a physiological increase in steroid hormone synthesis and a decrease in extramedullary hematopoiesis with age.

In TGP, either 0.5 % methylcellulose or corn oil works as vehicle, according to the solubility of the test drug. As a lot of data of both vehicles were being accumulated, the extraction of vehicle effects became possible. As we have already published (Takashima *et al.*, 2006), it was revealed that the extent and/or time course of expression of genes related to lipid metabolism was affected by corn oil, since rats received orally a high caloric intake in the morning when they usually do not eat.

These genes with reproducible expression patterns are now utilized for quality control of each expression data in the TGP. This could be applied for evaluation of data obtained in a different platform in the future. There are also many probe sets with quite poor reproducibility or large variance. If the cause of their variation is attributed to certain factors, like animal treatment or laboratory circumstances, such genes are in turn useful tools to evaluate inter-laboratory differences. It might be convenient to make a list of 'useless' genes that are excluded at the beginning in order to facilitate the analysis. However, care should be taken to exclude any genes that are absent in samples in the database even after a vast amount of data is accumulated, at least insofar as 'toxicity' is concerned. It is always possible that expression of such a gene is uniquely induced by a new drug.

Figure 20.7 shows the overview of the expression of the TNFRSF16 associated protein 1 in the first 35 chemicals analyzed with the A chip. Only thioacetamide and methapyrilene induced its expression after repeated administrations. It would be interesting to investigate the toxicological mechanism of these non-genotoxic carcinogens making this observation as a clue. The point here is that the simple accumulation of the vast amount of data is scientifically valuable and it is important to collect any observable changes as precisely as possible.

20.3 Construction of a Toxicity Prediction System Based on the TGP Database

The TGP system consists of basically three parts, i.e. the database itself that stores gene expression data with related pathology (scoring and the photo of HE staining), hematology, blood biochemistry and chemical information, the data analysis system that consists of the tools for up- and download of data, clustering, discriminant analysis, principal component analysis, gene- or compound list manager, etc. and the prediction system that is used when the expression data are uploaded.

In the summer of 2006, the final form of these systems has become operational. In the TGP database, *in vivo* data of 24 000 rats, expression data of about 24 000 'GeneChips' corresponding to ca. 700 000 000 probe sets, 2 880 000 measured test items, the data of 48 000 pathology specimens and various related information and reports are to be stored in their final form. In order to pick up useful data for toxicologically meaningful analysis from such a large scale of data, an efficient, toxicologist-friendly system is essential.

20.3.1 Analysis System

Let's see the previous omeprazole case again. The first one is pathology. In the single dose experiment, the significant and dose-dependent change was periportal eosinophility

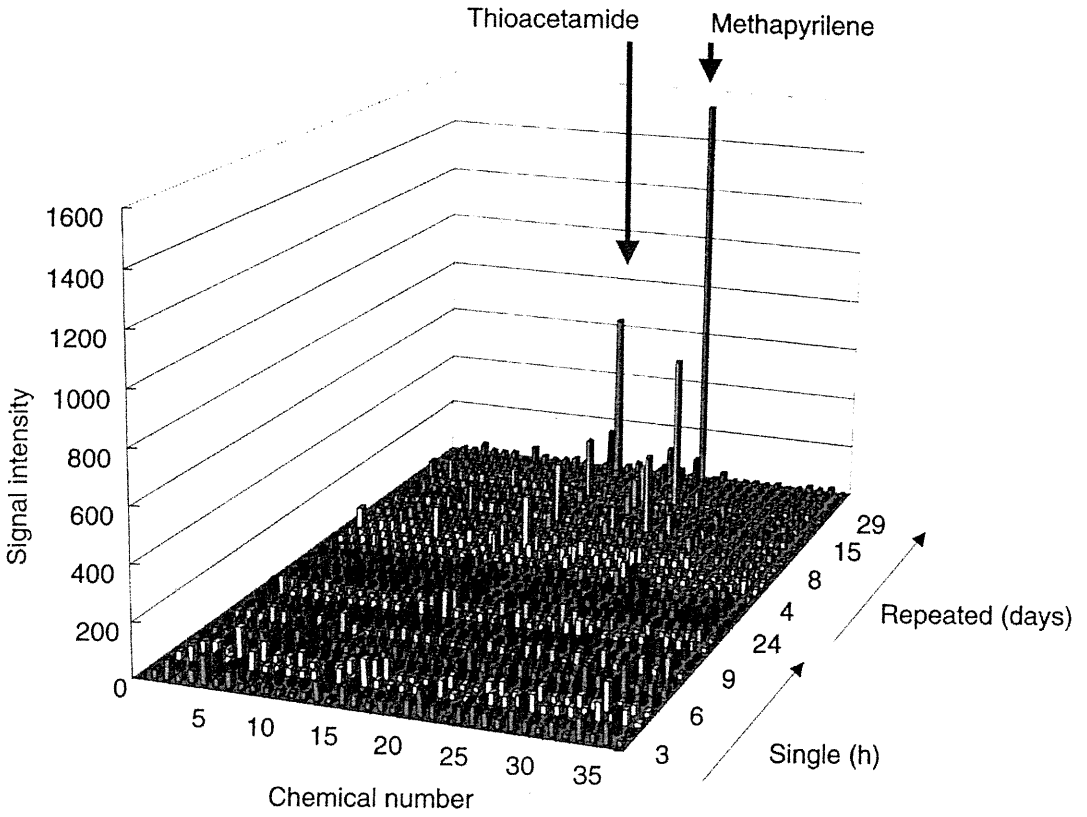


Figure 20.7 *Expression level of the TNFRSF16 associated protein 1 in the first 35 chemicals analyzed by using the RAE 230A chip. This gene was specifically up-regulated by repeated administration of thioacetamide (indicated by the arrow around the chemical number 17) and methapyrilene (indicated by the arrow around the chemical number 25). Expression data were normalized by mean value and multiplied by 500, and expressed as the mean of $N = 3$. On the time axis, sets of the data of control, low, middle and high doses are aligned in the order of 3, 6, 9 and 24 h after single doses, and 3, 7, 14 and 28 days of repeated doses*

observed at 6 and 9 h. In the repeated ones, centrilobular hypertrophy was evident with a peak at 2 weeks. Simultaneously measured test items were 43 in total, and 22 items showed some changes. As it is quite tedious to check each individually, the extent of the change to control value in each measure was converted into a semi-quantitative heat-map and depicted as Figure 20.8(a). It can be seen that omeprazole did not induce serious toxic changes in a single dose, whereas obvious hepatic hypertrophy, anemia and some mobilization in plasma lipid emerged by repeated administration.

The next one is the gene expression change in the liver. For each gene (probe sets), a graph of 4×4 matrix with $N = 3$ for each lattice can be drawn for single and repeated experiments. As it is again quite difficult to interpret the results consisting of more than 15 000 probe sets, checking one by one, a similar heat-map was depicted by a semi-quantitative conversion of the dose–response at each time point (Figure 20.8(b)). This is for the example of glutathione reductase. It is obvious that this gene was dose- and time-dependently up-regulated toward 24 h in the single dose, whereas the extent kept decreasing as the administration continued. Since glutathione reductase is known to involve oxidative stress responses, other genes

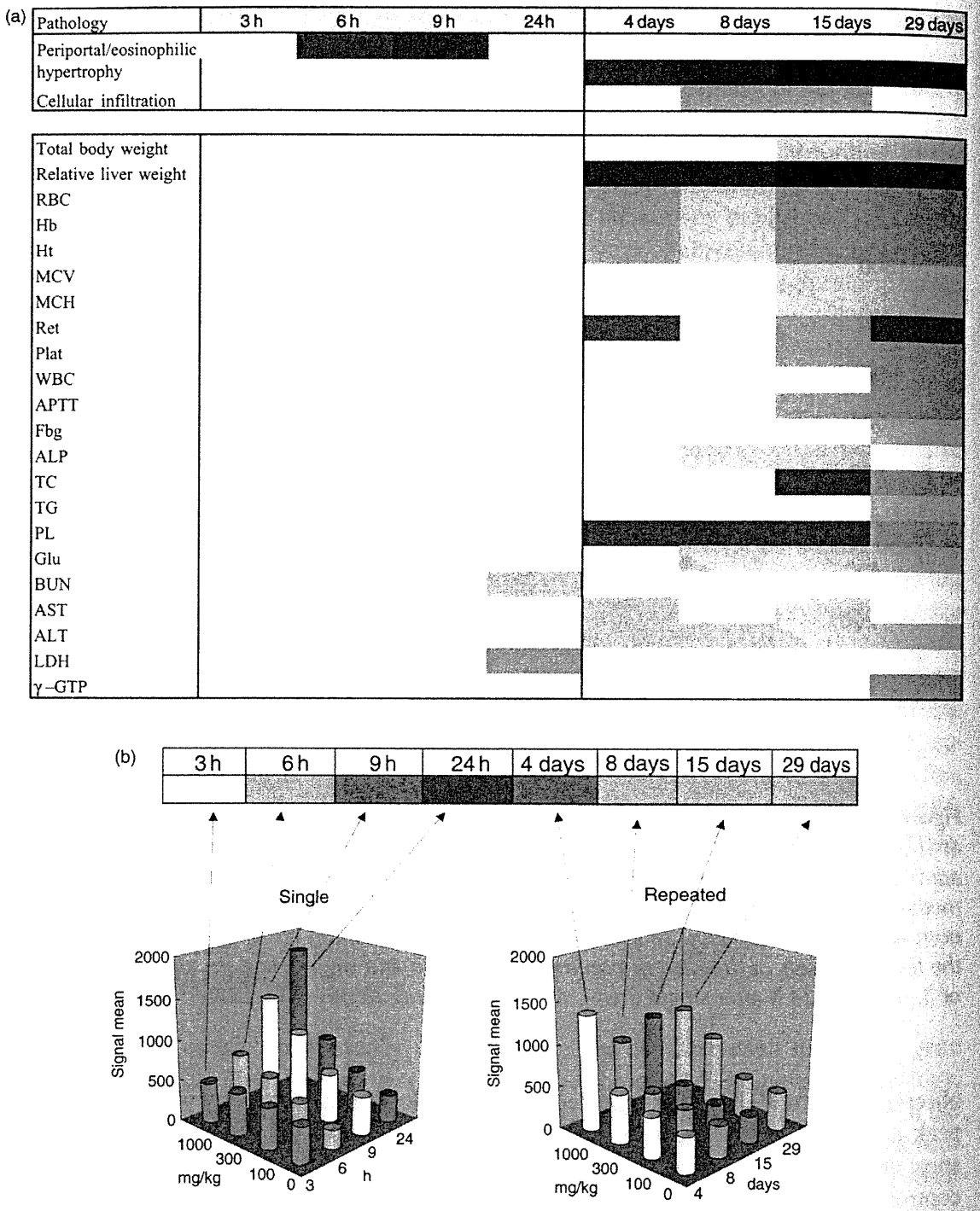
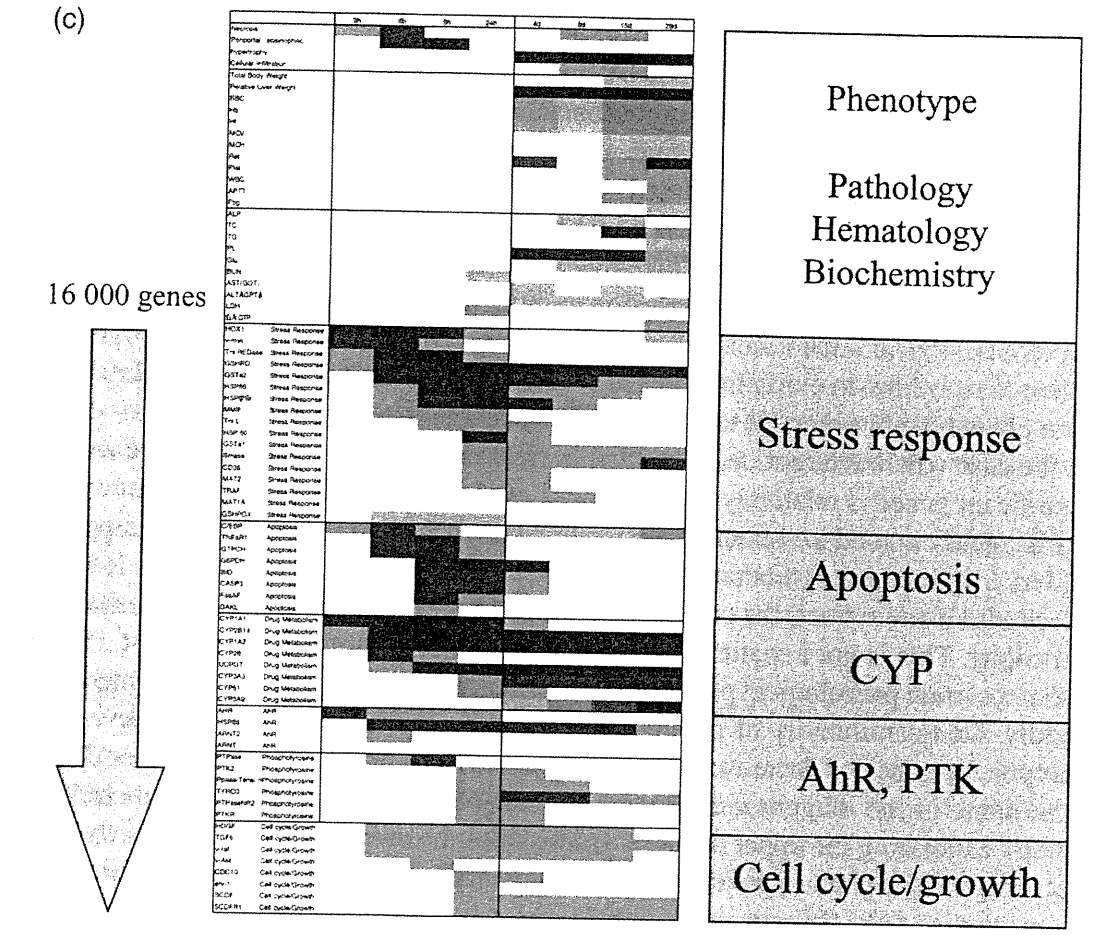


Figure 20.8 *Schematic representation of an image of data stored in the TGP database. (a) Heat-map of phenotype induced by omeprazole administered to rats as an example. The upper panel shows the pathological findings while the lower panel shows the changes in organ/body weight, plasma biochemistry and hematology. The actual data table is much larger than this, but the items without significant change are omitted and the data are converted to a semi-quantitative heat-map. (b) Heat-map conversion of the expression changes of glutathione reductase, 1369061_at. This way makes it convenient to overview time- and dose-dependent changes at a glance. (c) Heat-map expression of the gene expression changes together with the phenotype induced by omeprazole. Genes were categorized by their function and aligned by the order of the time when the first change appeared. This panel continues far down below. (d) The alignment of the panels of the chemicals prepared by the way described above. Practically, it continues in both directions of horizontal and vertical*



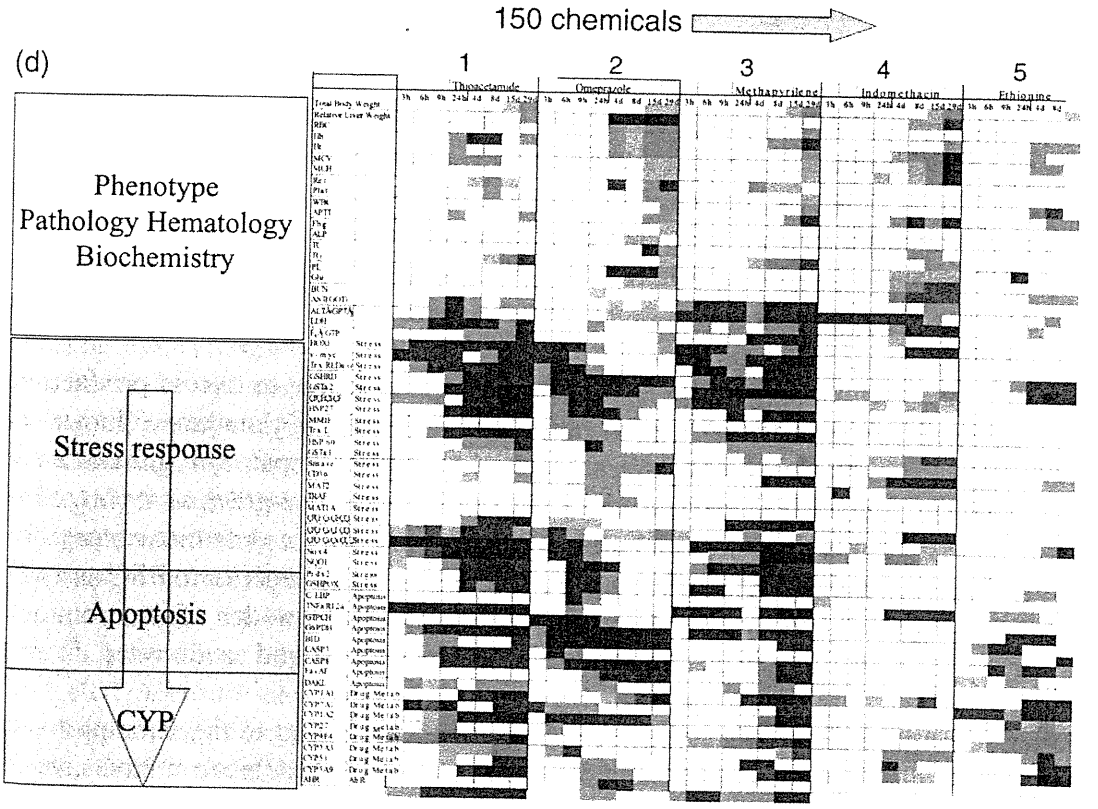


Figure 20.8 *(Continued)*

belonging to this category were examined and aligned in the order of their responding time to overview the sequence of chronological expression of the functional genes. Continuing this step for other categories, it produces a long heat-map as shown in Figure 20.8(c), which actually continues far down below. It might be possible to hypothesize a cascade of gene expression related to the effects of omeprazole on the liver, but the whole body of data is too large to reach an acceptable conclusion without the aid of computerized pathway tools.

Moreover, 150 in such a heat-map are lining horizontally (Figure 20.8(d)). The main problem here is how to extract the genes whose expression changes are correlated with a certain phenotype or potential toxicity by using the bioinformatics technique.

In the case where a target or phenotype is clear, such as PPAR agonists, the extraction of significant genes is relatively easy (Tamura *et al.*, 2006b). However, it is quite difficult to extract genes related to 'toxicity' with unknown mechanisms. For example, suppose one would try to extract genes related to above 'hypertrophy' caused by omeprazole. It would be unsuccessful if one extracts the commonly changed genes among the compounds that caused hypertrophy. This is not a matter of statistical technique but of pathology. First, it is quite rare that a certain pathological phenotype is attributed to a single toxicological mechanism. Secondly, the quantitativity of pathology scoring is poor. Thirdly, when the severity of a phenotype increases or decreases, its grading score does not always increase or decrease, but the name of the diagnosis changes. One cannot avoid this problem by including the diagnosis expressing the upper or lower grade, since the name does not necessarily express the severity of the phenotype alone. 'Phenotype anchoring' is a challenging issue in the toxicogenomics field (Moggs *et al.*, 2004) and it is also under investigation in our TGP. It is also expected that progress in 'toxicogenomics-oriented histopathology' should be an important field in toxicology.

We have therefore started with anchoring to clarify indicators in parallel. The TGP database contains representative drugs and hepatotoxicants, but it does not necessarily mean that their toxicological mechanisms are representative with respect to molecular biology. We then decided to perform additional single-dose experiments using compounds that possess clear molecular targets in order to investigate the relationship between molecular toxicity and gene expression changes. They included protein synthesis inhibitors, compounds related to infection/inflammation (lipopolysaccharide, TNFα, galactosamine), ER-stressor, and cytoskeleton disruptors. The results of phorone, a glutathione depletor, are presented.

As described above, hepatotoxicity of acetaminophen is due to excess production of active metabolite over the detoxification capacity of intracellular glutathione (James *et al.*, 2003). Therefore, any drugs that have a potential to deplete hepatocyte glutathione risk causing acetaminophen-type hepatotoxicity with overdosage. In a previous report, a list of marker genes for glutathione depletion was extracted using BSO, a glutathione biosynthesis inhibitor (Kiyosawa *et al.*, 2004). Phorone was considered to be superior to BSO as a model system, since the mechanism of glutathione depletion is similar to that of acetaminophen-type hepatotoxicants, i.e. it covalently binds to glutathione and is excreted from the cell.

Phorone, at 40, 120 or 400 mg/kg was administered according to the same protocol as the regular single-dose experiments and the measurement of glutathione contents was performed in addition to the regular tests. Phorone caused a marked but transient depletion of glutathione with maximal depletion occurring at 3 h; then it recovered and showed

an increase of glutathione at 24 h as a rebound. A significant increase in plasma AST was observed at 400 mg/kg, indicating hepatotoxicity. Expression data at this point were excluded from the work-up in order not to extract the expression changes secondary to hepatic injury. In the next step, genes whose expression were inversely correlated with hepatic glutathione contents for each rat were statistically extracted and filtered to get 130 probe sets. Principal component analysis of the chemicals stored in the database using these sets revealed that chemicals with a risk of glutathione depletion, such as bromobenzene and coumarin, in addition to acetamineophen, were clearly separated from other chemicals or controls toward the direction of PC1, suggesting that the list was a useful as 'marker gene list for risk assessment of glutathione depletion' (Kiyosawa *et al.*, in press).

Our present strategy is to prepare biomarker gene lists that are related to certain toxicological phenotypes, pathways, or any biologically meaningful factors, as many as possible using various procedures.

20.3.2 Toxicity Prediction System Based on Biomarker Gene Lists

In the general terminology, 'biomarker' is designated as one or a few biological measures quantitatively reflecting a certain biological change. However, this does not fit for the 'biomarker gene' in toxicogenomics. As discussed above, toxicology based on the transcriptome has various problems, such as poor statistics because of small *N*s compared with genes, the requirement to make the beta-error small, toxicity with uncertain time- or dose-dependency, etc. Although the quantitativity of microarrays has greatly improved in recent years, its quantitativity and reproducibility cannot be superior to the enzymes in serum or the metabolites in urine when a labile object, mRNA, is measured. It is thus dangerous to make a decision based on one or few marker genes, and it would be effective to use relatively large numbers of genes as a whole in order to make the assessment robust. A comprehensible example is shown in Figures 20.2(c) and 20.7(c). If heme oxygenase-1 alone is employed as a 'stress marker', the potential to overlook omeprazole must be quite high, but it should go down if a 'stress-responsive gene list' is employed. In this connection, the TGP is now trying to substantialize such gene lists.

When an assessment or prediction of toxicity is made by a list of multiple measures, it becomes necessary to summarize or quantify these measurements. Ideally, the quantification process should be optimized for each marker gene list. However this is practically difficult and thus a uniform system has to be created. In the TGP, a new scoring system was developed in one trial (Kiyosawa *et al.*, 2006). The score is calculated based on the ratio to control value (log 2) for each gene in the marker list and expressed as a heat-map. This scoring system has made it easy to overview the assessments of a target compound against many marker lists, or to overview the assessments of many compounds against a particular marker list. However, there are some problems in this system, i.e. the score is biased when the list contains a gene whose expression change is extremely large (e.g. CYP1A1) and changes are canceled when up- and down-regulated genes co-exist in the list. Therefore, another scoring system, e.g. effect size (the absolute value of the difference between means divided by the standard deviation) is also available in the TGP system.

Principal component analysis is a quite convenient tool to make a qualitative classification of compounds against a list of genes. As a prediction system, however, some quantitative

data would be favorable for the final output. Thus in our system, the following functions are added, i.e. when the user specifies a principal component with high contribution, the compounds are sorted by the value and the genes with large Eigenvector value are easily obtained. This gives an idea where the relative position of the test drug locates among the ones in the database, and suggests a candidate gene list for further investigation.

When a phenotype that can be judged as positive/negative is available, discriminant analysis is known to be powerful (Porter *et al.*, 2003), and prediction analysis of microarray (PAM) (Tibshirani *et al.*, 2002) has been firstly employed in the TGP. By a semi-automatic system of training and validation, the efficiency improves for the creation of discriminators. As above, the system exhibiting the prediction by PAM as quantitative scores (to show the relative position of a test drug among chemicals in the database) is under consideration. The TGP also includes the support vector machine (SVM) (Brown *et al.*, 2000) in the system.

Although the present system has not come to completion, the following picture of TGP use has emerged (Figure 20.9).

You have a candidate drug, X, which was administered to rats and 'GeneChip' data of the liver were obtained 24 h after dosing. The data are up-loaded to the TGP system and the marker viewer is activated to overview all the biomarker gene lists stored in the database. Alarms are noted for several markers (Figure 20.9(a)). Among them, one marker, M1, is selected, as this is highly related to the toxicological phenotype F1, if repeatedly administered. When PCA is performed using these marker genes, X is clearly separated in the direction of PC1 (Figure 20.9(b)). As the contribution of PC1 is found to be high, compounds are sorted by PC1 and their order is Y (high dose) > X > Z (high dose) > Y (middle dose) > ...., etc. From the analysis, it is predicted that X causes phenotype F1 when repeatedly dosed, and it requires a higher dose than Y but lower than Z. The gene list with a high Eigenvector is harvested for further analysis of the toxicological pathway. There is also another point. It is known that X is pharmacologically similar to compounds P and Q that are stored in the database. It is also known that P and Q cause phenotype F2 and actually that a marker gene list M2, predicting F2, exists in the database, whereas no alarm was noticed in the first survey. Then all the compounds are overviewed against M2. It is obvious that not only X but also P and Q show low scores because most of the marker genes in the list show transient expression changes and thus F2 is non-predictable using M2 at 24 h (Figure 20.9(d)). Therefore, it is suggested that an additional early time point is necessary to judge whether X has a similar property to P and Q.

This is just a simulation. The point here is that the prediction system of the TGP is not a simple output of a probability like a 'weather-forecasting system', but a supply of knowledge with suggestions for further investigation. As everybody knows, there is no drug without side effects. If a prediction like 'this drug is safe' appears, this must be a lie. Toxicologists do not want such a prediction, but want the information such as, 'What kind of phenotype would appear in what dose level and what are the toxicological mechanisms involved?'.

20.4 The Image of Toxicity Testing after the TGP Database is Established

At present, we say that the usage of the TGP database/prediction system is in the following condition. In the quite early stage of drug development, the database is used to select a lead compound among candidates. As the full-scale toxicity test is quite costly, safety assessment

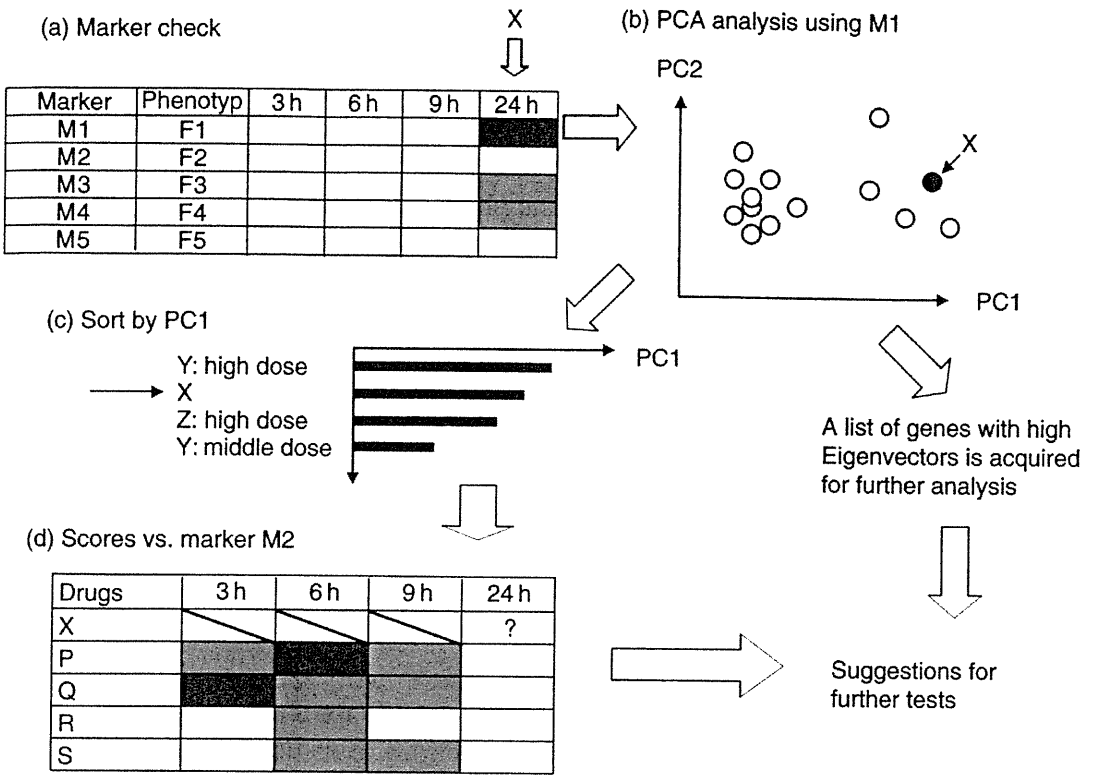


Figure 20.9 *Schematic representation of an image of toxicity prediction process in the TGP system. (a) The data of new drug X that are up-loaded to the TGP system are checked against several markers useful to predict phenotypes in repeated administration from single-dose experiments. One of the markers (M1) shows an alert. (b) PCA is performed using these marker genes, M1. X is clearly separated in the direction of PC1 with high contribution value. Then, the compounds are sorted by PC1, showing the order of Y (high dose) > X > Z (high dose) > Y (middle dose). The gene list with high Eigenvectors can be harvested for further analysis. (c) Drugs P and Q cause phenotype F2. The marker M2, predicting future phenotype F2, is available in the database. When compounds, including X, are overviewed against M2, not only X but also P and Q show low scores at 24 h while the latter two show high scores in this marker gene list at earlier periods. This indicates that prediction of F2 is inconclusive without the expression data in earlier stages*

of candidate drugs are usually done just before the clinical trial. If serious toxicity emerges at this stage, it might be necessary to return to the seeds, because toxicity is often inherent to the basic structure and thus never eliminated by minor modification. If the potential phenotype (when repeatedly dosed) is predictable in the early stage by gene expression data of a few numbers of experimental animals, it would effectively cut out time and cost for drug development. From another point of view, this also contributes to animal welfare by reducing the number of sacrifices.

Application of toxicogenomics to the toxicity test in the final candidate just before clinical trials seems to be promising to improve the predictivity of clinical side effects. In fact there is a trend to employ toxicogenomics and pharmacogenomics technology for regulatory science (Petit, 2004). In such a case, various issues regarding validation and

standardization of data acquisition and analysis in addition to the species difference will be problems that should be solved urgently.

The third field for toxicogenomics is post-marketing surveillance. One promising strategy against species difference would be the connection of the clinical data to the TGP database. As human *in vivo* experiments are impossible and the barrier between *in vivo* and *in vitro* is too high, the only way is to accumulate clinical phenomena that are related to the contents in the database. There is a movement to create databases of disease-related genotypes or SNPs, and it would be promising to make a functional network between these databases and the TGP to establish an integrated toxico/pharmacogenomics database.

In any event, the completion of the TGP database is not a goal, but a beginning for toxicogenomics study. Compared with the enrichment of the data, the analysis/prediction procedures are in the developing stage. It is our desire that toxicology together with systems biology rapidly advances by efficient use of this database and that it contributes in accelerating the development of more effective and safer drugs.

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H E T

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Effects of DMSO on gene expression in human and rat hepatocytes

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Abstract

Dimethyl sulfoxide (DMSO) is a very common organic solvent used for dissolving lipophilic substances, for example for in vitro cell-based assays. At the same time, DMSO is known to be cytotoxic at high concentrations. Therefore, it is important to define threshold concentrations of DMSO for cells but relevant data at the molecular level are very limited. We have focused on conducting microarray analyses of human and rat hepatocytes treated with more than 100 chemicals in attempts to identify candidate biomarker genes. In the present study, the effects of DMSO on gene expression and cytotoxicity were assessed in human cryopreserved hepatocytes and rat primary cultured hepatocytes. A cytotoxicity test with lactate dehydrogenase (LDH) activity demonstrated DMSO to be noncytotoxic up to a concentration of 2% (v/v) in both cases and there were only few effects on the gene expression profiles up to 0.5% (v/v). The observed differences from controls were considered to be of little toxicological importance, but still need to be taken into account in interpretation of findings when DMSO is used at high concentration.

Keywords

gene expression profile, DMSO, human cryopreserved hepatocytes, rat primary cultured hepatocytes, cytotoxicity

Introduction

A large-scale gene expression database, termed TG-GATES (Genomics Assisted Toxicity Evaluation System), has been established by the Toxicogenomics Project in Japan.[1] About 150 chemicals, mainly for medicinal use, were selected, and gene expression in rat liver, rat kidney, rat primary cultured hepatocytes, and human cryopreserved hepatocytes is being comprehensively analyzed using Affymetrix GeneChip system (Santa Clara, CA, USA). In the project, rat and human hepatocytes are treated with toxicological prototype drugs, in three dose-ranges, and samples are collected 2, 8, and 24 hr after a single treatment. One of the main aims of our project is to identify candidate biomarker genes to predict and/or diagnose toxicity.

The actual dose-ranges are set according to dose-finding studies. The maximum concentration was set at 10 mM in the case of a chemical with high solubility. The concentration of a chemical with low solubility is determined with reference to solubility in 0.1%

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(v/v) DMSO, which is the commonly used concentration for in vitro assays. However, in some cases, this means that the concentration is too low for the effects of the chemical to be adequately reflected in the gene expression profile. It is then necessary to increase the chemical concentration, but this also necessitates increasing the DMSO concentration to obtain a solution. Since DSMO is itself toxic at high concentration, this could result in misleading findings. Since there have been no reports of comprehensive gene expression profiles after treatment with DMSO, the present study was conducted using human cryopreserved hepatocytes and rat primary cultured hepatocytes.

There are several reports about influence of DMSO on expression of genes in cells. For example, it has differentiation-inducing effects on embryonic stem (ES) cells, which are most sensitive towards the cytotoxic effects of DMSO including Oct-4.[2] Klinken et al. also reported induction of differentiation in murine erythroleukemia (MEL) cells with alteration of proto-oncogene levels.[3] Similar findings have been described for HL60 cells.[4] As far as housekeeping genes are concerned, Nishimura et al. examined the effects of DMSO on the expression of beta-actin (Actb), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), beta-glucuronidase (Gusb), phosphoglycerate kinase 1 (Pgk1), peptidylprolyl isomerase A (Ppia), and transferrin receptor (Tfrc) mRNA in cultures of C2C12 myotubes and the mRNA levels of some housekeeping genes were affected by exposure of DMSO concentrations of 0.5% (v/v) or more.[5] The induction of some drug-metabolizing enzyme genes by DMSO has also been reported.[6-8]

Thus, for in vitro assays, it is important to define the threshold concentration of DMSO for cells in accordance with the endpoint. The aim of the present study was to identify the lowest concentration of DMSO at which no significant effects on gene expression profile were observed, with particular attention to candidate biomarker genes. For this purpose, human cryopreserved hepatocytes and rat hepatocytes were examined for effects of DMSO on gene expression with a DNA microarray system and cytotoxicity with a lactate dehydrogenase (LDH) leakage method.

Materials and methods

Cell culture and in vivo studies

Human cryopreserved hepatocytes, purchased from CellzDirect, Inc. (Durham, NC, USA), were recovered in cryopreserved hepatocyte-recovered medium (CellzDirect, Inc.) and seeded in HCM Bullekit® (Cambrex Corp., East Rutherford, NJ, USA) supplemented with 10% FBS (Invitrogen Corp., Carlsbad, CA, USA) in 6-well collagen type-1 coated plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a concentration of 1.2×10^6 cells/2 mL/well for 4 hours in a humidified atmosphere (37°C, 5% CO_2). After 4-hour culture, medium was replaced with HCM Bullekit® without FBS and human hepatocytes were cultured for 20 more hours.

At day 2, medium was replaced with HCM Bullekit® containing 0% (v/v), 0.1% (v/v), 0.5% (v/v), 0.75% (v/v), 1% (v/v), or 2% (v/v) DMSO (Kanto Chemical Co., Inc., Tokyo, Japan, purity > 99.7%), and the hepatocytes were cultured for 24 more hours in a humidified atmosphere (37°C, 5% CO_2).

At day 3, medium was collected for measurement of LDH activity and total RNA samples were collected for gene expression analysis. Extraction of total RNA was conducted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) before quantification with a spectrophotometer DU-7400 (Beckman Coulter, Fullerton, CA, USA) and assessment of ribosomal RNA integrity using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Rat primary-cultured hepatocytes, prepared from a 5-week-old male Spraque-Dawley rat (Charles River Japan Inc., Kanagawa, Japan), were seeded in HCM Bullekit® supplemented with 10% FBS in 6-well collagen type-1 coated plates at a concentration of 1.0×10^6 cells/2 mL/well for 2-3 hours in a humidified atmosphere (37°C, 5% CO_2). After 2-3 hours culture, medium was replaced with HCM Bullekit® without FBS and rat hepatocytes were cultured for 16 hours.

At days 2 and day 3, rat primary cultured hepatocytes were treated the same as human cryopreserved hepatocytes.

Gene expression analysis

Microarray analysis was conducted on three samples for each group using HGU133 plus 2.0 probe arrays and RG230 2.0 probe arrays (Affymetrix). The procedures were basically conducted following the manufacturer's protocol, as previously reported.[9,10] The obtained image files were analyzed with the Affymetrix data suite system, Microarray Suite 5.0 (MAS 5.0) and derived signal values were globally normalized and targeted to all probe sets equal to 500 before comparative analysis to examine gene expression differences between treatment and control samples.

Quantitative RT-PCR assay validation

To confirm the gene expression data using microarray, we conducted quantitative RT-PCR for some rat genes. Five genes (Ccl20, sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 [Sult1a1], Cyp1a1, carbonic anhydrase 2 [Car2], and Cyp2c12) were measured by real-time PCR using TaqMan® Gene Expression Assays and the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). cDNAs were generated from 12 ng of total RNA using Invtirogen reverse transcription reagents (SuperScript III Reverse Transcriptase (10,000 units), RNaseOUT, Random Primers, 10 mM dNTP, and 0.1 M DTT). Three replicates were run for each gene for each sample in a 384-well format plate.

Measurement of LDH activity

The collected medium was centrifuged at 1500 × *g* or more for 5 min at 4°C. About 0.8 mL of the supernatant was applied to a biochemical autoanalyzer TBA-200FR (Toshiba, Tokyo, Japan) with the UV-rate method.

Data analysis

Raw probe intensities in Affymetrix CEL files were normalized using the MAS 5.0 algorithm with default parameters. After the MAS 5.0 process, we selected each probe for which a detection call is "present" among all conditions in this study. The fold change values were calculated as the ratio of gene expression values between DMSO treatment data versus control data. To compare gene expression levels among five different DMSO concentrations, we normalized the expression data using Tukey's biweight method and converted them into signal/control $\log_2$ratios. We used the smoothing spline clustering method[11] to sort and identify gene expression patterns that were dependent on the DMSO concentration. To find significantly and differentially expressed gene sets between control and DMSO treatment data, gene set enrichment analysis (GSEA)[12] was performed. The gene set data for the human and the rat were extracted from the Kyoto encyclopedia of genes and genomes (KEGG) pathway database.[13]

Results

Gene expression analysis

In human cryopreserved hepatocytes, there were only 4 probe sets, which altered in expression ($p < 0.05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO compared with control (0% DMSO). There were 16 probe sets altered in expression at 0.5% (v/v) DMSO, 57 probe sets at 0.75% (v/v) DMSO, 133 probe sets at 1% (v/v) DMSO and 752 probe sets at 2% (v/v) DMSO. In rat primary cultured hepatocytes, there were only 1 probe set, which altered in expression ($p < 0.05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO compared with control (0% DMSO). There were 10 probe sets altered in expression at 0.5% (v/v) DMSO, 22 probe sets at 0.75% (v/v) DMSO, 67 probe sets at 1% (v/v) DMSO, and 497 probe sets at 2% (v/v) DMSO (Figure 1a-d).

The GSEA detected differentially expressed gene sets in each DMSO treatment data compared to the control data. We analyzed 165 and 195 gene sets for human and rat hepatocytes, respectively. The differentially expressed gene sets were shown in Table 1. In the case of human hepatocytes, the top 10 differentially expressed gene sets at 0.1% (v/v) DMSO were not within the top 10 at 1% (v/v) DMSO. Distinct gene set changes were observed between 0.75% (v/v) DMSO and 1% (v/v) DMSO. In rat primary cultured hepatocytes, of the top 10 gene sets detected at 0.1% (v/v) DMSO, only 2 gene sets were detected at 2% (v/v) DMSO. The data indicate that 0.1% (v/v) DMSO had distinctly less influence than higher DMSO concentration. From 0.5% (v/v) DMSO to 1% (v/v) DMSO, some overlapping gene sets like RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY were observed.

The alterations in expression of Phase I, II, III drug-metabolizing enzymes are summarized in Table 2. Of the total of 728 probe sets (Phase I: 295, Phase II: 325, Phase III: 108), 161 genes demonstrated significantly altered expression levels in human hepatocytes. In rat hepatocytes, of the total of 409 probe sets (Phase I: 170, Phase II: 183, Phase III: 56), 57 genes were significantly altered. The alteration in expression of most drug-metabolizing enzymes was not severe up to 0.75% (v/v) DMSO in both human and rat cases.

Cytotoxicity of DMSO

Human cryopreserved hepatocytes and rat primary cultured hepatocytes were treated with DMSO for 24 hours at five different concentrations in order to examine its cytotoxic effects. In both human and rat hepatocytes, no toxic effects were

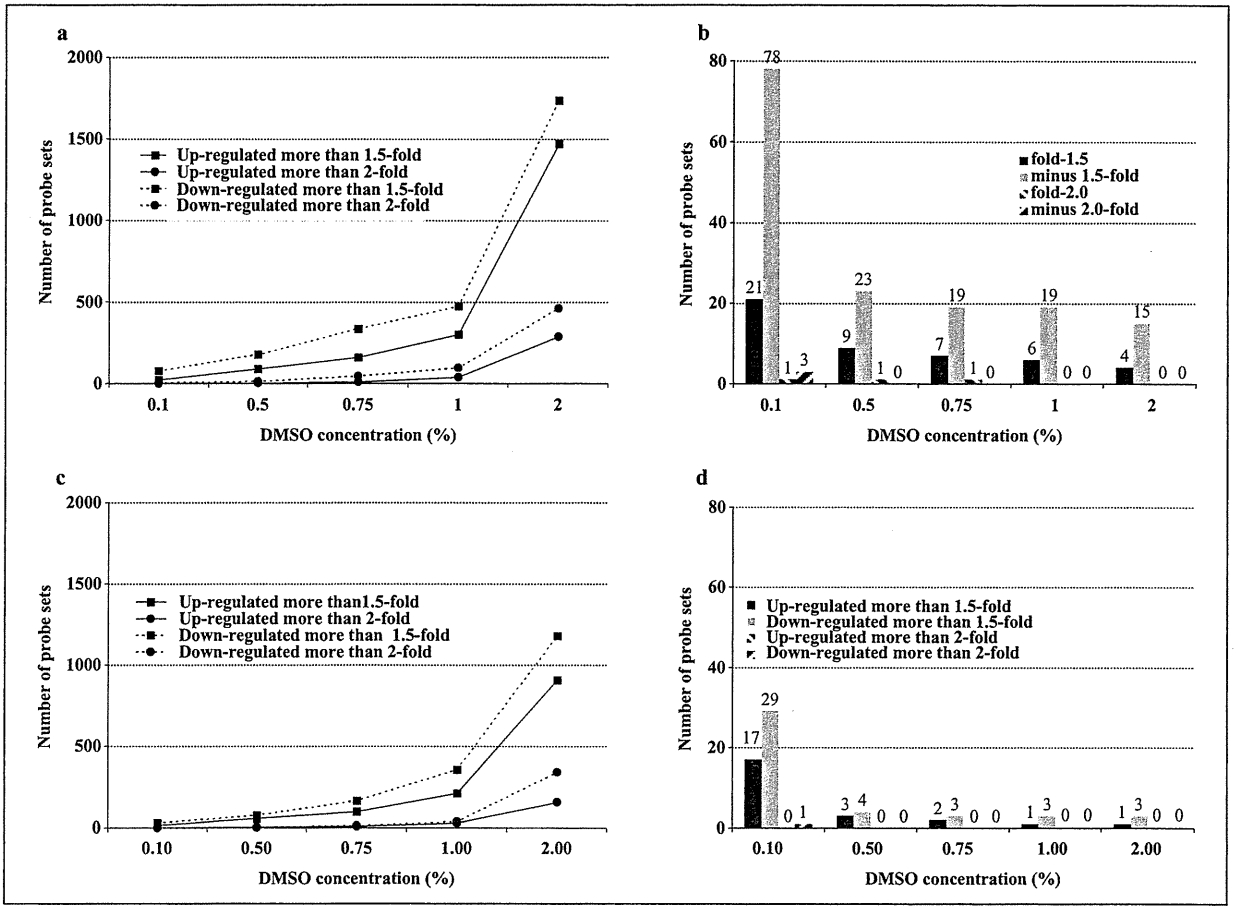

Figure 1. The numbers of differentially expressed genes (probe sets) in human cryopreserved hepatocytes (a) and rat primary cultured hepatocytes (c) are shown. Solid and dashed lines indicate up- and down-regulated genes, respectively. Circles and squares indicate 2.0- and 1.5-fold changes, respectively. The number of the probe sets was significantly increased between 1% (v/v) and 2% (v/v) dimethyl sulfoxide (DMSO). The numbers of differentially expressed genes (probe sets) commonly observed from 0.1% (v/v) DMSO to higher concentrations in human cryopreserved hepatocytes (b) and rat primary-cultured hepatocytes (d) are shown.

morphologically observed up to the concentration of 2% (v/v) DMSO.

In addition, the cytotoxicity of DMSO was examined by the conventional LDH test. LDH activity response curves for DMSO in human and rat hepatocytes are shown in Figure 2. Again, DMSO did not show any toxic effects up to the concentration of 2% (v/v).

Discussion

We have been focusing on identification of candidate biomarker genes to predict and/or diagnose toxicity in our project (TGP2, Toxicogenomics Informatics Project). Gene expression data using rat primary hepatocytes or human cryopreserved hepatocytes as well as gene expression data using rat liver are being analyzed. However, the effects of some chemicals were found to not be adequately reflected in the gene expression profile due to the low concentrations dictated by solubility in the set concentration of DSMO. The necessity to increase the DMSO concentration is the reason for the present study. The fact that there were not many probe sets with altered in expression up to 0.75% (v/v) DMSO is therefore very important.

In particular, there were remarkably few probe sets with altered in expression up to 0.5% (v/v) DMSO. As the concentration of DMSO increased, the number of probe sets with alteration and the magnitude of alteration increased. In human hepatocytes, there was only 1 probe set (histone cluster 1, H2bd; HIST1H2BD), which was upregulated ($p < .05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO, 4 probe sets

1 **Table 1.** The differentially expressed gene sets in human and rat hepatocytes are shown[a]

DMSO Con. (%)	Gene Set (KEGG Pathway ID and Name)	NOM *p* Value	FDR *q* Value	FWER *p* Value
0.1	HSA03010_RIBOSOME	0.00	0.00	0.00
	HSA03410_BASE EXCISION REPAIR	0.00	0.06	0.07
	HSA04110_CELL CYCLE	0.00	0.31	0.47
	HSA03050_PROTEASOME	0.02	0.24	0.48
	HSA03030_DNA REPLICATION	0.03	0.25	0.57
	HSA00760_NICOTINATE AND NICOTINAMIDE METABOLISM	0.03	0.21	0.58
	HSA00480_GLUTATHIONE METABOLISM	0.02	0.54	0.92
	HSA00240_PYRIMIDINE METABOLISM	0.02	0.48	0.92
	HSA03430_MISMATCH REPAIR	0.05	0.57	0.97
	HSA05012_PARKINSON's DISEASE	0.00	0.86	0.99
	HSA04020_CALCIUM SIGNALING PATHWAY	0.03	0.98	1.00
0.5	HSA03010_RIBOSOME	0.00	0.03	0.03
	HSA00590_ARACHIDONIC ACID METABOLISM	0.00	0.27	0.40
	HSA00591_LINOLEIC ACID METABOLISM	0.02	0.27	0.54
	HSA04742_TASTE TRANSDUCTION	0.04	0.20	0.54
	HSA00480_GLUTATHIONE METABOLISM	0.00	0.34	0.85
	HSA05012_PARKINSON's DISEASE	0.05	0.83	1.00
	HSA04080_NEUROACTIVE LIGAND-RECEPTOR INTERACTION	0.05	0.74	1.00
0.75	HSA00590_ARACHIDONIC ACID METABOLISM	0.00	0.02	0.02
	HSA03010_RIBOSOME	0.00	0.20	0.31
	HSA00591_LINOLEIC ACID METABOLISM	0.00	0.14	0.32
	HSA00190_OXIDATIVE PHOSPHORYLATION	0.00	0.16	0.46
	HSA05012_PARKINSON's DISEASE	0.00	0.15	0.50
	HSA00760_NICOTINATE AND NICOTINAMIDE METABOLISM	0.02	0.30	0.85
	HSA03050_PROTEASOME	0.04	0.42	0.95
	HSA05322_SYSTEMIC LUPUS ERYTHEMATOSUS	0.02	0.46	1.00
1	HSA04130_SNARE INTERACTIONS IN VESICULAR TRANSPORT	0.00	0.52	0.35
	HSA05322_SYSTEMIC LUPUS ERYTHEMATOSUS	0.00	0.29	0.38
	HSA05120_EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	0.00	0.44	0.65
	HSA04912_GNRH SIGNALING PATHWAY	0.00	0.37	0.69
	HSA00591_LINOLEIC ACID METABOLISM	0.02	0.39	0.80
	HSA00601_GLYCOSPHINGOLIPID BIOSYNTHESIS - LACTO AND NEOLACTO SERIES	0.02	0.36	0.84
	HSA04142_LYSOSOME	0.00	0.39	0.90
	HSA04010_MAPK SIGNALING PATHWAY	0.03	0.69	1.00
2	HSA04912_GNRH SIGNALING PATHWAY	0.00	1.00	0.76
	HSA05322_SYSTEMIC LUPUS ERYTHEMATOSUS	0.03	0.85	0.85
	HSA04916_MELANOGENESIS	0.00	0.89	0.91
	HSA04070_PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	0.03	0.98	0.98
0.1	RNO03420_NUCLEOTIDE_EXCISION_REPAIR	0.00	0.06	0.04
0.5	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.52	0.50
	RNO03420_NUCLEOTIDE_EXCISION_REPAIR	0.00	0.41	0.74
	RNO04623_CYTOSOLIC_DNA-SENSING_PATHWAY	0.00	1.00	1.00
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.83	1.00
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.69	1.00
	RNO05016_HUNTINGTON's_DISEASE	0.00	0.67	1.00
	RNO05012_PARKINSON's_DISEASE	0.02	0.98	1.00
	RNO00190_OXIDATIVE_PHOSPHORYLATION	0.02	1.00	1.00

(continued)

Table 1. (continued)

DMSO Con. (%)	Gene Set (KEGG Pathway ID and Name)	NOM *p* Value	FDR *q* Value	FWER *p* Value
0.75	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.81	0.49
	RNO05016_HUNTINGTON's_DISEASE	0.00	0.58	0.64
	RNO00071_FATTY_ACID_METABOLISM	0.00	0.44	0.71
	RNO00051_FRUCTOSE_AND_MANNOSE_METABOLISM	0.05	0.60	0.90
	RNO05012_PARKINSON's_DISEASE	0.00	0.60	1.00
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.55	1.00
	RNO03420_NUCLEOTIDE_EXCISION_REPAIR	0.00	0.50	1.00
	RNO04330_NOTCH_SIGNALING_PATHWAY	0.00	0.46	1.00
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.49	1.00
	RNO04120_UBIQUITIN_MEDIATED_PROTEOLYSIS	0.00	0.45	1.00
	RNO00330_ARGININE_AND_PROLINE_METABOLISM	0.04	0.54	1.00
1	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.05	0.02
	RNO05016_HUNTINGTON's_DISEASE	0.00	0.06	0.11
	RNO05012_PARKINSON's_DISEASE	0.00	0.10	0.24
	RNO00051_FRUCTOSE_AND_MANNOSE_METABOLISM	0.00	0.26	0.76
	RNO04330_NOTCH_SIGNALING_PATHWAY	0.00	0.28	0.85
	RNO00534_HEPARAN_SULFATE_BIOSYNTHESIS	0.00	0.33	0.92
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.35	1.00
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.32	1.00
	RNO00190_OXIDATIVE_PHOSPHORYLATION	0.00	0.33	1.00
	RNO04620_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.32	1.00
2	RNO00280_VALINE,_LEUCINE_AND_ISOLEUCINE_DEGRADATION	0.00	0.02	0.00
	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.03	0.04
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.02	0.04
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.05	0.13
	RNO04620_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.06	0.13
	RNO00071_FATTY_ACID_METABOLISM	0.00	0.12	0.35
	RNO04330_NOTCH_SIGNALING_PATHWAY	0.00	0.13	0.44
	RNO00600_SPHINGOLIPID_METABOLISM	0.00	0.12	0.53
	RNO04130_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	0.00	0.12	0.53
	RNO04622_RIG-I-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.12	0.56
	RNO04623_CYTOSOLIC_DNA-SENSING_PATHWAY	0.00	0.16	0.76
	RNO00640_PROPANOATE_METABOLISM	0.00	0.17	0.76
	RNO00100_STEROID_BIOSYNTHESIS	0.00	0.17	0.76
	RNO04142_LYSOSOME	0.00	0.16	0.76
	RNO04060_CYTOKINE-CYTOKINE_RECEPTOR_INTERACTION	0.00	0.17	0.81
	RNO04140_REGULATION_OF_AUTOPHAGY	0.00	0.19	0.86
	RNO05215_PROSTATE_CANCER	0.00	0.32	1.00
	RNO04660_T_CELL_RECEPTOR_SIGNALING_PATHWAY	0.00	0.34	1.00

[a]The pathway gene sets were extracted from KEGG pathway database and assessed using GSEA (gene set enrichment analysis). The GSEA can determine differentially expressed gene sets between two biological states or conditions. NOM-*p*val, FDR-*q*val and FWER-*p*val indicate nominal *p*-value, false discovery rate and family-wise error rate *p*-value, respectively.

(HIST1H2BD, BTB [POZ] domain containing 11 [BTBD11], chemokine [C-X-C motif] ligand [CXCL11], and another with no annotation) at 0.5% (v/v) DMSO. And there were 3 probe sets (endoplasmic reticulum aminopeptidase 1 [ERAP1], natural killer-tumor recognition sequence [NKTR], and cleavage and polyadenylation factor subunit, homolog [PCF11]), which were down-regulated ($p < 0.05$ and less than 1/2) at a concentration of 0.1% (v/v) DMSO, 12 probe sets (insulin-like growth factor binding protein 3 [IGFBP3], dehydrogenase/reductase (SDR family) member 9 [DHRS9], LIM and cysteine-rich domains 1 [LMCD1], solute carrier family 13 (sodium-dependent citrate transporter), member 5 [SLC13A5], IGFBP1, thyroid hormone responsive [THRSP], interleukin 1 receptor antagonist [IL1RN],

Table 2. Up- and down-regulated drug metabolizing enzymes in human and rat hepatocytes are shown[a]

Expression	Gene Symbol
Strongly up-regulated	AGXT2L1, BHMT, CYP2C8, CYP39A1, CYP3A4, CYP3A5, CYP4F3, CYP7B1, OAT, TAT
Up-regulated	ABAT, ABCC5, ABCC9, ABCD1, ABCD3, ABCD4, ADH6, ALDH1L1, ALDH2, ALDH3A2, ALDH5A1, ALDH6A1, ALDH7A1, ALDH9A1, BCAT1, BCAT2, BHMT2, COQ5, CYB5A, CYB5R4, CYP1B1, CYP20A1, CYP27A1, CYP2A6, CYP2A7, CYP2B6, CYP2B7P1, CYP2E1, CYP2U1, CYP4A11, CYP4A22, CYP4F11, DPYD, EPHX1, FMO3, FMO5, FTSJD2, GPT2, GPX4, GSTM2, HGSNAT, LRTOMT, MAOB, MSRB2, N6AMT2, NAT12, NAT15, PCMTD1, PCMTD2, PRMT2, PSAT1, SETD7, SLCO4C1, TPMT, TXNDC12, ZADH2
Strongly down-regulated	ABCA1, ABCC3, ADH1B, ADH1C, ADH4, AKR1B10, AKR1D1, ALDH1A1, ALDH1B1, ALPL, CYP2C18, CYP2C9, HNMT, METT10D, METTL12, METTL8, METTL9, NAT11, NAT8, PRMT1, SLCO1B1, SLCO1B3, SULT1A1, SULT1B1, TRMT6, UGT1A6, UGT2A3, UGT2B15, UGT2B28, UGT2B4
Down-regulated	ABCB10, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ADH5, AGXT2L2, AKR1A1, AKR1B1, AKR1C1, AKR1C4, AKR7A3, ALDH4A1, AOX1, ARD1A, AS3MT, ASMTL, COMTD1, COQ3, CYB5B, CYB5D1, CYB5R3, CYP51A1, DMAP1, DNMT1, FMO4, GPX1, GSTA1, GSTCD, GSTO1, HEMK1, HNMT, MAOA, METT5D1, METTL1, METTL11A, METTL13, METTL3, METTL5, MGST1, MTR, NAT10, NAT11, NAT13, NAT2, NAT5, NAT9, PEMT, PRMT5, RG9MTD1, RG9MTD2, RNMTL1, RRP8, SETD8, SHMT1, SULT1A1, SULT1A2, SULT2A1, TRDMT1, TRMT1, TRMT11, TRMT5, TRMT6, ZADH2
Up-regulated	Abca4, Abca5, Abcb1a, Aldh1a2, Aldh6a1, As3mt, Bhmt2, Ces3, Coq3, Cyp1a1, Cyp1a2, Cyp4a3, Cyp4f17, Cyp4f5, Gpx3, Gstm7, Gstt1, Gstt2, Nnmt, Pcmtd1, Pcmtd2, Slco3a1, Sult1a1, Tpmt, Trmt12
Down-regulated	Abcb11, Abcc2, Adh1, Akr1b8, Akr1c12, Akr1d1, Akr7a3, Ard1a, Bcat1, Comtd1, Cyp26b1, Cyp2b3, Cyp2c12, Cyp2c22, Cyp3a9, Fmo5, Gpt2, Gstm2, Gstt3, Maoa, Maob, Mettl0d, Mettl2, Mgst2, N6amt1, Prmt1, Psat1, Rrp8, Slco1a1, Slco1b2, Ugt2b17, Ugt2b36

[a]All drug metabolizing enzymes are classified using the smoothing spline clustering[11]. This method is able to classify a series data such as time series data. Several genes are shown multiple times due to redundant probe sets in the microarray.

chromosome 10 open reading frame 108 [C10orf108], palmdelphin [PALMD], and choroideremia-like (Rab escort protein 2) [CHML]) at 0.5% (v/v) DMSO. In rat hepatocytes, there was no probe set, which was up-regulated ($p < 0.05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO, 6 probe sets (Ccl2, radical S-adenosyl methionine domain containing 2 (Rsad2), Ccl20, Cxcl2, DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 [Ddx60], and Cxcl10) at 0.5% (v/v) DMSO and there was only 1 probe set (with no annotation), which was down-regulated ($p < 0.05$ and less than 1/2) at 0.1% (v/v) DMSO, and 4 probe sets (Car3, D4, zinc and double PHD fingers, family 3 [Dpf3], LOC681825, and another with no annotation) at 0.5% (v/v) DMSO. CXCL11 was up-regulated and IGFBP4 was down-regulated in common, but neither gene is reported to have any relation with DMSO.

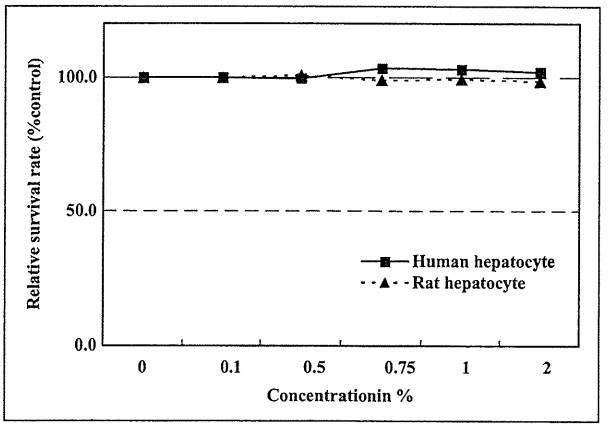

Figure 2. Lactate dehydrogenase (LDH) activity response curves for dimethyl sulfoxide (DMSO) in human cryopreserved hepatocytes and rat primary cultured hepatocytes. The experiments were conducted with hepatocytes in three independent wells ($n = 3$). Standard error bars are shown.

To determine whether an a priori defined set of genes shows statistically significant, concordant differences between control and DMSO treatment, GSEA was carried out. In human hepatocytes, a large gap was observed between 0.75% (v/v) and 1% (v/v) DMSO. And only 1 gene set was shared in common. In contrast, there were 3 gene sets in common

between 0.1% (v/v) and 0.5% (v/v) DMSO, 4 gene sets in common between 0.1% (v/v) and 0.75% (v/v) DMSO, and 4 gene sets in common between 0.5% (v/v) and 0.75% (v/v) DMSO. In rat hepatocytes, large gaps were observed between 0.1% (v/v) and 0.5% (v/v) DMSO and between 0.1% (v/v) and 0.75% (v/v) DMSO. There was only 1 gene set in common in each gap. In contrast, 6 gene sets were in common from 0.5% (v/v) to 1% (v/v) DMSO (Table 1).

With regard to Phase I, II, III drug-metabolizing enzymes, DMSO concentration-dependent probe sets were clustered with the smoothing spline clustering method, and 161 genes in human cryopreserved hepatocytes and 57 genes in rat primary cultured hepatocytes were altered in expression level, respectively (Table 2). However, the magnitude of alteration for expression of most drug-metabolizing enzymes was within 1 standard deviation in both cells.

In human hepatocytes, there were 66 enzymes, which were up-regulated, and 95 enzymes, which were down-regulated. Of 66 enzymes, 10 enzymes including CYP3A4 and CYP3A5, were strongly up-regulated enzymes and 56 enzymes including CYP1B1, CYP2A6 and CYP2E1 were moderately up-regulated. Of 95 enzymes, 30 enzymes including UGT1A6 were strongly down-regulated enzymes and 65 enzymes were moderately down-regulated. Nishimura et al. earlier investigated the gene expression of CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1, CYP3A4, CYP3A5, CYP3A7, UGT1A6, UGT1A9 and ABCB1 after 24 hours of exposure to 0.1% (v/v), 0.5% (v/v), and 2.5% (v/v) DMSO in primary culture of human hepatocytes.[6] Our results were in excellent agreement with their findings. Wilkening and Bader also reported the response after 24 hours exposure to 0%-1% (v/v) DMSO in primary human hepatocytes.[7] They found CYP3A4 but not CYP3A7 to be induced by DMSO and this also corresponds with our data. Furthermore, Choi et al. reexamined induction of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D8, CYP3A4, CYP3A5, UGT1A1, UGT1A4, UGT1A6, UGT1A9, and UGT2B7 after exposure to 1% (v/v) DMSO over 20 days in Huh7 cells, which was established from a 57-year-old male with a well-differentiated hepatocellular carcinoma in 1952.[8] In their report, all of the examined enzymes, expect for CYP1A1, showed significant increase in their expression by DMSO treatment. Since their experimental condition was very different from our own, we are not able to make an easy comparison, but CYP2B6, CYP2C8, CYP3A4, and CYP3A5 were induced by DMSO in both data sets. On the other hand, there is a discrepancy between the two concerning CYP2C9 and UGT1A6. In rat hepatocytes, there were 25 enzymes which were up-regulated and 32 enzymes which were down-regulated. Some enzymes including betaine-homocysteine methyltransferase 2 (Bhmt2), protein-L-isoaspartate (D-aspartate) *O*-methyltransferase domain containing 2 (Pcmtd2), aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase; Akr1d1), and methyltransferase 10 domain containing (Mett10D) were up-regulated or down-regulated in both human and rat hepatocytes. The magnitude of change of these transcripts in rat hepatocytes was often larger than that in human hepatocytes.

As for housekeeping genes, Nishimura et al. examined the effects of DMSO on the expression of Actb, Gapdh, Gusb, Pgk1, Ppia, and Tfrc mRNA in cultures of C2C12 myotubes.[5] They reported that Actb, Pgk1 and Tfrc were significantly ($p < 0.05$ and less than 1/2) decreased at 2.5% (v/v) DMSO after 24 hours. At 2% (v/v) DMSO, we found the above 6 genes to show similar expression change as their results.

To confirm the gene expression data using microarray, we conducted quantitative RT-PCR for some rat genes (Ccl20, Sult1a1, Cyp1a1, Car2, and Cyp2c12). Ccl20, which was up-regulated ($p < 0.05$ and 2.13-fold compared with control) in microarray analysis at a concentration of 0.5% (v/v) DMSO, showed up-regulation (dCt = −0.67) in expression in using quantitative RT-PCR. It was also confirmed that Sult1a1 and Cyp1a1 were up-regulated in quantitative RT-PCR analysis (dCt = −0,09 and dCt = −0.26, respectively) as well as in microarray analysis (1.59-fold and 1.84-fold) at 2% (v/v) DMSO. On the other hand, Car2 and Cyp2c12 showed down-regulation in both quantitative RT-PCR analysis and microarray analysis (eTable). The Quantitative RT-PCR data were well coincident with the microarray data.

As regards cytotoxicity, no toxic effects were observed up to the concentration of 2% (v/v) in either of the cells with either of the tests employed.

In conclusion, we showed that there are only very few probe sets altered in expression at doses up to 0.75% (v/v) DMSO in both human and rat hepatocytes. In particular, there are remarkably few probe sets altered in expression up to 0.5% (v/v) DMSO and the magnitude of alteration for expression of most drug-metabolizing enzymes was within 1 standard

deviation in both cells. Alteration of housekeeping genes was also very small. Furthermore, DMSO did not show any toxic effects up to the concentration of 2% (v/v) and there are few differences in effects of DMSO between human and rat hepatocytes with regard to change of transcripts. These results in human cryopreserved hepatocytes and rat primary cultured hepatocytes suggested that a DMSO concentration up to 0.5% (v/v) can be tolerated, although care must be taken in interpretation at higher concentrations.

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eTable 1. Validation of microarray data using TaqMan® real-time PCR assays