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Possible Implications of *In vitro* Assay System for Toxicity Evaluation using DNA Microarray

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Abstract

Toxicological profiles obtained from DNA microarray experiments are becoming increasingly important in toxicity evaluations. Many research groups are carrying out toxicogenomic studies to develop toxicological expression profiles of exposure to chemicals that can be applied to human and environmental monitoring. Although a wide range of *in vivo* and *in vitro* systems are used to obtain toxicological expression profiles, our *in vitro* assay system could provide important information on toxicity mechanisms. In this study, we treated the rat liver epithelial cell line WB-F344 with phenytoin (PT) or thioacetamide (TA) for 3 and 12 hr. We identified a total of 16,757 differentially expressed genes during the time courses of PT and TA treatments. Specific up-regulated genes at the early time point showed similar expression changes in response to both PT and TA. Also, at 3hr, *in vitro* PT and TA treatment predominantly gave rise to up-regulation of genes involved in apoptosis, signal transduction and transcriptional regulation. Therefore, these *in vitro* studies suggest that identifying specific expression patterns at early time points may be valuable in identifying pathways of toxic responses.

Keywords: Toxicogenomic, DNA microarray, Phenytoin, Thioacetamide, *In vitro*

Introduction

Toxicogenomics is a new area of research being developed to monitor specific gene expression patterns in response to toxicants[1]. Microarray technology has been used to predict toxicity based on specific expression signatures. Therefore, many research groups are conducting toxicogenomic studies and constructing toxicogenomic databases that contain gene expression patterns related to toxicity mechanisms induced by well-characterized compounds. These databases can be used as predictive tools to evaluate possible toxicity from uncharacterized chemicals[2-4].

Thioacetamide (TA) is a typical toxicant that induces acute liver injury and is an anticipated human carcinogen[5,6] because it is carcinogenic in experimental animal models. Phenytoin (PT) is an anticonvulsant drug that can be useful in the treatment of epilepsy[7,8]. However, it also has detrimental side effects including liver injury, leukocytosis and eosinophilia[9,10].

In this study, we used rat whole genomic DNA microarray to analyze the hepatotoxic effects of TA and PT *in vitro*. We found many regulated genes including apoptosis, signal transducer and transcriptional regulator genes. Early response genes from both chemical exposures show especially high similarity. Therefore, our study suggested that an *in vitro* assay system using DNA microarray might be suitable for early toxicity evaluations.

Results

Cytotoxicity Assay

WB-F344cells were exposed to doses of PT and TA ranging from 0.1 to 10,000 μM. PT and TA produced dose-dependent cell damage in WB cells. Doses of PT and TA were then chosen that produced 20% cell death as determined by MTT assay. Following 12 hrs of treatment with PT and TA, 20% cell death was observed with 1,000 μM PT and TA.

Gene Ontology Classification of Rat Whole Genome Microarray

For whole genomic expression analysis, we used an OpArray rat 27 K oligonucleotide chip that contains 26,962 oligos representing about 22,000 genes including ESTs. We confirmed the current state of annotation for this microarray through BLAST analyses with the *Rattus norvegicus* sequences as queries. We then classified the genes whose expressions were sig-

Table 1. Classification of significantly changed genes in all PT and TA treatment groups.

Category	Contents	PT Up (%)		TA Up (%)		PT Down (%)		TA Down (%)	
		3 hr	12 hr	3 hr	12 hr	3 hr	12 hr	3 hr	12 hr
Apoptosis regulator	520	19 (1.73)	0 (0)	7 (1.35)	0 (0)	0 (0)	2 (0.38)	1 (0.19)	2 (0.38)
Cell cycle	651	23 (3.53)	0 (0)	7 (1.08)	2 (0.31)	5 (0.77)	0 (0)	2 (0.31)	1 (0.15)
Development	4078	112 (2.75)	5 (0.12)	50 (1.23)	3 (0.07)	15 (0.37)	5 (0.12)	4 (0.10)	10 (0.25)
Enzyme	4922	115 (2.34)	4 (0.08)	42 (0.85)	6 (0.12)	9 (0.18)	4 (0.08)	1 (0.02)	15 (0.30)
Defence immunity	506	8 (1.58)	1 (0.20)	7 (1.38)	1 (0.20)	2 (0.40)	1 (0.20)	0 (0)	0 (0)
Motor activity	328	7 (2.13)	1 (0.30)	3 (0.91)	3 (0.91)	2 (0.61)	0 (0)	0 (0)	0 (0)
Nucleic acid binding	3343	90 (2.69)	4 (0.12)	36 (1.08)	4 (0.12)	6 (0.18)	2 (0.06)	5 (0.15)	6 (0.18)
Signal transducer	3045	47 (1.54)	3 (0.10)	22 (0.72)	4 (0.13)	8 (0.26)	5 (0.16)	9 (0.30)	13 (0.43)
Structual protein	640	28 (4.38)	0 (0)	9 (3.44)	1 (0.16)	3 (0.47)	1 (0.16)	0 (0)	1 (0.16)
Transport	2410	48 (2.00)	1 (1.41)	22 (0.91)	5 (0.21)	3 (0.12)	0 (0)	0 (0)	3 (0.12)
Transcription regulator	1513	42 (2.78)	2 (0.13)	18 (1.19)	1 (0.07)	4 (0.26)	0 (0)	2 (0.13)	2 (0.13)
Metabolism	5187	126 (2.43)	5 (0.10)	56 (1.08)	9 (0.17)	7 (0.13)	2 (0.04)	2 (0.04)	9 (0.17)

nificantly changed in all groups (Table 1).

Analysis of Microarray Expression Data with Hierarchical Clustering

In order to identify genes regulated at each time point, we performed hierarchical clustering based on the expression profiles of triplicate hybridizations of the 3 hr and 12 hr time points (Figure 1). Genes with signals that changed by two-fold among triplicate hybridizations were selected. The expression pattern was analyzed by mean intensity. Clustering data clearly show that the early response genes from both chemical exposures were very similar.

Expression Pattern Analysis Based on Elapsed Time after PT and TA Administration

In order to estimate the major gene expression profiles, expression pattern analyses were performed based on elapsed time after PT and TA administration. In the up-regulated cluster, the number of genes in each pattern point was as follows: PT, 589; TA, 324 (Figure 2A) and PT, 29; TA, 25 (Figure 2B). In the down-regulated cluster, the number of genes in each point was as follows: PT, 9; TA, 115 (Figure 2C) and PT, 52; TA, 21 (Figure 2D).

Hepatotoxicant-induced Gene Expression Changes at 3 hr Time Point

We analyzed specific 2-fold up-regulated gene expression at each time point. We found many hepatotoxicity-related genes were up-regulated at 3 hr and down-regulated at 12 hr by both PT and TA treatments. These analyses indicated that the identification of genes associated with response to PT and TA at 3 hr is more valuable to understanding hepatotoxicity. The genes of interest fall into three main categories including apoptosis (Table 2A, B), signal transducers (Table 2C, D) and transcriptional regulators (Table 2E, F). Lists of some gene intensities in each category for PT and TA are shown in Table 2.

Discussion

We classified the genes whose expressions were significantly changed after PT and TA treatment in the two time groups. Gene classification and annotation were performed on the basis of gene function (Table 1). After analyzing differentially expressed genes, we found that more genes induced by PT and TA at the 3 hr time point were usually up-regulated than at the 12 hr time point (Table 1). Therefore, we assumed that early expression pattern analysis might be important in measuring the toxic effects of hepatotoxicants *in vitro*.

Hierarchical clustering was also performed to monitor the closely expressed genes based on the microarray data (Figure 1). The early response genes from 3 hr exposure to both chemicals are more similar than genes induced from 12 hr exposure. Also, we sorted specific expression patterns in up-regulated and down -regulated genes (Figure 2). Figure 2A pattern groups contain more differentially expressed genes than Figure 2B pattern groups. These results also clearly show that monitoring possible toxic effects at an early time point is very important in an *in vitro* system[11].

Category classification of genes affected by PT and TA treatments showed that most of the genes were related to apoptosis and cell signaling (Table 2). The apoptosis category includes cell death signal genes[12] (Tnfrsf21, tumor necrosis factor receptor superfamily, member 21; Pdcd11, programmed cell death protein 11; Casp 11, Caspase 11; Casp 12, Caspase 12). The signal transducer category genes show similar expres-

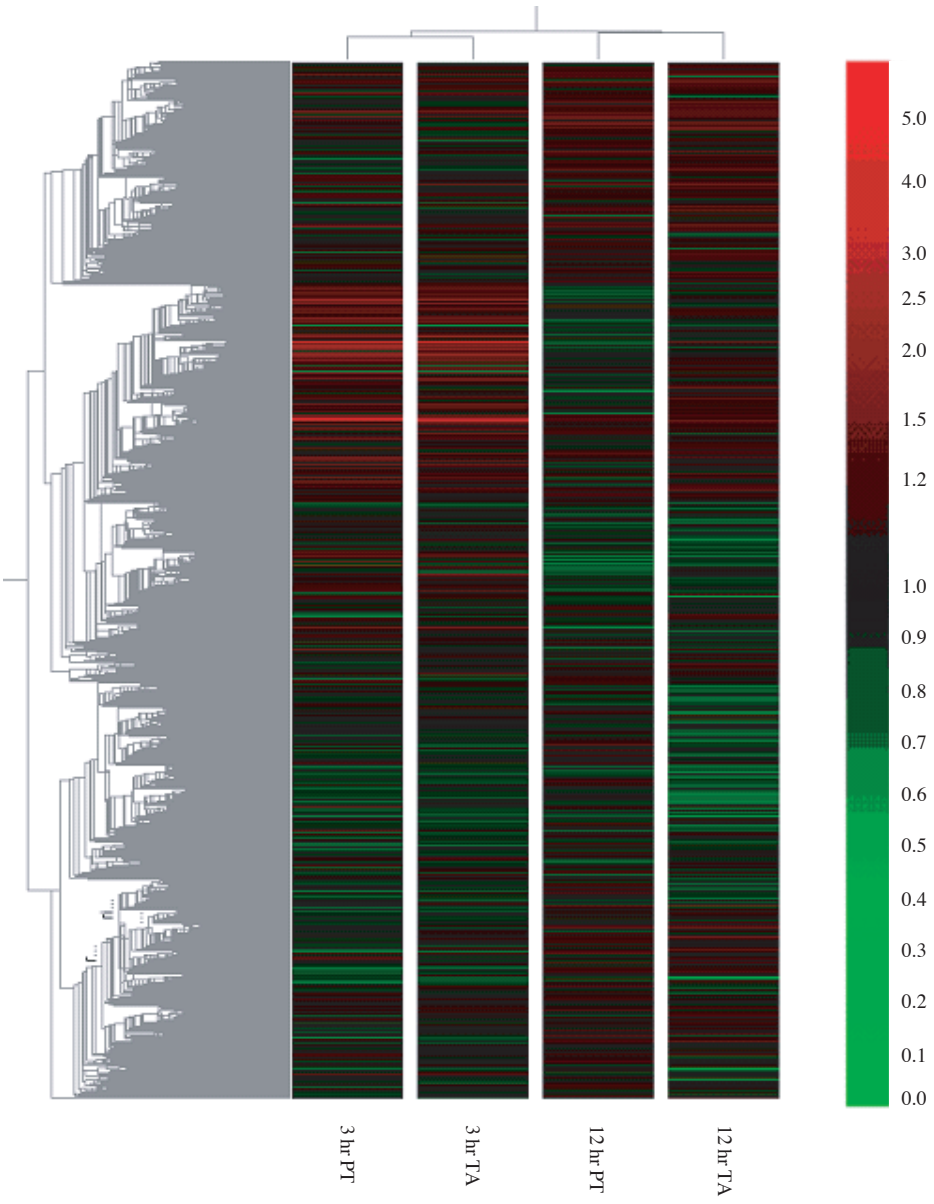

Figure 1. Hierarchical clustering analysis from PT and TA treatments at two time points. Gene expression data are expressed as fold of control values, with the range of change represented by colors at the right of the cluster images.

sion patterns for both PT and TA treatments and includes Hnrpm (Heterogeneous nuclear ribonucleoprotein M); Hnrpm is an RNA binding protein that interacts with carcinoembryonic antigen. In the transcriptional regulator category, many genes were differentially expressed following both chemical treatments including Vav1 (vav 1 oncogene), which stimulates the exchange of guanyl nucleotides by a member of the Rho family of GTPases[13]. This gene also plays a role in T-cell and B-cell development and can activate the c-Jun N-terminal kinase-1 (JNK1) pathway[14]. According to our microarray analyses, expressed genes induced by PT and TA treatments have similar expression signatures at the early time point. Therefore, these results suggest that identification of specific expression patterns at early time points might be valuable for identifying pathways of toxic responses *in vitro*.

Materials and Methods

Cell Culture

WB-F344 (WB) cells, a kind gift from Dr J. Trosko of Michigan State University (East Lansing, MI, USA), were cultured in D-media (Formula No. 78-5470EF, Gibco BRL, Grand Island, NY, USA) containing 3 mL/L penicillin-streptomycin-neomycin solution

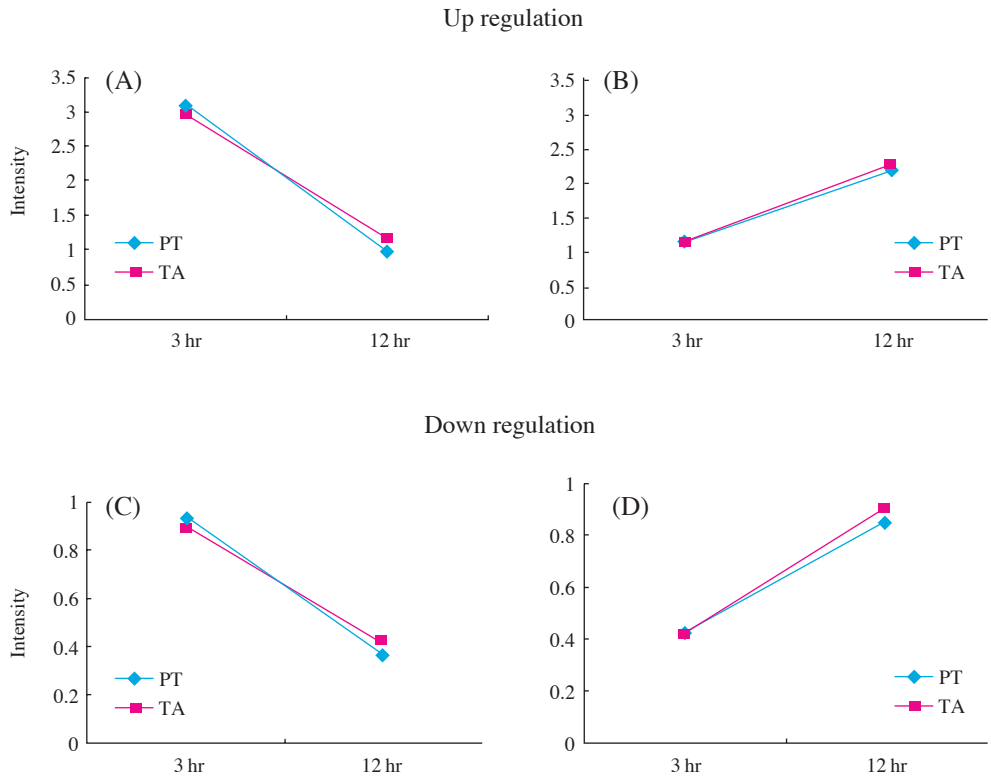

Figure 2. Expression pattern analysis based on elapsed time after PT and TA administration.

(Gibco BRL, Grand Island, NY, USA) and 5% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Cells were incubated in a 37°C humidified incubator containing 5% CO_2 and 95% air. Cells were grown in 75-mm tissue culture plates, and the culture medium was changed every other day.

Cell Viability Assay

The cytotoxic effects of phenytoin (PT, Aldrich Sigma, St. Louis, MO) and thioacetamide (TA, Aldrich Sigma, St. Louis, MO) in WB cells were measured by MTT assay, which is based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, cells were seeded at a density of 2×10^5 cells /mL in 24-well microplates and incubated overnight. The cells were then treated with various concentrations of PT and TA or its vehicle (ethanol or deionized water) for 12 hrs. At the end of this period, 100 μL of MTT stock solution (5 mg/mL, Sigma) was added to each well and incubated for 4 hrs at 37°C. The supernatant was removed, and 500 μL of DMSO was added to each well to solubilize the water-insoluble purple formazan crystals and then transferred into 96-well microplates for reading. The absorbency at 570 nm was measured with EL800 microplate reader (BIO-TEK Instrument, Winooski, VT). All measurements were performed in triplicate. Results are expressed as the percent proliferation with respect to vehicle control group.

RNA Isolation

WB cells were seeded at a density of 2×10^5 cells/mL in 100-mm tissue culture dishes and incubated overnight at 37°C, 5% CO_2. Upon reaching confluency, cells were dosed for 1, 3, 6, 12 and 24 hrs. Following the appropriate incubation period, WB cells were washed three times with PBS and scraped into 1 mL of TRIzol Reagent™ (Invitrogen) per dish. RNA was extracted from four individual culture incubations and equal amounts of RNA from each extraction were pooled. Total RNA was extracted according to the manufacturer's instructions. The quantity of RNA in each sample was measured by spectrophotometry, and the intensity was determined with the Bioanalyzer (Agilent Technologies, CA, USA).

Microarray Hybridization

We used the OpArray Rat genome 27 K oligonucleotide chip (Operon Biotechnologies GmbH, Cologne, Germany) consisting of 26,962 oligos repre-

Table 2. Classification of of up-regulated genes based on time points.

A. Apoptosis (PT)

Gene name	ID	3 hr	12 hr
Tumor necrosis factor receptor superfamily, member 21	XM_236992	3.974	0.892
Presenilin 2	NM_031087	3.869	1.024
DNA-damage inducible transcript 3	NM_024134	3.754	0.996
Vascular endothelial growth factor A	NM_031836	3.731	1.388
Tribbles homolog 3	NM_144755	3.123	1.375
Poly(rC) binding protein 4	XM_343468	2.931	0.996
Protein kinase C, epsilon	NM_017171	2.457	0.984
DNA-damage inducible transcript 3	NM_024134	2.447	1.165
Programmed cell death protein 11	XM_219966	2.39	1.088
Eukaryotic translation initiation factor 5A	XM_213368	2.383	0.967
Oncostatin M	NM_001006961	2.341	1.213
Amyloid beta (A4) precursor protein	NM_019288	2.322	0.944
Nuclear protein 1	NM_053611	2.256	1.152
Caspase 12	NM_130422	2.177	1.522
Myelocytomatosis viral oncogene homolog (avian)	NM_012603	2.168	0.998
Retinoic acid receptor, alpha	NM_031528	2.167	0.68
Caspase 11	NM_053736	2.15	1.547
Caspase 12	NM_130422	2.142	1.414
Growth arrest and DNA-damage-inducible 45 beta	NM_001008321	2.048	0.986

B. Apoptosis (TA)

Gene name	ID		
Tumor necrosis factor receptor superfamily, member 21	XM_236992	2.872	1.153
Tnf receptor-associated factor 2	XM_231032	2.645	1.171
Bcl2-associated athanogene 3	NM_001011936	2.259	1
Eukaryotic translation initiation factor 5A	XM_213368	2.105	1.36
Heat shock 70 kDa protein 5 binding protein 1	NM_178021	2.087	1.103
Programmed cell death protein 11	XM_219966	2.085	1.161
Poly(rC) binding protein 4	XM_343468	2.025	1.346

C. Signal transducer (PT)

Gene name	ID		
Heterogeneous nuclear ribonucleoprotein M	NM_053876	9.418	1.045
Glutamate receptor, metabotropic 4	NM_022666	8.532	0.581
Melatonin receptor 1B	XM_345899	6.329	0.885
Retinoic acid early transcript 1L	XM_218058	6.098	1.191
Olfactory receptor 877	NM_001000707	6.093	0.839
Slit homolog 3	NM_031321	5.787	1.38
Ephrin A2	XM_234903	5.742	NO DATA
Olfactory receptor 1413	NM_214821	5.716	0.851
Endothelin receptor type B	NM_017333	5.445	0.722
Thyroid hormone receptor associated protein 4	CK475406	5.414	0.77
Growth arrest specific 6	NM_057100	5.042	0.655
PDZ domain containing 1	NM_031712	4.644	1.051
Olfactory receptor 1624	NM_001000089	4.592	0.848
Tumor necrosis factor receptor superfamily, member 21	XM_236992	3.974	0.892
Presenilin 2	NM_031087	3.869	1.024
Vascular endothelial growth factor A	NM_031836	3.731	1.388
Cortactin isoform B	NM_021868	3.097	0.807

Table 2. Continued.

Gene name	ID	3 hr	12 hr
D. Signal transducer (TA)			
Heterogeneous nuclear ribonucleoprotein M	NM_053876	7.061	1.473
Glutamate receptor, metabotropic 4	NM_022666	5.23	0.885
Melatonin receptor 1B	XM_345899	5.091	1.194
Olfactory receptor 877	NM_001000707	4.825	1.281
Retinoic acid early transcript 1L	XM_218058	4.489	1.838
Thyroid hormone receptor associated protein 4	CK475406	3.985	1.015
Ephrin A2	XM_234903	3.939	1.283
Slit homolog 3	NM_031321	3.905	NO DATA
Olfactory receptor 1413	NM_214821	3.904	1.076
PDZ domain containing 1	NM_031712	3.893	0.973
Olfactory receptor 1624	NM_001000089	3.663	1.337
Endothelin receptor type B	NM_017333	3.609	1.2
Growth arrest specific 6	NM_057100	2.918	1.064
Tumor necrosis factor receptor superfamily, member 21	XM_236992	2.872	1.153
Neuropilin 1	NM_145098	2.652	1.377
Tnf receptor-associated factor 2	XM_231032	2.645	1.171
G protein-coupled receptor 157	NM_001012107	2.616	1.084
Low density lipoprotein receptor	NM_175762	2.578	1.342
Heterogeneous nuclear ribonucleoproteins MTF-like 2	NM_024363	2.556	1.23
E. Transcription regulator (PT)			
Ankyrin repeat domain 32	XM_226614	8.74	0.936
Vav 1 oncogene	NM_012759	6.169	0.799
Upstream binding transcription factor, RNA polymerase I	XM_340913	5.137	0.999
Ribosomal protein S10	NM_031109	4.957	1.054
Pre-B-cell leukemia transcription factor interacting protein 1	XM_227420	4.514	1.078
WD40 protein Ciao1	NM_001008766	4.039	1.318
DNA-damage inducible transcript 3	NM_024134	3.754	0.996
Id3, dominant negative helix-loop-helix protein	NM_013058	3.341	0.771
Flightless I homolog	NM_001008279	3.295	0.928
Ribosomal protein S6 kinase, polypeptide 4	XM_342004	3.157	1.019
Tribbles homolog 3	NM_144755	3.123	1.375
Heterogeneous nuclear ribonucleoprotein A/B	NM_031330	3.072	0.884
Ribosomal protein S6 kinase, polypeptide 4	XM_342004	2.903	0.883
Kruppel-like factor 15	NM_053536	2.86	1.174
MAD homolog 4	NM_019275	2.743	0.877
Similar to TGF-beta 1 induced transcript 4, isoform 1	L25785	2.679	1.182
Activating transcription factor 3	NM_012912	2.572	1.25
Retinoic acid induced 14	NM_001011947	2.563	1.059
Wolf-Hirschhorn syndrome candidate 1-like 1	XM_214371	2.516	0.939
LIM domains containing 1	XM_236734	2.511	0.825
Aryl hydrocarbon receptor nuclear translocator-like	NM_024362	2.507	1.105

senting about 22,000 genes including ESTs. Microarray experiments were performed according to the manufacturer's protocol. For microarray hybridization, total RNA from untreated WB control cells was pooled and used for hybridization and each total RNA sample (30 μg) was labeled with Cyanine 3 (Cy3)- or Cy5-conjugated dCTP (GE healthcare, Piscataway, NJ, USA) by reverse transcription using reverse tran-

Table 2. Continued.

Gene name	ID	3 hr	12 hr
F. Transcriptional regulator (TA)			
Ankyrin repeat domain 32	XM_226614	8.74	5.916
WD40 protein Ciao1	NM_001008766	4.039	4.453
Ribosomal protein S10	NM_031109	4.46	4.432
Vav 1 oncogene	NM_012759	6.169	4.171
Ribosomal protein S6 kinase, polypeptide 4	XM_342004	3.157	3.373
Ribosomal protein S10	NM_031109	4.957	3.333
Heterogeneous nuclear ribonucleoprotein A/B	NM_031330	3.072	3.287
Id3, dominant negative helix-loop-helix protein	NM_013058	3.341	2.838
Upstream binding transcription factor, RNA polymerase I	XM_340913	5.137	2.736
Chain A, Solution Structure Of The Bola-Like Protein	XM_345942	2.416	2.431
MAD homolog 4	NM_019275	2.743	2.382
Wolf-Hirschhorn syndrome candidate 2	NM_001008339	2.183	2.343
Zinc finger protein 367	NM_001012051	2.272	2.277
Silent mating type information regulation 2, homolog 6	XM_234931	1.84	2.224
Heterogeneous nuclear ribonucleoprotein D	NM_024404	2.308	2.163
Heterogeneous nuclear ribonucleoprotein A/B	NM_031330	1.649	2.09
Programmed cell death protein 11	XM_219966	2.39	2.085
Proteasome 26S subunit, non-ATPase, 9	NM_130430	2.097	2.067
Pbx/knotted 1 homeobox	NM_001013074	2.244	2.039
RAB2, member RAS oncogene family	NM_031718	2.088	2.017
Minichromosome maintenance deficient 2 mitotin	XM_232168	1.912	2.001

scriptase, SuperScript II (Invitrogen, Carlsbad, California). The fluorescently labeled cDNAs were mixed and hybridized simultaneously to the OpArray rat 27 K oligo microarray. We hybridized TA-treated samples three times per time point (3 hr and 12 hr). Processed slides were scanned with an Axon 4000B Scanner (Axon, CA, USA) using laser excitation of the two fluorophores at 532 and 635 nm for the Cy3 and Cy5 labels, respectively. The scanned images for each slide were analyzed using the GenePix Pro 5.1 software (Axon Instruments).

Data Management

The raw intensity data was globally normalized by intensity-dependent normalization using LOWESS method and then normalized by with-print-tip group normalization method for each print-tip; 48 tips were used to make the OpArray Rat genome 27 K microarray. Statistical software was used to determine the mean of triplicate experimental data. Microarray data management was performed with GeneSpring 7.2 software (Agilent Technologies). Comparison of present genes, fold-change determinations and various clustering analyses were performed. The gene expression values for each array were normalized to their respective median value. All clustering analyses were performed using standard correlations. Fold-change filters included the requirement that the genes be present at levels at least 200% of controls for up-regulated genes and at levels lower than 50% of controls for down-regulated genes.

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