

## FORUM

# The Principles and Practice of Toxicogenomics: Applications and Opportunities

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### INTRODUCTION

The last decade has seen major developments in large-scale genome sequencing and in the development of technical platforms to support this. Several genomes, such as yeast (DeRisi *et al.*, 1997), have now been completely sequenced and the sequencing of the entire human genome is anticipated to be completed early in this decade, as a result of major effort in both the academic and industrial sectors. The availability of both the sequence information for many thousands of genes and, in many cases, physical clones of the coding regions of these genes, has allowed the construction of gene microarrays that enable quantitative measurement of the transcriptional activity of potentially tens of thousands of genes in biological samples (Schena *et al.*, 1995). Microarray technology promises to revolutionize investigative biology (Khan *et al.*, 1999), including drug discovery (Marton *et al.*, 1998). Application of genomics to toxicology, *toxicogenomics*, may also yield a number of substantial dividends, including assisting predevelopment toxicology by facilitating more rapid screens for compound toxicity; allowing compound selection decisions to be based on safety as well as efficacy; the provision of new research leads; a more detailed appreciation of molecular mechanisms of toxicity; and an enhanced ability to extrapolate accurately between experimental animals and humans in the context of risk assessment. In this article, we provide a brief overview of microarray technology and its applications to mechanistic and predictive toxicology research, outlining both the strengths and limitations of this approach.

#### Microarray Technology Platforms

DNA chips, or microarrays, allow quantitative comparisons of the expression levels of potentially thousands of individual genes between different biological samples. This facilitates, for

instance, comparisons of normal tissue with diseased, and of control with toxicant-treated cell lines. Construction of arrays involves the immobilization of DNA sequences (either cDNA sequences or oligonucleotides), corresponding to the coding sequence of genes of interest, on a solid support such as a glass slide or nylon membrane (Bowtell, 1999). The mRNA prepared from cells or tissues can be labeled and hybridized, usually in the form of a reverse transcribed cDNA copy, to a microarray and visualized using phosphorimager scanning or other appropriate methodologies. Subsequent analysis using appropriate software allows determination of the extent of hybridization of the labeled probes to the corresponding arrayed cDNA spots, and a comparison of control with test samples permits quantitative assessment of changes in gene expression associated with treatment (Brown and Botstein, 1999).

Many commercial microarrays are available, varying from those comprising several hundred genes (usually immobilized on a nylon membrane and probed with radiolabeled cDNA) to those harboring tens of thousands of oligonucleotide sequences (immobilized on glass slides and analyzed using dual fluorescent-probe technology). These arrays can be either “broad spectrum” or custom designed to profile particular tissues, biological pathways, or even disease states. In this regard, microarrays designed to profile genes involved in response to toxic insult have been developed by commercial vendors, pharmaceutical companies, and academic institutions (see, for example, Nuwaysir *et al.*, 1999).

#### Possibilities and Caveats

A major part of the developmental cost of every successful new pharmaceutical or agrochemical product is the recovery costs of compounds that have failed in development, due to potential or observed toxicity. The application of toxicogenomics to mechanistic and predictive toxicology will enable the identification of more effective markers with the potential for

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adverse health effects, thus highlighting possible “show-stopping” toxicity earlier in a compound’s development, and potentially enhancing the predictive power of *in vitro* model systems (Davila *et al.*, 1998). This may prove particularly valuable if toxicogenomics can improve our understanding of processes such as nongenotoxic carcinogenesis or reproductive toxicity, which normally require both long-term animal assays and significant amounts of chemical to be detected accurately.

Clearly, the possibility of toxicogenomics giving an early alert to potential adverse effects at low levels of exposure, while powerful, raises many issues in the context of interpreting gene expression changes with respect to hazard and risk assessment. It is reasonable to predict that in the first instance, accurate interpretation of expression changes will only be possible when toxicogenomics analyses are conducted as part of a larger experimental design to understand *observed* toxicity at the physiological, histological, and/or biochemical levels. Also, there is a vast amount of information already available on *in vivo* toxic responses to compounds, particularly in rodents. Strategically designed toxicogenomics experiments will allow the exploitation and annotation of some of these data.

As microarrays only profile gene expression at the mRNA level, this technology alone cannot identify corresponding changes in the level of functional protein. Additionally, protein modifications such as phosphorylation, which may be critical for the function of many proteins (and indeed entire biochemical pathways such as signal transduction), cannot be detected directly by microarray analysis, although indirect detection is likely where the protein modifications result in downstream changes in gene expression. The complementary technology of *proteomics* may help resolve issues around such differential protein modification, leading to changes in the activity of gene products. Briefly, proteomics allows the separation of the total protein complement of cell extracts, consisting usually of thousands of different proteins, by 2D polyacrylamide gel electrophoresis. Stained gels are scanned and analyzed with bioinformatics software, in order to compare protein extracts of cells in different physiological states. Compounds with particular toxicological effects will induce changes in the proteome, producing a signature proteomic expression profile (for example, see Anderson *et al.*, 1996). The throughput of proteomics is currently much lower than that of genomic microarrays, largely due to the requirement for mass spectroscopy analysis (or similar technologies) of differentially expressed proteins to determine identity. Recent reports have suggested, however, that microarray-based approaches to protein detection may overcome this limitation (Lueking *et al.*, 1999). Protein function can also be affected by mutation or polymorphism of the encoding gene (without influencing transcript levels), and such events cannot be resolved directly by transcript profiling experiments. Methodologies such as pharmacogenetics are being developed to define polymorphic responses. Nevertheless, it is reasonable to assume that mechanistic events will give rise to

diagnostic changes in the transcription of other genes that can be detected by microarrays. However, careful and informed interpretation of microarray results will obviously be required to “backtrack” to the important initiating events, under such circumstances.

### Toxicogenomics Applications

In general terms, the applications of toxicogenomics can be characterized into two broad and overlapping classes: *mechanistic* or *investigative research* and *predictive toxicology*.

The biological relevance of the experimental system for transcript profiling is clearly of major importance where a mechanistic understanding of a toxic process or a mode of action is required. In all likelihood, the toxic endpoint is known in advance (at a physiological, histological, and/or biochemical level) and an appropriate test system (*in vitro* or *in vivo*) can be designed to model the endpoint as closely as possible. An example of such an endpoint, non-genotoxic carcinogenesis, is usually evaluated in the context of long-term cancer bioassays in rodents (Chhabra *et al.*, 1990). Toxicogenomic applications may help to identify surrogate markers for the development of this phenotype, and indeed the exposure of rodent hepatocytes to the nongenotoxic carcinogen phenobarbital has been studied, using both microarray and gel-based expression technologies. In excess of 300 genes have been identified where expression is modulated by this compound (Rodi *et al.*, 1999). Many other toxic endpoints could be profiled using these methods, with combinatorial approaches such as transgenic or knockout models (Ryffel, 1997) potentially providing insights into the role of specific genes.

The possibility that a specific group or class of compounds (grouped by toxic endpoint, mechanism, structure, target organ etc.) may induce signature patterns of gene expression changes is the basis for the application of toxicogenomics to predictive toxicology. The use of these technologies to analyze genome-wide changes in mRNA expression following treatment of *in vitro* systems with known reference toxicants may permit the identification of diagnostic gene expression patterns. Pattern recognition may, in turn, allow the design and construction of *miniarrays*, customized to detect specific toxicity endpoints or pathways. The throughput requirements of this approach will almost certainly necessitate employing *in vitro* culture systems. While *in vitro* systems have practical advantages, there are major drawbacks to consider. Even where appropriate cells *in vitro*, such as primary hepatocytes, are available, compound-induced changes in transcription may not necessarily reflect accurately the response of the corresponding organ *in vivo*. In addition, availability of appropriate cell lines may be limited (although where mechanistic information is not sought, generic cell lines may still be of value), and in some cases, metabolism may be required to produce the active chemical reactive species, although often this can be accomplished by pre-incuba-

TABLE 1  
Broad Gene Classes Included on the *ToxBlot* Microarrays

Cancer	Immunology	Endocrinology and neurobiology	Investigative toxicology	Predevelopment toxicology	Safety assessment
Apolipoprotein genes	Basic transcription factors	Basic transcription factors	Acetyl CoA pathway	Bcl/Bax family	Bcl/Bax family
Basic transcription factors	Cell adhesion molecules	CYP	Bcl/Bax family	CYP	CYP
Bcl/Bax family	Cell surface receptors	Drug metabolism	Drug metabolism	Acetyl CoA pathway	Drug metabolism
CYP genes	Chemokines	Extracellular matrix	Immediate early genes	Ion channels	GST
Caspases	Extracellular matrix	GABA receptors/transport	GST	GST	Heat shock proteins
cdc/cdk s	Heat shock proteins	Heat shock proteins	CYP	Histones	Liver acute-phase markers
Cyclins	Interleukins	Ion channels	Basic transcription factors	Heat shock proteins	Markers for GI tract physiology
GST	Metalloproteinases	Neurotransmitter-metabolising enzymes	Steroid hormone receptors	Steroid regulated genes	Oxidative-stress markers
Heat shock proteins		Neurotrophic factors/receptors			Steroid regulated genes
Immediate early genes		Peptide hormones			Thyroid hyperplasia markers
Interleukins		Steroidogenesis/aromatase			
Matrix metalloproteinases		Steroid hormone receptors			
Steroid hormone receptors		Steroid regulated genes			

tion with metabolically active cell extracts. Importantly, where the toxicity is specific to species, strain, sex, or route of administration, *in vitro* modeling is unlikely to be fully diagnostic. Nevertheless, even if these systems were able to detect potential adverse health effects of only a small subset of development compounds, their application in predevelopment toxicology screening would be of substantial benefit in providing an early view of compound safety in advance of traditional studies.

Development of reference data sets to allow a “pattern recognition” approach to toxicology is likely to require the application of complex computer algorithms and statistical approaches. For example, statistical clustering techniques have been applied to microarray data to analyze the temporal patterns of gene expression that characterize serum-responsiveness and wound repair (Iyer *et al.*, 1999), and to distinguish cancerous tissue from normal tissues and cell lines (Alon *et al.*, 1999). The building of reference data sets, possibly by comparison of microarray output across different laboratories, will require consistency in data analysis and format. A number of resources exist in both the academic and commercial sectors for such purposes (Bassett *et al.*, 1999). One example, the software platform *ArrayDB*, has been developed at The National Human Genome Research Institute. The system facilitates the storage, retrieval, and analysis of microarray data along with information linking some 15,000 genes to public domain sequence and pathway databases (Ermolaeva *et al.*, 1998).

#### Custom Toxicology Microarray Construction: *ToxBlot*

In addition to the use of high-density “discovery” arrays from commercial vendors, our laboratories have a program of

in-house custom toxicology array design and manufacture: *ToxBlot*. A custom approach allows the selection of the genes to be represented on the array, permitting arrays to be focused on areas of particular interest to mechanistic or investigative toxicology research programs. (See Table 1). While the major costs of custom array manufacture are capital investment in appropriate technologies, once cDNA sets have been amplified, purified, and verified, the unit array cost is relatively modest, being in the order of \$20 per array. For the construction of the *ToxBlot* arrays, approximately 2000 sequences of human or murine origin were identified as being of potential relevance to various forms of toxicity or normal cell regulatory or signaling pathways. In-house cDNA clone sets, of both public domain and proprietary origin, were used as the source material for array construction. Following selection, PCR amplification, purification, and (where appropriate) sequence verification, the representative cDNA sequences were immobilized on nylon membranes. Both a human and a mouse *ToxBlot* array have been constructed, in each case comprising approximately 2400 cDNA sequences, spanning about 600 genes of the relevant species. Reproducibility is ensured by each gene being represented by 4 individual spots on each array and the inclusion of two non-overlapping cDNAs for each gene (in duplicate) wherever possible. To aid in the interpretation of differential gene-expression results, we have assembled a database on gene function, distribution, and known allelic variations for each represented gene. In this regard, public-domain databases such as the GeneCards system developed at the Weizmann Institute (Rebhan *et al.*, 1997), or the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (<http://www.genome.ad.jp/kegg/>) can provide valuable supplementary information.

The *ToxBlot* series of arrays are now in wide use in our

laboratories and some examples of current applications are outlined below:

### *Endocrine Disruption*

Concern that some environmental compounds (of natural and man-made origin) may affect estrogen receptor function, with consequences for normal endocrine function, has been a topic of debate among both the general public and the scientific community. Ligand-mediated estrogen receptor (ER) activation, followed by activation (or repression) of estrogen receptor-responsive genes has a complex mode of action which is not captured by many of the current *in vitro* screens for estrogen action. Recent research from our laboratory and others has indicated that the precise DNA sequence of the estrogen-response elements within the promoter of estrogen responsive genes may have a great influence on ER action (Pennie *et al.*, 1998, Vanacker *et al.*, 1999). Hence assays which measure the activation of a single gene product as a marker for estrogenicity may be misleading. Toxicogenomics analysis of ER action should help resolve pathways of gene regulation involved in estrogen action and lead to the development of more sophisticated *in vitro* assays for ER activation. In this regard, the combination of suppression subtractive hybridization (Diatchenko *et al.*, 1999) and cDNA construction has been employed to characterize the differences between ER-positive and -negative cell lines (Yang *et al.*, 1999). Our own laboratory is employing *ToxBlot* microarrays to characterize gene expression changes that take place in cultured cells exposed to natural estrogens (e.g., estradiol), synthetic (e.g., diethyl stilbestrol), and phytoestrogens (e.g., genestein). By treatment of appropriate cell lines (e.g., the ER + ve breast carcinoma cell lines T47D and MCF7) with estrogens at multiple doses and at a variety of time points, a pattern of consistent gene expression changes has begun to emerge that may permit evaluation of endocrine disruption at the molecular level. In addition, the use of cell lines of uterine origin, such as the human line SKUT (Bamberger *et al.*, 1997), may aid the understanding of gene expression changes in particular target cells and possibly assist prediction of endocrine disruption, thus reducing the number of animals used to predict this endpoint by traditional methods such as the uterotrophic assay (Odum *et al.*, 1997). Transcript profiling in these systems clearly demonstrates the need to profile gene expression changes at multiple time and dose points, as individual responsive genes can vary considerably in the kinetics of their regulation.

### *Hepatocyte Toxicity*

Many liver cell models retain tissue-specific characteristics in culture and have proved useful in, for instance, cytotoxicity and genotoxicity assays, drug-drug interactions, and mode of action studies (Guillouzo, 1998). The characterization of cyto-

toxicity in cultured hepatocytes provides an ideal model for profiling induced changes in gene expression. A number of hepatotoxicants has been characterized for cytotoxicity in cultured human hepatocyte cell lines (such as HepG2), including ethanol (Neuman *et al.*, 1993), paracetamol (Nicod *et al.*, 1997), hydrogen peroxide (Yang, C.-F. *et al.*, 1999) and carbon tetrachloride (Dai and Cederbaum, 1995). At appropriate doses, these compounds all cause liver necrosis *in vivo* (and cytotoxicity in HepG2 cells), induce periportal hepatocyte proliferation, cause elevation of enzyme levels (e.g., cypP4502E1) and modulate oxidative stress and lipid peroxidation. *ToxBlot* microarrays have been employed to characterize the gene expression changes induced in HepG2 cells when they are exposed to varying concentrations of these 4 cytotoxicants. By calculating cytotoxicity over a range of concentrations (based on release of cellular LDH), we were able to select appropriate high and low doses for each compound (set at 40% and 25% LDH release, respectively). Transcript profiling at these selected doses for all 4 compounds (at a fixed time point) revealed genes that were up- or down-regulated consistently by different compounds. These investigations permit the identification of patterns of gene changes, which may prove to be diagnostic for hepatotoxicity.

### *Bone Marrow Toxicity of Insulin-Sensitizing Compounds*

As discussed above, one of the most important applications of toxicogenomics is to provide information on safety early in the development process, to assist in compound selection. We have used microarrays for transcript profiling in parallel with a program to develop novel insulin-sensitizing agents as therapies for non-insulin-dependent diabetes mellitus (NIDDM). Such a program provides a useful "proof of concept" opportunity to use transcript profiling to obtain information for safety evaluation, for several reasons. First, the existing therapeutic agents for NIDDM, the thiazolidinediones (TZDs), have undesirable toxic properties that limit their clinical application. In particular, TZD administration to both rats and dogs results in bone marrow fat deposition (adipogenesis), impaired hematopoiesis, and anemia (Deldar *et al.*, 1993; Williams *et al.*, 1993). Thus, there is an incentive to develop alternative therapeutic agents with a more favorable toxic profile. Second, the TZDs are known to activate a nuclear receptor, the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ; Lambe and Tugwood, 1996), which represents a potential mechanism for both the toxic and therapeutic effects of these compounds, and assists the design and interpretation of array experiments. Third, a reliable *in vitro* system is available for evaluating adipogenic potential of compounds, using human bone-marrow stromal-cell culture (Gimble *et al.*, 1996).

This *in vitro* model has been used to investigate adipogenesis caused by a number of insulin sensitizing compounds, including TZDs. Differentiation of the stromal cells along an

adipogenic pathway develops over 7–10 days and fat deposition can be detected at this stage. The goal was to characterize transcriptional changes that precede this, and whether such changes are specific for TZDs. To this end, a custom *ToxBlot* array was designed that comprised genes associated with hematopoiesis, osteogenesis and adipogenesis in the bone marrow, together with genes known to be regulated by PPAR $\gamma$ . The final array consisted of approximately 2400 individual “spots”, representing 802 discrete genes, and this is being employed currently to examine changes in gene transcription of TZD and non-TZD compounds at adipogenic concentrations.

### Future Issues and Opportunities: A Perspective

The examples cited above serve to illustrate the ways in which the application of toxicogenomics can contribute significantly to our understanding of toxic processes. The potential utility of this new technology has necessarily stimulated a lively debate about how best genomics can be applied to the toxicological sciences and what, in practice, may be achieved. The debate is vigorous and essential and one that is continued here in considering several of the issues posed by the current and future application of toxicogenomics.

The first of these is that of “certainty”: a question posed commonly as to what extent we can be certain that the changes in gene expression detected by microarray technology are *real* and, if real, whether the altered gene expression necessarily translates into protein production. There is of course no *certainty*, as such, on either count. In the context of identifying new research leads, or changes that may provide clues to a particular toxic mechanism, it is probably best to regard the results of microarray analyses as the springboard to more detailed and more focused investigations (using other experimental approaches) that would confirm (or otherwise) the robustness of the changes observed. Furthermore, it is certainly not the case that induced changes in gene expression, associated with no altered protein production, are of no interest or are without relevance. This is particularly not so if these gene expression changes are proven to represent reliable, sensitive, and selective markers of a toxic process or the actions of a particular class of toxicant. This introduces a broader issue, of course, relating to the certainty of biological relevance. There is a comfort in understanding the molecular basis for any normal or adverse biological process, but if we are to make full use of toxicogenomics, and the results of microarray technologies, it is likely that we will have to accept greater intellectual discomfort and learn to embrace data that display a much more holistic view of biological changes, which cannot necessarily be reconciled into known and discrete mechanistic pathways.

Although to make full use of toxicogenomics, at least for some applications, an acceptance of a frameshift in data analysis will be necessary, one thing that remains unchanged is the

absolute requirement for appropriate experimental design. The principles of good experimental design and the need for appropriate controls and consideration of single variables remain critical. The results of toxicogenomic analyses will be only as valuable as the rigor of the experiment from which they derive. The old adage—*the best research comes from asking Nature simple questions, one at a time*—is no less true for microarray analyses. The answers that toxicogenomics can provide may be considerably more complex, but the questions we pose should be no less simple and specific than those asked using other experimental approaches. In this context, it is important that genomic and proteomic technologies are not blighted by precipitate or inappropriate interpretation of poor quality data from badly designed investigations.

Another important aspect of experimental biology is the need to understand and acknowledge the limitations of any approach. There has been, in the toxicological sciences, and particularly in predictive toxicology, a desire for *validation*, which in this context can be defined as a level of confidence that a particular method or test has the sensitivity and selectivity to permit conclusions to be drawn about the likely properties of a chemical. While these are important attributes of any test method, of at least equivalent and probably of greater importance is an appreciation of the constraints and limitations of the approach. Ultimately, test methods are the tools we employ as toxicologists to aid us in making decisions about the likely properties of chemicals in biological matrices and the potential for adverse health effects. It is a great arrogance to believe that we are able to design any experimental approach that will reflect accurately all the myriad effects xenobiotics may induce. No single test method will ever be absolute or comprehensive for all toxic endpoints, anymore than one single drug is ever likely to treat all disease. It is for this reason that the experience and expertise of toxicologists and their ability to accurately interpret the results of tests remain the final arbiter. The same is true of toxicogenomics. It is a tool that provides data that require interpretation—not an end in itself.

It is important that microarrays are tailored to the needs of the experimental system. At one extreme, where the intention is to identify novel genes of relevance to a toxic process, the use of an extensive array incorporating as many sequences, including those of unknown function, will be the favored approach. In contrast, for more discrete objectives such as, for instance, interrogating the potential of chemicals to induce a particular toxic response, it may be more appropriate to use an array comprising a smaller number of carefully selected genes of known function. There is a middle ground of course. As described above, the *ToxBlot* incorporates 600 genes of diverse function, and it is being used to explore different biological and toxicological responses. This serves to combine the benefits of functional genomics, while retaining the opportunity, to some extent, to explore unexpected changes in gene expression.

In this regard, it may be argued that potentially the most valuable aspect of genomics is that it will encourage, and indeed require, toxicologists to consider biological responses in a more holistic fashion. It is inevitable that when there exists opportunity only to evaluate changes in the expression of very few genes, those genes will be selected carefully, based upon our knowledge, expectations, and/or prejudice. What toxicogenomics offers and demands of us, is that consideration be given also to changes in the expression of genes that were believed to be of no apparent relevance. This will, and has already, yielded some surprises. Perhaps the most exciting advances in defining toxicological mechanisms and identifying new research leads will derive from the inter-disciplinary and more integrated toxicology that genomics will undoubtedly encourage.

Toxicogenomics is not a promise for the future, it is a tool that is available to us now, and which, if used correctly and within the guiding principles of good experimental biology, will bring huge dividends. Concern has been voiced already that a potential problem is the misinterpretation, or over-interpretation, of genomic analyses, particularly in the context of determining product safety. It must be recognized that the interaction of xenobiotics with biological systems will in many instances result in some changes in gene expression, even under circumstances where such interactions are benign with respect to adverse effects. The challenge again is to ensure that sound judgment and the appropriate toxicological skills and experience are brought to bear on the data generated, so that toxicologically relevant changes in gene expression are distinguished from those that are of no concern.

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