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Toxicity Testing in the 21st Century: Bringing the Vision to Life

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In 2007, the U.S. National Academy of Sciences released a report, *Toxicity Testing in the 21st Century: A Vision and a Strategy*, that envisions a not-so-distant future in which virtually all routine toxicity testing would be conducted in human cells or cell lines *in vitro* by evaluating cellular responses in a suite of toxicity pathway assays using high-throughput tests, that could be implemented with robotic assistance. Risk assessment based on results of these types of tests would shift towards the avoidance of significant perturbations of these pathways in exposed human populations. Dose-response modeling of perturbations of pathway function would be organized around computational systems biology models of the circuitry underlying each toxicity pathway. *In vitro* to *in vivo* extrapolations would rely on pharmacokinetic models to predict human blood and tissue concentrations under specific exposure conditions. All of the scientific tools needed to affect these changes in toxicity testing practices are either currently available or in an advanced state of development. A broad scientific discussion of this new vision for the future of toxicity testing is needed to motivate a departure from the traditional high dose animal-based toxicological tests, with its attendant challenges for dose and species extrapolation, towards a new approach more firmly grounded in human biology. The present paper, and invited commentaries on the report that will appear in *Toxicological Sciences* over the next year, are intended to initiate a dialog to identify challenges in implementing the vision and address obstacles to change.

Key Words: toxicity testing; toxicity pathways; *in vitro-in vivo* extrapolations; perturbations; high-throughput assays.

This contribution describes a new vision for toxicity texting with environmental agents as outlined in a National Academy of Sciences report, *Toxicity Testing in the 21st Century: A Vision and A Strategy*. The report was released in June 2007, representing the work of a committee of 22 individuals: Daniel Krewski (*Chair*), Daniel Acosta, Jr, Melvin Andersen, Henry Anderson, John Bailar III, Kim Boekelheide, Robert Brent, Gail Charnley, Vivian Cheung, Sidney Green, Karl Kelsey, Nancy Kerkvliet, Abby Li, Lawrence McCray, Otto Meyer, D. Reid Patterson, William Pennie, Robert Scala, Gina Solomon, Martin Stephens, James Yager, Jr, and Lauren Zeise. The NRC project director was Ellen Mantus. The full report is available online from the National Academy Press at www.nas.edu. This perspective reflects the views of two of the committee members on the future of toxicity testing, and has not been reviewed by the full committee. The views of the authors have been refined since the publication of the NRC report through a series of over 25 presentations at scientific forums in North America and Europe, as well as direct feedback from various stakeholders.

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In recent decades, health protection agencies and the public alike have experienced increasing frustration with the failure of toxicity testing to provide timely, relevant information to support informed regulation of environmental agents. Current toxicity testing strategies rely primarily on the observation of adverse health responses in laboratory animals treated with high doses of these agents. Inferences about risks to human populations based on such observations require uncertain extrapolations, first from high doses to environmental levels that are usually orders-of-magnitude lower than those used in the animal studies, and then from animals to humans. These traditional toxicity testing approaches date back some 30–60 years, and were developed at a time when knowledge of biology—and of the organization of signaling pathways in biological organisms in particular—was primitive. Although there have been incremental improvements in toxicity testing over the years, there has been no comprehensive evaluation of the manner in which advances in cellular and molecular biology might be exploited to improve toxicity testing practices.

The U.S. Environmental Protection Agency (EPA) and the U.S. National Institute of Environmental Health Sciences (NIEHS) asked the U.S. National Research Council (NRC) to provide guidance on new directions in toxicity testing, incorporating new technologies such as genomics and computational systems biology into a new vision for toxicity testing. The NRC convened an expert committee in 2004 to address this charge. The committee produced two reports over the ensuing three years. The committee's interim report (NRC, 2006) provided an overview of testing methods and of proposed new approaches under consideration to incrementally improve traditional approaches. The final report of the toxicity testing committee (NRC, 2007) outlined design criteria for a modern approach to toxicity testing. In choosing among various toxicity
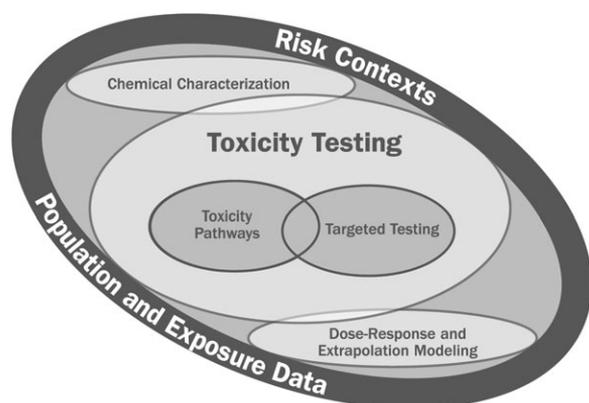
FIG. 1. Schematic of the components required in the vision for toxicity testing in the 21st century. The key elements in this proposal are related to toxicity testing, which includes the types of *in vitro* tests and short-term *in vivo* tests to evaluate perturbations on toxicity pathways, and dose-response and extrapolation modeling, which provides the requisite tools for interpreting toxicity testing results for assessing human health risk assessment. Reproduced from the NRC report (NRC, 2007) with permission.

testing options, the NRC committee sought to define a paradigm that would (1) achieve broad coverage of chemicals, chemical mixtures, outcomes, and life stages, (2) reduce the cost and time required for toxicity testing, (3) develop a more robust scientific basis for assessing health effects of environmental chemicals, and (4) minimize use of animals in testing. These criteria led to the new framework for toxicity testing outlined here. The committee considered four options for the future of toxicity testing (NRC, 2007): the preferred option, emphasized in this Perspective, entails a major overhaul of current practice.

TOXICITY TESTING AND TARGETED IN LIFE STUDIES

The overall toxicity testing framework developed by the committee has four main components: chemical characterization, toxicity pathways and targeted testing, dose-response and extrapolation modeling, and population-based and human exposure data (Fig. 1). The report envisages greatly expanded use of *in silico* methods for estimating or predicting physical and possible toxicological properties of compounds in the chemical characterization component; the population-based component emphasizes the need for ongoing human health surveillance and the linkage of population studies to discoveries about toxicity pathways with biomonitoring and biomarker surveillance activities.

The transformative parts of the new toxicity testing paradigm lie in the nature of the proposed toxicity tests and the manner in which results from these tests would be organized to support human health risk assessment. The suite of tools and methodologies that would be involved in developing these new toxicity testing protocols and in interpreting the results of these tests spans a broad spectrum within modern biology

TABLE 1
Toxicity Testing Tools and their Application in Risk Assessment

Tool	Application
High-throughput screens	Efficiently identify critical toxicity pathway perturbations across a range of doses and molecular and cellular targets
Stem cell biology	Develop *in vitro* toxicity pathway assays using human cells produced from directed stem cell differentiation
Functional genomics	Identify the structure of cellular circuits involved in toxicity pathway responses to assist computational dose-response modeling
Bioinformatics	Interpret complex multivariable data from HTS and genomic assays in relation to target identification and effects of sustained perturbations on organs and tissues
Systems biology	Organize information from multiple cellular response pathways to understand integrated cellular and tissue responses
Computational systems biology	Describe dose-response relationships based on perturbations of cell circuitry underlying toxicity pathway responses giving rise to thresholds, dose-dependent transitions, and other dose-related biological behaviors
PBPK models	Identify human exposure situations likely to provide tissue concentrations equivalent to *in vitro* activation of toxicity pathways
Structure-activity relationships	Predict toxicological responses and metabolic pathways based on the chemical properties of environmental agents and comparison to other active structures
Biomarkers	Establish biomarkers of biological change representing critical toxicity pathway perturbations

(Table 1); some of these are elaborated below. This vision for toxicity testing is built on defining dose-response relationships for toxicity pathway perturbations that would be expected to lead to adverse health outcomes if the perturbations were maintained *in vivo* at a sufficient level of intensity and for a period of sufficiently long duration. Central to the committee's vision is the elucidation of toxicity pathways, which are simply normal biological signaling pathways that may be perturbed by chemical exposures. Pathway testing would require a suite of tests that could identify the range of significant perturbations of human biology that might occur as a result of chemical exposure (Fig. 2). These assays would be conducted using robotic-assisted, high-throughput screens (Inglese *et al.*, 2006, 2007; Xia *et al.*, 2008). Ideally, these assays would be conducted in human cells, cell lines or tissues. The use of a comprehensive array of *in vitro* tests of toxicity pathway responses to identify relevant biological perturbations using cellular and molecular systems based on human biology would markedly reduce the need for whole animal testing, and
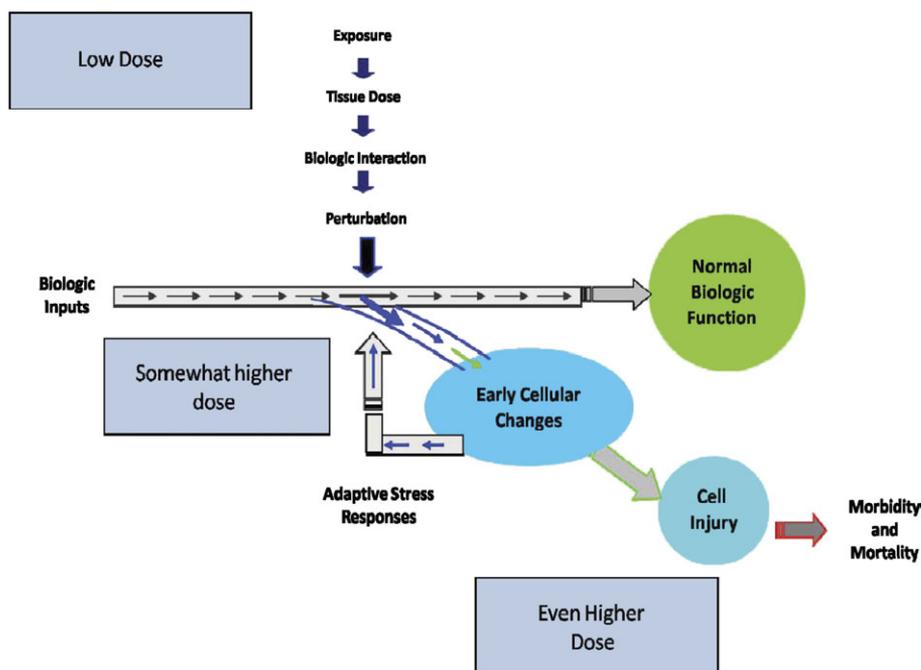
FIG. 2. Representation of progressive activation of toxicity pathways from perturbation of initial targets, through activation of stress controlling pathways, to overtly toxic responses (apical endpoints). Biologic responses are viewed as results of an intersection of exposure and biologic function. The intersection results in perturbation of biologic pathways. When perturbations are sufficiently large or when the host is unable to adapt because of underlying nutritional, genetic, disease, or life-stage status, biologic function is compromised, and this leads to toxicity and disease. The circuitry affected by the chemical is expected to determine shapes of dose-response relationships for these perturbations. Adapted from Andersen *et al.* (2005).

provide much stronger, mechanistically based, predictive tools for human health risk assessment.

Obviously, challenges exist in ensuring that the suite of assays provides sufficient coverage of toxicity pathways so as to capture the broad range of possible pathway perturbations. The identification and characterization of these pathways is not the sole province of the toxicology community; most research into these pathways comes from, and will continue to arise from, contemporary cell biology. These cell-signaling pathways are being enumerated through mechanistic studies from front-line biologists interested in responses of cells and organisms to various stressors. Accounting for metabolism in biological systems *in vitro* remains a difficult problem in developing *in vitro* tests (Coecke *et al.*, 2006). Until prediction of metabolism can be more reliably accomplished through computational toxicology and *in vitro* testing, some degree of highly targeted testing using whole animals may be needed to identify metabolites, prioritize metabolites for high-throughput testing, and to assist in developing pharmacokinetic (PK) models for the distribution of test compounds and their metabolites in humans. If such short term in life toxicology studies were pursued, they should be designed to give improved mechanistic evaluations of tissue responses. For example, this enhanced information content might be collected by examining changes in gene transcript levels in key tissues at the end of in life studies that might last two to four weeks. In

this manner, shorter-term dose-response studies in small numbers of rodents could be used to identify target tissues and describe the nature of pathways affected by exposure to toxic environmental substances.

DOSE-RESPONSE AND EXTRAPOLATION MODELING

How will results from a comprehensive suite of toxicity pathways inform quantitative risk assessments for environmental agents? In this new strategy, low dose and interspecies extrapolations are not expected to be as problematic as in the current paradigm. The use of high-throughput testing for a suite of assays provides a means to characterize dose-response relationships over a very broad range of doses. For practical reasons, including animal usage, sensitivity of apical assays, and cost, a very limited number of doses are generally used in most animal studies. Results from high-throughput based studies will permit an evaluation of responses from high concentrations down to, and lower than, environmentally relevant concentrations, thereby permitting the definition of dose ranges resulting, or not resulting, in significant alterations of normal biological function. However, new challenges will arise in understanding the mechanistic bases for the dose-response behavior of the toxicity pathway assays used, in calibrating expected blood/tissue concentrations in humans

against the vitro concentrations used in the toxicity pathway assays, and understanding the linkages of early perturbations to more downstream, apical responses. Other testing challenges arise just due to the diversity of physical properties of environmental agents. Most compounds tested in high-throughput systems to date have readily dissolved in dimethyl sulfoxide; the broader universe of environmental agents includes volatile materials, poorly soluble lipophilic compounds, particulate materials, and mixtures.

Mechanistic dose-response evaluations will be possible using computational systems biology to describe the biological circuitry of toxicity pathways. Pathway models are being investigated by a number of research groups, using an engineering approach to understand the circuitry and response patterns of cellular pathways (see Alon 2006, 2007). In a user's guide to systems biology, Aldridge *et al.* (2006) describes current quantitative approaches to modeling cellular response networks. As toxicity pathway circuitry becomes better understood over time, it will be possible to create computational systems biology models for expected dose-response relationships for each of the assays used for toxicity testing following similar principles. PK models—especially physiologically based pharmacokinetic (PBPK) models (Bouvier d'Yvoire *et al.*, 2007; Reddy *et al.*, 2005)—will be needed to determine environmental exposure levels giving rise to human tissue concentrations comparable to those associated with perturbations of toxicity pathways *in vitro*. The process of linking *in vitro* concentrations to those expected *in vivo* has been an active area of investigation in Europe, where scientists working on alternatives to animal testing having developed PBPK (or "biokinetic") models to assist the interpretation of *in vitro* data for human health risk assessment (DeJongh *et al.*, 1999).

"IMPROVEMENTS TO TOXICITY TESTING," RATHER THAN "ALTERNATIVES TO ANIMAL TESTING"

This new vision focuses on replacing current toxicity testing procedures with suites of toxicity pathway assays that can be done rapidly and efficiently using modern robotics. In current initiatives by the European Center or Validation of Alternative Methods (ECVAM—http://ecvam.jrc.cec.eu.int/), the Inter-agency Coordinating Committee for Validation of Alternative Methods (ICCVAM—http://iccvam.niehs.nih.gov/), and other groups, many of the *in vitro* test panels are being developed to be predictive of the outcome of acute dosing, repeat dose or target specific toxicity tests in laboratory animals (Clemedson *et al.*, 2006; Prieto *et al.*, 2006; Spielmann *et al.*, 2000). The current emphasis in the U.S. EPA ToxCast program (Dix *et al.*, 2007), which uses a variety of high-throughput tests and computational methods, is on prioritization of compounds for targeted testing in animals. Nonetheless, the tools and approaches being developed by ECVAM, ICCVAM, and

ToxCast will be important for achieving the long-term vision for transforming toxicity testing. The proposal from the NRC report replaces high dose animal tests with *in vitro* evaluations of perturbations of human biology by environmental agents and extrapolating from *in vitro* perturbations of human biology to *in vivo* human exposure conditions. Although not designed as an "alternative to animal testing," the proposed toxicity testing framework would require very few animals, and, over time, greatly reduce animal use for routine testing. Optimally, this new approach would provide the data to support a human health risk assessment in weeks rather than years, and facilitate the generation of the large volumes of toxicity test data required to implement comprehensive regulatory risk assessment programs, such as the REACH program in Europe, without increasing the use of laboratory animals (Blaauboer and Andersen, 2007).

Understanding the relationships between early perturbations and more integrated apical responses will require co-ordination of *in vitro* and *in vivo* studies in the near term. At present, toxicity testing is done *in vivo* at high doses using experimental animals, with mechanistic research conducted to assist in the interpretation of the findings. In contrast, the vision of the NRC committee utilizes mechanistically based, high-throughput tests to identify critical perturbations of toxicity pathways, and to establish conditions under which these perturbations are expected to be without significant consequences for human health. Interpretive research on the relationship of simpler pathway responses observed *in vitro* to more integrated, apical responses *in vivo* could then be pursued to place these early responses into context.

NATIONAL AND INTERNATIONAL COOPERATION

Three federal U.S. agencies with responsibilities for health-related research—the EPA, the NIEHS National Toxicology Program (NTP), and the National Institutes of Health Chemical Genomics Center—have recently announced a memorandum of understanding to conduct research necessary to make the NRC committee vision for the future of toxicity testing strategy a reality. Collaboration among these organizations will be essential in establishing a national commitment to develop the scientific foundation of the vision. This collaboration was described in a policy forum in Science (Collins *et al.*, 2008) which highlighted research activities in these agencies to (1) develop high-throughput test methods, (2) identify key toxicity pathways, (3) pursue targeted testing in short-term *in vitro* tests, and (4) develop dose-response and extrapolation models. Evaluating new approaches for toxicity testing has been an important emphasis of EPA and NTP (Dix *et al.*, 2007; NTP, 2004). In addition to the cooperation of US agencies, implementation of this vision will benefit greatly from broader discussions and co-operation among international bodies in North America, Europe and Asia, including ICCVAM, ECVAM, and Japanese Center for Validation of Alternative Methods.

IMPLICATIONS FOR DRUG SAFETY AND EFFECTIVENESS

Although designed to assess the toxicity of environmental agents, the vision for toxicity testing from the NRC is also relevant in other areas, notably drug safety evaluation. The pharmaceutical industry already makes extensive use of several core components of the vision, including *in silico* (Kruhlak *et al.*, 2007) and *in vitro* (Houck and Kavlock, 2008) screens to efficiently identify drugs that may pose serious health risks as early on as possible in the drug development process. The identification of molecular targets that underlie phenotypic response (a process known as "target deconvolution") is an important component of phenotype-based drug design; the elucidation of such targets is also important for target-based drug design, in which validated targets serve as a starting point for the development of screening assays to identify candidate drugs for further development (Terstappen *et al.*, 2007). The mapping of toxicity pathways as the vision is implemented should provide important clues about molecular targets relevant to both phenotype- and target-based drug designs.

Dose-response extrapolation, cell circuitry modeling, and PK model development, representing elements of the NRC committee vision that are related to predicting human responses to environmental agents, could provide a more quantitative basis for many areas of safety assessment. As the circuitry of response pathways is described in quantitative terms and the molecular components of response become better characterized, these new approaches could provide information about genetic polymorphisms that could affect adverse drug reactions, as well as identifying the genetic basis for idiosyncratic drug responses that occur in only a very small number of patients.

CHALLENGES IN IMPLEMENTATION

Given the design criteria outlined in the report, it seems inevitable that toxicity testing will evolve in the direction of the NRC committee vision: it is more a question of when, rather than if, the anticipated changes will come. Nonetheless, there are considerable obstacles in implementing the vision. Establishing the science base on which the vision rests, including the mapping of toxicity pathways and the development of high-throughput *in vitro* screens to identify important pathway perturbation, will occur over an extended period of time at considerable expense: the committee estimated that implementation of this vision would likely require an investment on the order of $1 billion over a period of 10–20 years.

Although final implementation of the vision will require a suite of toxicity pathway assays providing broad coverage of endpoints and targets, the first steps will likely focus on developing examples involving specific toxicity pathway assays to show how they will be developed, validated, and applied for target identification and dose-response modeling of the observed pathway perturbations. These early examples will likely focus on prototype compounds, tested in assays that query the known targets of the environmental agent, evaluating, for example, estrogenic compounds in a human cell system that sensitively responds to such compounds. These prototype assays will be test beds for pathway elucidation and development of computational systems biology models for mechanistically motivated dose-response modeling along with PBPK models for *in vitro* to *in vivo* extrapolations. The NRC report provided two broad-brush cases of the manner in which data from multiple pathway assays might be utilized in a risk context—one for a reactive vapor and another for an estrogenic agonist. However, these examples lacked specific details because the assays and extrapolation models are still under development.

The committee emphasized the use of human cells and human cell lines in future toxicity tests without detailing specifics of the test methods for using these cells and cell lines. The last 3 years have witnessed an explosion in understanding of stem cell differentiation to specific phenotypes and methods to de-differentiate these stem cells to lineage specific adult cells (Dimos *et al.*, 2008; Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Wernig *et al.*, 2007). Fetal amniotic stem cells are not tumorigenic, expand extensively without feeders, double in 36 h, and maintain long teleomers and normal karyotype (DeCoppi *et al.*, 2007); a variety of differentiated cell types have already been derived from human AFS cells. Progress in this key field has already outpaced expectations. Initiatives in regenerative medicine, guided by advances in stem cell biology, will also provide tools, methods, and test systems for evaluating toxicity pathway responses in human cells, cell lines, and cell-tissue aggregates. Another area, modeling of cell response modeling and cell-signaling circuitry using computational systems biology, is also progressing more rapidly than expected, largely through the interest in biomedical engineering and development of systems biology centers both in the United States and internationally. It is encouraging to see the rapid advances in key methodologies that are required for implementation—stem cell biology (e.g., DeCoppi *et al.*, 2007), computational modeling of cellular response pathways (Alon, 2007; Muzzey and Oudenaarden, 2006), and high data content assays for cellular level responses that can be scaled to rapid high-throughput platforms (Inglese *et al.*, 2007).

OVERSEEING THE TRANSFORMATION

The collaborative research program among the National Institutes of Health, NIEHS, and EPA described by Collins *et al.* (2008) is a welcome step towards U.S. interagency cooperation, but still falls short of a full commitment to move

forward aggressively with implementing the NRC committee vision. (As described, this collaborative research program is still more oriented towards predicting high dose animal outcomes, rather than on developing in vitro test methodologies to evaluate potential risks of chemicals to humans over a broad range of exposures. Nonetheless, these efforts will be essential in developing the science base on which the NRC vision ultimately rests. At the same time, it is essential to emphasize the need to move away from the current view of high dose animal tests as the "gold standard" for toxicological risk assessment towards tests that examine human biology at relevant human exposure levels.) Although the toxicity testing committee envisioned such a program as residing within a publically funded federal institute, a multisector partnership involving both government and industry, including the chemical, pharmaceutical, and consumer product industries, would broaden the base of funding, and involve both regulators and the regulated communities in a common research effort of mutual interest and benefit. Because toxicity testing and risk assessment of environmental agents transcends national boundaries, the path forward should be charted in consultation with the international scientific and regulatory communities.

Professional associations in the United States, especially the Society of Toxicology and the Society for Risk Analysis, will need to become engaged in debating and fine-tuning the implementation strategy. The National Academies of Sciences could play a role in implementing the vision by providing scientific direction, monitoring progress, and ensuring consideration of new technologies that could further support the vision (An approach of this kind was successfully pursued by the NRC Committee for Research Priorities for Airborne Particulate Matter. Following the articulation and endorsement of a 13 year $440 million program of research focusing on ten research priorities on the health effects of ambient fine particulate matter in 1998 [NRC, 1998], the committee produced a series of reports evaluating progress and refining the vision for that program over the next 6 years; NRC, 2004.) Mid-course corrections will no doubt be required, and will benefit from input from all stakeholders.

TOXICOLOGY/RISK ASSESSMENT EDUCATION

Although training of personnel was not extensively discussed in the NRC committee report, the overhaul of current toxicity testing methods and tools for interpretation of test results will require significant revision of the curricula currently used to train students for careers in toxicology. This re-direction is especially important in what today might be called translational toxicology, which focuses on how testing results and interpretive tools are used to make population health risk management decisions regarding hazardous environmental agents. This discipline intersects toxicology, human health risk assessment and public health. The shift in emphasis

is away from high dose hazard identification studies and enumeration of possible *in vivo* effects to an emphasis on approaches to understand the underlying biology of toxicity pathway circuits and how they become perturbed by environmental agents. Education in design of appropriate high-throughput assays and the quantitative methods for dose-response assessment of these perturbations will be essential. Human stem cell biology will likely be a point of emphasis in the development of many of the assays that are likely to populate the suite of toxicity tests used to evaluate pathway perturbations. Functional genomics, computational biology, and bioinformatics will need to become core disciplines in future toxicology training curricula. In concert, traditional programs organized around apical endpoints such as end organ responses, that is, neurotoxicity, and reproductive and developmental toxicity, etc., will become less common.

CONCLUSIONS

Toxicity testing has reached a tipping point. The tools and technologies for conducting biological research on cell-signaling pathways are evolving rapidly, providing a wealth of insights into the design characteristics of cell circuitry that co-ordinate cellular stress responses and maintain function of normal pathways in the presence of exogenous compounds, acting as agonist or antagonists of normal biological function. Developing toxicity testing approaches based on a suite of toxicity pathway assays will have to be revisited as new biological knowledge accumulates, such as current research on gene regulation by small interfering RNA species or on transgenerational responses associated with genomic methylation. Nonetheless, the overall strategy outlined in the NRC report will only be strengthened with the accumulation of this knowledge and the expansion of our understanding of the relationship between cellular perturbations and organism level health outcomes.

This call for change from the NRC committee ultimately reflects the need for toxicity testing to better inform risk management decision making with respect to environmental agents (Krewski *et al.*, 2007). The vision articulated by the NRC committee offers the promise of efficiently testing the large numbers of agents to which we are potentially exposed, providing new information that, once the scientific foundation on which the vision rests has been established, will provide an improved basis for human health risk assessment. From the perspective of ensuring adequate protection of public health, it is important that the NRC committee's vision be subject to broad discussion and debate within the toxicology and risk assessment community, as well as in the broader biomedical research community concerned with public and population health. Krewski *et al.* (2009) have provided a more risk assessment-oriented perspective on the NRC report. A commitment to implement the vision with the involvement

of the full scientific community will accelerate the transformative paradigm shift in toxicity testing elaborated by the NRC committee. Implementation of the vision will provide a firmer scientific foundation for human health risk assessment, increase the coverage of the universe of environmental agents requiring toxicity testing, and markedly reduce the use of experimental animals in toxicity testing. This revolution is overdue and requires international attention to ensure its success.

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