

Cite this: *Nanoscale*, 2019, **11**, 22907

Microarray-assisted size-effect study of amorphous silica nanoparticles on human bronchial epithelial cells†

Yang Li, ^{‡a,b} Junchao Duan,^{‡a,b} Xiangyuan Chai,^c Man Yang,^{a,b} Ji Wang,^{a,b} Rui Chen^{*a,b} and Zhiwei Sun^{*a,b}

Amorphous silica nanoparticles (SiNPs) are not only abundant in nature, but also the second largest engineering nanomaterials in terms of annual output. Respiratory exposure is the main route for SiNPs to enter the human body. A large number of studies have focused on the respiratory toxicity of SiNPs and demonstrated that SiNPs could induce pulmonary tissue damage, inflammation, fibrosis, and even the malignant transformation of bronchial epithelial cells, while the size-dependent toxicity of SiNPs and their underlying biological mechanisms remain unclear. In this regard, a transcriptomics study would be conducive to gaining a better understanding of the toxic mechanism. In the present study, microarray analysis was performed to investigate the genome-wide transcriptional alteration induced by different sizes of SiNPs in human primary bronchial epithelial cells (BEAS-2B). To determine the effect of the particle size on the toxicity, nanoparticles of two sizes (41 nm and 61 nm) and submicron particles of one size (206 nm) were introduced. The bioinformatics analysis results indicated that: (1) the number of differentially expressed genes in the three SiNP-treated groups increased with the particle size decreasing; (2) the genes involved in the immune and inflammatory response, gene expression, signal transduction, endoplasmic reticulum stress, oxidative stress, cell metabolism, and cell proliferation were gradually upregulated with the particle size decreasing, while the genes related to the morphological development of the respiratory system were gradually downregulated with the particle size decreasing; (3) the modes of action of the two nanoparticles overlapped with each other to some degree, and there existed many different modes compared to those from the submicron particles; (4) both the silica nanoparticles affected the pathways associated with the cell entry of silica nanoparticles, autophagy and lysosomal dysfunction, endoplasmic reticulum stress, inflammatory response, DNA damage, and gene expression, as well as apoptotic resistance and cancer. To the best of our knowledge, this is the first study that has reported the alteration trend of gene expression profiles with the change in silica particle size. Our study provides a great deal of information on the toxic mechanisms underlying the respiratory toxicity induced by SiNPs, and can also serve as an experimental basis for the toxicity and safety evaluation of silica nanoparticles.

Received 26th August 2019,
Accepted 10th November 2019

DOI: 10.1039/c9nr07350g

rsc.li/nanoscale

1. Introduction

With the development of nanotechnology, engineering nanomaterials have attracted increasing attention due to their favorable physical and chemical properties. SiNPs, as one of the key nanotechnological products, have been used in a variety of

commercial products, such as cosmetics, toothpaste, food processing and production, electronic devices, paints, and plastics.¹ Additionally, owing to their good biocompatibility, high stability, and easy surface modification, SiNPs have shown potential for nanomedicine applications as biosensors, biological imaging agents, drug delivery carriers, *etc.*² It was reported that SiNPs were among the top three engineering nanomaterials referenced in nanotech-based consumer products,³ and their annual global production has exceeded 1.5 million tons.⁴ However, their mass production and wide application means a high frequency of exposure to the occupational population as well as the general population, so more attention should be paid to the adverse health effects of amorphous SiNPs. The Organization for Economic Cooperation and

^aSchool of Public Health, Beijing, 100069, China^bBeijing Key Laboratory of Environmental Toxicology, Capital Medical University, Beijing, 100069, China. E-mail: zwsun@ccmu.edu.cn, ruichen@ccmu.edu.cn^cXuanwu Hospital, Capital Medical University, Beijing, 100053, China

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c9nr07350g

‡Authors contributed equally.

Development (OECD) and the National Institute of Environmental Health Sciences (NIEHS) have already included amorphous SiNPs in the priority list of nanomaterials requiring urgent toxicological evaluation.⁵

The general population is mainly exposed to SiNPs through inhalation, ingestion, dermal contact, or injection, while the occupational population is mainly exposed through inhalation and dermal contact. Compared with the general population exposure, occupational exposure has the characteristics of a high exposure dose, high exposure frequency, and long exposure duration. Besides, respiratory exposure is the main route for SiNPs to enter the human body. Thus, a growing number of studies have focused on the respiratory toxic effect of SiNPs. Several *in vivo* studies have shown that the inhalation of SiNPs could cause the apoptosis and necrosis of bronchiolar epithelial cells and alveolar epithelial cells, and could induce pulmonary tissue damage, inflammation, and fibrosis.^{6–8} In recent years, researchers have also explored related mechanisms concerning the respiratory toxicity of SiNPs using cell lines *in vitro*. Lin *et al.* exposed human lung epithelial cells (A549) to SiNPs and observed an abnormal increase of intracellular ROS, leading to oxidative stress and apoptosis of the cells.⁹ The findings of Skuland *et al.* indicated that SiNPs resulted in a pro-inflammatory response in two different human bronchial epithelial cell lines (BEAS-2B and HBEC3-KT), and the combined triggering of NF- κ B and the p38/TACE/TGF- α /EGFR signaling pathway seemed to be important for the release of pro-inflammatory cytokines.^{10,11} Zhao *et al.*⁸ reported an induced autophagic flux blockage in A549 cells after exposure to SiNPs, with the results demonstrating the repressive effect of SiNPs on lysosomal acidification, contributing to decreased autophagic degradation, thus leading to apoptosis. Li *et al.*¹² maintained that the downregulation of the PI3K/Akt/mTOR pathway should be responsible for the SiNPs causing autophagy dysfunction in BEAS-2B cells. In a study by Wu *et al.*,¹³ endoplasmic reticulum (ER) stress was emphasized as an important toxic mechanism of SiNP-induced apoptosis in human pulmonary alveolar epithelial cells (HPAEPiC). In summary, existing studies have demonstrated that SiNP-induced respiratory system injury is mainly derived from oxidative stress, endoplasmic reticulum stress, autophagic dysfunction, and an inflammatory response, while the underlying complex biological mechanisms remain incompletely understood.

A thorough and systematic investigation of the changes of gene expression profiles would be beneficial for better elucidating the potential molecular mechanism of the respiratory toxicity caused by SiNPs. Toxicogenomics has already shown advantages in nanotoxicity assessment and related mechanism research.¹⁴ Thus, genome-wide transcriptional analysis was introduced herein to analyze the adverse effects of SiNPs on BEAS-2B cells in this study. The characterization and size-dependent cytotoxicity of three different sizes of SiNP (SiNP-40, SiNP-60, and SiNP-200) were first determined. Then, microarray analysis and bioinformatics analysis were performed, which included differential gene expression analysis,

significant gene ontology (GO) analysis, series test of cluster of gene ontology (STC-GO) analysis, significant pathway analysis, and signal-net analysis. Finally, quantitative RT-PCR analysis was used to verify the obtained microarray data. Our study unfolded new evidence referring to the adverse effects of SiNPs on BEAS-2B cells, and also provided considerable in-depth information for the further investigation of how SiNPs affect human pulmonary health.

2. Experimental section

2.1. Preparation of amorphous SiNPs

Amorphous SiNPs were prepared using the Stöber method as described in our previous study.¹⁵ The particle size was increased by increasing the amount of the catalyst (NH₄OH) and silica precursor (tetraethyl orthosilicate, TEOS), and by decreasing the amount of water in the reaction mixture regardless of the solvents used for the synthesis.¹²

2.2. Characterization of amorphous SiNPs

The morphology and average size of the SiNPs were observed using transmission electron microscopy (TEM) (JEOL JEM2100, Japan). The purity of the SiNPs was assessed by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Thermo Fisher Scientific ARL 3520, Switzerland). A zeta electric potential granulometer (Malvern Nano-ZS90, UK) was used to determine the hydrodynamic size and zeta potential of the SiNPs in DMEM culture medium (Gibco, USA).

2.3. Cell culture and SiNPs exposure

The human bronchial epithelial cell line BEAS-2B was purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China). The cells were maintained in DMEM culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin, and cultured at 37 °C in a 5% CO₂ humidified environment.

For the experiments, the cells were seeded in culture plates at a density of 1×10^5 cells per mL and allowed to attach for 24 h, and then treated with SiNPs suspended in DMEM culture medium of 25 μ g mL⁻¹ for another 24 h. The suspension of SiNPs was dispersed by a sonicator (160 W, 20 kHz, 5 min) (Bioruptor UDC-200, Belgium), and then added to BEAS-2B cells immediately.

2.4. Cell viability analysis

The effect of the different sizes of SiNPs on cell viability was detected by a WST-8 cell counting kit (CKK-8) according to the manufacturer's instructions. BEAS-2B cells were exposed to 25 μ g mL⁻¹ of SiNPs for 24 h. Then, 10 μ L CKK-8 solution was added into each well, and the cells were incubated for an additional 1 h at 37 °C. The optical density at 450 nm was detected by a microplate reader (Thermo Multiscan MK3, USA).

2.5. Total RNA extraction and microarray analysis

Gene expression was analyzed using BEAS-2B cells exposed to $25 \mu\text{g mL}^{-1}$ of each SiNPs through microarray analysis, with unexposed cells used as the normal control. Each group had three replicate samples tested, and for each sample, the experiment was performed in triplicate as technical replicates. For Affymetrix microarray profiling, the total RNA was isolated from 1×10^6 cells from the control and SiNP-treated groups using TRIzol reagent (Invitrogen, Carlsbad, Canada) and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The amount and quality of RNA were determined using a UV-Vis spectrophotometer (Thermo, NanoDrop 2000, USA) at an absorbance of 260 nm. The mRNA expression profile was measured using Human Transcriptome Array 2.0 (Affymetrix GeneChip, USA), which contains 44 699 gene-level probe sets. The microarray analysis was performed using Affymetrix Expression Console Software (version 1.2.1). Raw data (CEL files) were normalized at the transcript level using the robust multi-array average method (RMA workflow). The median summarization of transcript expressions was calculated. The gene-level data were then filtered to include only those probe sets that were in the 'core' metaprobe list, which represents RefSeq genes. Data from the microarray analysis (ECL files) discussed in this article were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository and are available through the accession number GSE127962 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127962>).

2.6. Bioinformatics analysis

2.6.1. Differentially expressed mRNA analysis. For the microarray data analysis, differentially expressed genes were identified based on the random variance model (RVM) *t*-test. Also, the differentially expressed genes were considered to be up- or downregulated with at least $p < 0.05$. Genes with similar expression patterns often facilitate overlapping functions. Accordingly, cluster analysis of the gene expression patterns was performed using Cluster and Java Treeview software.

2.6.2. Gene ontology and pathway analysis. Gene ontology (GO) analysis was applied to analyze the main function of the differentially expressed genes according to the GO, as the key functional classification of the National Center for Biotechnology Information (NCBI), which can organize genes into hierarchical categories and can uncover the gene regulatory network on the basis of biological processes and molecular function. Specifically, a two-sided Fisher's exact test and chi-square test were used to classify the GO category, and the false discovery rate (FDR) was calculated to correct the *P*-value; the smaller the FDR, the smaller the error in judging the *P*-value.

Pathway analysis was used to determine the significant pathways of the differential genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and Reatome databases. Fisher's exact test was performed to select the statistically significant pathway, and the threshold of significance was considered as $p < 0.05$.

2.6.3. Series test of cluster of gene ontology (STC-GO) analysis. The series test of cluster (STC) algorithm of gene expression dynamics was used to profile the gene expression series and to identify the most probable set of clusters generated by the effect of the SiNP size. This method was used as it could explicitly take into account the dynamic nature of gene expression profiles during clustering and as it allowed identifying the number of distinct clusters. Differential expression genes were selected in a logical sequence according to RVM (Random variance model) corrective ANOVA. Significant profiles had a higher probability than expected in the Fisher's exact test and multiple comparison test, and the threshold of significance was considered as $p < 0.05$.

2.6.4. Gene signal transduction networks. Gene signal transduction networks (Signal-net), based on the KEGG database on the interactions between different gene products and the theory of network biology, were established to illustrate the intergene signaling between differentially expressed genes. The networks were stored and presented as graphs, with the degree defined as the link number of one node with all of the other nodes. Genes with higher degrees occupied more important positions within the network. In addition, the properties of genes were described by Betweenness Centrality (BC) measures, reflecting the intermediary capacity of a node to modulate other interactions between nodes. Finally, the purpose of the signal transduction network analysis was to locate the core key regulatory genes that had a stronger capacity to modulate adjacent genes.

2.7. Quantitative RT-PCR analysis

Total RNA was extracted from 1×10^6 cells from the control and SiNP-treated groups using TRIzol reagent (Invitrogen, Carlsbad, Canada) according to the manufacturer's protocol. Equal amounts of total RNA from each sample were reverse transcribed using Thermoscript reverse transcriptase (Invitrogen) using oligo (dT) and random hexamer primers. The qRT-PCR reaction was monitored using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA) and was run with three biological repeats and three technical repeats. All the levels were normalized to β -actin (18S levels were similar) and fold induction was calculated by setting the control conditions to 1.

2.8. Statistical analysis

Statistics for the microarray and bioinformatic analysis were performed by one-way analysis of variance (ANOVA) using Affymetrix® Expression Console™ TAC (Affymetrix® Expression Console™), followed by the least significant difference (LSD) test. Statistics for cell viability and qRT-PCR were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Data are expressed herein as the mean $\pm$ S.D. One-way analysis of variance (ANOVA) was used for the comparison of multiple groups. Pairwise comparison in multiple groups was conducted with the least significant difference (LSD) test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Characterization of SiNPs

The morphology, average size, hydrodynamic diameter, zeta potential, and purity were detected to characterize the amorphous SiNPs with different sizes. As shown in Fig. 1A–C, the three amorphous SiNPs, namely SiNP-40, SiNP-60, and SiNP-200, were spherical or ellipsoidal, and the average sizes were 41.26 ± 3.78 , 61.51 ± 7.93 , and 206.31 ± 18.70 nm, respectively. The hydrodynamic sizes and zeta potentials were measured in DMEM culture medium at 5 min and 24 h to reflect the dispersion of SiNPs throughout the experiments. As shown in Table 1, all three SiNPs possessed larger hydrodynamic diameters in the dispersion medium than their original size, which could result from the van der Waals force and hydrophobic interaction between the SiNPs and the surrounding medium. The polydispersity indexes (PDI) of all three SiNPs were about 0.1, which showed that these particles could maintain relatively good dispersity in DMEM cell culture medium. The zeta potential results manifested that the three SiNPs possessed highly negative charge, indicating a fairly

stable suspension in DMEM culture medium. The purity of the three SiNPs was higher than 99.9% as detected by ICP-AES.

3.2. Size-dependent cytotoxicity of SiNPs

Cell viability was previously examined after BEAS-2B cells were exposed to the three sizes of SiNPs at various concentrations (6.25 , 12.5 , 25 , 50 , and $100 \mu\text{g mL}^{-1}$) for 6, 12, and 24 h in our earlier study.¹² In order to compare the effect of the particle size on the toxicity in this microarray analysis, BEAS-2B cells were exposed to $25 \mu\text{g mL}^{-1}$ of the three SiNPs for 24 h in the following experiments. As shown in Fig. 1D, all three SiNPs inhibited cell proliferation significantly compared with the control. The cell viability of the SiNP-40-, SiNP-60-, and SiNP-200-treated groups was 93.57%, 82.29%, and 68.15%, respectively, which indicated that the smaller SiNPs exhibited a higher toxic effect.

3.3. Differential gene expression induced by SiNPs

Genome-wide transcriptional analysis was performed to investigate the possible gene expression changes in BEAS-2B cells induced by the SiNPs with different sizes using the Affymetrix

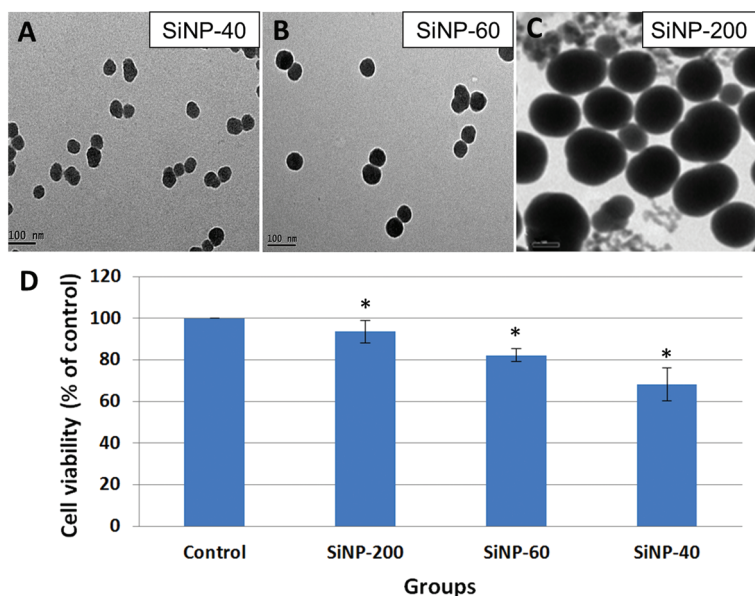


Fig. 1 Characterization and cytotoxicity of SiNPs with different sizes. TEM images showing SiNP-40 (a), SiNP-60 (b), and SiNP-200 (c) were spherical or ellipsoidal, with the original diameter of 41.26, 61.51, and 206.31 nm, respectively. (d) Cell viability was measured after BEAS-2b cells exposure to $25 \mu\text{g mL}^{-1}$ of SiNPs with different sizes for 24 h. Data are expressed as the mean $\pm$ S.D. from three independent experiments. * $p < 0.05$ compared with the control group using ANOVA.

Table 1 Hydrodynamic size and zeta potential of SiNPs in the DMEM culture medium

SiNPs	Hydrodynamic size (nm)		PDI		Zeta potential (mV)	
	5 min	24 h	5 min	24 h	5 min	24 h
SiNP-40	98.37 ± 4.51	99.03 ± 2.18	0.136 ± 0.008	0.124 ± 0.010	-35.05 ± 1.16	-34.31 ± 0.34
SiNP-60	110.17 ± 3.13	109.50 ± 4.73	0.112 ± 0.010	0.108 ± 0.013	-32.82 ± 0.59	-32.82 ± 0.59
SiNP-200	202.70 ± 1.25	206.87 ± 2.19	0.095 ± 0.018	0.086 ± 0.026	-36.63 ± 0.31	-37.48 ± 1.12

GeneChip (Human Transcriptome Array 2.0), which contains 44 699 gene-level probe sets. The results manifested that, compared with the control group, 1330 (933 upregulated genes and 397 downregulated genes), 603 (375 upregulated genes and 228 downregulated genes), and 341 (163 upregulated genes and 176 downregulated genes) significantly differentially expressed genes were triggered by SiNP-40, SiNP-60, and SiNP-200, respectively. Treatment-independent clustering histograms based on the differentially expressed genes revealed the changes of gene expression patterns between the SiNP-treated groups and the control (Fig. 2A–C). Putting all the differentially expressed genes in the three treatment groups together, a clear particle size-dependent change of gene

expression pattern could be seen, as shown in Fig. 2D. The top 10 differentially expressed upregulated and downregulated genes in the three SiNP-treated groups are listed in Tables 2 and 3, respectively.

3.4. GO analysis of the differential gene expression induced by SiNPs

GO analysis was introduced to find the main gene functions affected by SiNP-40, SiNP-60, and SiNP-200. The top 20 significantly changed up- and downregulated GOs of the differentially expressed genes are listed in Table 4. As shown, in the top 40 significantly changed GOs, there were 15 same significantly changed GOs between the SiNP-40- and SiNP-60-treated

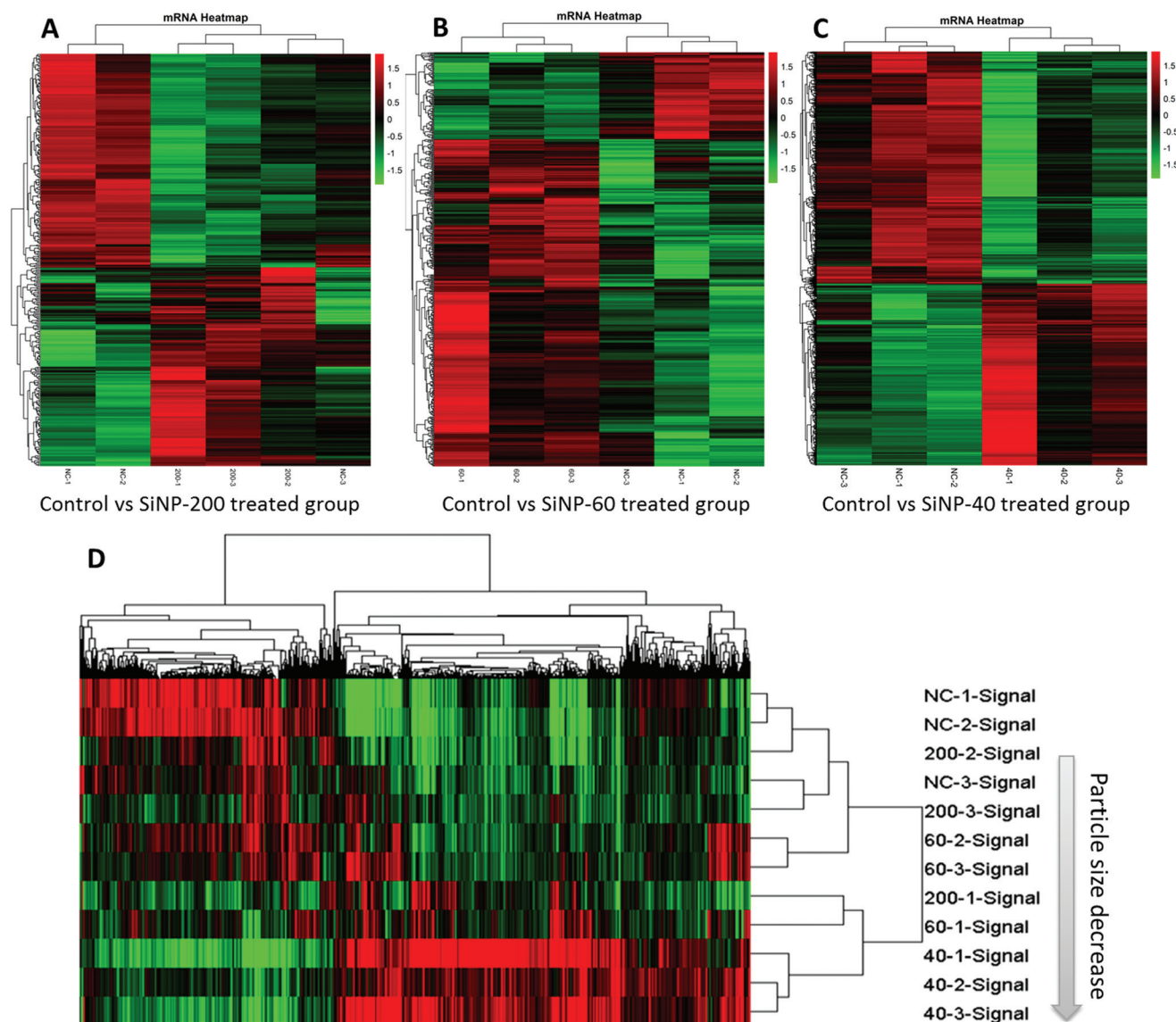


Fig. 2 Hierarchical cluster analysis of the differentially expressed genes triggered by SiNP-40 (a), SiNP-60 (b), and SiNP-200 (c) compared with the control group. (d) Hierarchical cluster analysis of all the differentially expressed genes in the three SiNPs-treated groups. The analysis result manifested that the gene expression pattern of BEAS-2b cells changed in a particle size-dependent manner. *P*-Values < 0.05. The relative gene log₂ expression changes were expressed by a color gradient intensity scale. Red color indicates upregulation, and green color indicates downregulation of mRNAs expression. Each row represents a single mRNA and each column represents a separate sample.

Table 2 Top 10 differentially expressed upregulated genes between the SiNP-treated groups and the control in BEAS-2B cells

SiNPs	Gene name	Geom mean of intensities		Fold-change	<i>p</i> -Value	Related significant GO
		SiNP-treated group	Control group			
SiNP-40	MMP1	60.71	4.34	13.99	0.0000031	Proteolysis; blood coagulation; virus–host interaction; extracellular matrix disassembly; extracellular matrix organization
	NARS	120.61	27.48	4.39	0.0142228	tRNA aminoacylation for protein translation; gene expression; translation; tRNA aminoacylation for protein translation; gene expression
	DNAJA1	159.24	37.18	4.28	0.0115635	Protein folding; response to unfolded protein; spermatogenesis; response to heat; androgen receptor signaling pathway; DNA damage response, detection of DNA damage
	CSDE1	224.14	53.75	4.17	0.0194631	Regulation of transcription, DNA-dependent; male gonad development; nuclear-transcribed mRNA catabolic process, no-go decay; regulation of transcription, DNA-dependent
	RAB31	243.5	61.81	3.94	0.0232852	GTP catabolic process; small GTPase mediated signal transduction; receptor internalization; cellular response to insulin stimulus; Golgi to plasma membrane protein transport
	SDCBP	146.77	38.43	3.82	0.013641	Protein targeting to membrane; substrate-dependent cell migration, cell extension; Ras protein signal transduction; synaptic transmission; actin cytoskeleton organization
	IL8	195.12	51.36	3.8	0.0010846	Angiogenesis; response to molecules of bacterial origin; cellular component movement; inflammatory response; immune response; activation of signaling protein activity involved in unfolded protein response
	HSP90AA1	142.15	37.53	3.79	0.0135181	G2/M transition of mitotic cell cycle; mitotic cell cycle; neuron migration; skeletal muscle contraction; ATP catabolic process; mitochondrial transport
	DDX5	256.75	68.91	3.73	0.0209044	Negative regulation of transcription from RNA polymerase II promoter; regulation of alternative mRNA splicing, <i>via</i> spliceosome; <i>in utero</i> embryonic development; transcription, DNA-dependent; cell growth
	HSP90B1	205.1	55.76	3.68	0.0086771	response to hypoxia; toll-like receptor signaling pathway; protein folding; activation of signaling protein activity involved in unfolded protein response; protein transport
SiNP-60	HSP90AB1	517.6	195.32	2.65	0.0366057	placenta development; protein folding; response to unfolded protein; positive regulation of protein binding
	PSMC2	130.26	51.2	2.54	0.0450757	G1/S transition of mitotic cell cycle; protein polyubiquitination; mitotic cell cycle
	DNAJA1	91.46	37.34	2.45	0.0443343	Protein folding; response to unfolded protein; spermatogenesis; response to heat; androgen receptor signaling pathway; DNA damage response, detection of DNA damage
	HSP90AA1	89.45	37.68	2.37	0.0025324	G2/M transition of mitotic cell cycle; mitotic cell cycle; neuron migration; skeletal muscle contraction; ATP catabolic process; mitochondrial transport
	CSDE1	127.44	53.98	2.36	0.0251007	Regulation of transcription, DNA-dependent; male gonad development; nuclear-transcribed mRNA catabolic process, no-go decay; regulation of transcription, DNA-dependent
	NARS	64.03	27.6	2.32	0.0338064	tRNA aminoacylation for protein translation; gene expression; translation; tRNA aminoacylation for protein translation; gene expression
	HSP90B1	126.97	55.99	2.27	0.0089799	Response to hypoxia; toll-like receptor signaling pathway; protein folding; activation of signaling protein activity involved in unfolded protein response; protein transport
	HSPA8	737.73	325.49	2.27	0.0417112	Transcription, DNA-dependent; mRNA processing; protein folding; post-Golgi vesicle-mediated transport; response to unfolded protein
	SDCBP	83.93	38.58	2.18	0.0409171	Protein targeting to membrane; substrate-dependent cell migration, cell extension; Ras protein signal transduction; synaptic transmission; actin cytoskeleton organization
	HSPA9	159.48	75.83	2.1	0.0448056	Protein folding; protein export from nucleus; protein targeting to mitochondrion; negative regulation of apoptotic process
SiNP-200	HSP90AB1	500.97	194.51	2.58	0.04974	Placenta development; protein folding; response to unfolded protein; positive regulation of protein binding
	SUPT16H	55.98	22.17	2.52	0.03891	DNA replication; DNA repair; nucleosome disassembly; transcription from RNA polymerase II promoter
	DHX9	49.06	20.3	2.42	0.02553	mRNA splicing, <i>via</i> spliceosome; circadian rhythm; RNA splicing; gene expression; cellular response to heat
	HSP90B1	123.1	55.76	2.21	0.019863	Placenta development; protein folding; response to unfolded protein; positive regulation of protein binding
	IQGAP1	92.36	43.2	2.14	0.044886	Regulation of cytokine production; energy reserve metabolic process; signal transduction; small GTPase mediated signal transduction; negative regulation of dephosphorylation
	CKAP5	40.44	19.06	2.12	0.029282	G2/M transition of mitotic cell cycle; mitotic cell cycle; spindle organization; mitosis; establishment or maintenance of microtubule cytoskeleton polarity
	XRCC5	107.45	51.67	2.08	0.039441	Telomere maintenance; DNA repair double-strand break repair; double-strand break repair <i>via</i> nonhomologous end joining; DNA recombination

Table 2 (Contd.)

SiNPs	Gene name	Geom mean of intensities		Fold-change	<i>p</i> -Value	Related significant GO
		SiNP-treated group	Control group			
	FTSJ3	151.79	72.96	2.08	0.044094	RNA methylation; rRNA processing; methylation; ribosome biogenesis; RNA methylation
	PSMD1	31.64	15.51	2.04	0.029586	G1/S transition of mitotic cell cycle; protein polyubiquitination; mitotic cell cycle; antigen processing and presentation of peptide antigen <i>via</i> MHC class I
	SNRNP200	114.11	57.14	2	0.048628	<i>cis</i> assembly of pre-catalytic spliceosome; mRNA splicing, <i>via</i> spliceosome; mRNA splicing, <i>via</i> spliceosome; mRNA splicing, <i>via</i> spliceosome

Table 3 Top 10 differentially expressed downregulated genes between the SiNP-treated groups and the control in BEAS-2B cells

SiNPs	Gene name	Geom mean of intensities		Fold-change	<i>p</i> -Value	Related significant GO
		SiNPs treated group	Control group			
SiNP-40	HIST1H3F	405.84	989.58	0.41	0.0184901	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	HIST1H3A	101.41	225.19	0.45	0.0235292	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	HIST1H2AB	506.71	1113	0.46	0.0160593	Nucleosome assembly
	HIST1H3E	865.86	1681.12	0.52	0.008364	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	HIST1H3J	503.85	963.74	0.52	0.0131037	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	BOK	124.62	230.45	0.54	0.0040555	Apoptotic process; induction of apoptosis; activation of cysteine-type endopeptidase activity involved in apoptotic process
	HIST1H2BI	210.98	392.61	0.54	0.0300564	Nucleosome assembly; defense response to bacterium
	HIST1H4F	7.14	13.03	0.55	0.0164686	Telomere maintenance; chromatin organization; nucleosome assembly; CENP-A containing nucleosome assembly at centromere
	PARD6A	42.02	74.54	0.56	0.0091128	Cell cycle; transforming growth factor beta receptor signaling pathway; viral reproduction; cell junction assembly
	HIST1H4A	73.5	131.44	0.56	0.0237258	Telomere maintenance; chromatin organization; nucleosome assembly; CENP-A containing nucleosome assembly at centromere
SiNP-60	HIST1H3F	580.97	993.67	0.58	0.0433713	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	HIST1H2AB	654.06	1117.6	0.59	0.0293484	Nucleosome assembly
	MVD	111.86	170.65	0.66	0.0257484	Cholesterol biosynthetic process; cholesterol biosynthetic process; cholesterol biosynthetic process; positive regulation of cell proliferation
	HIST1H4B	43.52	59.61	0.73	0.0464964	Telomere maintenance; chromatin organization; nucleosome assembly; CENP-A containing nucleosome assembly at centromere
	IGHV1-45	6.11	8.25	0.74	0.0025443	—
	HIST1H3E	26.4	35.12	0.75	0.0236087	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	HTR1B	19.84	26.13	0.76	0.0261584	G-protein coupled receptor internalization; G-protein coupled receptor signaling pathway; G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger
	HIST1H4I	12.85	16.56	0.78	0.0422065	Telomere maintenance; chromatin organization; nucleosome assembly CENP-A containing nucleosome assembly at centromere
	HIST1H2AL	159.68	200.92	0.79	0.0147292	Nucleosome assembly
	KRTAP12-4	13.08	16.53	0.79	0.0226738	—
SiNP-200	HIST1H3F	563.84	989.58	0.57	0.028461	Nucleosome assembly; blood coagulation; S phase; regulation of gene silencing
	ISG15	67.9	95.34	0.71	0.016123	Cell-cell signaling; virus-host interaction; cytokine-mediated signaling pathway
	ID3	28.18	39.51	0.71	0.016376	Negative regulation of transcription from RNA polymerase II promoter; metanephros development; regulation of DNA replication; transcription, DNA-dependent
	C9orf62	10.37	14.19	0.73	0.001641	—
	CXorf67	13.9	18.94	0.73	0.013549	—
	FZD9	13.27	18.17	0.73	0.044329	Vasculature development; nervous system development; neuroblast proliferation; brain development
	TRBV20OR9-2	9.94	13.4	0.74	0.017915	—
	LSM7	114.92	154.33	0.74	0.047164	Nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay; mRNA splicing, <i>via</i> spliceosome; gene expression
	DUX4L2	45.06	59.43	0.76	0.009888	Transcription, DNA-dependent
	DCAF8L2	4.87	6.39	0.76	0.020117	—

Table 4 Significantly changed up- and downregulation GOs of differentially expressed genes induced by SiNPs in BEAS-2B cells

GOs	SiNP-40	SiNP-60	SiNP-200
Up regulation GOs	Nucleosome assembly	Protein heterotetramerization	Olfactory bulb interneuron differentiation
	Telomere organization	Chromatin silencing	Positive regulation of cell cycle arrest
	Chromatin silencing at rDNA	Telomere organization	Keratinization
	DNA replication-dependent nucleosome assembly	Detection of chemical stimulus involved in sensory perception of smell	Eating behavior
	Protein heterotetramerization	DNA replication-dependent nucleosome assembly	Phospholipase C-activating G-protein coupled receptor signaling pathway
	Negative regulation of gene expression, epigenetic	Chromatin silencing at rDNA	Positive regulation of bone mineralization
	Positive regulation of gene expression, epigenetic	Nucleosome assembly	Negative regulation of axon extension
	Regulation of gene silencing	Keratinocyte differentiation	Negative regulation of cAMP biosynthetic process
	Cellular protein metabolic process	Peptide cross-linking	Neuron development
	Negative regulation of megakaryocyte differentiation	Negative regulation of gene expression, epigenetic	Negative regulation of cell differentiation
	Chromatin silencing	Positive regulation of gene expression, epigenetic	Histamine metabolic process
	Gene silencing by RNA	Cell-cell signaling	Antigen processing and presentation of peptide or polysaccharide antigen <i>via</i> MHC class II
	CENP-A containing nucleosome assembly	G-protein coupled receptor signaling pathway	Neural crest cell migration involved in heart formation
	Telomere capping	Cellular protein metabolic process	Negative regulation of gliogenesis
	DNA-templated transcription, initiation	Negative regulation of protein processing	Cerebellar granular layer maturation
	Chromatin organization	Regulation of gene silencing	Radial glia guided migration of cerebellar granule cell
	DNA replication-independent nucleosome assembly	CENP-A containing nucleosome assembly	Female sex determination
	Antibacterial humoral response	Negative regulation of cAMP biosynthetic process	Immature T cell proliferation in thymus
	Innate immune response in mucosa	Sensory perception of smell	Non-canonical Wnt signaling pathway <i>via</i> MAPK cascade
Down regulation GOs	Keratinization	Protein import into nucleus	l-Methylmalonyl-CoA metabolic process
	Neutrophil degranulation	Response to unfolded protein	Negative regulation of growth
	Protein folding	Neutrophil degranulation	Cellular response to zinc ion
	Negative regulation of growth mRNA splicing, <i>via</i> spliceosome	Protein folding	Plasma membrane repair
	Cell-cell adhesion	Glycosphingolipid metabolic process	Protein localization to cytoplasmic stress granule
	Cellular response to zinc ion	Positive regulation of apoptotic process	Mitotic nuclear division
	Viral process	Negative regulation of thymocyte apoptotic process	mRNA splicing, <i>via</i> spliceosome
	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	Negative regulation of growth	Centriole-centriole cohesion
	rRNA processing	Cellular response to lipopolysaccharide	Protein targeting to ER
	PERK-mediated unfolded protein response	ATF6-mediated unfolded protein response	Ciliary basal body docking
	Protein targeting to Golgi	Chaperone mediated protein folding requiring cofactor	Response to metal ion
	Cellular response to cadmium ion	Regulation of cellular response to heat	DNA duplex unwinding
	Positive regulation of apoptotic process	Chaperone-mediated protein complex assembly	Cell-cell adhesion
	Response to metal ion	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	Mitochondrial transport
	Negative regulation of cell proliferation	Vascular endothelial growth factor production	Negative regulation of translational initiation
	Response to unfolded protein	Chaperone-mediated autophagy	Hematopoietic progenitor cell differentiation
	Ribosome assembly	Telomerase holoenzyme complex assembly	Spindle organization
	Transferrin transport	Regulation of pro-B cell differentiation	Nucleosome disassembly
	Toxin transport	Positive regulation of protein oligomerization	Double-strand break repair
	tRNA aminoacylation for protein translation	Regulation of I-kappaB kinase/NF-kappaB signaling	Chromosome segregation
		DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	G2/M transition of mitotic cell cycle

group, including nucleosome assembly, telomere organization, chromatin silencing at rDNA, DNA replication-dependent nucleosome assembly, protein heterotetramerization, negative regulation of gene expression, positive regulation of gene expression, regulation of gene silencing, cellular protein metabolic process, chromatin silencing, CENP-A containing nucleosome assembly, neutrophil degranulation, negative regulation of growth, maturation of SSU-rRNA from tricistronic rRNA transcript, and positive regulation of apoptotic process; while there were 4 same significantly changed GOs between the SiNP-40- and SiNP-200-treated groups, including keratinization, mRNA splicing, cell-cell adhesion, and cellular response to zinc ion; and there was only one same significantly changed GO between the SiNP-60- and SiNP-200-treated groups, which was negative regulation of the cAMP biosynthetic process.

3.5. STC and STC-GO analysis of differential gene expression induced by SiNPs

According to the different signal intensity change tendency of the genes under different situations, a set of unique model

expression tendencies were identified using the STC strategy. To profile the differentially expressed genes and select the significant gene clusters with parallel expression trends caused by the SiNPs with different sizes, 26 different gene expression trends were analyzed, from which 8 significant clusters were identified (Fig. 3A). The genes belonging to significant clusters were further submitted to GO analysis for functional classification. As shown in Fig. 3B and C, 354 differentially expressed genes were classified into profile 26 and 667 differentially expressed genes were classified into profile 17, in which the genes were gradually upregulated with the particle size decreasing. Related important GOs belonging to these two profiles included the immune response, inflammatory response, positive regulation of interleukin-8 production, negative regulation of transcription, signal transduction by p53 class mediator (negative regulation of DNA damage response), cytokine-mediated signaling pathway, cellular protein metabolic process, endoplasmic reticulum unfolded protein response, response to oxidative stress, cell cycle arrest, regulation of mitotic cell cycle, negative regulation of apoptotic process,

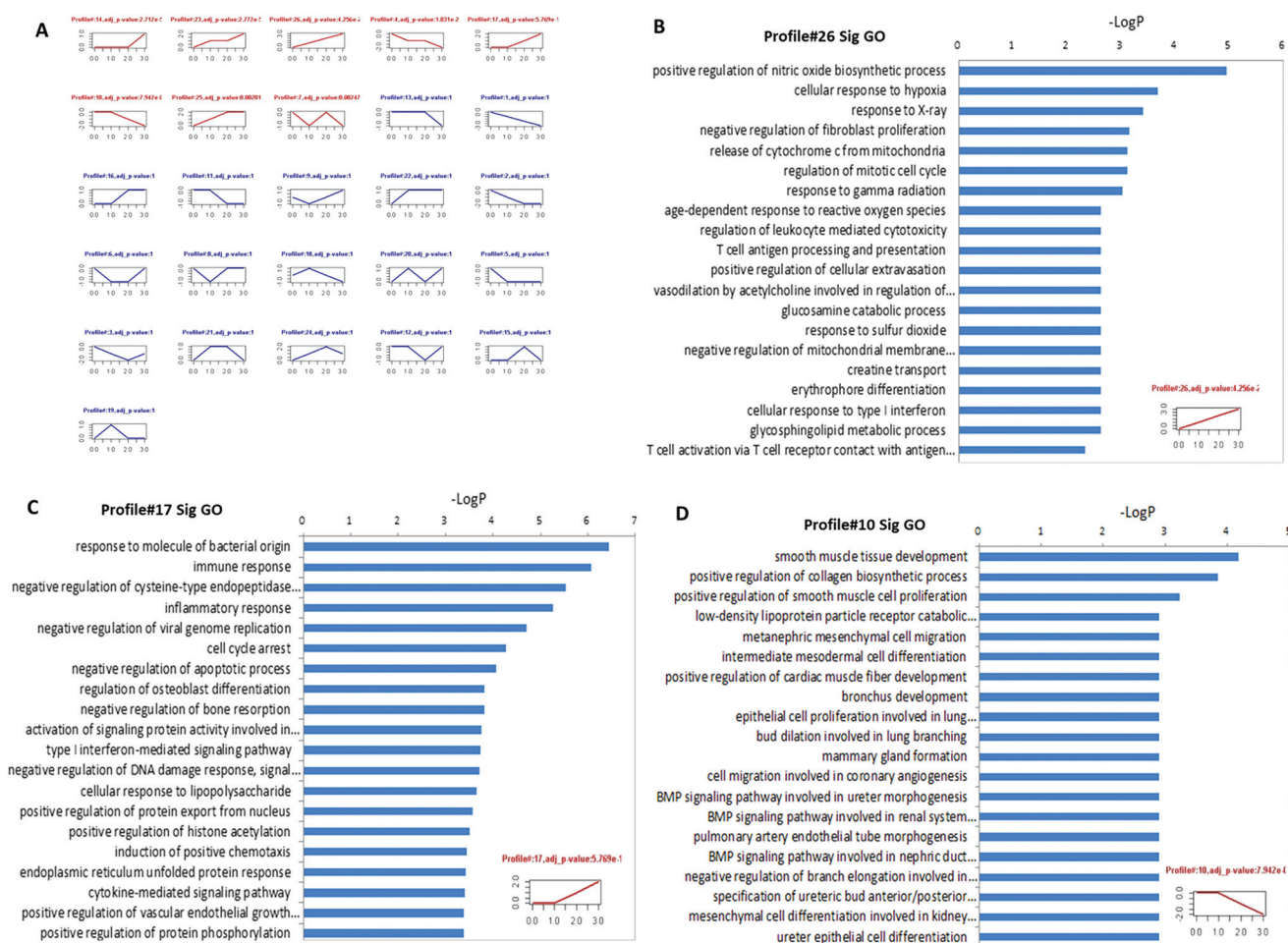


Fig. 3 STC analysis and STC-GO analysis. (a) In total, 26 differential mRNA expression trends were analyzed, and 8 significant trends were plotted over the color part; (b) STC-GO analysis of significant profile 26; (c) STC-GO analysis of significant profile 17; (d) STC-GO analysis of significant profile 10.

negative regulation of mitochondrial membrane potential, and the response to silicon dioxide. There were also 284 differentially expressed genes classified into profile 10 (Fig. 3D), in which the genes were gradually downregulated with the particle size decreasing. Related important GOs belonging to this profile included bronchus development, epithelial cell proliferation involved in lung morphogenesis, bud dilation involved in lung branching, cell envelope organization, trachea development, and the positive regulation of branching involved in lung morphogenesis.

3.6. Pathway analysis of the differential gene expression induced by SiNPs

The significant pathways were then analyzed according to the functions and interactions of the differential genes based on the KEGG database. Compared with the control, there were 54 significant pathways in the SiNP-40-treated group, including 15 upregulation pathways and 39 downregulation pathways; 32 significant pathways in the SiNP-60-treated group, including 5 upregulation pathways and 27 downregulation pathways; and 11 significant pathways in the SiNP-200-treated group, including 4 upregulation pathways and 7 downregulation pathways (Fig. 4).

In addition, the significant pathways could be further classified into top and middle categories according to the KEGG database. The middle categories of the significant pathways are displayed by Pie chart in Fig. 5. As shown, the significant pathways in the SiNP-40-treated group were involved in 21 categories, namely cancers, infectious diseases, glycan biosynthesis and metabolism, transport and catabolism, immune system, translation, carbohydrate metabolism, cell growth and death, folding, sorting and degradation, immune diseases, signaling molecules and interaction, signal transduction, cellular community – eukaryotes, digestive system, endocrine and metabolic diseases, excretory system, lipid metabolism, nervous system, replication and repair, substance dependence, and transcription; while the significant pathways in the SiNP-60-treated group were involved in 19 categories, namely infectious diseases, cancers, immune system, folding, sorting and degradation, immune diseases, signal transduction, transport and catabolism, carbohydrate metabolism, cell growth and death, digestive system, drug resistance, endocrine and metabolic diseases, endocrine system, glycan biosynthesis and metabolism, lipid metabolism, replication and repair, sensory system, signaling molecules and interaction, and substance dependence; and the significant pathways in the SiNP-200-treated group were involved in 11 categories, namely cancers, carbohydrate metabolism, cellular community – eukaryotes, digestive system, excretory system, metabolism of cofactors and vitamins, nervous system, neurodegenerative diseases, signaling molecules and interaction, transcription, and translation.

3.7. Signal-net analysis of the key genes triggered by SiNPs

Based on the significant GO and pathway analysis, signal-net analysis was introduced to screen the key genes involved in SiNP-induced toxicity in BEAS-2B cells. The result manifested

that there was a total number of 270 key genes involved in the transduction network. Fig. 6 shows the interaction relationship of 80 genes with a degree greater than or equal to 10, and Table 5 lists the top 20 genes ranked by degree in the single-net, which were *hsp90ab1* (Homo sapiens heat shock protein 90 kDa alpha), *hspb1* (Homo sapiens heat shock 27 kDa protein 1), *hsp90aa1* (Homo sapiens heat shock protein 90 kDa alpha), *krt9* (Homo sapiens keratin 9), *sqstm1* (Homo sapiens sequestosome 1), *cct8* (Homo sapiens chaperonin containing TCP1), *hsp1* (Homo sapiens heat shock 105 kDa/110 kDa protein 1), *ranbp2* (Homo sapiens RAN binding protein 2), *hsp90b1* (Homo sapiens heat shock protein 90 kDa beta), *rbmx12* (Homo sapiens RNA binding motif protein), *hspa4* (Homo sapiens heat shock 70 kDa protein 4), *dsp* (Homo sapiens desmoplakin), *map1b* (Homo sapiens microtubule-associated protein 1B), *znf638* (Homo sapiens zinc finger protein 638), *acta2* (Homo sapiens actin, alpha 2), *cdk4* (Homo sapiens cyclin-dependent kinase 4), *hspe1* (Homo sapiens heat shock 10 kDa protein 1), *nedd4* (Homo sapiens neural precursor cell expressed), *ctsa* (Homo sapiens cathepsin A), and *prpf39* (Homo sapiens PRP39 pre-mRNA processing factor 39 homolog).

3.8. Quantitative RT-PCR verification

To verify the microarray analysis results, the expression of 7 genes were detected using qRT-PCR. Fig. 7A exhibits the expression of two significantly upregulated genes and one significantly downregulated gene selected from profile 26, profile 17, and profile 10 based on STC analysis, which were related to the antioxidant response (*sod2*), immune and inflammatory response (*il8*), and mitotic cell cycle (*cenpf*), respectively. As shown, the expression of *sod2* and *il8* increased obviously with the particle size decreasing, while the expression of *cenpf* decreased gradually as the function of the SiNP size. Fig. 7B exhibits the expression of 4 key regulatory genes selected from Signal-net analysis, which were *hsp90ab1*, *hsp90b1*, *hsp90aa1* and *hspa4*. These genes encoded heat shock proteins (HSP) and were related to cell stress, signal transduction, protein folding and degradation, and morphological evolution and tumor formation. As indicated, the SiNPs increased the expression of HSP-related genes, and the effect of the two nanosized particles was more obvious. The trends of gene expression were similar with that indicated in the microarray analysis. Thus, very good consistency was obtained between the microarray analysis and qRT-PCR verification.

4. Discussion

Amorphous silica is an abundant compound found in nature.¹⁶ Nowadays, with the extensive application of amorphous SiNPs, it has become the second largest engineering nanomaterial in terms of annual output.⁴ Inhalation is the main route for SiNPs to enter the human body. A large number of experimental studies have shown that SiNPs possess respiratory toxicity, but knowledge of the underlying

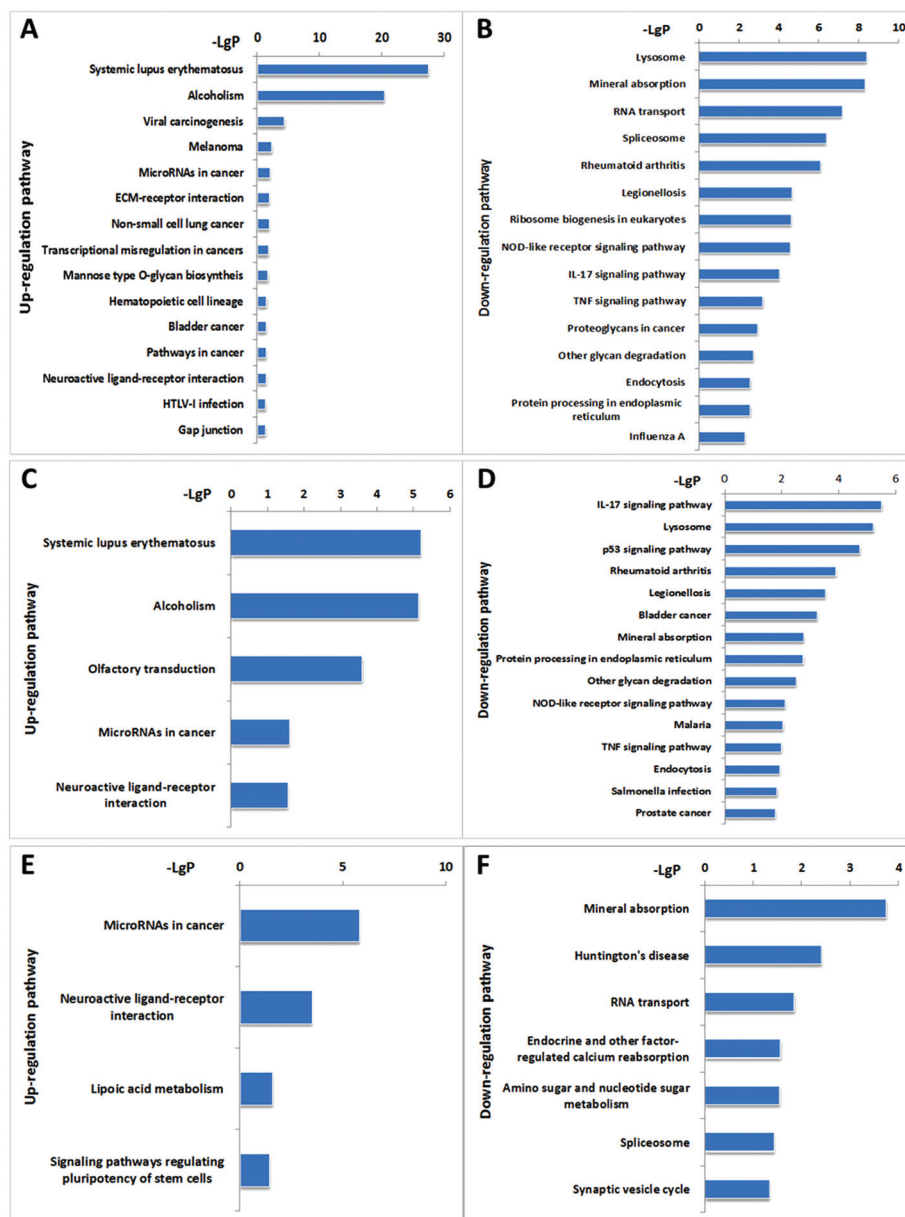


Fig. 4 Significantly changed pathways of the differentially expressed up- and downregulated genes based on the KEGG database. (a) Significant upregulation pathways in the SiNP-40-treated group; (b) top 15 significant downregulation pathways in the SiNP-40-treated group; (c) significant upregulation pathways in the SiNP-60-treated group; (d) top 15 significant downregulation pathways in the SiNP-60-treated group; (e) significant upregulation pathways in the SiNP-200-treated group; (f) significant downregulation pathways in the SiNP-200-treated group. $-\text{Lg}P$, negative logarithm of the P value. x -Axis denotes that a larger number corresponds to a smaller P -value.

mechanisms is still limited. The unbiased and high throughput nature of omics platforms, such as microarrays, has led to them playing an important role in the identification of potential toxicity pathways induced by emerging nanomaterials.^{17,18} In this study, microarray analysis and corresponding bioinformatics analysis were introduced to investigate the toxic mechanism of SiNPs with different sizes in BEAS-2B cells. Based on our previous study, BEAS-2B cells were exposed to $25 \mu\text{g mL}^{-1}$ of three SiNPs for 24 h in the present experiment. As indicated in Fig. 1D, cell viability declined gradually with the particle

size decreasing. Additionally, even BEAS-2B cells exposed to SiNP-40, the most toxic silica nanoparticles among the particles we tested, could maintain a relatively high viability, which was nearly 70% at this exposure condition. Therefore, our microarray analysis was carried out when the cells were still in a relatively good state. According to the reports, during production, the concentration of amorphous SiNPs in the atmosphere is about 11 mg m^{-3} .^{19,20} As calculated by multiple-path particle dosimetry (MPPD) model software, the predicted deposition concentration in the human tracheal-bronchial

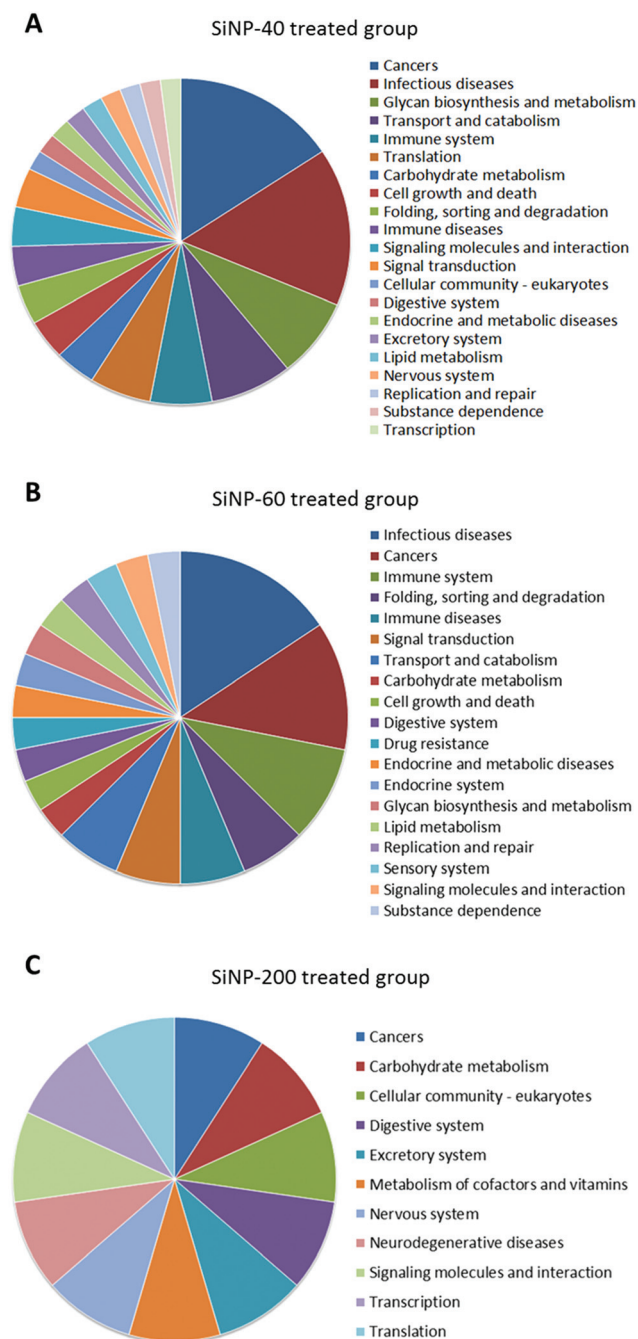


Fig. 5 Middle categories of significant pathways in the SiNP-40-treated group (a), SiNP-60-treated group (b), and SiNP-200-treated group (c) based on the KEGG database.

(TB) region of SiNPs-40, SiNPs-60, and SiNPs-200 was 0.73, 0.49, and 0.24 $\mu\text{g cm}^{-2}$, respectively, after 8 h exposure at the real concentration of 11 mg m^{-3} and 16 h of clearance. In this study, 10 mm Petri dishes were used, the bottom area of which was 55 cm^2 , and the total exposure volume was 10 mL. It could be calculated that the dose of 25 $\mu\text{g mL}^{-1}$ corresponded to a concentration of 4.5 $\mu\text{g cm}^{-2}$ on the surface of the culture dishes. Thus, the experimental dose used in this study was about 6 to 19 times the predicted deposition concentration in

the human TB region of silica nanoparticles. The baseline set of MPPD inputs is shown in Table S1.†

In a nanotoxicology study, the particle size is considered as one of the most critical factors affecting the biological effects of nanoparticles. It is well accepted that at the nanoscale (1–100 nm), with the particle size decreasing, the number of surface molecules of particles increases exponentially, which makes the reactivity of nanoparticles increase sharply.²¹ Within the organisms, small-sized nanoparticles are more likely to avoid the clearance mechanism and be retained in the body for a long time, which facilitates the biodistribution of nanoparticles and greatly increases the chance of tissue damage.²¹ Our group and other researchers identified the size-dependent toxic effects of SiNPs *in vitro* and *in vivo*.^{21–25} Yet, there are many conflicting results about the relationship between SiNP size and toxic effect due to the complexity of biological organisms.^{26,27} In the present study, three sizes of silica particles were used, two nanoparticle (SiNP-40 and SiNP-60) and one submicron size (SiNP-200), to evaluate whether the genome scale changes induced by SiNPs are particle size dependent. The results of our microarray analysis demonstrated that all three SiNPs could affect the expression of many important functional genes (Tables 2 and 3). Also, the number of differentially expressed genes in the three SiNP-treated groups increased with the SiNP size decreasing, which first confirmed the function of the particle size at the gene expression level. Then, changes in the gene expression patterns were compared using treatment-independent clustering analysis, which is an exploratory analysis method that can classify samples according to the difference in gene expression patterns. The result in Fig. 2D also displayed a clear particle size-dependent change in the three SiNP-treated groups. Waters *et al.*¹⁷ used 7 and 500 nm amorphous SiNPs to compare the effects of different surface concentrations on the gene expression profiles of macrophages, and demonstrated that the gene expression change induced by SiNPs was dependent primarily on surface area of the particles. It is known that at the same mass concentration, smaller nanoparticles possess a larger surface area; therefore, the author's conclusion was consistent with the present study.

Based on the bioinformatics technology, the differentially expressed genes in each treatment group were systematically analyzed to further compare the different effects of SiNPs with different sizes on the BEAS-2B cells. GO is the key functional classification of NCBI and GO analysis can organize genes into hierarchical categories and uncover the gene regulatory network on the basis of the biological process and molecular function.^{28,29} As shown in Table 4, differentially expressed genes induced by the three SiNPs were involved in many important cellular biological processes. In the top 40 significantly changed GOs (20 upregulated GOs and 20 downregulated GOs), there were 15 same significantly changed GOs between the SiNP-40- and SiNP-60-treated group, which could mainly be classified into the following functions: chromatin structure formation, gene expression, gene silencing, protein synthesis and metabolism, cell growth, and cell death; while

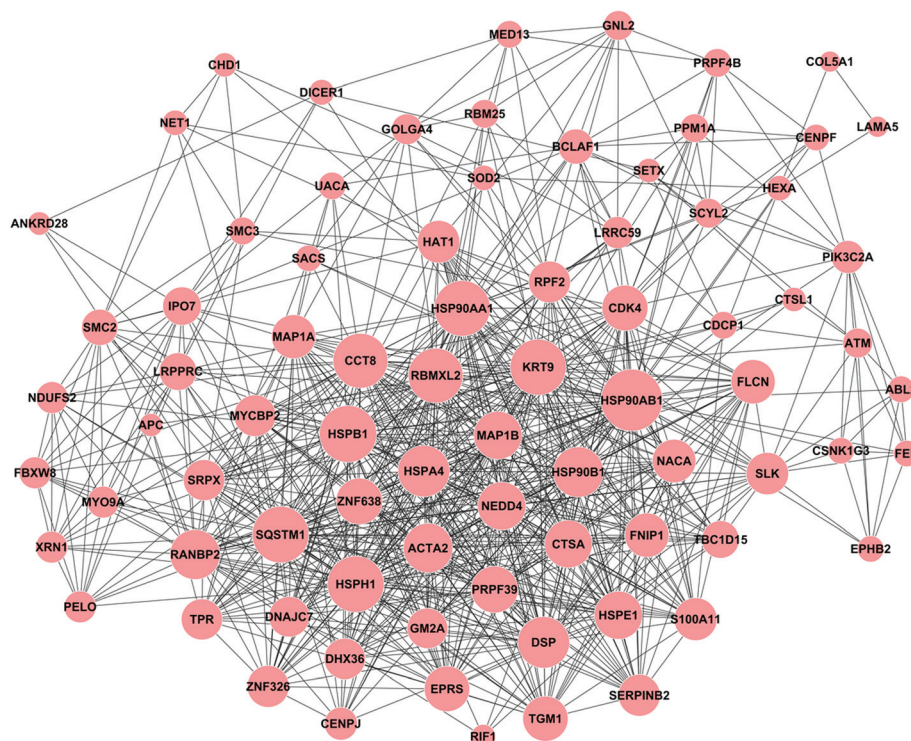


Fig. 6 Signal-net analysis of the differentially expressed genes induced by SiNPs. Circles represent genes, and area of the circle represents the degree of genes.

Table 5 Top 20 genes ranked by degree in Signal-net analysis

Gene name	Description	Style	Degree	Indegree	Outdegree
HSP90AB1	Homo sapiens heat shock protein 90 kDa alpha (cytosolic), class B member 1 (HSP90AB1), transcript variant 2, mRNA	Up	68	33	36
HSPB1	Homo sapiens heat shock 27 kDa protein 1 (HSPB1), mRNA	Up	62	31	31
HSP90AA1	Homo sapiens heat shock protein 90 kDa alpha (cytosolic), class A member 1 (HSP90AA1), transcript variant 1, mRNA	Up	55	27	29
KRT9	Homo sapiens keratin 9 (KRT9), mRNA	Down	55	34	21
SQSTM1	Homo sapiens sequestosome 1 (SQSTM1), transcript variant 2, mRNA	Up	55	30	25
CCT8	Homo sapiens chaperonin containing TCP1, subunit 8 (theta) (CCT8), mRNA	Up	50	29	21
HSPH1	Homo sapiens heat shock 105 kDa/110 kDa protein 1 (HSPH1), mRNA	Up	47	19	28
RANBP2	Homo sapiens RAN binding protein 2 (RANBP2), mRNA	Up	46	24	22
HSP90B1	Homo sapiens heat shock protein 90 kDa beta (Grp94), member 1 (HSP90B1), mRNA	Up	46	21	26
RBMXL2	Homo sapiens RNA binding motif protein, X-linked-like 2 (RBMXL2), mRNA	Down	45	22	23
HSPA4	Homo sapiens heat shock 70 kDa protein 4 (HSPA4), mRNA	Up	39	16	23
DSP	Homo sapiens desmoplakin (DSP), transcript variant 2, mRNA	Up	39	21	18
MAP1B	Homo sapiens microtubule-associated protein 1B (MAP1B), mRNA	Up	37	16	21
ZNF638	Homo sapiens zinc finger protein 638 (ZNF638), transcript variant 2, mRNA	Down	35	17	18
ACTA2	Homo sapiens actin, alpha 2, smooth muscle, aorta (ACTA2), transcript variant 1, mRNA	Up	35	20	15
CDK4	Homo sapiens cyclin-dependent kinase 4 (CDK4), mRNA	Up	34	19	15
HSPE1	Homo sapiens heat shock 10 kDa protein 1 (chaperonin 10) (HSPE1), nuclear gene encoding mitochondrial protein, mRNA	Up	34	13	21
NEDD4	Homo sapiens neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase (NEDD4), transcript variant 1, mRNA	Up	34	16	18
CTSA	Homo sapiens cathepsin A (CTSA), transcript variant 1, mRNA	Up	33	16	17
PRPF39	Homo sapiens PRP39 pre-mRNA processing factor 39 homolog (<i>S. cerevisiae</i>) (PRPF39), mRNA	Up	31	17	14

there were only 4 same significantly changed GOs between the SiNP-40- and SiNP-200-treated group, and one same significantly changed GO between the SiNP-60- and SiNP-200-treated

groups among the top 40 significantly changed GOs. This indicated the modes of action of the two nanoparticles (SiNP-40 and SiNP-60) overlapped with each other to some degree, and

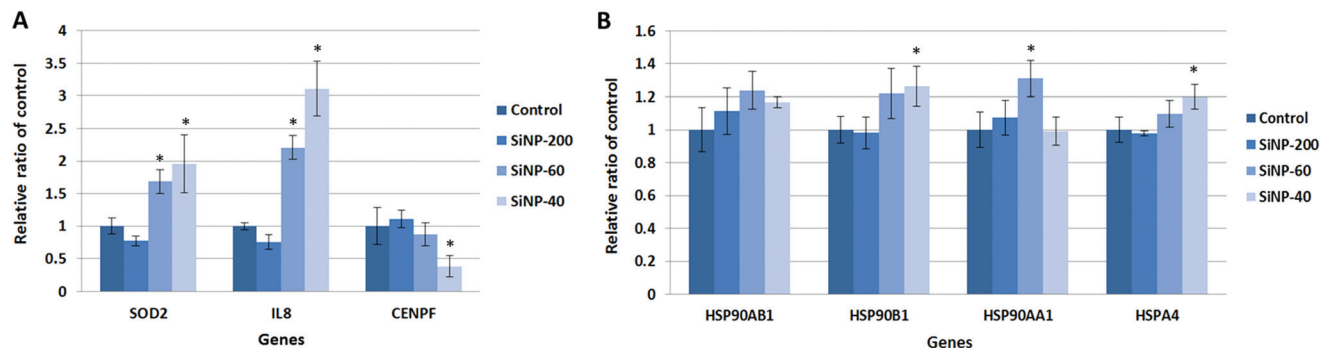


Fig. 7 qRT-PCR analysis of the key genes in the effect of SiNPs with different sizes. (a) qRT-PCR verification of the genes in the STC-GO analysis; (b) qRT-PCR verification of the key regulatory genes in the signal-net analysis. Data are expressed as the mean $\pm$ S.D. from three independent experiments. * $p < 0.05$ compared with the control group using ANOVA.

many differences existed with that of the submicron particles (SiNP-200). Napierska *et al.*³⁰ investigated the cytotoxic effects of a series of SiNPs with different sizes (16, 19, 60, 104, and 335 nm) on endothelial cells, and also observed the difference between nanoparticles and fine particles. Compared with nanometer-sized particles, two fine silica particles (104 and 335 nm) showed quite a lower cytotoxic response.

In order to profile the significant gene clusters and select related GOs with parallel expression trends induced by the different sizes of SiNPs, STC-GO analysis was then performed. As shown in Fig. 3, among 26 different kinds of gene expression trends, there were 3 significant clusters in which the gene expression trend was related to the silica particle size. In profiles 26 and 17, the genes were gradually upregulated with the particle size decreasing, with the related GOs involved in the immune and inflammatory response, gene expression, signal transduction, endoplasmic reticulum stress, oxidative stress, cell metabolism, cell proliferation, apoptosis, *etc.* In profile 10, the genes were gradually downregulated with the particle size decreasing, and related important GOs mainly referred to morphological development of respiratory system. To further verify the results of the microarray analysis, three key genes were selected from the above significant clusters for PCR validation. *Sod2* belonged to profile 26 and was associated with oxygen homeostasis, superoxide metabolic process, mitochondrial respiratory chain, response to reactive oxygen species, response to oxidative stress, and so on.^{31,32} *Il8* belonged to profile 17 and played an important role in the inflammatory response, immune response, signal transduction, cellular response to tumor necrosis factor, endoplasmic reticulum unfolded protein response, negative regulation of cell proliferation, and so on.³³ *Cenpf* belonged to profile 10 and was closely related to mitotic control, such as mitotic cell cycle, chromosome segregation, mitotic spindle assembly checkpoint, cell proliferation, cell differentiation, *etc.*³⁴ The PCR results verified the accuracy of our microarray analysis. The expression trends of the three genes agreed with the STC classification (Fig. 7A). Moreover, the above results significantly extend previous studies that reported that smaller silica nanoparticles induced more severe toxic effects, such as oxi-

dative damage,^{35,36} inflammatory response,^{11,37} genotoxicity,³⁸ cell cycle arrest,^{39,40} aberrant mitosis,⁴¹ inhibition of stem cell differentiation,⁴² and cell death.^{43,44}

Pathway analysis was used to determine the significant pathway of differential genes induced by SiNPs. The number of significant pathways in each treated group increased as the particle size decreased. These significant pathways were further sorted into middle categories according to the KEGG database. As indicated in Fig. 4, all three SiNPs significantly upregulated several pathways related to cancer, such as microRNAs in cancer, non-small cell lung cancer, transcriptional misregulation in cancers, and pathways in cancer. In addition, the effect of the two nanoparticles on the signaling pathway was more similar than the effect of the submicron particles. Important signaling pathways altered by both two silica nanoparticles included: (1) endocytosis, which is in regard to the cell entry of silica nanoparticles; (2) lysosomal signaling, which is related to autophagy and lysosomal dysfunction; (3) protein processing in endoplasmic reticulum, which is involved in endoplasmic reticulum stress; (4) IL-17 signaling pathway, nod-like receptor signaling pathway, as well as TNF signaling pathway, which play key regulatory roles in inflammatory response; (5) non-homologous end-joining and RNA degradation, which is associated with DNA damage and gene expression; (6) p53 signaling pathway, which is critical to apoptotic resistance and cancer. It is widely accepted that SiNPs could accumulate in cells through not only passive diffusion but also the active endocytotic pathway.^{25,45} In addition, besides oxidative stress SiNPs could also induce prolonged autophagy dysfunction^{8,46,47} and endoplasmic reticulum stress^{13,48} which were thought to be essential to the subsequent inflammatory response, genotoxicity and cell death. Therefore, many of the transcriptional changes stimulated by SiNPs herein were in accord with the important events associated with the toxic effect. Transcriptomic studies investigating the regulation of genes under specific treatments could be successfully used to define molecular alterations and predict the toxicity.⁴⁷ Moreover, the emerging omics science also provides a toolbox of assays for understanding the underlying mechanisms of the nanotoxicity of SiNPs.^{49,50} For instance, in a pre-

viously published study, our group first demonstrated that chronically low-dose amorphous SiNPs exposure resulted in the malignant transformation and tumorigenesis of BEAS-2B cells due to the inactivation of p53 and the aberration of the p53 signaling pathway.⁵¹ This experimental clue was precisely derived from the results of the microarray analysis.

Signal-net analysis could display the intergene signaling of the differentially expressed genes, and was constructed based on the interaction database from KEGG. In the global signaling network, the degree of a gene is related with its modulate capacity, whereby the higher the degree, the stronger the modulate ability. The top 20 genes ranked by degree are listed in Table 5; according to the gene ontology biological process, znf638 and prpf39 were relevant to transcriptional regulation and mRNA processing; krt9, dsp, map1b, and acta2 were relevant to cytoskeleton organization; ranbp2 and cdk4 were relevant to cell cycle and cell proliferation; cct8, nedd4, and ctsa were relevant to cellular protein metabolic process, protein ubiquitination, and intracellular signal transduction; qsqtm1 was relevant to the response to stress, intracellular signal transduction, autophagy, and apoptotic process. Thus, gene transcription, signal transduction, cytoskeleton organization, cell cycle, autophagy and apoptosis related genes played key regulatory role in the toxic effect of SiNPs in BEAS-2B cells. Besides, it is worth noting that seven out of the top 20 genes encoded proteins belonged to the heat shock protein (HSP) family, namely hsp90ab1, hspb1, hsp90aa1, hsp1, hsp90b1, hspa4, and hspe1. Moreover, in all three SiNP-treated groups, genes encoding HSP90 proteins (hsp90aa1, hsp90ab1, and hsp90b1) were also ranked in the top 10 differentially upregulated genes (Table 2). This indicated that SiNPs significantly affected the expression of HSPs, which further played a crucial role in regulating the toxicity induced by SiNPs. The expression of 4 HSP coding genes was verified by qRT-PCR (Fig. 7B). As reported, HSPs are highly conserved molecular chaperones that are synthesized and expressed by cells in response to stress conditions.⁵² This kind of protein performs essential functions in activity regulation and in the initial folding of a large number of kinases.⁵³ HSP90, the most prominent member of the HSP family, was closely related to the maturation, conformational stabilization, and activity of various oncogenic client proteins, such as p53, ErbB2, Bcr-Abl, Akt, Her-2, Cdk4, Cdk6, and Raf-1.⁵¹ Many researchers have observed the high expression of HSP90 in various human cancers, such as breast, lung, colon, prostate, and leukemia.^{54,55} Although amorphous silica was sorted by the International Agency for Research on Cancer (IARC) as 'not classifiable as to its carcinogenicity to humans' (Group 3) due to insufficient toxicological and epidemiological evidence, our previous study did demonstrate that SiNPs induced the malignant transformation of BEAS-2B cells⁵⁰ and the formation of polyploid and aneuploid in other cell lines.⁴¹ This microarray analysis sheds new light on the toxic mechanism study of SiNPs. Therefore, the relationship between HSP upregulation, especially HSP90, and the toxicity as well as carcinogenicity induced by SiNPs deserves further investigation.

5. Conclusions

Taken together, the gene expression profile alteration caused by the different sizes of SiNPs (SiNP-40, SiNP-60, and SiNP-200) in BEAS-2B cells was investigated. Our study, for the first time, reports the size-dependent effect of SiNPs at the gene expression level. The results of microarray analysis indicated that the number of differentially expressed genes in the three SiNP-treated groups changed as a function of particle size. Further GO and STC-GO analysis manifested that the genes involved in the immune and inflammatory response, gene expression, signal transduction, endoplasmic reticulum stress, oxidative stress, cell metabolism, cell proliferation, and apoptosis were gradually upregulated with the particle size decreasing, while the genes related to morphological development of the respiratory system were gradually downregulated with the particle size decreasing. However, it was observed that the modes of action of the two nanoparticles (SiNP-40 and SiNP-60) overlapped with each other to some degree, and many differences existed between them and that of the submicron particles (SiNP-200). Pathway analysis suggested that both the silica nanoparticles affected the pathways associated with the cell entry of silica nanoparticles, autophagy and lysosomal dysfunction, endoplasmic reticulum stress, inflammatory response, DNA damage and gene expression, as well as apoptotic resistance and cancer, which were in accord with the important events associated with the toxic effects published previously. In signal-net analysis, the key role of the HSPs-related genes in regulating SiNP toxicity was noticeable, which has not been reported by other researchers. Hence, transcriptomic investigation herein could not only facilitate the toxicity prediction, but also provide new experimental clues for the mechanism study of SiNPs. Meanwhile, our study could serve as an experimental basis for the toxicity and safety evaluation of silica nanoparticles.

Conflicts of interest

The authors declare no conflict of interests at personal and/or organization level.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81571130090, 81673204, and 81602875). The authors would like to thank Weiping Tang of Cnkingbio biotechnology Co. Ltd. for bioinformatics assistance.

References

- 1 S. Murugadoss, D. Lison, L. Godderis, S. Van Den Brule, J. Mast and F. Brassinne, *Arch. Toxicol.*, 2017, **91**, 2967–3010.
- 2 L. Tang and J. Cheng, *Nano Today*, 2013, **8**, 290–312.

- 3 C. Liljenström, D. Lazarevic and G. Finnveden, *Silicon-based nanomaterials in a life-cycle perspective, including a case study on self-cleaning coatings*, 2013, ISBN: 978-91-7501-942-0.
- 4 World Health Organization, *WHO Guidelines on Protecting Workers from Potential Risks of Manufactured Nanomaterials*, 2017, ISBN-13: 978-92-4-155004-8.
- 5 Organisation for Economic Co-operation and Development (OECD), *List of Manufactured Nanomaterials and List of Endpoints for Phase One of the Sponsorship Programme for the Testing of Manufactured Nanomaterials*, Revision, 2010.
- 6 W. S. Cho, M. Choi, B. S. Han, M. Cho, J. Oh and K. Park, *Toxicol. Lett.*, 2007, **175**, 24–33.
- 7 M. Yang, L. Jing, J. Wang, Y. Yu, L. Cao and L. Zhang, *Int. J. Nanomed.*, 2016, **11**, 6217–6228.
- 8 X. Zhao, S. Wei, Z. Li, C. Lin, Z. Zhu and D. Sun, *Cell Death Dis.*, 2019, **10**, 127.
- 9 C. Lin, X. Zhao, D. Sun, L. Zhang, W. Fang and T. Zhu, *Sci. Rep.*, 2016, **6**, 21133.
- 10 T. Skuland, J. Ovreivik, M. Låg, P. Schwarze and M. Refsnes, *Toxicol. Appl. Pharmacol.*, 2014, **279**, 76–86.
- 11 M. Låg, T. Skuland, A. Godymchuk, T. H. T. Nguyen, H. L. T. Pham and M. Refsnes, *Basic Clin. Pharmacol. Toxicol.*, 2018, **122**, 620–632.
- 12 Q. Li, H. Hu, L. Jiang, Y. Zou, J. Duan and Z. Sun, *Toxicol. Res.*, 2016, **5**, 1216–1228.
- 13 T. Wu, S. Zhang, X. Liang, K. He, T. Wei and Y. Wang, *Toxicol. In Vitro*, 2019, **56**, 126–132.
- 14 L. Galluzzi, L. Chiarantini, E. Pantucci, R. Curci, J. Merikhi and H. Hummel, *Nanomedicine*, 2012, **7**, 393–409.
- 15 W. Wang, Y. Li, X. Liu, M. Jin, H. Du and Y. Liu, *Int. J. Nanomed.*, 2013, **8**, 3533–3541.
- 16 D. Napierska, L. C. Thomassen, D. Lison, J. A. Martens and P. H. Hoet, *Part. Fibre Toxicol.*, 2010, **7**, 39.
- 17 K. M. Waters, L. M. Masiello, R. C. Zangar, B. J. Tarasevich, N. J. Karin and R. D. Quesenberry, *Toxicol. Sci.*, 2009, **107**, 553–569.
- 18 V. Kodali, M. H. Littke, S. C. Tilton, J. G. Teeguarden, L. Shi and C. W. Frevert, *ACS Nano*, 2013, **7**, 6997–7010.
- 19 M. P. Sutunkova, S. N. Solovyeva, B. A. Katsnelsona, V. B. Gurvich, L. I. Privalova and I. A. Minigalieva, *Toxicology*, 2017, **384**, 59–68.
- 20 S. Oh, B. Kim and H. Kim, *Ind. Health*, 2014, **52**, 152–162.
- 21 G. Oberdörster, E. Oberdörster and J. Oberdörster, *Environ. Health Perspect.*, 2005, **113**, 823–839.
- 22 Y. Li, L. Sun, M. Jin, Z. Du, X. Liu and C. Guo, *Toxicol. In Vitro*, 2011, **25**, 1343–1352.
- 23 Z. Du, D. Zhao, L. Jing, G. Cui, M. Jin and Y. Li, *Cardiovasc. Toxicol.*, 2013, **13**, 194–207.
- 24 T. Kaewamatawong, N. Kawamura, M. Okajima, M. Sawada, T. Morita and A. Shimada, *Toxicol. Pathol.*, 2005, **33**, 743–749.
- 25 I. Y. Kim, E. Joachim, H. Choi and K. Kim, *Nanomedicine*, 2015, **11**, 1407–1416.
- 26 X. Lu, J. Qian, H. Zhou, Q. Gan, W. Tang and J. Lu, *Int. J. Nanomed.*, 2011, **6**, 1889–1901.
- 27 D. Breznan, D. D. Das, J. S. O'Brien, C. MacKinnon-Roy, S. Nimesh and N. Q. Vuong, *Nanotoxicology*, 2017, **11**, 223–235.
- 28 M. Ashburner, C. A. Ball, J. A. Blake, D. Botstein, H. Butler and J. M. Cherry, *Nat. Genet.*, 2000, **25**, 25–29.
- 29 Gene Ontology Consortium, *Nucleic Acids Res.*, 2006, **34**, D322–D326.
- 30 D. Napierska, L. C. Thomassen, V. Rabolli, D. Lison, L. Gonzalez and M. Kirsch-Volders, *Small*, 2009, **5**, 846–853.
- 31 P. Tokarz, T. Płoszaj, Z. Regdon, L. Virág and A. Robaszkiewicz, *Free Radicals Biol. Med.*, 2019, **131**, 218–224.
- 32 A. Ashtekar, D. Huk, A. Magner, K. M. D. La Perle, L. Boucai and L. S. Kirschner, *J. Clin. Endocrinol. Metab.*, 2018, **103**, 4135–4145.
- 33 V. T. Baron, R. Pio, Z. Jia and D. Mercola, *Br. J. Cancer*, 2015, **112**, 755–764.
- 34 M. Shahid, M. Y. Lee, H. Piplani, A. M. Andres, B. Zhou and A. Yeon, *Cell Cycle*, 2018, **17**, 2802–2818.
- 35 P. Ariano, P. Zamburlin, A. Gilardino, R. Mortera, B. Onida and M. Tomatis, *Small*, 2011, **7**, 766–774.
- 36 S. E. Lehman, A. S. Morris, P. S. Mueller, A. K. Salem, V. H. Grassian and S. C. Larsen, *Environ. Sci.: Nano*, 2016, **3**, 56–66.
- 37 Z. Du, D. Zhao, L. Jing, G. Cui, M. Jin and Y. Li, *Cardiovasc. Toxicol.*, 2013, **13**, 194–207.
- 38 D. M. Brown, J. Varet, H. Johnston, A. Chrystie and V. Stone, *J. Nanopart. Res.*, 2015, **17**, 410.
- 39 F. Wang, F. Gao, M. Lan, H. Yuan, Y. Huang and J. Liu, *Toxicol. In Vitro*, 2009, **23**, 808–815.
- 40 S. Bancos, D. L. Stevens and K. M. Tyner, *Int. J. Nanomed.*, 2014, **10**, 183–206.
- 41 Y. Li, L. Jing, Y. Yu, Y. Yu, J. Duan and M. Yang, *Part. Part. Syst. Charact.*, 2015, **32**, 636–645.
- 42 M. V. Park, W. Annema, A. Salvati, A. Lesniak, A. Elsaesser and C. Barnes, *Toxicol. Appl. Pharmacol.*, 2009, **240**, 108–116.
- 43 Y. Ye, J. Liu, J. Xu, L. Sun, M. Chen and M. Lan, *Toxicol. In Vitro*, 2010, **24**, 751–758.
- 44 H. Yang, Q. Y. Wu, C. S. Lao, M. Y. Li, Y. Gao and Y. Zheng, *Genet. Mol. Res.*, 2016, **15**, DOI: 10.4238/gmr.15039005.
- 45 Y. Li, Y. Yu, J. Duan, Z. Li, W. Geng and L. Jiang, *Part. Part. Syst. Charact.*, 2016, **33**, 664–674.
- 46 J. Wang, Y. Yu, K. Lu, M. Yang, Y. Li and X. Zhou, *Int. J. Nanomed.*, 2017, **12**, 809–825.
- 47 C. Xi, J. Zhou, S. Du and S. Peng, *Inflammation Res.*, 2016, **65**, 325–341.
- 48 C. Guo, R. Ma, X. Liu, Y. Xia, P. Niu and J. Ma, *Chemosphere*, 2018, **210**, 183–192.
- 49 M. Yazdimamaghani, P. J. Moos, M. A. Dobrovolskaia and H. Ghandehari, *Nanomedicine*, 2019, **16**, 106–192.
- 50 A. Wilmes, A. Limonciel, L. Aschauer, K. Moenks, C. Bielow and M. O. Leonard, *J. Proteomics*, 2013, **79**, 180–194.
- 51 C. Guo, J. Wang, M. Yang, Y. Li, S. Cui and X. Zhou, *Nanotoxicology*, 2017, **11**, 1176–1194.

- 52 A. Vidyasagar, N. A. Wilson and A. Djamali, *Fibrog. Tissue Repair*, 2012, **5**, 7.
- 53 M. Grimes, B. Hall, L. Foltz, T. Levy, K. Rikova and J. Gaiser, *Sci. Signaling*, 2018, **11**, eaaq1087.
- 54 A. Haque, Q. Alam, M. Z. Alam, E. I. Azhar, K. H. Sait and N. Anfinan, *Curr. Pharm. Des.*, 2016, **22**, 2947–2959.
- 55 H. Ledford, *Nature*, 2011, **472**, 273.