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Genetic Toxicity Test of *o*-Nitrotoluene by Ames, Micronucleus, Comet Assays and Microarray Analysis

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Abstract

o-Nitrotoluene is used to synthesize artificial dyes and raw materials of urethane resin. In this study, we have carried out *in vitro* genetic toxicity tests and microarray analysis to understand the underlying mechanisms and the mode of action of toxicity of *o*-nitrotoluene. TA1535 and TA98 cells were treated with *o*-nitrotoluene to test its toxicity by basic genetic toxicity test. Ames and two new *in vitro* micronucleus and COMET assays were applied using CHO cells and L5178Y cells, respectively. In addition, microarray analysis of differentially expressed genes in L5178Y cells in response to *o*-nitrotoluene was analyzed using Affymatrix genechip. The result of Ames test was that *o*-nitrotoluene treatment did not increase the mutations both in base substitution strain TA1535 and in frame shift TA98. *o*-Nitrotoluene has not increased micronuclei in CHO cells. But *o*-nitrotoluene increased DNA damage in L5178Y cell. Two-hundred two genes were initially selected as differentially expressed genes in response to *o*-nitrotoluene by microarray analysis and forty four genes among them were over 2 times of log fold changed. These forty four genes could be candidate biomarkers of genetic toxic action of *o*-nitrotoluene related to induction of mutation and/or induction of micronuclei and DNA damage. Further confirmation of these candidate markers related to the DNA damage will be useful to understand the detailed mechanism of action of *o*-nitrotoluene.

Keywords: *o*-Nitrotoluene, Ames test, COMET, MN assay, Microarray, S9 fraction

o-Nitrotoluene is used to synthesize artificial dyes and raw materials of urethane resin. It is a high production chemical and caused tumor formation at multiple sites. A study of *o*-nitrotoluene-induced mouse intestinal tumors indicate that the *Wnt*/β-catenin signaling pathway, K-ras/MAPKinase pathway and p53 pathway, and cyclin D1 play a role in large intestinal tumor development following chemical exposure[14]. And a recent study was identified genes that are relevant to the development of cancer. The categories of functions related to cancer development included binding activity, growth and proliferation, cell cycle progression, apoptosis, and invasion and metastasis[11]. Gene expression studies of human mesothelioma and mesothelioma cell lines revealed activation of similar signaling pathways[9,16].

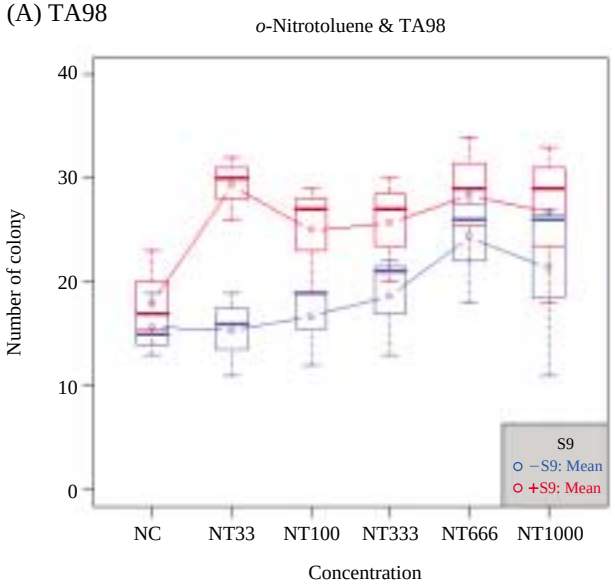
o-Nitrotoluene (3.0 to 1,000 μg/plate) was not mutagenic in *Salmonella typhimurium* strain TA98, TA100, TA1535, or TA1537, with or without aroclor-induced rat or hamster liver S9[7,12]. Significantly increased SCE frequencies were observed in cultured CHO cells treated with *o*-nitrotoluene with S9; an equivocal response was seen without S9[5,12]. Due to *o*-nitrotoluene-induced cell cycle delay in the trial without S9, an extended culture time was employed to permit accumulation of sufficient second-division metaphase cells for analysis. *o*-Nitrotoluene did not induce chromosomal abbreviation (Abs) in cultured CHO cells with or without S9[5,12].

Although the genetic toxicity and carcinogenicity of *o*-nitrotoluene has been reported, no further study has not been carried out to find out the underlying mechanism and mode of action of carcinogenicity of *o*-nitrotoluene. In this study, we have tested *o*-nitrotoluene using Ames test, *in vitro* micronuclei assay in CHO cells, single cell gel/comet assay in L5178Y cells, microarray analysis of gene expression profiles in L5178Y cells in order to find out biomarker genes in response to carcinogenicity of *o*-nitrotoluene.

o-Nitrotoluene had not Induced Gene Mutations in both TA98 and TA1535 Strains

The numbers of revertant colonies was assessed as a measure of gene mutation in both TA98 and TA1535 strains exposed to different concentrations of *o*-nitrotoluene (Figure 1). In TA98 strain, the numbes of revertant colonies of 1.0 μg 2-nitrofluorene treated bacteria in the absence of S9 was 564 ± 15.1 and the

(A) TA98



(B) TA1535

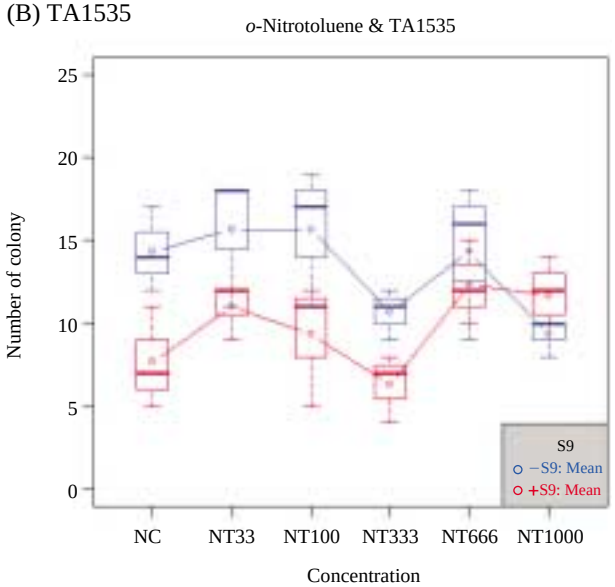


Figure 1. Individual data on the mutagenicity of *o*-nitrotoluene tested in strain TA98 and TA1535. The Ames test was performed by the pre-incubation test method[6] with or without metabolic activation using *Salmonella typhimurium* strains TA98 and TA1535 as described in methods. The data represent averages from three experiments with triplicate plates per dose. NC: negative control. (−S9: 1.5 μg/plate sodium azide, +S9: 10 μg/plate 2-aminofluorene).

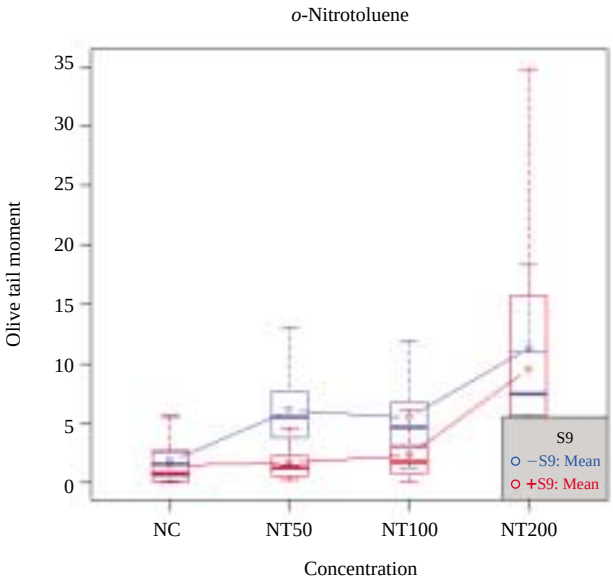


Figure 2. Comet assay for *o*-nitrotoluene usingL5178Y mouse lymphoma cells. The Olive Tail Moments were measured using comet assay according to Singh *et al.*[15] with slight modification as described in methods. The Olive Tail Moments of L5178Y mouse lymphoma cells exposed to 25, 50, 100 μg/mL *o*-nitrotoluene for 2 h. Negative control was medium. Positive controls were MNNG (100 μM) in the absence of S9 and BaP (50 μM) in the presence of S9 metabolic activation system, respectively. Data are means, boxplots. NC: negative control.

MF of cells exposed to 10 μg 2-aminofluorene in the presence of S9 was 399.7±10.50. The positive control chemicals, 2-nitrofluorene and 2-aminofluorene generated large increases in revertant. The numbers of revertant colonies of solvent control bacteria were 15.67±3.06 in the absence of S9 and 18.00±4.58 in the presence of S9. The numbers of revertant colonies (33, 100, 333, 666, 1,000 μg) treated bacteria were 15.33±4.04, 16.67±4.04, 18.67±4.93, 24.33±5.69, 21.33±8.96 in the absence of S9, 29.33±3.06, 25.00±5.29, 25.67±5.13, 28.33±6.03, 26.67±7.77 in the presence of S9, respectively. *o*-Nitrotoluene treatments was not increased in numbers of revertant colonies in TA98 with or without S9. In TA1535 strain, the numbers of revertant colonies of 1.5 μg sodium azide treated cells in the absence of S9 was 12.00±2.00 and the numbers of revertant colonies of bacteria exposed to 10 μg 2-aminofluorene in the presence of S9 was 20.67±5.03. The positive control chemicals, sodium azide and 2-aminofluorene resulted increases in revertant numbers. The numbers of revertant colonies of solvent control bacteria were 7.67±3.06 in the absence of S9 and 14.33±2.52 in the presence of S9. The numbers of revertant colonies of o-nitrotoluene (33, 100, 333, 666, 1,000 μg) treated bacteria were 11.00±1.73, 9.33±3.79, 6.33±2.08, 12.33±2.52, 11.67±2.52 in the absence of S9, 15.67±4.04, 15.67±4.16, 10.67±1.53, 14.33±4.73, 9.33±1.15 in the presence of S9, respectively. *o*-Nitrotoluene treatments was not increased in revertant

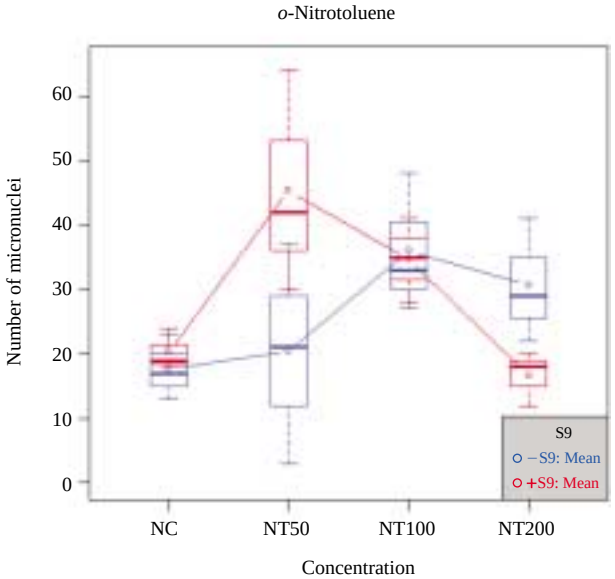


Figure 3. Micronucleus formation induced by *o*-nitrotoluene in CHO-K1 cells. The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech[3] with modification and the recommendation of the 3rd International Workshop on Genotoxicity Testing[10] as described in methods. CHO-K1 cells were grown in 24-well plates and treated with *o*-nitrotoluene (50, 100, 200 μg/mL) or cyclophosphamide (2.5, 5, 10 μg/mL) for 4h with or without S9. Cells were stained with 0.24 mM acridine orange and micronuclei were scored under the fluorescence microscope at 1000 magnification. Data are means, boxplots. NC: negative control (cyclophosphmide 2.5, 5 and 10 μg/mL).

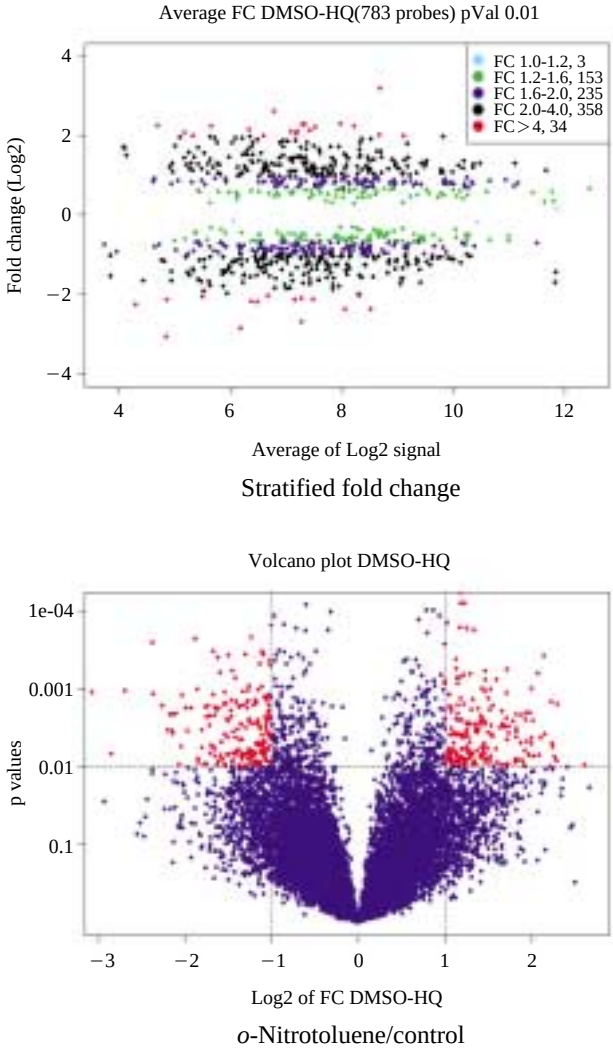


Figure 4. Microarray analysis for L5178Y cells treated with *o*-nitrotoluene. The microarray analysis was performed according to Affymetrix Inc.[1] with modification as described in methods. L5178Y mouse lymphoma cells were treated with *o*-nitrotoluene (200 μg/mL), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). Profiles were analyzed by Volcano Plot.

numbers in TA1535 with or without S9. *o*-Nitrotoluene had not increases therefore considered to have provided clear evidence of nonmutagenic activity of *o*-nitrotoluene in both TA98 and TA1535.

o-Nitrotoluene Induced DNA Damage in L5178Y Cells

The Olive Tail Moment was assessed as a measure of DNA damage in the comet assay in L5178Y mouse lymphoma cells exposed to different concentrations of *o*-nitrotoluene (50-200 μg/mL) for 2 h (Figure 2). The Olive Tail Moment of MNNG-treated cells (100 μM, positive control in the absence of S9) was 4.13 ±3.08 and the Olive Tail Moment of cells exposed to B[a]P (50 μM, positive control in the presence of S9 metabolic activation system) was 2.79±2.17. The Olive Tail Moment of control cells was 1.89±1.64 in the absence of S9 and 1.89±1.64 in the presence of S9. Cells were exposed to 50, 100, 200 μg/mL *o*-nitrotoluene for 2 h. The Olive Tail Moment induced by *o*-nitrotoluene was 6.87±6.51, 5.99±5.27, 13.1± 16.64 in the absence of S9 and 1.81±1.81, 2.45± 2.45, 10.34±11.82 in the presence of S9, respectively. It thus caused an increase in DNA damage in comparison to the solvent control.

o-Nitrotoluene had not Induced Micronuclei in CHO-K1 Cells

CHO-K1 cell cultured RPMI medium and treated with cyclophosphamide in the presence of S9. As expected, numbers of micronuclei were induced to be 2.5, 5, 10 μg/mL CPA 41.0±9.0, 39.0±11.0, 39.0±

8.0 in the absence of S9 and 60.0±7.0, 82.0±11.0, 107.0±15.0 respectively. Cells were exposed 50, 100, 200 μg/mL *o*-nitrotoluene for 4h. Numbers of micronuclei of *o*-nitrotoluene treated cells were 45.3 ±17.2, 34.7±6.5, 16.7±4.2 in the absence of S9 and 20.3±17.0, 36.0±10.8, 30.7±9.6 in the presence of S9, respectively. Increase in the numbers of micronuclei with *o*-nitrotoluene treatment was not observed (Figure 3).

Microarray Analysis of Differentially Expressed Genes with *o*-Nitrotoluene Treatment in L5178Y Cells

Gene expression profiling offers a powerful approach for identifying differentially expressed gene and identifying mechanism.

Differentially expressed genes from L5178Y cells treated with *o*-nitrotoluene (200 μg/mL) was analyzed by microarray using Affymetrix Mouse Genome 430 2.0 GeneChip arrays. Two-hundred two genes were specifically regulated and their fold of change was greater than 2 of log formation. Among them forty four genes were selected after the *t*-tests and performed Volcano plot analysis (Figure 4). Figure 5 showed the results of clustering analysis of *o*-nitrotoluene regulated genes. Table 1 showed pathway information of expressed genes which were increased by *o*-nitrotoluene treatment. If these genes expression and pathway would be related to carcinogenicity of *o*-nitrotoluene, it would need further study.

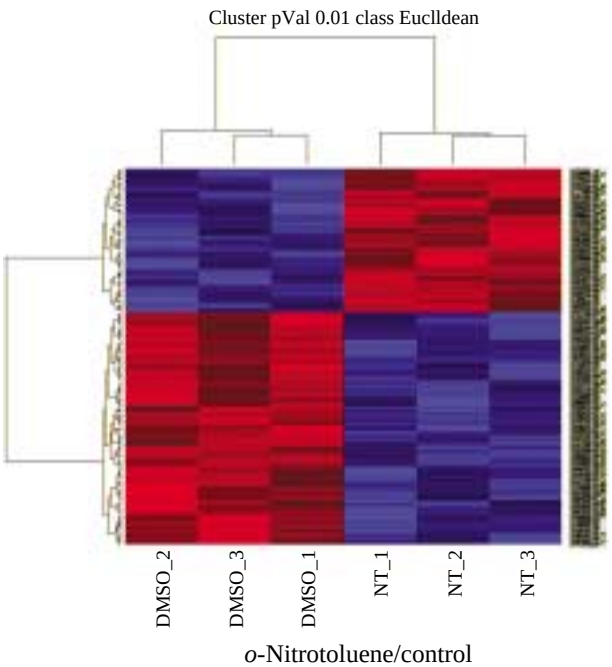


Figure 5. Hierarchical clustering for *o*-nitrotoluene. The microarray analysis was performed according to Affymetrix Inc.[1] with modification as described in methods. The data were analyzed by hierarchical clustering, and green represents down regulation of the transcripts; black, no change; red, up regulation of the transcript. DMSO: dimethylsulfoxide, NT: *o*-nitrotoulene.

Discussion

Genetic toxicity of *o*-nitrotoluene was verified through Ames test, Comet assay, *In vitro* micronucleus assay. Differentially expressed genes by *o*-nitrotoluene were analyzed by microarray analysis.

Ames test that was performed using *Salmonella typhimurium* strains TA98 and TA1535 for *o*-nitrotoluene is negative. *In vitro* micronucleus assay was carried out using CHO-K1 cell and three different concentrations with and without metabolic activation. The result of assay was negative response, which is not *o*-nitrotoluene increased micronucleus in CHO cell.

Comet assay was performed in L5178Y mouse lymphoma cell with and without metabolic activation. In conducting *in vitro* Comet assay, care should be taken to avoid conditions that would lead to positive results that do not reflect genotoxicity but arise from DNA damage (i.e., DSB) associated with cytotoxicity[3,17]. *o*-Nitrotoluene caused an increase in DNA damage and the result was confirmed with three replicate experiment.

As a test for genetic toxicity, the Comet assay can be used to identify possible human mutagens and carcinogens[2]. However, a perfect correlation between chemicals positive in this test and carcinogenicity is not expected. The correlation would be expected to

Table 1. Pathway information of expressed gene by *o*-Nitrotoluen treatment.

KEGG pathway	Involved genes count	Involved genes/ Total count (%)	Modified fisher exact P-value, Ease score
MMU00230:PURINE METABOLISM	6	3.08%	0.041847*
MMU00230:PURINE METABOLISM	2	1.03%	0.076192.
MMU04130:SNARE INTERACTIONS IN VESICULAR TRANSPORT	3	1.54%	0.061563.
MMU04010:MAPK SIGNALING PATHWAY	8	4.10%	0.05812.

depend on chemical class and on the mechanism of carcinogenicity involved[13].

The cell was treated *o*-nitrotoluene and the gene expression profile from microarray was analyzed data manipulation and preprocessing. Then hierarchical clustering was conducted with meaningful genes.

The gene expression profile provides us a better understanding of underlying mechanisms for *o*-nitrotoluene-induced carcinogenicity. Integration of gene expression changes with known pathological changes can be used to provide mechanistic scheme for *o*-nitrotoluene-induced carcinogenicity related DNA damage.

Methods

Materials

o-Nitrotoluene, 2-aminofluorene, 2-nitrofluorene, sodium azide, 1-methyl-3-nitro-1-nitrosoguanidin (MNNG), benzo(a)pyrene and cyclophosphamide were obtained from Sigma chemical Co.(St. Louis, MO, USA). The S9 fraction was purchased from Moltox® S9 (Canbiotech, USA).

Ames Test

The Ames test was performed by the pre-incubation test method[6] with or without metabolic activation using *Salmonella typhimurium* strains TA98 and TA1535. The tester strains were cultured overnight in 0.8% oxide nutrient broth at 37˚C. To the 0.1 mL of bacterial suspension, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) or 0.5 mL of S9 was added and then 0.1 mL of *o*-nitrotoluene (33, 100, 333, 666, 1,000 μg/plate) or positive control chemicals such as 2-aminofluorene, 2-nitrofluorene and sodium azide were added and incubated for 20 min at 37˚C. After incubation, 2.0 mL of top agar was added to mix and the mixture was poured onto a minimal glucose agar plate. 48 hours after the incubation at 37˚C, the numbers of revertant colonies were counted[8].

Comet Assay

Comet Assay was carried out according to Singh *et al*. with slight modification[13,15]. L5178Y mouse lymphoma cells were grown at 37˚C in a 5% CO_2 incubation. L5178Y mouse lymphoma cells were seeded in 12 well plates (1×10^6 cells/mL) and were exposed to 50, 100, 200 μg/mL *o*-nitrotoluene for 2 h. Positive controls were 100 μM 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) in the absense of S9 metabolic activation, 50 μM benzo(a)pyrene (BaP) in the presence of S9 metabolic activation. 20 μL of cell suspension were mounted in 1% agarose on slide glass. Slides were immersed in a cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1.5 h at 4˚C and then for 20 min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13). Slides were electrophoresed and neutralized using Tris buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 μg/mL). Cells were analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Andor Bioimaging Division, Nottingham, UK).

In vitro Cytokinesis Block Micronucleus Assay

The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech[4] with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing[10]. CHO-K1 cells were grown in 24-well plates and treated with *o*-nitrotoluene (50, 100, 200 μg/mL) or cyclophosphamide (2.5, 5, 10 μg/mL) for 4 h with or without S9. After the treatment, cells were washed with PBS and further incubated for 20 h in the medium containing 3 μg/mL cytochalasin B. Cells were harvested and spread on glass slide, and fixed with 100% methanol for 5 min and stained with 0.24 mM acridine orange in 1/150 M phosphate buffer (pH 6.8) for 3 min. Micronuclei were scored under the fluorescence microscope at 1000 magnification.

Microarray

The microarray analysis was performed according to Affymetrix Inc.[1] with modification. L5178Y mouse lymphoma cells were plated in RPMI-1640 medium into 12-well plate (1×10^6 cells/mL). After 24 h of treatment with *o*-nitrotoluene (200 μg/mL), total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and purified by a RNeasy mini kit (QIAGEN, Hilden, Germany). Total RNA (1 μg) was amplified using the Affymetrix one-cycle cDNA synthesis protocol. For each array, 15 μg of amplified biotin-cRNAs was fragmented and hybridized to the Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) for 16 h at 45˚C in a rotating hybridization oven. Slides were stained with streptavidin/phycoerythrin and washed for antibody amplification. Arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037).

Statistical Analysis

All numerical data were expressed as the average of the values obtained ± S.D. and their significance determined by conducting a paired Student's *t*-test.

Acknowledgements

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