database. Overall, 1456 and 959 genes were found to be the putative targets of the down- and up-regulated miRNAs, respectively. Analysis of the ontology of these target genes was performed to generate putative biological processes for these genes,⁸ and thus, a possible role for these miRNAs (Tables S6 and S7†). The functions of the target genes were found to fall into several categories including defense, cell migration, development, growth, locomotion, reproduction, metabolism, longevity, transport, and signal transduction (Fig. 3a).

Furthermore, signaling pathways mediated by the genes that were predicted to be targeted by the dysregulated miRNAs were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. In nematodes, the up-regulated miRNAs were found to influence 105 signaling pathways, while the down-regulated miRNAs influenced 95 signaling pathways (signaling pathways with gene counts more than 4 have been shown in Fig. 3b, Tables S8 and S9†). These signaling pathways were predominantly related to oxidative stress response, Wnt, TGF-beta, PI3 kinase pathway, VEGF, Parkinson disease, Alzheimer disease-amyloid secretase pathway, p53, apoptosis, Notch signaling pathways, and so on (Fig. 3b). Identifying these important signaling pathways increases our understanding of the molecular mechanisms of action during miRNA regulation of coal combustion derived PM_{2.5} toxicity.

Loss-of-function mutations of candidate miRNAs altered responses to coal combustion related PM_{2.5} in nematodes

Loss-of-function miRNA mutants are powerful tools to study the function of miRNAs in the *in vivo* *C. elegans* model.³² Due to signaling pathways mediated by the dysregulated miRNAs including the oxidative stress response and neurodegeneration, such as Parkinson's disease and Alzheimer disease in

Fig. 3b, intestinal ROS production and locomotion in nematodes with loss-of-function mutations in candidate miRNAs were assessed compared to wild-type N2 nematodes after exposure to PM_{2.5}. For intestinal ROS production, it was found that nematodes with loss of function of *mir-230*, *mir-251*, or *mir-35* had higher levels of ROS when exposed to PM_{2.5} compared to wild-type N2, while the reverse occurred in *mir-231* and *mir-232* mutants (Fig. 4a). Similarly, in terms of locomotion, it was noted that *mir-230*, *mir-251*, or *mir-35* mutants exposed to PM_{2.5} had significantly less head thrash and body bend than wild-type N2, while *mir-232* or *mir-231* had significantly more head thrash and body bend (Fig. 4b). In contrast, nematodes with loss of function of *mir-62*, *mir-83*, or *mir-234* had intestinal ROS production and locomotion approximately equal to wild-type N2 after exposure to PM_{2.5} (Fig. 4a and b). Under normal conditions, nematodes with a loss of function in these miRNAs displayed no significant changes in intestinal ROS production or locomotion compared to wild-type N2 (Fig. 4a and b). Therefore, the miRNAs of *mir-35*, *mir-230*, *mir-231*, *mir-232*, and *mir-251* are likely involved in the control of coal combustion related PM_{2.5} toxicity.

SMK-1 acted as a potential target for *mir-231* during regulation of coal combustion related PM_{2.5} toxicity

When determining the functions of specific miRNAs, the functions of the predicted target genes can provide insights.^{33,34} In this study, the TargetScan database was used to predict the targets of *mir-231* to delineate the molecular mechanisms behind this miRNA's regulation of the response to PM_{2.5}. Ninety-three putative targets were identified, including *smk-1*, which encoded an evolutionarily conserved protein ortholog of the mammalian SMEK (suppressor of MEK null) protein.³⁵

In the *mir-231* mutant nematodes, it was found there was significant up-regulation of *smk-1* expression, suggesting that *mir-231* is a negative regulator of *smk-1* (Fig. 5a). Upon exposure to PM_{2.5}, there was an even further increase in *smk-1* expression levels in *mir-231* mutants compared to wild-type N2 nematodes (Fig. 5a).

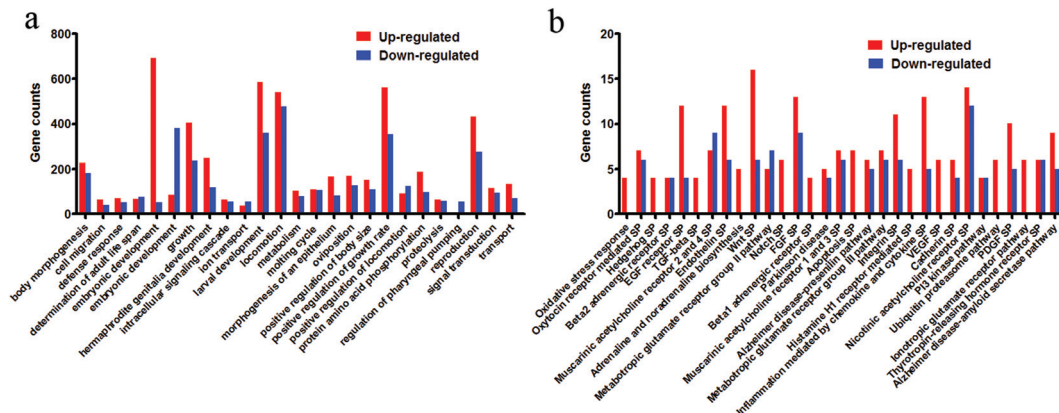


Fig. 3 Gene ontology terms and signaling pathways based on predicted targets of dysregulated miRNA induced by coal combustion related PM_{2.5}. (a) Gene ontology terms. Gene counts >40. (b) KEGG signaling pathways. Gene counts >4. SP: signaling pathway.

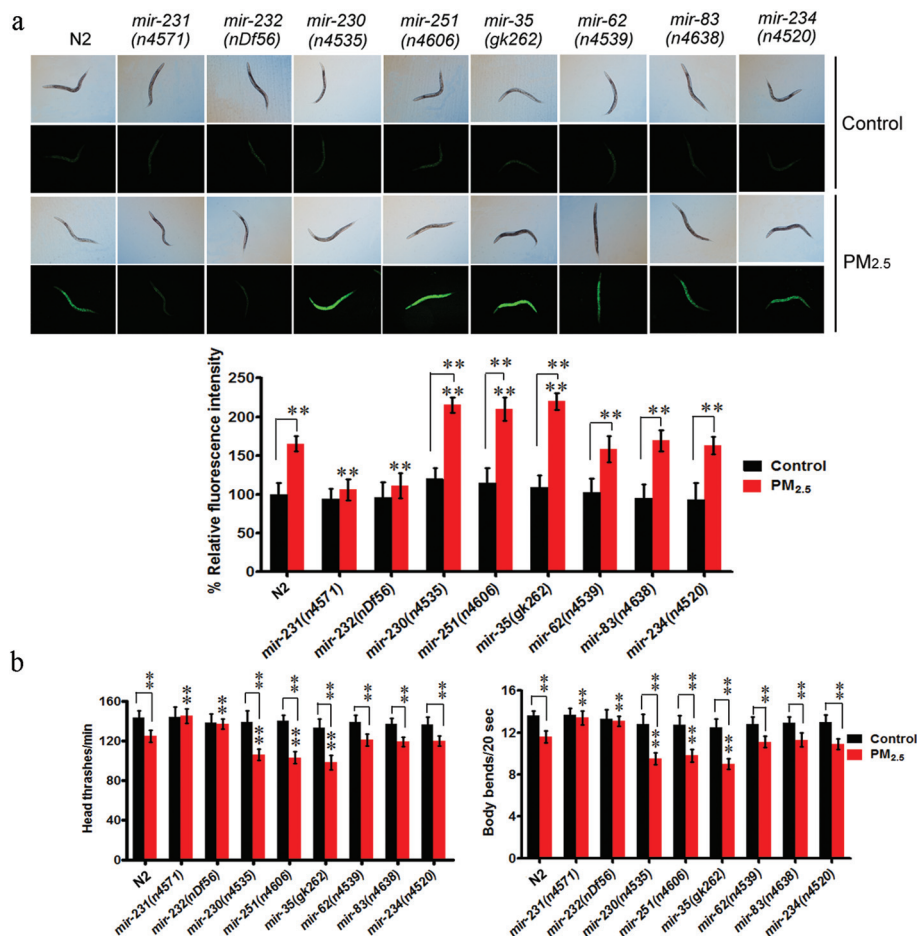


Fig. 4 Confirmation of miRNAs involved in the control of coal combustion related PM_{2.5} toxicity. (a) Comparison of intestinal ROS production. (b) Comparison of locomotion behavior. The concentration of coal combustion related PM_{2.5} was 1 mg L⁻¹. Prolonged exposure was performed from L1-larvae to young adults at 20 °C in the presence of food. Bars represent mean ± SD. ***P* < 0.01 vs. N2 (if not specially indicated).

When intestinal ROS production and locomotion were evaluated in the *smk-1*(*mn156*) mutant after PM_{2.5} exposure, it was found that this mutant had much higher levels of intestinal ROS production, as well as significantly less locomotion, compared to wild-type N2 nematodes (Fig. 5b and c). These data suggest that *smk-1* mutation may induce a susceptibility of nematodes to coal combustion related PM_{2.5} toxicity.

Genetic interaction between *mir-231* and *smk-1* during the regulation of coal combustion related PM_{2.5} toxicity

Compared to the wild-type N2 nematodes, when exposed to PM_{2.5}, the *mir-231* mutant had a significant decrease in the induction of intestinal ROS and an increase in locomotion, while the reverse phenotypes were seen in the *smk-1*(*mn156*) mutant (Fig. 6). Importantly, ROS production and locomotion in a double mutant of *mir-231* (n4571);*smk-1*(*mn156*) paralleled those in the *smk-1*(*mn156*) single mutant nematodes (Fig. 6). These results suggest that the effects of a *mir-231* mutation on coal combustion derived PM_{2.5} toxicity, in the induction of intestinal ROS production and suppression of locomotion, can be abrogated by mutating *smk-1*. Overall, these results suggest

SMK-1 is a target of *mir-231* during the regulation of coal combustion derived PM_{2.5} toxicity.

Mutation of *smk-1* altered the expression of genes required for the response to oxidative stress in coal combustion related PM_{2.5} exposed nematodes

Due to exposure to PM_{2.5}, the expression of some genes required for the response to oxidative stress in wild-type N2 nematodes becomes dysregulated, including *sod-1*, *sod-2*, *sod-3*, *sod-4*, *ctl-3*, *mev-1*, *isp-1* and *clk-1*.¹³ Upon comparing PM_{2.5} exposed wild-type and *smk-1* mutant nematodes, it was found that *sod-3*, *sod-4* and *ctl-3* gene expression were significantly decreased in the *smk-1* mutant compared to wild-type N2 nematodes (Fig. 7a). In contrast, the expression of the *sod-1*, *sod-2*, *isp-1*, *clk-1* and *mev-1* genes was not significantly different in the *smk-1* mutant compared to that in the wild-type N2 nematodes upon exposure to PM_{2.5} (Fig. 7a). Moreover, after PM_{2.5} exposure, the transcriptional expressions of *sod-3*, *sod-4*, and *ctl-3* were significantly increased in *mir-231* mutant nematodes, and the transcriptional expressions of *sod-3*, *sod-4*, and *ctl-3* were significantly decreased in the double

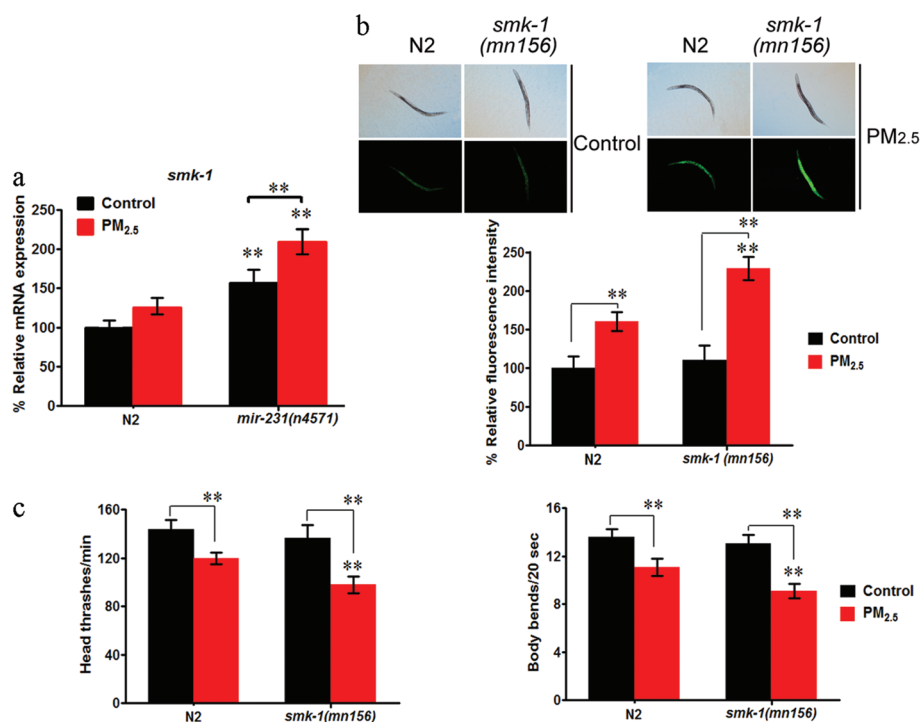


Fig. 5 Effect of *smk-1* mutation on coal combustion related PM_{2.5} toxicity in nematodes. (a) Expression pattern of *smk-1* gene in *mir-231* mutant nematodes. (b) Effect of *smk-1* mutation on intestinal ROS production in coal combustion related PM_{2.5} exposed nematodes. (c) Effect of *smk-1* mutation on locomotion behavior in coal combustion related PM_{2.5} exposed nematodes. The concentration of coal combustion related PM_{2.5} was 1 mg L⁻¹. Prolonged exposure was performed from L1-larvae to young adults at 20 °C in the presence of food. Bars represent mean ± SD. ***P* < 0.01 vs. N2 (if not specially indicated).

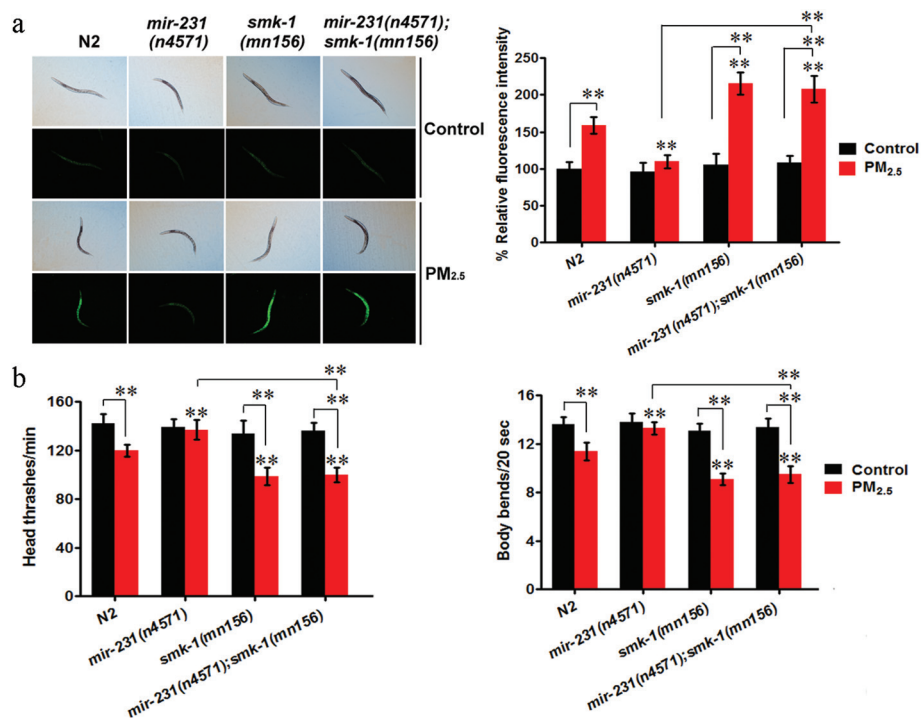


Fig. 6 Genetic interaction of *mir-231* and *smk-1* in regulating coal combustion related PM_{2.5} toxicity. (a) Genetic interaction of *mir-231* and *smk-1* in regulating coal combustion related PM_{2.5} toxicity in inducing intestinal ROS production. (b) Genetic interaction of *mir-231* and *smk-1* in regulating coal combustion related PM_{2.5} toxicity in decreasing locomotion behavior. The concentration of coal combustion related PM_{2.5} was 1 mg L⁻¹. Prolonged exposure was performed from L1-larvae to young adults at 20 °C in the presence of food. Bars represent mean ± SD. ***P* < 0.01 vs. N2 (if not specially indicated).

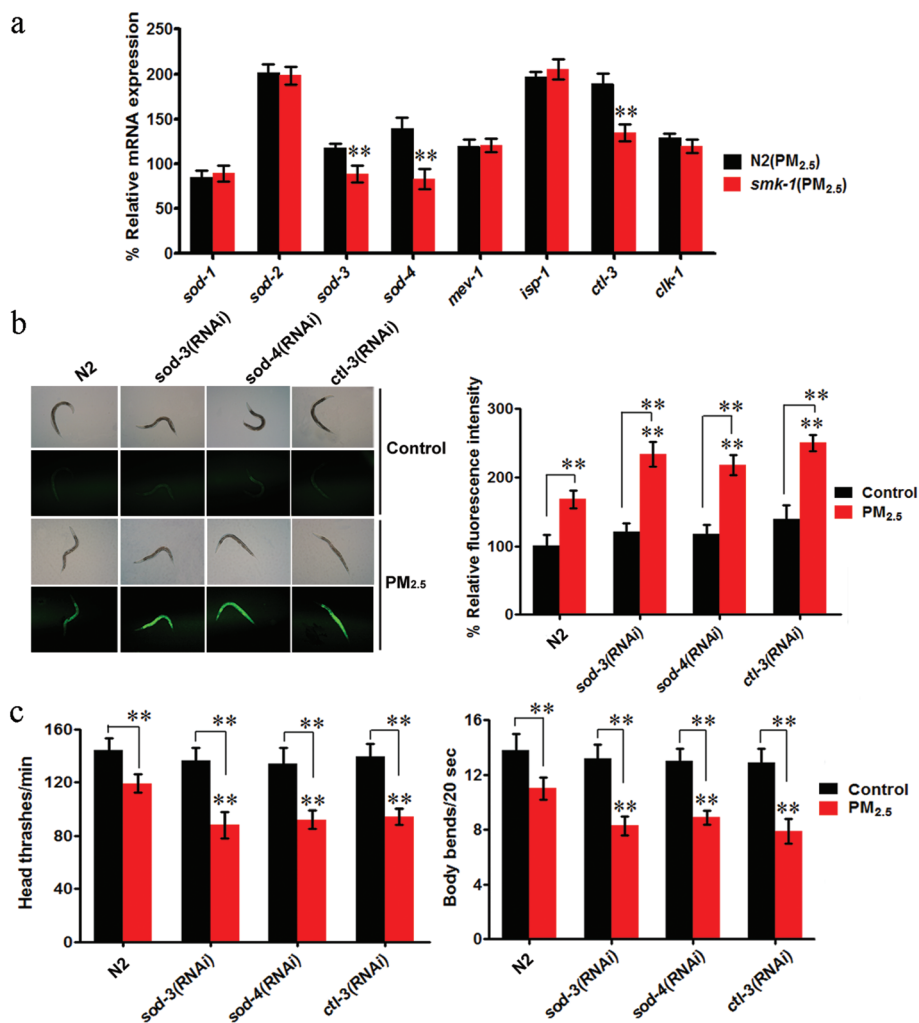


Fig. 7 Oxidative stress related genes acted as downstream regulators of *smk-1* in the regulation of coal combustion related PM_{2.5} toxicity. (a) Expression pattern of genes required for the control of oxidative stress in coal combustion related PM_{2.5} exposed wild-type and *smk-1* mutant nematodes. (b) Effect of RNAi knockdown of *sod-3*, *sod-4* or *ctl-3* on toxicity of coal combustion related PM_{2.5} in inducing intestinal ROS production. (c) Effect of RNAi knockdown of *sod-3*, *sod-4* or *ctl-3* on toxicity of coal combustion related PM_{2.5} in decreasing locomotion behavior. The concentration of coal combustion related PM_{2.5} was 1 mg L⁻¹. Prolonged exposure was performed from L1-larvae to young adults at 20 °C in the presence of food. Bars represent mean ± SD. ***P* < 0.01 vs. N2 (if not specially indicated).

mutant of *mir-231;smk-1* (Fig. S1†). These results imply that SOD-3, SOD-4 and CTL-3, which encode an iron/manganese superoxide dismutase, an extracellular Cu²⁺/Zn²⁺ superoxide dismutase and a catalase, respectively, may be important downstream targets of SMK-1 during PM_{2.5} toxicity.

SOD-3, SOD-4, and CTL-3 participated in the regulation of coal combustion related PM_{2.5} toxicity

To determine the functions of SOD-3, SOD-4, and CTL-3 during the control of PM_{2.5} toxicity, RNAi knockdown was performed in *C. elegans* for the *sod-3*, *sod-4* and *ctl-3* genes. Under normal conditions, RNAi knockdown of *sod-3*, *sod-4*, or the *ctl-3* gene did not significantly alter intestinal ROS production or locomotion (Fig. 7b and c). However, after exposure to PM_{2.5}, nematodes with RNAi knockdown of the *sod-3*, *sod-4*, or *ctl-3* gene had a significantly higher induction of intestinal ROS

production and a decrease in locomotion compared to the wild-type N2 (Fig. 7b and c). Therefore, SOD-3, SOD-4 and CTL-3 confer protection to nematodes against coal combustion related PM_{2.5} toxicity in nematodes.

Discussion

To date, the functional toxicogenomics has been widely performed to investigate the possible molecular targets for certain toxicants. However, we still know little about the molecular basis for miRNAs in the regulation of coal combustion related PM_{2.5} toxicity. Herein, we employed the *in vivo* *C. elegans* assay system to perform the SOLiD sequencing analysis in order to identify the possible miRNA targets for coal combustion related PM_{2.5} after prolonged exposure. Based on

the SOLiD sequencing results, we identified 25 differentially expressed miRNAs in coal combustion related PM_{2.5} exposed nematodes. Some of the dysregulated miRNAs in coal combustion related PM_{2.5} exposed nematodes were confirmed by the qRT-PCR assay (Fig. 2c). Our results provide important *in vivo* miRNAs targets for coal combustion related PM_{2.5} in organisms.

For the identified potential miRNA targets of coal combustion related PM_{2.5}, the gene ontology analysis suggested that they might be involved in the control of a series of important biological processes (Fig. 3a). Moreover, the KEGG analysis further demonstrated that the identified potential miRNA targets of coal combustion related PM_{2.5} might be associated with some important signaling pathways (Fig. 3b). These results provide important clues for further revealing the molecular mechanisms of coal combustion related PM_{2.5} toxicity or response of organisms to coal combustion related PM_{2.5}.

To confirm the exact roles of candidate dysregulated miRNAs in coal combustion related PM_{2.5} exposed nematodes, we used the available mutants to perform the toxicity assessment using intestinal ROS production and locomotion behavior as the endpoints. After coal combustion related PM_{2.5} exposure, we found that 5 (*mir-231*, *mir-232*, *mir-230*, *mir-251*, and *mir-35*) among the examined 8 miRNA mutants had an altered toxicity in inducing intestinal ROS production and in decreasing locomotion behavior in nematodes (Fig. 4). These data confirm the possible crucial roles of *mir-231*, *mir-232*, *mir-230*, *mir-251*, and *mir-35* in the regulation of coal combustion related PM_{2.5} toxicity. In *C. elegans*, *mir-231* is the homologue of human miR-99 and miR-556, *mir-232* is the homologue of human miR-302 and miR-519, and *mir-251* is the homologue of human miR-26.³⁶ Moreover, our results imply that the involvement of miRNAs in the regulation of PM_{2.5} toxicity should not be concluded only based on the expression pattern of miRNAs.

In this study, we further focused on the *mir-231* to determine the underlying molecular mechanism for its involvement in the control of coal combustion related PM_{2.5} toxicity. At least three lines of evidence were raised to indicate the role of SMK-1 acting as the molecular target for *mir-231* in the regulation of coal combustion related PM_{2.5} toxicity. First, after coal combustion related PM_{2.5} exposure, *mir-231* mutation increased the expression of *smk-1* (Fig. 5a). Second, the *smk-1* mutant had the opposite phenotypes of intestinal ROS production and locomotion behavior to those in the *mir-231* mutant after coal combustion related PM_{2.5} exposure (Fig. 5b and c). Third, *smk-1* mutation suppressed the resistance of the *mir-231* mutant to coal combustion related PM_{2.5} toxicity in inducing intestinal ROS production and decreasing locomotion behavior (Fig. 6). Previous study has suggested that SMK-1 activity is essential for the control of longevity and resistance to oxidative and UV-induced damage.³⁵ Our study indicates a novel function of SMK-1 in the regulation of PM_{2.5} toxicity. Moreover, our data suggest that *mir-231* acts as the direct upstream regulator for SMK-1 in regulating biological processes in nematodes (Fig. 8).

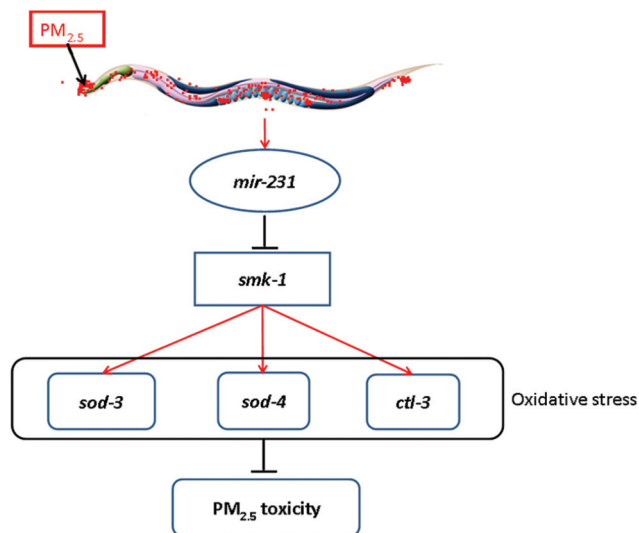


Fig. 8 A diagram showing the molecular mechanism for *mir-231* in the regulation of coal combustion related PM_{2.5} toxicity.

In this study, we further identified the SOD-3, SOD-4 and CTL-3 as the downstream targets for SMK-1 in the regulation of coal combustion related PM_{2.5} toxicity. On one hand, after coal combustion related PM_{2.5} exposure, *smk-1* mutation decreased the expression levels of *sod-3*, *sod-4* and *ctl-3* genes (Fig. 7a). On the other hand, RNAi knockdown of *sod-3*, *sod-4*, or *ctl-3* genes induced a susceptibility to coal combustion related PM_{2.5} toxicity in inducing intestinal ROS production and in decreasing locomotion behavior (Fig. 7b and c). Therefore, the raised *mir-231*-SMK-1-SOD-3/SOD-4/CTL-3 signaling cascade may provide an important molecular basis for the role of oxidative stress in the induction of coal combustion related PM_{2.5} toxicity in organisms (Fig. 8). That is, after exposure, coal combustion related PM_{2.5} can increase the expression of *mir-231*. The increased *mir-231* may further suppress the protection function of SOD-3, SOD-4, and CTL-4 against the activation of oxidative stress by inhibiting the SMK-1 expression.

Conclusions

In conclusion, using the SOLiD sequencing technique, we obtained the dysregulated miRNA profiling induced by coal combustion related PM_{2.5} in the *in vivo* assay system of *C. elegans*. Based on the determination of the expression pattern and functional analysis, we confirmed that 5 miRNAs (*mir-231*, *mir-232*, *mir-230*, *mir-251*, and *mir-35*) were involved in the control of coal combustion related PM_{2.5} toxicity. Among these 5 miRNAs, we further identified SMK-1 as the molecular target for *mir-231* in the regulation of coal combustion related PM_{2.5} toxicity. Moreover, we raised a signaling cascade of *mir-231*-SMK-1-SOD-3/SOD-4/CTL-3 to explain the possible important molecular basis for the role of oxidative stress in the induction of coal combustion related PM_{2.5} toxicity in organisms.

Conflict of interest

None of the authors have any conflicting interests.

Acknowledgements

This study was supported by the grant from National Natural Science Foundation of China (21577016), and Fundamental Research Funds for the Central Universities in China (2242016 K41033).

References

- C. A. Pope, R. T. Burnett, M. J. Thun, E. E. Calle, D. Krewski, K. Ito and G. D. Thurston, *J. Am. Med. Assoc.*, 2002, **287**, 1132–1141.
- S. D. Procto, K. L. Dreher, S. E. Kelly and J. C. Russell, *Toxicol. Sci.*, 2006, **90**, 385–391.
- J. Lewtas, *Mutat. Res.*, 2007, **636**, 95–133.
- J. K. Chan, J. G. Charrier, S. D. Kodani, C. F. Vogel, S. Y. Kado, D. S. Anderson, C. Anastasio and L. S. Van Winkle, *Part. Fibre Toxicol.*, 2013, **10**, 34.
- Y. Jie, H. Houjin, M. Xun, L. Kebin, Y. Xuesong and X. Jie, *Braz. J. Med. Biol. Res.*, 2014, **47**, 982–989.
- F.-F. Wang, C.-M. Geng, W.-D. Hao, Y.-D. Zhao, Q. Li, H.-M. Wang and Y. Qian, *Biomed. Environ. Sci.*, 2016, **29**, 107–116.
- S. Brenner, *Genetics*, 1974, **77**, 71–94.
- I. Antoshechkin and P. W. Sternberg, *Nat. Rev. Genet.*, 2007, **8**, 518–532.
- M. C. Leung, P. L. Williams, A. Benedetto, C. Au, K. J. Helmcke, M. Aschner and J. N. Meyer, *Toxicol. Sci.*, 2008, **106**, 5–28.
- Y.-L. Zhao, Q.-L. Wu, Y.-P. Li and D.-Y. Wang, *RSC Adv.*, 2013, **3**, 5741–5757.
- D.-Y. Wang, *Toxicol. Res.*, 2016, **5**, 1003–1011.
- Y.-L. Zhao, Z.-Q. Lin, R.-H. Jia, G.-J. Li, Z.-G. Xi and D.-Y. Wang, *J. Hazard. Mater.*, 2014, **274**, 106–114.
- L.-M. Sun, Z.-Q. Lin, K. Liao, Z.-G. Xi and D.-Y. Wang, *Sci. Total Environ.*, 2015, **512–513**, 251–260.
- R.-L. Yang, Y.-L. Zhao, X.-M. Yu, Z.-Q. Lin, Z.-G. Xi, Q. Rui and D.-Y. Wang, *Toxicol. Res.*, 2015, **4**, 333–343.
- L.-M. Sun, Q.-L. Wu, K. Liao, P.-H. Yu, Q.-H. Cui, Q. Rui and D.-Y. Wang, *Chemosphere*, 2016, **144**, 2392–2400.
- R.-L. Yang, Q. Rui, L. Kong, N. Zhang, Y. Li, X.-Y. Wang, J. Tao, P.-Y. Tian, Y. Ma, J.-R. Wei, G.-J. Li and D.-Y. Wang, *Toxicol. Res.*, 2016, **5**, 1097–1105.
- L. P. Lim, N. C. Lau, E. G. Weinstein, A. Abdelhakim, S. Yekta, M. W. Rhoades, C. B. Burge and D. P. Bartel, *Genes Dev.*, 2003, **17**, 991–1008.
- D. P. Bartel, *Cell*, 2004, **116**, 281–297.
- Y.-L. Zhao, Q.-L. Wu and D.-Y. Wang, *RSC Adv.*, 2015, **5**, 92394–92405.
- Y.-L. Zhao, Q.-L. Wu and D.-Y. Wang, *Biomaterials*, 2016, **79**, 15–24.
- Y.-L. Zhao, R.-H. Jia, Y. Qiao and D.-Y. Wang, *Nanomedicine*, 2016, **12**, 735–744.
- Y.-L. Zhao, J.-N. Yang and D.-Y. Wang, *Sci. Rep.*, 2016, **6**, 23234.
- Z.-H. Zhuang, M. Li, H. Liu, L.-B. Luo, W.-D. Gu, Q.-L. Wu and D.-Y. Wang, *Sci. Rep.*, 2016, **6**, 32409.
- S. Donkin and P. L. Williams, *Environ. Toxicol. Chem.*, 1995, **14**, 2139–2147.
- Y. Qiao, Y.-L. Zhao, Q.-L. Wu, L.-M. Sun, Q.-L. Ruan, Y.-Y. Chen, M. Wang, J.-A. Duan and D.-Y. Wang, *PLoS One*, 2014, **9**, e91825.
- C.-J. Shu, X.-M. Yu, Q.-L. Wu, Z.-H. Zhuang, W.-M. Zhang and D.-Y. Wang, *RSC Adv.*, 2015, **5**, 8942–8951.
- Z.-H. Zhuang, Y.-L. Zhao, Q.-L. Wu, M. Li, H.-C. Liu, L.-M. Sun, W. Gao and D.-Y. Wang, *PLoS One*, 2014, **9**, e85482.
- Y.-P. Li, Y.-X. Li, Q.-L. Wu, H.-Y. Ye, L.-M. Sun, B.-P. Ye and D.-Y. Wang, *PLoS One*, 2013, **8**, e71180.
- Y.-L. Zhao, Q. Liu, S. Shakoor, J.-R. Gong and D.-Y. Wang, *Toxicol. Res.*, 2015, **4**, 270–280.
- Z.-F. Liu, X.-F. Zhou, Q.-L. Wu, Y.-L. Zhao and D.-Y. Wang, *RSC Adv.*, 2015, **5**, 94257–94266.
- Q.-L. Wu, Y.-L. Zhao, G. Zhao and D.-Y. Wang, *Nanomedicine*, 2014, **10**, 1401–1410.
- E. A. Miska, E. Alvarez-Saavedra, A. L. Abbott, N. C. Lau, A. B. Hellman, S. M. McGonagle, D. P. Bartel, V. R. Ambros and H. R. Horvitz, *PLoS Genet.*, 2007, **3**, e215.
- S. Lall, D. Grün, A. Krek, K. Chen, Y.-L. Wang, C. N. Dewey, P. Sood, T. Colombo, N. Bray, P. Macmenamin, H.-L. Kao, K. C. Gunsalus, L. Pachter, F. Piano and N. Rajewsky, *Curr. Biol.*, 2006, **16**, 460–471.
- D. P. Bartel, *Cell*, 2009, **136**, 215–233.
- S. Wolff, H. Ma, D. Burch, G. A. Maciel, T. Hunter and A. Dillin, *Cell*, 2006, **124**, 1039–1053.
- C. Ibanez-Ventoso, M. Vora and M. Driscoll, *PLoS One*, 2008, **3**, e2818.