Transcriptional profiling reveals gene expression changes associated with inflammation and cell proliferation following short-term inhalation exposure to copper oxide nanoparticles

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Short-term inhalation exposure to copper oxide nanoparticles induces gene expression changes associated with inflammation and cell proliferation in rat bronchoalveolar epithelium

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Running title: Pulmonary responses to copper oxide nanoparticles: a transcriptomics study.
Highlights

- CuO NPs triggered inflammation in the lungs of rats following short-term inhalation exposure.
- qPCR and protein analysis demonstrated upregulation of pro-inflammatory mediators following exposure.
- Microarray analysis showed that pathways linked to cell proliferation and inflammation were affected.
- Cell proliferation in bronchoalveolar epithelium was confirmed based on Ki67 expression.
- The oncoprotein, ECT2 and the chemokine, CCL2 were upregulated at the gene and protein level.
- These findings suggest novel biomarkers and could guide the development of AOPs for NPs.
Abstract

Our recent studies revealed a dose-dependent pro-inflammatory response to copper oxide nanoparticles (CuO NPs) in rats following short-term inhalation exposure for five consecutive days. Here, using the same model, an upregulation of pro-inflammatory markers was observed. To investigate this further, transcriptomics approaches were applied to assess global gene expression in lung tissues obtained 1 and 22 days post-exposure from rats exposed to clean air, or 6 h equivalent doses of 3.3 mg/m$^3$ (low dose, LD) and 13.2 (high dose, HD) mg/m$^3$. Microarray analyses yielded about 1000 differentially expressed genes in HD rats and 200 in LD compared to the clean air control group, and less than 20 after the recovery period. Pathway analysis indicated cell proliferation/survival and inflammation as the main processes triggered by exposure and upregulation of epithelial cell transforming protein 2 (Ect2), a known oncogene, was also noted. ECT2 protein was also upregulated in NP-exposed lungs and localized in alveolar epithelial cells in HD rats, in hyperplasic foci based on Ki67 expression, a marker of cell proliferation. The gene encoding monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) was also upregulated and this was confirmed by immunohistochemistry. However, no aberrant DNA methylation of inflammation associated genes was observed. In summary, we show that inhalation of CuO NPs in rats causes upregulation of the oncprotein ECT2 and the chemokine CCL2, along with other pro-inflammatory markers, and proliferation in bronchoalveolar epithelium even after a short-term exposure. Thus, pathways known to be associated with neoplastic processes were affected in this model.

Keywords: transcriptomics; oncogene; inflammation; epithelial cell hyperplasia; nanoparticles
1. Introduction

Copper oxide (CuO) nanoparticles (NPs) are manufactured for a broad range of industrial applications due to their unique features regarding electricity and heat conductivity, biocidal properties and others. As such, they are commonly found in many commercial products, such as electronic equipment, paints and processed wood (for an overview, see Karlsson et al., 2015). As for many other nanomaterials, inhalation is a route of exposure of major concern in the occupational setting (Kuhlbusch et al., 2011). In fact, there is a general concern regarding the effects of micro- and nano-sized particulate matter in air, due to potential links with human respiratory diseases (Seaton et al., 1995). However, while several recent in vitro studies have provided evidence that CuO NPs are particularly potent in comparison to other metal oxides in terms of triggering cytotoxicity and genotoxicity (reviewed in: Ivask et al., 2014; Karlsson et al., 2015; and see also: Karlsson et al., 2008; Perreault et al., 2012; Di Bucchianico et al., 2013, for recent examples), less is known about the potential health risks of CuO NPs following inhalation.

Effective prediction of health risks associated with exposure to xenobiotics requires an understanding of the mechanisms by which a given agent induces toxicity and/or elicits a defensive response. However, assessing the risk from exposure to engineered nanomaterials is still a challenge, and a deeper knowledge of the underlying biological mechanisms of nanomaterials is required. High-throughput omics-based approaches to address mechanisms of toxicity are emerging as an integrated component of a new systems biology or ‘systems toxicology’ approach to risk assessment of substances including nanomaterials (Sturla et al., 2014; Costa and Fadeel, 2016). One of the main advantages of omics-based approaches is the fact that omics data sets are amenable to advanced computational analysis enabling both quantification and statistical validation. Furthermore, using global omics approaches, such as transcriptomics, proteomics, or metabolomics, or a combination of these approaches, novel insights into the underlying mechanisms of nanomaterials can be obtained in an unbiased manner, and subtle (low-dose) effects or effects not readily captured by traditional toxicity...
assays can be determined (see, for example, Lucafò et al., 2013; Feliu et al., 2015; Gioria et al., 2016). However, while omics tools have provided unprecedented opportunities to record global changes occurring at the molecular level (reviewed in Costa and Fadeel, 2016), the application of omics data in risk assessment of nanomaterials is still in its infancy (Labib et al., 2016; Nikota et al., 2016). Nonetheless, as pointed out by Wilson et al. (2013), transcriptomics data can support risk assessment by corroborating existing information on mode or mechanism of action; moreover, as chemical risk assessment moves away from a single mechanism of action approach toward a toxicity pathway-based paradigm, omics data could be used to understand the complexities of pathways affected by chemicals which will impact human health risk assessment. In a recent case study to evaluate the utility of toxicogenomics in the risk assessment of benzo[a]pyrene, a well-studied carcinogen, Moffat et al. (2015) concluded that global gene expression analysis may serve as a relatively fast and cost-effective tool for hazard identification and preliminary evaluation of potential carcinogens, and their carcinogenic potency.

In a recent, short-term inhalation study of CuO NPs in rats, organ burden and pulmonary toxicity was assessed (Gosens et al., 2016). The results revealed acute lung inflammation and cellular damage in rats following exposure to CuO NPs for 5 days, with most alterations resolved at 22 days post-exposure. In the present study, we aimed at further investigating the mechanisms behind the responses to inhaled CuO NPs reported by Gosens et al. (2016). Specifically, we focused on: i) assessing selected pro-inflammatory mediators in the lungs and the liver following short-term inhalation exposure to CuO NPs; ii) analysing changes in the transcriptome in lungs of rats following CuO NP exposure and recovery; iii) unravelling the main molecular pathways underlying exposure to CuO NPs; iv) integrating molecular pathways with phenotypical changes, to validate the results. These studies, combining transcriptomics approaches with conventional toxicity end-points, may aid in the development of adverse outcome pathways (AOPs) and could assist in health risk assessment strategies for these NPs.
2. Materials and methods

2.1. Nanomaterials

The CuO nanomaterial was supplied as a nano-sized (15-20 nm according to information provided by the manufacturer) black powder from PlasmaChem GmbH (Berlin, Germany). For detailed characterization by transmission electron microscopy (TEM), to determine the primary particle size, and X-ray-excited Auger electron spectroscopy (XE-AES), to assess the chemical state of the copper, refer to Gosens et al. (2016). While most of the material (core and surface) was in the CuO form, the latter analysis revealed a non-negligible presence of copper hydroxides, resulting from exposure to air. The particle size was found to be 10 nm (Gosens et al. 2016).

2.2. Animals

The animal study was performed as described in Gosens et al. (2016). Adult male (8 week-old) Wistar Unilever outbred rats (strain HsdCpb:WU) were obtained from Harlan Netherlands b.v. (Horst, Netherlands). Rats (332 ± 22 g) were acclimatized for two weeks in Macrolon type IV cages (2-5 individuals per cage) with food and water supplied ad libitum. Exposure and tissue collection were conducted at the National Institute for Public Health (RIVM) at Bilthoven, The Netherlands, under permit number 201300190. Experimental procedures complied with the European directive on the protection of animals for scientific purposes (Directive 2010/63/EU).

2.3. Experimental design

The exposure was conducted using the $C \times T$ protocol (as described in OECD Test Guideline 403) considering a 6 h exposure equivalent per day (the maximum recommended), where $C$ is the concentration (set at 10 mg/m$^3$) of exposure and $T$ is the duration (time) of exposure. In brief, rats were exposed nose-only to a target concentration of 10 mg/m$^3$ and the dose was subsequently expressed as 6 h concentration equivalents of 0, 0.6, 2.4, 3.3, 6.3, and 13.2
7 mg/m³ CuO NPs. Controls were exposed to clean air. Exposure was done during 5 consecutive days, with assessment in two groups, namely 1 day after the final exposure at day 6 (T₆) and after a recovery period of 22 days thus being sampled at day 28 (T₂₈). The animals collected at T₆ and T₂₈ are henceforth referred to as “exposed” and “recovery” groups, respectively. At each sampling time, animals (five per experimental treatment and time-point) were euthanized and lung samples were collected for analyses as seen below. For qPCR and protein analysis, liver tissue was harvested in addition to lung tissue. For the microarray study and subsequent validation studies, animals exposed to the 6 h equivalent doses of 3.3 and 13.2 mg NPs / m³, were analyzed, and we refer to these doses as “low” (LD) and “high” (HD) doses, respectively.

2.4. Histochemical analysis

The lungs were infused via the trachea with neutral aqueous phosphate-buffered 4% solution of formaldehyde as a fixative under a constant pressure of 20 cm water for 1 h. Sections (5 µm thick) were then obtained from lungs fixed in neutral-buffered formalin and embedded in paraffin. Following deparaffination and rehydration the sections were treated with a tetrachrome histochemical procedure to highlight fibres (to detect potential fibrosis) and mucins that combines Alcian Blue and van Gieson’s Elastic Stain. Briefly, lung sections were stained with Alcian Blue (pH 2.5) for acidic polysaccharides for 30 min; Weigert’s Iron Haematoxylin for 10 min (differentiated in ferric chloride), rinsed in 95% v/v ethanol to remove excess iodine and counterstained with van Gieson’s Acid Picro-Fuchsin for 2 min (Martins et al., 2015). After rinsing, dehydration and clearing with xylene, the slides were mounted in DPX resinous medium. Analysis was performed with a Nikon Eclipse TE2000 microscope (Nikon Corporation). Cu was localized in lung histological sections through the Rubeanic Acid (dithiooxiamide) method. In brief, sections were deparaffinated and rehydrated as described above and incubated overnight at 37°C in dithiooxiamide working solution (final concentration 0.005% in 10% m/v sodium acetate from a 0.1% stock solution in absolute ethanol), rinsed in water and counterstained with Nuclear Fast Red. All washing steps and material rinsing were performed with ultra-pure grade water (>16.2 Ω.cm) to avoid Cu cross contamination.
2.5. Immunohistochemistry

Tissue sections were deparaffinized and brought to water, rinsed in phosphate-buffered saline (PBS) and permeabilized for 15 min in 0.1% Triton X-100 in PBS. After rinsing with PBS, the sections were blocked with Image-iT FX signal enhancer (Thermo Scientific) for 30 min and again washed. Sections were incubated overnight with primary antibodies at 4°C, washed in PBS and incubated with the AlexaFluor 488 fluorochrome-tagged goat anti-rabbit IgG polyclonal antibody (Thermo Scientific) for 2.5 h in the dark at room temperature. The sections were then rinsed and mounted with DAPI-containing VectaShield (Vector Laboratories) and analysed immediately. Rabbit anti-rat ECT2 polyclonal antibody (Santa Cruz Biotechnology ref. sc-1005), rabbit anti-rat CCL2 antibody (Invitrogen ref. PA-34505) and the rabbit anti-rat Ki-67 (Invitrogen ref. PA5-16785) were employed as primary antibodies, on the basis of the microarray results, with the working concentrations of 2 µg/mL, 20 µg/mL and 2 µg/mL, respectively. Ki-67 expression was used as a marker of cell proliferation (Schlüter et al., 1993). For each marker, sections from 3-5 individuals per condition were analyzed and representative images are shown.

2.6. PCR analysis of gene expression

RNA was extracted from tissues using the Mag MAX total RNA isolation kit (Ambion) according to the manufacturer's instructions. The RNA content was measured using a NanoDrop 2000 (Thermo Scientific), adjusted to the required concentration and cDNA produced using the High capacity RNA-to-cDNA kit (Applied Biosystems). Real time PCR was conducted using a 7900 RT fast PCR system, in 384-well plates with SDS 2.3 software (Applied Biosystems). TaqMan Kits (rat) with FAM dye (Applied Biosystems) were used including the following primers: Rn00562055 m1 (tumour necrosis factor alpha, TNF-α), Rn00580432 m1 (interleukin 1 beta, IL1β), Rn00565482 m1 (interleukin 1 receptor, IL-1RI), Rn00586403 m1 (chemokine cxcl2, MIP-2), Rn00563409 m1 (interleukin 10, IL10), Rn00577994 g1 (glutathione peroxidase, GSHpox), Rn00563754 m1 (FAS ligand, FASL), Rn00564227 m1 (ICAM-1), Rn00821759 g1
(Metallothionein 1A, MT1A), Rn00588658 g1 (Metallothionein 3, MT3), Rn01531734 g1 (Metallothionein 4, MT4) and the house keeping gene, Rn01479927 g1 (Ribosomal Protein - Large P2, Rplp2). Data are expressed as the fold increase for each gene relative to Rplp2. A 1.8-fold increase or decrease in gene expression was used as cut-off value. Results were analysed using SigmaPlot 6 with analysis of variance (ANOVA) followed by t-test (significance: 95%).

2.7. Cytokine protein analysis

Frozen lung or liver tissue (approximately 5 mm x 5 mm pieces), obtained from the exposed animals, was defrosted and placed in 1.5 mL Eppendorf tubes. The homogenisation process was carried out on ice. Two hundred and fifty microliters of lysis buffer (containing 150 mM NaCl, 50 mM Tris buffer pH 8.0, 1% triton and 1 Sigma antiprotease tablet) was added to each tube and the tissue finely chopped using surgical scissors. Homogenisation was completed using a VWR VDI 12 hand held homogeniser (setting number 3) for 20 s. Samples were then rapidly frozen in liquid N₂ and defrosted at room temperature after which they were sonicated for 20 s in a sonic bath. Samples were centrifuged at 4500 g for 4 min at 4°C and the supernatant transferred to clean tubes, labelled and stored at -80°C until required. Homogenate samples were diluted 1:20 in PBS and the protein content measured using fluorescamine (1.5 mg in 10 mL DMSO). A series of BSA standards ranging from 1000 µg/mL to 0 µg/mL in PBS were prepared. Twenty-five microliters of standard or diluted homogenate was pipetted into a 96-well plate and 25 µL of stock fluorescamine solution added to each well. The plate was placed on a shaker for 2 min, after which the plate was read on a plate reader set to measure at 360 nm excitation and 450 nm. The protein content of each sample was calculated using a linear regression obtained from the standard curve. The cytokine measurements were carried out using the Magpix (BioRad) technology. The master mix of coated magnetic beads was prepared based on the selected cytokines (IL-1α, IL-1β, IL-6, IL-12, IL-13, TNF-α and MIP-2). The beads were added to duplicate wells on a 96-well plate and diluted tissue homogenates were added. A series of cytokine standards were included. After
incubation, the beads were washed and treated with detection antibody, followed by another
series of washes. Finally, phycoerythrin (PE) labelled streptavidin was added. The
fluorescence obtained in each well was measured using a Magpix plate reader, and the
concentration of the respective cytokines was calculated from the standards. Data were
analysed by ANOVA using a general linear model and Tukey’s post test. Significance was set
at 5%.

2.8. Microarray hybridization
Total RNA was extracted from individual rat lung samples using the AllPrep DNA/RNA Mini kit
for animal tissue (Qiagen), following the manufacturer’s instructions. Total RNA was quantified
using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and RNA quality and
integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). All samples
showed RNA integrity numbers (RIN) above 7, which is regarded as adequate for microarray
analyses (Schroeder et al., 2006). Details of the microarray procedures can be found in
Halappanavar et al. (2015). In brief, labelled cDNAs and cRNAs were synthesized using the
Linear Amplification Kit (Agilent Technologies) from 200 ng of total RNA per individual rat lung
sample or Universal Rat Reference RNA (Agilent Technologies). T7 RNA polymerase-
transcribed cRNA from experimental samples were labelled with Cyanine-5 and UMRR was
labelled with Cyanine-3. An equimolar amount of UMRR cRNA was mixed with each
experimental cRNA and was hybridized to Agilent SurePrint G3 Rat 8 × 60K microarrays
(Agilent). After washing, microarray slides were scanned in a G250B scanner (Agilent). Data
was retrieved using the Feature Extraction 10.7.3 software (Agilent). Data analysis is described
below.

2.9. DNA methylation analysis
The level of methylation in inflammation-related genes was determined by real-time
polymerase chain reaction (RT-PCR) using a PCR array designed for 22 rat genes, based on
the principle of impaired transcription of methylated genomic DNA that has been cleaved by
restriction enzymes at methylation sites. DNA extraction was performed as described above (Qiagen). The EpiTect Methyl II PCR Array was used, coupled with the Epitect Methyl II DNA restriction kit (Qiagen), following manufacturer instructions. Analysis was run on a 7500 model qRT-PCR system (Applied Biosystems), using the RT² SYBR Green qPCR Mastermix (Qiagen). The results are expressed as the percentage of methylated nucleotides per gene sequence.

2.10. Bioinformatics analysis

All statistics and computational analyses were performed using the software R 3.2 (Ihaka and Gentleman, 1996) and the significant threshold was set at $\alpha = 0.05$. Analysis of DNA methylation data was conducted through the $t$-test adapted for multiple comparisons by applying the Bonferroni correction for FDR, Pearson's product-moment correlation $r$ statistics and heatmaps (on log-transformed data). Microarray data were analysed as described in Halappanavar et al. (2015). Briefly, from a randomized block design (Kerr and Churchill 2007) data were normalized through Locally Weighted Scatterplot Smoothing (LOWESS) regression modelling. The Microarray Analysis of Variance (MAANOVA) package for R (Wu et al., 2003) was used to determine the statistical significance of the differentially expressed genes (DEGs). The $F$s statistic with residual shuffling (Cui et al., 2005) was used to test between-treatment effects and to obtain $p$-estimates, which were corrected for False Discovery Rate (FDR) for multiple testing (Benjamini and Hochberg 1995). Fold-change ratios were based on the least-square means. Any given microarray probe (gene) was considered to be expressed if the signal was above background in at least 4 of 5 samples in one or more experimental conditions and differential regulation was considered significant if the FDR-corrected $p$-value was $< 0.05$ and the normalized fold change (NFR) $> 2$. Hierarchical clustering was achieved through the Ward-Pearson’s method using the EMA package for R (Servant et al., 2010). Venn diagrams were obtained using Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Data were deposited in the NCBI Gene Expression Omnibus Database under accession number GSE86390.
Gene ontology (GO) enrichment and KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway analyses (Kanehisa et al., 2014) on DEGs were performed using the software DAVID (Huang et al., 2009a, 2009b). Fold-enrichment was considered significant if > 1.5. The significance of the pathways was estimated through a modification of Fisher’s Exact Test termed EASE (Expression Analysis Systematic Explorer) score (α < 0.05) available through DAVID (Hosack et al., 2003). Comparative causal networks pathway analysis (Krämer et al., 2014) was performed on differentially expressed genes using the Ingenuity Pathway Analysis (IPA) software (content version 24718999) software (license from Ingenuity Systems, Redwood City, CA). The outputs were filtered by p-value (α) < 0.05 and activation Z-score > 2 or < -2.

3. Results

3.1 Real time PCR and protein analysis of lung and liver tissue

In a recent study (Gosens et al., 2016), rats were exposed to CuO NPs for 5 days, with 1- and 22-day post-exposure evaluations of (sub)acute toxicity. To further evaluate whether exposure to CuO NPs triggers an inflammatory response, we determined the expression of a panel of pre-selected pro-inflammatory markers in the lungs (the primary target organ) and in liver (as NPs have been shown to cross the air-lung barrier and translocate to distal organs, including the liver). Exposure to CuO NPs for 5 days resulted in a dose-dependent increase in the expression of mRNA for CXCL2 (or MIP-2α), IL1-β and TNF-α in lung tissue, with results achieving statistical significance at a 6 h equivalent dose of 3.3 mg/m³ for CXCL2 and IL-1β, while a 6 h equivalent dose of 13.2 mg/m³ was required to increase TNF-α (Table S1). For the liver tissue, Mt1a was found to be downregulated 1.9 fold at a 6 h equivalent dose of 3.3 mg/m³ (Table S1). No changes in any of the other genes examined were observed in the liver (not shown). We also determined the protein expression of a panel of pro-inflammatory markers (Brown et al., 2014). For the lung tissue, IL-1α was significantly increased at the 0.6, 3.3 and
13.2 mg/m³ 6 h equivalent doses compared with the control (Table S2). This pattern was similar for IL-1β, although there was no significant increase at the 6 h equivalent dose of 3.3 mg/m³. No statistically significant increase was observed at any concentration for the TNF-α protein. In the case of CXCL2 (MIP-2α), the protein level was significantly greater than the control at 6 h equivalent doses of 0.6, 3.3 and 13.2 mg/m³. For the liver tissue, CuO NPs did not cause a significant increase in any of the cytokines investigated. Instead, at 6 h equivalent doses of 6.3 and 13.2 mg/m³, there was a significant reduction in the amount of the IL-1β protein (Table S2).

3.2. Microarray analysis following short-term inhalation exposure to CuO NPs

Based on our previous study, which demonstrated acute lung inflammation in rats exposed to CuO NPs for 5 days (Gosens et al., 2016), and on the results above, showing that pro-inflammatory markers were upregulated in the lungs, but not in the liver, after 5 days of exposure, we proceeded to further examine lung tissue samples for changes in gene expression using cDNA microarrays. Of approximately 30,000 probes on the microarray slide, 1038 were found to be significantly dysregulated in at least one experimental condition, relative to control, most of which corresponded to up-regulation of gene expression (Fig. 1). The largest number of DEGs was found at 1-day post-exposure in the lungs of rats exposed to a 6 h equivalent dose of 13.2 mg/m³ (HD), whereas the lowest number was observed in rats exposed to a 6 h equivalent dose of 3.3 mg/m³ (LD) and allowed to recover for 22 days (Fig. 1). The highest normalized fold change (NFR = 87) recorded for any single gene in the lungs of rats exposed to HD 1-day post exposure was for the c-c motif chemokine ligand 2 (Ccl2) (NM_001105822), encoding for CCL2, also termed monocyte chemoattractant protein-1, MCP-1 (Table 1). Metallothioneins 1A and 2A (Mt1a, Mt2a), whose upregulation is an anticipated response to increased levels of intracellular metal ions from NPs (Tuomela et al., 2013), were both upregulated in HD rats (4.6 and 3.4 fold, respectively), but only Mt1a in LD animals (2-fold).
3.3. Gene ontology enrichment and pathway analysis of the transcriptomics data

Gene ontology (GO) analyses of the microarray data revealed that of 24 genes related to inflammation (GO:0002526), 23 were upregulated in the lungs of rats exposed to 13.2 mg/m³ NPs 1-day post-exposure (NFR ranging between 2 and 11), with the exception of the Klotho gene (NM_031336.1), which was downregulated in animals subjected to this treatment. Interestingly, lipopolysaccharide (LPS)-induced inflammation in rat lungs has previously been associated with downregulation of the Klotho gene (Ohyama et al., 1998). On the other hand, animals sampled at day 6, but subjected to the lowest dose, yielded only 10 deregulated genes from the same gene ontology, with consistently lower NFR values (2 and 4). Only one of the genes associated with immune response was significantly dysregulated in animals exposed for 28 days and only at the highest dose, namely complement component 4 binding protein alpha (C4bpa) (NM_012516), NFR = 2. We then retrieved twenty-five pathways from the same gene list following KEGG analyses, of which 10 fitted the significance criteria (Table 2), altogether comprising approximately 100 genes (Fig. 2). Overall, the mRNA levels of these genes yielded a similar pattern between animals exposed to either dose and collected at 1-day post-exposure, thus forming a cluster distinct to the animals collected following the recovery period (22-day post-exposure), which was mainly defined by upregulation of gene expression. The majority of the genes at 1-day post-exposure were related to immune response and cell cycle (Fig. 2), in accordance with the results from the GO analysis, which showed that the most significant pathways were related to cell cycle and chemokine signalling pathways.

Pathway analysis performed using IPA yielded more detailed information on the molecular pathways potentially activated by exposure to aerosolized CuO NPs. When comparing the disease and function pathways between the treatments, the most significantly activated (Z-score > 2) related to cell proliferation and inflammation, the exception being the pathway ‘concentration of lipids’ in animals collected at 1-day post-exposure (Fig. 3A). The results integrate pathways obtained from both in vivo and in vitro experiments with pulmonary models.
or whole-lung tissue. The pathways related to ‘cell proliferation of carcinoma cell lines’ were
the most significantly activated of nine pathways ($Z \approx 3$). None of the pathways were found to
be activated in animals sampled after recovery (i.e., 22-day post-exposure). These
observations prompted a search for genes associated with epithelial lung cell/cancer cell
proliferation, the most significant of which was epithelial cell transforming protein (Ect2,
BC168962), which was consistently present in the aforementioned pathways. The expression
of this oncogene attained NFR 3.34 and 7.46 relative to control, in LD and HD rats at 1-day
post-exposure, respectively, having returned to control levels following recovery, 22 days post-
exposure. Ccl2 was also consistently present in pathways related to inflammation such as cell
migration, as well as cell proliferation pathways (Fig. 3B), hence yielding a significant overlap
with Ect2.

Using the IPA tool, the genes most significantly related to the upstream regulation of the
identified pathways were Nos2 and Insig1. The former reached a positive Z-score of 3.6
(activation) in HD animals at 1-day post-exposure (not significant in any other treatment),
whereas the latter attained a Z score of -3.8 (deactivation) in animals subjected to either dose
(Fig. 3A).

3.4. Histopathological analysis and immunostaining for ECT2, Ki67 and CCL2
Next, histopathological evaluation and immunohistochemistry was performed to validate the
microarray results. We noted an intrusion of immune cells into alveoli (indicating lung alveolitis)
at 1-day post-exposure in a seemingly dose-response manner, returning to levels similar to
control animals after recovery (Fig. S1), in line with the previous study by Gosens et al. (2016).
The inflammatory response tended to be focal in the lungs of animals exposed to either dose,
although the foci were more disseminated and larger in animals exposed to the highest dose
(13.2 mg/m$^3$). Overall, the two most significant alterations found in alveolar epithelia were
intrusion of inflammatory cells (monocytes and macrophages) and proliferation of epithelial
cells (the constituents of alveolar septa), in either case exhibiting the same dose-response
pattern after the five days of exposure (Fig. S1A,C,E), subsequently returning to the normal condition, similar to the control animals (Fig. S1B,D,E). Additionally, epithelial cell hyperplasia was accompanied by hypertrophy of cells, thus contributing to the thickening of septa. Cell death was limited to necrotic macrophages in the most severe inflammatory foci in HD rats, but was never diffuse. Emphysema and fibrosis were rare or absent in the lungs of rats from either treatment (data not shown). The latter findings are also fully in line with the previous study by Gosens et al. (2016) where emphysema was rare and fibrosis was absent. Alcian Blue staining indicated increased secretion of mucous in bronchial epithelia in HD-exposed animals, accompanied by moderate proliferation of goblet cells, but without any signs of hyperplasia (data not shown). Copper deposits were occasionally found in HD rats at 1-day post-exposure, being rare in LD animals and absent in the lungs of control animals or in the recovery group. These deposits were occasionally found in alveoli (extracellular deposits) or, more frequently, in immune cells (Fig. S1E).

We then determined the expression of Ki67 in the lung in order to corroborate increased cell proliferation, as well as the detection of the ECT2 and CCL2, which were both predicted to be upregulated based on the microarray data. The ECT2 protein was successfully localized in alveolar epithelia (Fig. 4) being present in the nuclei of cells of samples from all experimental treatments, including controls. This pattern of expression was expected, since ECT2 is known to regulate DNA synthesis and cytokinesis (Tatsumoto et al., 1999), processes which occur normally in type II alveolar cells. Interestingly, ECT2 was found to be increased in foci of hyperplastic cells, low-to-moderate in the case of LD-exposed animals at day-1 post-exposure, and strong expression was noted in the case of rats exposed to the HD at the same time-point (Fig. 4C,E). In contrast, the expression of ECT2 after the recovery period displayed a similar pattern to that of controls (Fig. 4B,D,F). In the HD animals at day-1 post-exposure, the ECT2 protein was also localized in the cytoplasm, yielding a strong fluorescent signal, whereas this less prominent in the LD animals The same pattern was noted for Ki67, a marker of cell proliferation (Fig. 5). When compared to controls (Fig. 5A), LD and HD rats yielded a strong
signal for the Ki67 protein in a clear dose-response manner in hyperplasic foci (Fig. 5C,E), thereby confirming proliferation of alveolar epithelial cells, whereas after recovery, the epithelial expression returned to control values (Fig. 5B,D,F). Similarly, CCL2 (Fig. 6) was overexpressed in the lungs of LD and HD rats and yielded a strong signal in both epithelial and immune cells, mostly in hyperplasic foci (Fig. 6C,E), where CCL2 localized mostly within immune cells in controls (Fig. 6A). CCL2 expression was higher in HD than in LD day-1 post-exposure, suggesting a dose-response, and normalized after recovery (i.e., at day-22 post-exposure) (Fig. 6B,D,F). Thus, the expression of CCL2 and ECT2 in lungs of rats exposed to CuO NPs was in agreement with the levels of transcripts of the respective genes (see Table 1).

3.5. No evidence for epigenetic events underlying the gene expression results

Next, a targeted epigenetics study was conducted to address the potential role of DNA methylation in the regulation of inflammatory responses following exposure of rat lungs to CuO NPs. To this end, DNA methylation of a selected panel of inflammation-related genes was investigated using a PCR array. However, the levels of gene methylation were overall low (typically totalling < 1%) and highly variable for all the genes that were investigated (Fig. S2A). Among the 22 genes, only Fas-associated death domain (Fadd) yielded a significant difference versus the controls (Fig S2A), in LD animals evaluated at 1-day post-exposure, suggesting increased methylation (Fig. S2B). Nonetheless, no correlation was found between Fadd gene methylation and gene expression, as determined by our microarray analysis. When combining the methylation levels of all the analysed genes, no significant differences were found between treatments, in spite of a trend towards reduced DNA methylation in animals exposed to HD at 1-day post-exposure. Overall, only marginal effects of DNA methylation on inflammation could be inferred and no significant correlations were found between gene expression results retrieved from microarray experiments and the percentage of gene methylation (Pearson r, p > 0.05).
4. Discussion

In the present study, short-term inhalation exposure of rats to CuO NPs resulted in upregulation of pro-inflammatory cytokines and chemokines in the lungs, while no such changes were detected in the liver. We therefore focused subsequent studies on the lungs, the primary target organ in this model. Global gene expression analyses were performed on the lungs of rats exposed via inhalation for 5 consecutive days to two doses (low and high) of CuO NPs. Pathway analysis of the transcriptomics data showed that cell proliferation/survival and inflammation in rat lungs were the main processes affected by inhalation exposure to CuO NPs. Validation studies confirmed the upregulation of specific molecules, i.e., the oncoprotein, ECT2, and the chemokine, CCL2, in the lungs, while epithelial cell hyperplasia and cell proliferation was evidenced by conventional histological analysis and expression of the proliferation marker, Ki67. Importantly, while lung inflammation is in line with our recently published findings on short-term inhalation exposure to CuO NPs (Gosens et al., 2016), microarray analysis identified molecular responses that may serve to explain the mode of action of the CuO NPs. In particular, the finding of epithelial cell proliferation is relevant as alveolar type II cells are known to be able to initiate lung adenocarcinoma, a major form of non-small cell lung cancer (Lin et al., 2012). However, one should not infer from these observations that a short-term inhalation exposure to CuO NPs would translate into lung cancer in rats or humans, as the lung pathology and gene expression changes returned to normal after the recovery period. Nevertheless, the present findings suggest that further study of the impact of long-term exposure to CuO NPs on neoplastic processes is warranted. Overall, it is important to note that the molecular pathways captured by the current transcriptomics approach were largely related to, and validated by, conventional toxicological end-points. This, therefore, serves to validate, in a broad sense, the use of such approaches for the assessment of NP effects.

Oxidative stress has previously been implicated as a major cause of CuO NP-induced cell death in both in vitro and in vivo models (Fahmy and Cormier, 2009; Jing et al., 2015).
However, our KEGG and IPA analyses of the microarray data did not yield evidence for pathways related to oxidative stress and genes related to oxidative stress were not among the most significantly dysregulated genes, nor did we detect significant changes in genes or pathways related to the promotion (or inhibition) of cell death. The latter results are supported by the paucity of apoptotic cells in alveolar epithelia of exposed animals. It should be noted that several markers of cell damage were detected in the broncho-alveolar lavage (BAL) fluid obtained from rats at 1-day post-exposure in our previous study, including elevated levels of lactate dehydrogenase (LDH) and total cellular protein, both markers of general cell damage, as well as elevated gamma-glutamyl transferase (GGT) levels, a marker for alveolar type II epithelial cell and/or bronchiolar secretory cell damage (Gosens et al., 2016). The changes in cell damage markers returned to baseline levels after the recovery period. However, cellular damage is not necessarily reflected at the transcriptional level and the present results in combination with our previous study underline the value of applying more than one method or end-point when evaluating biological responses to NPs. The fact that cell death related genes or pathways were not affected in the present study is in apparent contrast to the findings of Hanagata et al. (2011), who noted that genes related to cell cycle and cell death were deregulated in A549 lung carcinoma cells exposed to CuO NPs. Notwithstanding the fact that in vitro models may not capture the orchestrated in vivo responses to NPs, it should also be noted that the A549 cell line is derived from a lung adenocarcinoma and may not be a faithful model of normal bronchial epithelium (Feliu et al., 2015) and that the study by Hanagata et al. (2011) was designed to assess acute toxicity, using relatively high doses and a short exposure time (24 h), in contrast to our in vivo study in which rats were exposed for 5 consecutive days to a range of doses (low to high) (Gosens et al., 2016). Moreover, expression of genes related to metal homeostasis and detoxification, namely the metallothioneins, Mt1a and Mt2a, was only modestly upregulated according to our microarray study, in comparison to genes involved in inflammation-related pathways. Furthermore, using real-time PCR, we noted a minor decrease in the expression of Mt1a in liver tissue in the low-dose exposure group, but not at the higher dose, while no changes were observed in the lung tissue. These results, together
with the scant amount of copper deposits evidenced by our histochemical analysis, indicated that the Cu burden may be rapidly mitigated and that this may not be the direct causative trigger of the deleterious effects. In fact, in our previous study, we could show that the Cu was cleared completely from the lungs within the recovery period suggesting that the clearance rate is faster than expected for poorly soluble particles (Gosens et al., 2016). We also noted here and in our previous study (Gosens et al., 2016) that common markers of lung injury such as emphysema and lung fibrosis were essentially absent in rats exposed to CuO NPs. Instead, our results suggest that the adverse outcomes of inhalation exposure of CuO NPs may be linked to the induction of inflammation and a concomitant proliferation of lung alveolar epithelial cells.

We found that short-term exposure via inhalation to CuO NPs promoted overexpression of the ECT2 oncoprotein whose overexpression has previously been proposed as a prognostic biomarker for early-stage lung adenocarcinoma (Murata et al., 2014). ECT2 expression was also associated with a poor prognosis for patients with non-small-cell lung cancers, and the induction of exogenous expression of ECT2 in fibroblasts or fibroblast-like cells promoted cellular invasive activity (Hirata et al., 2009). To this effect are added inflammation-related responses that we could also verify histologically. In fact, even though the alterations to lung bronchoalveolar tissue in rats exposed to CuO NPs were found to be reversible at 22 days post-exposure, the lesions were distributed as foci where infiltration of inflammatory cells and hyperplasia coincided, thus suggesting a link between the two effects: inflammation and proliferation of alveolar epithelial cells. The chemokine CCL2 (MCP-1) is a known chemotactic agent for monocytes and has been found to play a key role in mediating lung inflammation, for instance, following hypoxia (Chao et al., 2011), cigarette smoking (Koth et al., 2010), and LPS challenge (Mercer et al., 2014). Moreover, CCL2 expression and recruitment of monocytes-macrophages are correlated with poor prognosis and metastatic disease in human breast cancer (Qian et al., 2011). The protein is secreted by a broad range of cells, which explains its localization within both immune and epithelial cells in the present study. Previous work has
shown that welding-related metal oxide nanoparticles trigger the production of pro-inflammatory cytokines and chemokines, including CCL2, in macrophage-like THP.1 cells (Andujar et al., 2014), while Brown et al. (2014) reported that silica NPs, but not iron NPs, are potent inducers of CCL2 (MCP-1) secretion in the murine J774A.1 macrophage cell line. Moreover, Driscoll et al. (1996) reported that 90-day inhalation exposure to carbon black resulted in a dose-dependent upregulation of MCP-1 mRNA with increased inflammation and alveolar epithelial cell hyperplasia. In the present work, a prominent (almost 90-fold) upregulation of the Ccl2 gene was observed in HD-exposed animals. Additionally, the upregulation of CCL2 protein expression evidenced here supports a role of this molecule in the regulation of inflammatory processes following short-term inhalation to CuO NPs. Interestingly, in a recent bioinformatics study of transcriptomics data in public repositories, Ccl2 was identified as a member of a group of genes related to pulmonary disease (fibrosis) following exposure to several different nanomaterials (Williams and Halappanavar, 2015).

Overall, these findings show that CCL2 may have a ubiquitous role in the activation of inflammatory responses (in the lungs) following NP challenge, specifically inhalation exposure of CuO NPs. In fact, the combination of lung alveolar hyperplasia and inflammation (phenotype level) and expression of Ccl2 and Ect2 (genotype level) may suggest a novel biomarker ‘footprint’ and could also guide the development of AOPs that could reflect inhalation exposure to NPs.

Epigenetic mechanisms of toxicity of nanomaterials including changes to DNA methylation, histone modification and microRNA expression have been postulated although the evidence is scant (Shyamasundar et al., 2015). Patil et al. (2016) recently uncovered epigenetic alterations including DNA methylation changes in a lung fibroblast cell line in response to TiO2 and ZnO NPs. Other recent studies demonstrated that alterations in gene-specific methylation corresponded with a pulmonary inflammatory response to multi-walled carbon nanotubes in mice after 7 days of exposure, but not after acute (24 h) exposure (Brown et al., 2016). Moreover, modest epigenetic effects including global genome methylation changes have also
been described in the lungs of mice exposed to CuO NPs after only 24 h, at doses that caused
significant cellular damage in the lungs as evidenced by LDH release (Lu et al., 2016).
However, in the present study, the inflammatory response could not be explained by aberrant
DNA methylation of inflammation-related genes, although a non-significant trend towards
hypomethylation in the HD group at 1-day post-exposure (but not at recovery) was observed.

Taken together, the present study has confirmed that a short-term exposure via inhalation to
CuO NPs causes dose-dependent inflammation in the lungs of rats and we could show that
this coincides with proliferation of bronchoalveolar epithelium and upregulation of the
oncoprotein ECT2 and the chemokine CCL2. Importantly, in spite of the known role of both
ECT2 and CCL2 in lung neoplastic disease, as discussed above, no studies to date have been
dedicated to their combined effects or interactions in tumorigenesis, nor to their putative role
in malignant transformation in bronchoalveolar epithelia following (long-term) exposure to NPs.
The current transcriptomics study has shown that omics-based approaches provide a means
to achieve a comprehensive and unbiased view of nanomaterial effects in living organisms and
may shed light on the mode of action of NPs, provided that these tools are combined with
adequate phenotypic anchoring of the data with assessment of conventional end-points of
toxicity.

Conflicts of interest
The authors do not declare any conflicts of interest.

Supplementary data to this article can be found online at http://............................
The transparency document associated with this article can be found in the online version.

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exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in

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expression profiling of immune-competent human cells exposed to engineered zinc oxide or


**Figure legends**

**Fig. 1.** Microarray analysis of rat lung tissue following inhalation exposure to CuO NPs. Venn diagrams of downregulated (A) or upregulated (B) genes that comply with at least 2-fold normalized change relatively to controls and FDR-corrected $p < 0.05$, for each experimental treatment, *i.e.*, animals exposed to a high dose (HD) or low dose (LD) of CuO NPs for 5 days and analyzed 1 day after the last exposure (‘exposure’) or after a recovery period of 22 days (‘recovery’).

**Fig. 2.** Heatmap illustrating the findings from the KEGG pathway analysis, which retrieved 98 genes pertaining to the pathways listed in Table 2. Cluster analysis was done though Ward’s method employing Pearson’s $r$ as metric. Colour scale was trimmed at $|1.5|$ to enhance contrast.

**Fig. 3.** Ingenuity Pathway Analysis (IPA) results. A) Comparative analysis of disease and function pathways between the four experimental conditions, *i.e.*, exposure for 5 days to a 6 h equivalent dose of 13.2 (HD) and 3.3 (LD) mg/m$^3$ CuO NPs, respectively, plus recovery for 22 days after exposure to HD and LD for 5 days, and significant molecules integrating potential upstream regulation. B) Transcriptional network of disease and function pathways retrieved from rats exposed to HD for five days. Significance thresholds were set at $Z > |2|$ and $p < 0.05$.

**Fig. 4.** Expression of the oncoprotein, ECT2 in lung alveoli of control animals or rats exposed to CuO NPs for 5 days at the indicated doses and analyzed 1 day post-exposure and after a recovery period of 22 days (day 28). Lungs of rats exposed to clean air as a control (A,B) and after a low dose exposure (C,D) or after a high dose exposure (E,F). Note the expression of ECT2 in the cytoplasm of epithelial cells (*), especially in the lungs of HD animals. Scale bars: 25 µm.
Fig. 5. Expression of the cell proliferation marker, Ki67 in lung alveoli of control animals or rats exposed to CuO NPs for 5 days and analyzed 1 day post-exposure and after a recovery period of 22 days (day 28). Lungs of rats exposed to clean air as a control (A,B) and after a low dose exposure (C,D) or after a high dose exposure (E,F). Arrows examplify probe signals. Scale bars: 25 µm.

Fig. 6. Expression of the chemokine, CCL2 in lung alveoli of control animals or rats exposed to CuO NPs for 5 days and analyzed 1 day post-exposure and after a recovery period of 22 days (day 28). Lungs of rats exposed to clean air as a control (A,B) and after a low dose exposure (C,D) or after a high dose exposure (E,F). Arrows examplify probe signals. Scale bars: 25 µm.
### Table 1. Summary of the most significantly up- and downregulated genes.

<table>
<thead>
<tr>
<th>Exposure, 3.3 mg/m³</th>
<th>Accession</th>
<th>Gene</th>
<th>Gene name</th>
<th>FDR</th>
<th>NFC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulation</td>
<td>NM_130741</td>
<td>Len2</td>
<td>Lipocalin 2</td>
<td>= 0</td>
<td>19.3</td>
<td>Iron uptake and transport</td>
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<tr>
<td>Downregulation</td>
<td>NM_012666</td>
<td>Tac1</td>
<td>Tachykinin 1</td>
<td>= 0</td>
<td>-8.9</td>
<td>Intracellular signalling (cell adhesion-related)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Exposure, 13.2 mg/m³</th>
<th>Accession</th>
<th>Gene</th>
<th>Gene name</th>
<th>FDR</th>
<th>NFC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulation</td>
<td>NM_00110582</td>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2 / Monocyte chemoattractant protein 1</td>
<td>= 0</td>
<td>87.3</td>
<td>Pro-inflammatory response</td>
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<tr>
<td>Downregulation</td>
<td>NM_012666</td>
<td>Tac1</td>
<td>Tachykinin 1</td>
<td>= 0</td>
<td>-12.7</td>
<td>Intracellular signalling (cell adhesion-related)</td>
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<table>
<thead>
<tr>
<th>Recovery (3.3 mg/m³)</th>
<th>Accession</th>
<th>Gene</th>
<th>Gene name</th>
<th>FDR</th>
<th>NFC</th>
<th>Remarks</th>
</tr>
</thead>
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<tr>
<td>Upregulation</td>
<td>NM_134418</td>
<td>Gp2</td>
<td>Glycoprotein 2 (zymogen granule membrane)</td>
<td>0.9194</td>
<td>3.0</td>
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<tr>
<td>Downregulation</td>
<td>NM_012543</td>
<td>Dbp</td>
<td>D site of albumin promoter (albumin D-box) binding protein</td>
<td>= 0</td>
<td>-4.3</td>
<td>Circadian rhythm regulation</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Recovery (13.2 mg/m³)</th>
<th>Accession</th>
<th>Gene</th>
<th>Gene name</th>
<th>FDR</th>
<th>NFC</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>Upregulation</td>
<td>NM_012543</td>
<td>Dbp</td>
<td>D site of albumin promoter (albumin D-box) binding protein</td>
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<td>Downregulation</td>
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<td>Complement component 4 binding protein, a</td>
<td>= 0</td>
<td>2.2</td>
<td>Immune-related</td>
</tr>
</tbody>
</table>

Rats were exposed via inhalation for 5 days to a low or high dose and analysis was done at day-1 post-exposure ('exposure') and after a recovery period of 22 days ('recovery').

FDR-p, false discovery rate-corrected p-value; NFC, normalized fold change relatively to controls.
Table 2. KEGG pathway analyses of significantly deregulated genes.

<table>
<thead>
<tr>
<th>KEGG pathway term</th>
<th>No. Genes</th>
<th>% Coverage</th>
<th>Fold Enrichment</th>
<th>Benjamini-corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rno04110:Cell cycle</td>
<td>17</td>
<td>2.27</td>
<td>2.969</td>
<td>0.022*</td>
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<tr>
<td>rno04062:Chemokine signalling pathway</td>
<td>20</td>
<td>2.67</td>
<td>2.574</td>
<td>0.016*</td>
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<tr>
<td>rno00900:Terpenoid backbone biosynthesis</td>
<td>6</td>
<td>0.80</td>
<td>9.432</td>
<td>0.012*</td>
</tr>
<tr>
<td>rno04610:Complement and coagulation cascades</td>
<td>12</td>
<td>1.60</td>
<td>3.773</td>
<td>0.009*</td>
</tr>
<tr>
<td>rno04940:Type I diabetes mellitus</td>
<td>10</td>
<td>1.34</td>
<td>3.668</td>
<td>0.037*</td>
</tr>
<tr>
<td>rno04261:NOD-like receptor signalling pathway</td>
<td>10</td>
<td>1.34</td>
<td>3.550</td>
<td>0.039*</td>
</tr>
<tr>
<td>rno05332:Graft-versus-host disease</td>
<td>9</td>
<td>1.20</td>
<td>3.884</td>
<td>0.036*</td>
</tr>
<tr>
<td>rno04612:Antigen processing and presentation</td>
<td>12</td>
<td>1.60</td>
<td>3.001</td>
<td>0.033*</td>
</tr>
<tr>
<td>rno04142:Lyososome</td>
<td>14</td>
<td>1.87</td>
<td>2.633</td>
<td>0.035*</td>
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<tr>
<td>rno04606:Cytosignificant kine-cytokine receptor interaction</td>
<td>19</td>
<td>2.54</td>
<td>2.123</td>
<td>0.045*</td>
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<td>rno04623:Cytosolic DNA-sensing pathway</td>
<td>8</td>
<td>1.07</td>
<td>3.827</td>
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<tr>
<td>rno04514:Cell adhesion molecules (CAMs)</td>
<td>15</td>
<td>2.00</td>
<td>2.231</td>
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<td>rno04620:Toll-like receptor signalling pathway</td>
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<td>rno05330:Allograft rejection</td>
<td>8</td>
<td>1.07</td>
<td>3.322</td>
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<td>rno00982:Drug metabolism</td>
<td>9</td>
<td>1.20</td>
<td>2.751</td>
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<td>rno00760:Nicotinate and nicotinamide metabolism</td>
<td>5</td>
<td>0.67</td>
<td>4.784</td>
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<td>rno04710:Circadian rhythm</td>
<td>4</td>
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<td>rno05322:Systemic lupus erythematosus</td>
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<td>rno00100:Steroid biosynthesis</td>
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<td>rno03320:PPAR signalling pathway</td>
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<td>rno05416:Viral myocarditis</td>
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<td>rno00072:Synthesis and degradation of ketone bodies</td>
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<td>rno00650:Butanoate metabolism</td>
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<td>3.335</td>
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<td>rno04914:Progesterone-mediated oocyte maturation</td>
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*Significant pathways (EASE p < 0.05 and fold enrichment > 1.5).
Fig. 2
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<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<td><img src="image3.png" alt="Image" /></td>
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<tr>
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Fig. 5

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<td><img src="image" alt="D" /></td>
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<tr>
<td><strong>High dose (13.2 mg/m³)</strong></td>
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Note: Images show cellular structures under different conditions.
Fig. 6

<table>
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<tr>
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<th>Recovery</th>
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<tbody>
<tr>
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<tr>
<td>Low dose (6.3 mg/m³)</td>
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<td>High dose (13.2 mg/m³)</td>
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