Toxicogenomics and Cell-based Assays for Toxicology

Weida Tong¹, Hong Fang² and Donna Mendrick¹

¹Division of Systems Toxicology, National Center for Toxicological Research (NCTR), U.S. Food and Drug Administration (FDA), 3900 NCTR Road, Jefferson, AR 72079, USA
²Z-Tech, an ICF International Company at FDA’s National Center for Toxicological Research, Jefferson, AR 72079, USA

Subject areas: Toxicogenomics

Author contribution: WT and HF wrote the first draft version of the manuscript and DM completed the manuscript.

*Correspondence and requests for material should be addressed to W.T. (Weida.tong@fda.hhs.gov).

Editor: Sun Choi, Ewha Womans University, Republic of Korea

Received July 27, 2009; Accepted July 31, 2009; Published July 31, 2009

Citation: Tong w., et al. Toxicogenomics and Cell-based Assays for Toxicology. IBC 2009, 1(3):10, 1-5. doi:10.4051/ibc.2009.3.0010

Funding: This work is funded by the FDA internal project, no external funding is involved.

Competing interests: All authors declare no financial or personal conflict that could inappropriately bias their experiments or writing.

Disclaimer: The views presented in this article do not necessarily reflect those of the US Food and Drug Administration.

Copyright: This article is licensed under a Creative Commons Attribution License, which freely allows to download, reuse, reprint, modify, distribute, and/or copy articles as long as a proper citation is given to the original authors and sources.

Keywords: toxicogenomics, cell-based assay, microarrays, MAQC

SYNOPSIS

Toxicity is usually investigated using a set of standardized animal-based studies which, unfortunately, fail to detect all compounds that induce human adverse events and do not provide detailed mechanistic information of observed toxicity. As an alternative to conventional toxicology, toxicogenomics takes advantage of currently advanced technologies in genomics, proteomics, metabolomics, and bioinformatics to gain a molecular level understanding of toxicity and to enhance the predictive power of toxicity testing in drug development and risk/safety assessment. In addition, there has been a renewed interest, particularly in various government agencies, to prioritize and/or supplement animal testing with a battery of mechanistically informative in vitro assays. This article provides a brief summary of the issues, challenges and lessons learned in these fields and discuss the ways forward to further advance toxicology using these technologies.
Introduction

Toxicogenomics (TGx) is an emergent discipline of toxicology made possible by large genomic research projects and other rapidly emerging biomarker technologies. It has been a robust area since the concept was introduced in 1999 (Nuwaysir et al. 1999) (Figure 1). TGx began with an emphasis on assessing toxicity using gene expression profiling which was encouraged by the high throughput nature of microarray technology. Quickly, its scope was expanded by the inclusion of proteomic and metabolomic technologies which were expanding rapidly in toxicology. The potential utility and adoption of a growing arsenal of emerging molecular technologies for toxicology research were quickly realized, including Next Generation Sequencing (NGS) and Genome Wide Association Study (GWAS). Subsequently, the scope of TGx was further expanded with the inclusion of emerging biomarker technologies and bioinformatics tools to identify and characterize mechanisms of action of known and suspected toxicants as well as to determine predictive biomarkers for risk and safety assessment.

More recently, there has been renewed interest to investigate a new generation of high-throughput and high-content screening (HTS/HCS) cell-based assays in the field of toxicology. The assays are designed to assess functional responses related to the specific mechanisms that might be important to the expression of toxicity. The rationale behind these assays is that the functional response of cells provides a better understanding of the toxicity observed in animals and/or humans. Since HTS/HCS cell-based assays simultaneously measure multiple toxicity endpoints, they might be able to detect different aspects related to the onset of cell stress, thus increasing the capacity of predicting specific toxicities.

Both TGx and cell-based assays have played an important role in the pharmaceutical industry (e.g., target identification, detecting possible toxicity in the early stage of drug development and providing molecular-level insights leading to a mechanistic basis for prioritizing drug candidates). The rapid advancement and adoption of both disciplines in drug discovery and development also results in a number of opportunities and challenges for regulatory agencies. The US Food and Drug Administration (FDA)’s Critical Path Initiative has identified pharmacogenomics, including the investigation of toxicity, as a major opportunity to advance medical product development (http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/). Similarly, the US Environmental Protection Agency (EPA) has been developing the ToxCast program that uses a battery of cell-based assays along with TGx methods to screen environmental chemicals (Dix et al. 2007). In 2008, the National Research Council Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment released a report on application of TGx technologies to predictive toxicology and risk assessment (http://www.nap.edu/catalog.php?record_id=12037). They concluded that the application of genetics, genomics metabolomics and proteomics to the study of toxicology may transform the “current observation-based approaches into predictive Science”.

This article will discuss key topics related to TGx (defined herein as the application of genomics to toxicity) and cell-based assays with emphasis on the current thinking and rationale for assessing their technical performance and practical use. Specifically, the issues and challenges associated with these topics are emphasized so that the reader can gain an overall perspective on the preferred approaches to apply the existing technology. The ways and means for TGx and cell-based studies are evolving rapidly, and the authors ardently expect that the snapshot of current methods outlined here will soon be refined or replaced with new innovations; paving the way for a paradigm shift in toxicological sciences.

Figure 1. Number of Pubmed publications indexed by keyword ‘toxicogenomics’, grouped by year.

Toxicogenomics

Differential expression

One of the most common TGx approaches is to identify a list of genes (or proteins, metabolites if one uses proteomics or metabolomics, respectively) that are differentially expressed between two or more conditions (e.g., treated versus control). Often, these so-called differentially expressed genes (DEGs) are subsequently used to identify potentially altered pathways, gene/protein functions, and/or regulatory networks to understand the underlying mechanisms of physiological response. These types of descriptive TGx studies are abundant in the literature and continue to dominate the research in hypothesis generation. This type of study is hampered by the partial knowledge of pathways and gene annotations. Given the broad and sometimes contrary information available in the literature, the interpretation can be imprecise. Care should be taken to avoid the phenomena of looking for a black cat in a dark room where there is no cat yet finding one anyway. For example, not all changes in gene expression are necessarily related to a toxic effect even when a toxic dose of a compound is applied in the study. Careful experimental design and proper analysis techniques are needed in order to distinguish between expression patterns due to undesired toxic responses and those caused by just simple homeostatic adjustments and/or therapeutic effects (Gatzidou et al. 2007).

Another challenge is associated with how the differential expression is statistically determined. Five years ago, the debate over the discrepancy in DEGs identified by different gene expression microarray platforms clouded the utility of this approach (Marshall 2004). The microarray technology, along with proteomics and metabolomics, allows measurement of thousands of endpoints in a single experiment, which enhances throughput over older methods but this poses a great challenge to correctly identify the true changes (i.e., minimize false positives) and demonstrate reproducibility across labs and platforms. Gene expression and metabolite flux is interdependent through a number of complex networks and pathways comprised of feedback loops, which challenges most classical statistical methods that often assume the independence of interactions such that individual expression constitutes a null hypothesis test. Several approaches have been introduced to deal with this difficulty (Benjamini et al. 2001; Tusher et al. 2001; Reiner et al. 2003). In reality, the gene-gene interdependency can not be accurately estimated. Thus, all the methods either over- or under-estimate the degree of interdependency of the genes, which results in a biased statistical estimation. This likely contributes to observations of poor
Molecular signatures and predictive toxicology

TGx-based predictive toxicology aims to identify molecular signatures that can be used to make inferential predictions that a discrete toxicological endpoint will manifest from a specific toxic exposure. As depicted in Figure 2, signature identification usually starts with a set of gene expression data (called the training set) from two or more distinct endpoint groups (e.g., genotoxic carcinogens versus non-genotoxic carcinogens). Next, a machine learning method is used to correlate gene expression patterns with toxicity classes to develop a predictive model (i.e., classifier or molecular signature). This area of study is becoming more affordable as the price for microarrays continues to decrease making larger experiments feasible. The focus is mainly on developing TGx signatures based on the short term in vivo or in vitro assays that predict the toxic effects that are normally measured in long-term and expensive bioassays. A study by Thomas et al., for example, used a short term TGx study to develop a gene signature for predicting whether a compound would induce lung tumors (Thomas et al. 2007). It this signature is validated, it could be an alternative to the traditionally-used and expensive two year rodent bioassays.

It has become relatively easy to develop a statistically sound model (i.e., molecular signature) using training data. The current challenge is in developing a model with the capability to predict accurately the classification of untested toxicants, i.e., a model that is not overfit. The authors specifically advocate an iterative approach to enhance the confidence of the models through alternating between incorporating new data in the models and using the models to choose new compounds to assay. As depicted in Figure 3, the process starts with initial sets of compounds for model development. Next, the preliminary models are used prospectively to define a set of compounds that may further improve the model’s robustness and predictive capability. These new compounds are assayed, and the data are then used to challenge and refine the models. Several benefits accrue from the integration of the experimental and modeling efforts. For example, immediate feedback can be given to guide chemical compound selection. As the models evolve, the scientists can select the compounds for subsequent testing, based on considerations of known toxicity potential, chemical structural diversity and dose range. Each new assay data point from the lab becomes a challenge to the evolving model; the result is either further confirmation of its validity or identification of a limitation. Failure of the model also provides important information, such as identification of the need for new data based on rational limitations of the model. Regardless of the cause for the model failure, a research hypothesis is spawned with each iteration, which should lead to new data and/or an improved training set, and an

Figure 2. Signature development process contains two steps. During the training stage, a machine learning method is used to determine a toxic signature that discriminates the treated samples from the controls, with an internal validation process often included in this step. In the validation stage, the signature is challenged by "unknown" samples that have not been utilized during the training stage.
improvement in understanding the mechanism. The recently completed second phase of the MAQC project emphasizes this approach and the lessons learned have been summarized in several manuscripts that are, at the time of this writing, being reviewed by Nature Biotechnology (http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/).

Cell-based assays – A renewed interest in toxicology

Cell-based assays are still being used actively for lead screening and target identification in drug discovery. However, their utility in toxicology has been questionable and their potential not fully realized until recently. The reasons, in part, are that cellular phenotypes often correlate poorly with animal and human pathology. For example, most cell-based ADME/Tox assays currently used in drug discovery rely on engineered cells that often provide results with relatively poor prediction of the responses in living systems. The renewed interest of cell-based assays in toxicology is largely due to the current advance in sensitive detection, automated fluid handling and imaging, which enable simultaneously quantitative and efficient analysis of different mechanisms involved in cytotoxicity. Given the fact that in vitro cellular phenotypes correlate poorly with those found in animal and human pathologies, the current strategy is focused on combining functional responses of multiple cell types in vitro with toxicities seen in animals and/or humans using machine learning approaches.

For example, the EPA ToxCast program intends to screen and prioritize a large number of environmental chemicals (mainly industrial agents and pesticides) using a battery of cell-based assays with the option of including omics assays (Dix et al. 2007). The initial step is to use sets of chemical compounds (~1,000) that have extensive animal toxicity data to develop and verify toxicity signatures (i.e., patterns of in vitro assay data correlated with specific toxicity endpoints). Phase I of ToxCast (320 chemicals) included nine in vitro assays measuring a total of 524 features of cellular phenotypes. In the first ToxCast Summit (http://www.epa.gov/NCCT/toxcast/summit.html), the preliminary results of these features as signatures for predicting various in vitro animal toxicity endpoints were discussed. Currently, the Phase II chemical set is being compiled and will include on the order of 100 human drugs with documented human toxicities.

The NIH Chemical Genomics Center (NCGC) in collaboration with National Toxicology Program (NTP) and EPA launched the Tox21 program (http://www.alttox.org/ttc/overarching-challenges/way-forward/austin-kavlock-tice). The program combines the industrial-scale quantitative HTS and informatics platform at the NCGC with the animal toxicity expertise of the NTP and the computational toxicology expertise of the EPA (Collins et al. 2008) to develop a set of in vitro assays that can be used to generate toxicity signatures to both better predict human toxicity and reduce the need for animal testing. The compound library to be assayed in the Tox21 assay panel will contain all drugs approved by FDA (including those approved and subsequently withdrawn), as well as the majority of drugs approved by regulatory agencies in the EU, Canada, and Japan. This compilation (the NCGC Pharmaceutical Collection) represents an unprecedented resource for systematically studying the in vitro correlates of various toxicities. A subset of 3000 of these compounds has been screened across approximately 50 assays to date, covering a range of phenotypes and molecular pathways and targets. Crucial for conclusions about toxicity screening in Tox21 is not performed in the usual single-concentration format. Rather, all screens in the Tox21 program are performed at 15 different concentrations from ~1 nM – 100 uM, producing concentration-response relationships for all compounds in all assays. Screening of the Tox21 library is expected to continue for several years with at least 2 new screens added per month, and the Tox21 library is expected to grow beyond 10,000 chemicals as procedures are streamlined and the screening and informatics capacity of Tox21 grows.

We have initiated a Liver Toxicity Knowledge Base (LTKB) project at the FDA. The project aims to provide focused knowledge and bioinformatics tools for liver toxicity. Some 200 compounds (most being drugs) were studied. Both rat primary hepatocytes and HepG2 cells were used for in vitro toxicant-induced evaluation, involving four time and eight dose points. Cellular alterations were assessed using multiple pathway-related endpoint measurements from the assay including apoptosis, peroxisomal proliferation, phospholipidosis, mitochondrial function, and DNA damage. In addition, the DNA microarray experiments using the Affymetrix rat chip will also be conducted for these compounds using rat primary hepatocytes to generate gene expression data along with targeted proteomics and metabolomics studies.

A way forward

The current challenges as well as opportunities in TGs lie in the integration of multi-omics data to address toxicity at the systems level; i.e., integration at the gene, protein and metabolite levels to
assist in the identification of biological context as the perturbed pathways or functions are in the center stage of systems toxicology. In the absence of data integration, lists of genes, proteins, and metabolic products that are differentially expressed between sample groups are only lists, providing one level of information regarding biological cause and context. Integrating different omics data types provides the ability to elicitate biological context such as the perturbed functions, signaling pathway components, transcription-factor mechanisms of action, gene regulatory networks, and post-translational modifications, among many others. Moreover, when different data types lead to the same hypothesis (data triangulation) with weight of evidence, both reliability and validity are enhanced.

Regarding the HTS/HCS cell-based assays, while the potential of this approach is apparent, its utility needs to be further validated. Several disparities clearly exist between the physiological environments of cells, particularly transformed cells, compared to those found in tissues in situ. Thus, it would be preferable to use cells freshly derived from tissue, for example, primary hepatocytes as they more closely resemble the phenotypes found in the animal and human liver. However, a major limitation of any primary cell for HTS/HCS is the low throughput nature of their isolation and potential experiment to experiment variability. One prospective area is to use human stem cells that have the potential to differentiate in vitro into various specialized cell types for toxicity study, although many practical considerations still remain in this field such as developing the knowledge to differentiate these cells into adult-type hepatocytes. In summary, the continual advances in miniaturization (e.g., enabling the use of very small amounts of compound), imaging (e.g., moving from current fixed cell endpoint assay to live cell readouts), sample automation, and stem cell research will enhance our capability for the early identification of toxicity.

Huge efforts have been focused on TGx and cell-based assays for the past ten years with the hope of discovering novel insights into mechanisms of toxicity and the identification of biomarkers that can be used to identify such adverse events in animals and humans. As a result, a large number of TGx studies have been performed and some of the resulting data have been deposited in the public domain. We are living in an exciting, data rich era, which calls for innovative bioinformatic approaches to utilize this ever growing amount of information through meta-analysis and text mining for knowledge base development. Data standards and ontology development/maturati on will play an increasing role to guide data curation, mining and analysis, which will help the development of a content-centric knowledge base. Such a knowledge base will spawn hypotheses to develop new studies to address the current gaps and lead to further improvements in research. New data and information generated from these studies will further enrich the knowledge base. Such knowledge bases could also be important for the regulatory agencies for use as a first tier of information to determine the need for additional studies from the sponsors to support any safety concerns. Cumulatively, current and future studies will address not only the adverse events for drugs and environmental chemicals, but also show promise to be able to lead new discovery in the prevention of such adverse events.

References


