Electronic Supplementary Material (ESI) for Toxicology Research.

SUPPLEMENTAL MATERIAL

Synergistic Effects of Particulate Matter ($PM_{2.5}$) and Sulfur Dioxide (SO_2) on Neurodegeneration via the MicroRNA-Mediated Regulation of Tau Phosphorylation

*Tingting Ku[1], Minjun Chen[1], Ben Li , Yang Yun, Guangke Li, Nan Sang**

College of Environment and Resource, Research Center of Environment and Health, Shanxi University, Taiyuan, Shanxi, PR China 030006

[1] These authors contributed equally to this work

* Corresponding author. Tel.: +86-351-7011932

Fax: +86-351-7011932

E-mail: sangnan@sxu.edu.cn

Mailing address: Nan Sang

College of Environment and Resource,

Shanxi University, Taiyuan, Shanxi 030006

People's Republic of China

TABLES

Table S1. Analysis of individual chemical-concentration responses

	Nonlinear regression Model [A]	Formula [B]	NOEC [C]
$PM_{2.5}$(μg/mL)	Langmuir	$y=1/(a + b*x^{c-1})$	10
SO_2 derivatives(μg/mL)	Langmuir	$y=1/(a + b*x^{c-1})$	1

[A] Nonlinear regression functions employed in this study according to the “best fit” approach.

[B] y is the mean effect; a, b and c are model parameters; and x is the concentration of the test chemical.

[C] The largest tested concentration that produced effects not significantly different from those of untreated controls. Determined using the Dunnett test.

Table S2. Predicted potential targets for miR-337-5p using four bioinformatics software namely Targetscan, RNA22, RNAhybird and miRMap

Accession No.	Gene ID	Gene
NM_030595	26422	Nbea
NM_028017	108123	Napg
NM_020007	56758	Mbnl1
NM_026521	68036	Zfp706
NM_026367	67769	Gpatch2
NM_025965	107513	Ssr1
NM_172785	237256	Zc3h12d
NM_008862	18767	Pkia
NM_054064	114872	Psg29
NM_007900	13605	Ect2
NM_177798	327826	Frs2
NM_173447	270190	Ephb1
NM_182993	72961	Slc17a7
NM_007611	12369	Casp7
NM_177814	238988	Erc2
NM_011753	22688	Zfp26
NM_030266	269180	Inpp4a
NM_145516	226971	Plekhb2
NM_001038999	11980	Atp8a1

NM_001177881	71306	Mfap3l
NM_028185	72290	Lsm11
NM_008095	14467	Gbas
NM_198300	208922	Cpeb3
NM_028815	74201	Cep97
NM_133781	12283	Cab39
NM_001043354	225998	Rorb
NM_010181	14119	Fbn2
NM_010566	16331	Inpp5d
NM_001081286	14107	Fat1
NM_010560	16195	Il6st
NM_001033475	382620	Tmed8
NM_016961	26420	Mapk9
NM_008860	18762	Prkcz
NM_001008542	17859	Mxi1
NM_028859	74297	1700106J16Rik
NM_178446	245945	Rbm47
NM_194061	330286	D630045J12Rik
NM_023794	104156	Etv5
NM_001163672	26420	Mapk9
NM_001110218	319468	Ppm1h
NM_001033385	544696	Tbc1d32

NM_027652	28042	Ept1
NM_145221	72993	Appl1
NM_172992	68770	Phtf2
NM_021506	59009	Sh3rf1
NM_001081420	230234	BC026590
NM_172468	209131	Snx30
NM_013464	11622	Ahr
NM_009974	13000	Csnk2a2
NM_028712	74012	Rap2b
NM_030132	78581	Utp23
NM_029561	76273	Ndfip2
NM_173390	215819	Nhsl1
NM_001013777	382867	Zfp488
NM_177388	338365	Slc41a2
NM_025943	66573	Dzip1
NM_177351	235386	Hykk
NM_018814	54604	Pcnx
NM_015767	50500	Ttpa
NM_183216	329065	Scd4
NM_008102	14528	Gch1
NM_172632	225724	Mapk4
NM_026331	67712	Slc25a37

NM_028234	381626	Rbm33
NM_029658	76566	Fam101b
NM_016721	29875	Iqgap1
NM_001040426	207596	Thsd4
NM_023053	65960	Twsg1
NM_001081309	75669	Pik3r4
NM_023764	54473	Tollip
NM_172964	268970	Arhgap28
NM_009793	12326	Camk4
NM_028722	74026	Msl1
NM_009616	11492	Adam19
NM_175312	101148	B630005N14Rik
NM_145505	226252	Fam160b1
NM_031494	27081	Zfp275
NM_201226	72946	Lrrc47
NM_011865	23983	Pcbp1
NM_001199084	232341	Wnk1
NM_133352	107358	Tm9sf3
NM_008740	18195	Nsf
NM_181750	226412	R3hdm1
NM_024203	67544	Fam120b
NM_146156	230809	Pdik1l

NM_011642	22062	Trp73
NM_148925	17281	Fyco1
NM_197990	69399	1700025G04Rik
NM_001033210	102502	Pls1
NM_001163006	433771	Minos1
NM_001038609	17762	Mapt
NM_001039080	56516	Rbms2
NM_008636	17764	Mtf1
NM_007581	12297	Cacnb3
NM_001159965	78255	Ralgps2
NM_001205339	107272	Psat1
NM_183138	194388	Tet3
NM_133906	74570	Zkscan1
NM_001195031	94212	Pag1
NM_001134457	385658	Nxpe3
NM_001163793	320827	C530008M17Rik
NM_001167578	1E+08	Tcp10c
NM_028943	74442	Sgms2
NM_001162977	230971	Megf6
NM_029528	76156	Fam131b
NM_001171147	22601	Yap1
NM_178716	238799	Tnpo1

NM_013742	27267	Cars
NM_001172123	207181	Rbms3
NM_026696	68364	0610030E20Rik
NM_001024837	110532	Adarb1
NM_001037758	12234	Btrc
NM_001044386	22764	Zfx

TEXT

Animals and exposure

Healthy male C57BL/6 mice were housed in four stainless steel cages and maintained under standard conditions (24 ± 2°C, 50 ± 5% relative humidity) with a 12-hour light-dark cycle. The animals were divided into control group, SO_2 group (0.5 mg/m^3), $PM_{2.5}$ group (0.075 mg/m^3, approximately 1 mg/kg), and SO_2 (0.5 mg/m^3) & $PM_{2.5}$ (0.075 mg/m^3) group, each of which contained six mice. SO_2 dynamic inhalation exposures and $PM_{2.5}$ intranasal instillations were performed according to the following methods. For inhalation exposure, the mice were dynamically exposed to 0.5 mg/m^3 SO_2 in a inhalation chamber for 6 h/day for 28 days. The SO_2 gas was diluted with air at the intake port of exposure chamber to obtain the desired concentration, and the mixed gas was uniformly distributed among the whole chamber by two perforated gas dispersion plates, with one being placed on the intake port and the other one on gas outlet connected to an aspirator pump. The SO_2 concentration in the exposure chamber was measured using a real-time SO_2 monitor (Wandi, China), and exhausted gas was absorbed by an alkali absorption device. For instillation exposure, the mice were exposed with $PM_{2.5}$ at 1 mg/kg every other day for 28 days. Correspondingly, vehicle control animals were continually exposed to filtered air using the same protocol in the other exposure chamber. During the exposure period, the animals, together with their cages, were placed in the exposure chamber and the mice were as free as they are under normal circumstances, except for access to water and food. After the treatments, the mice were sacrificed 18 h after their last exposure. Their cortices were removed, quickly frozen in liquid nitrogen and stored at -80°C until further analysis.

Real-time quantitative reverse transcription-PCR analysis

Briefly, total RNA was isolated, quantified and synthesized to complementary DNA (cDNA) using TRIzol Reagent (Invitrogen, USA) and a reverse transcription kit (Qiagen Biotechnology, Germany) according to the manufacturer's instructions. The cDNA product was stored at -20 °C until use. The forward primer sequence for mmu-mir-337-5p was 5'-CGATATGCAGGAGTTGATT-3' and the internal control mmu-mir-202 miRNA was 5'-TTGAACCCTTTTCCATCTGA-3'. The two miRNAs share the same reverse primer, and they were all obtained with a miScript SYBR Green PCR kit (Qiagen Biotechnology Germany). Each 20 μl PCR reaction contained 2 μl of cDNA (a 6-fold dilution of the original cDNA product), 10 μL of QuantiTect SYBR Green, 7 μL of RNase-free H_2O, and 0.5 μL of each primer. The real-time PCR was run according to a three-step method. The reaction conditions were 95°C for 3 min and 40 cycles of 95°C for 20 s, 55°C for 20 s and 72°C for 20 s in a qTOWER 2.2 Real-Time PCR (Analytik Jena AG, Jena, Germany). Each cDNA sample was run in triplicate, and the relative quantification of the gene expression was determined by using mmu-mir-202 as an internal control.

Luciferase reporter assay

Two psiCHECK-2 vectors (Promega) were constructed to carry a Mapt 3′UTR DNA fragment containing the indicated miR-337-5p binding site with the following primers: 5'-TCAGGCTCCCAGGGCAGTCAATAA-3' (forward) and 5'-TATTCA GAGT AATAACTTTATTTCC-3' (reverse). In addition, two matched mutants were constructed to disrupt the putative miR-337-5p binding sites in the 3'-UTR of MAPT by using KOD Plus neo DNA polymerase (ToYoBo). The first primers: 5'-

GGGCAAAGGGAGGTCTTGGAGTGTACGGCAATAGCCATCTTGTAACCCCATTCATGA-3' (forward) and 5'-TCATGAATGGGGTTACAAGATGGCTATTGCCGTACACTCCAAGACCTCCCT TTGCCC-3' (reverse) the second primers: 5'-ACCCTCCTGATGAACTGTAGCCTG TCGGCAATCTGTGCTGGGCATGATCTCCAGTGC-3' (forward) and 5'-GCACTGGAGATCATGCCCAGCACAGATTGCCGACAGGCTACAGTTCATCAGGAGGGT-3' (reverse). Both the wild-type and mutated plasid were cloned into the site of the psiCHECK-2 vector downstream of the luciferase gene with PCR. HEK293T cells were plated into 24-well plates (2×10^4/well) in DMEM with high glucose containing 10% FBS at 24 h before transfection. Either the wild-type or mutant 3'-UTR vector (0.5μg) and either 50 nM of the miR-337-5p mimic or 100 nM of the miR-337-5p inhibitor were transfected into the cells with Lipofectamine 2000 (Invitrogen). Another 48 h later, the cells were collected and suspended in Passive Lysis Buffer (Promega), and their luciferase activities were monitored with the reagents in the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). All experiments were performed in triplicate.