Minireview

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Current Progress of Next Generation Battery of Toxicology - Cellular and Molecular Toxicology, and Toxicogenomics

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Introduction

The detection and the regulation of synthetic chemicals and the establishment of toxicity that may pose a genetic hazard in our environment are subjects of great concern because of its close correlation between environmental contamination and human health. Since the tens of thousands of man-made chemicals that have been introduced into the environment in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified.

Toxicology has been defined as the qualitative and quantitative study of the adverse effects of xenobiotics on living organisms. Moreover, modern toxicology goes beyond the study of the adverse effects of exogenous agents to the study of cellular and molecular effects of toxicants using molecular biological tools. Toxicology is a multidisciplinary field, and an important science that impacts both environmental health regulation and the development and practice of medicine (Klaassen, 2001). Classical toxicological tools require hundreds of animals and provide little information with respect to mechanism(s). For example, descriptive studies in genetically inbred animals do not explain genetic and biological differences in the human population that influence individual response to drugs and environmental xenobiotics.

Recently, several new methods for the detection of genetic damages *in vitro* and *in vivo* were introduced according to the rapid progress in toxicology combined with cellular and molecular biology (Ryu, 1999c, 2002a,b). Among these methods, the single cell gel electrophoresis (comet assay) which can be detected DNA damages in cell level (Mckelvey-Martin *et al.*, 1993; Singh *et al.*, 1988, 1991, 1994; Ryu *et al.*, 1997; Tice *et al.*, 2000), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Ryu *et al.*, 1999b), FISH (fluorescence in situ hybridization) (Hayashi *et al.*, 1994), PRINS (primed in situ hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991; Ryu *et al.*, 1998a,b, 1999a, 2000, 2001c) or *lac* Z (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on cellular and molecular toxicological approaches. Also, *in vivo* supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining (Hayashi *et al.*, 1990; Ryu *et al.*, 2001b) was introduced instead of mouse bone marrow micronucleus assay. Moreover, the rapid progress in cellular and molecular biology, like many other branches of biomedical research, toxicology is now experiencing a renaissance fueled by the application of "omic" technologies to gain a better understanding of the biological basis of toxicology of drugs and other environmental factors (Aederma and McGregor, 2002; Hamadeh and Afsari, 2004).

In this review on current toxicology, at first, the rapid progress of cellular and molecular toxicological tools, and then concept, approaches and applications of toxicogenomics will be described.

Cellular and Molecular Toxicology

Since the remarkable progress in cell biology and biochemistry, the use of cell lines, enzymes, construction of genes *etc* became more easier to handle in the laboratories. In this respect, classical toxicology moved to cellular and molecular toxicology at present, and will move to toxicogenomics (Newton *et al.*, 2004) in the near future. For example, genetic toxicology has great changes in methodology and sensitivity. Generally, the carcinogenicity including genotoxicity is one of the potential toxicity that may consider for the human health. It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated

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with carcinogenic potency (Meselson and Russel, 1977). Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement (Zimmermann, 1971) which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes (Radman *et al.*, 1982). Several classical genotoxicity assay systems with rapidity and reliability have been introduced and practically applied (Ames *et al.*, 1973; Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity (Ishidate and Odashima, 1977; Radman *et al.*, 1982; Hayashi *et al.*, 1990, 1994; Ryu *et al.*, 1993a,b, 1997, 1999b) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b, 2001b). Cytogenetic studies on mammalian cells *in vivo* (Schmid, 1975; Hayashi *et al.*, 1990, 1994) as well as *in vitro* (Ishidate and Odashima, 1977) have also been widely used as a screening method for DNA-attacking substances.

However, these kinds of toxicity evaluation tools cannot elucidate the mode and/or mechanism of actions of chemicals, especially carcinogens and mutagens. Moreover, many scientists try to develope more precise, convenient and sensitive techniques for the detection of DNA damages as an index of carcinogenicity. Several new cellular and molecular genotoxicity assay systems are summarized briefly as follows.

Single Cell Gel Electrophoresis (Comet) assay : Since Ostling and Johanson (1984) introduced microelectrophoretic technique, Singh *et al.* (1988) have modified and improved the microgel electrophoresis technique to evaluate DNA damage in single cells under alkaline conditions. The comet assay is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells at least 3 major basic protocols (Ostling and Johanson, 1984; Singh *et al.*, 1988, 1991, 1994, 1995; Olive *et al.*, 1993, 1994).

If the agent can cause the strand breakage, we can see the extent of tail from the head (nucleus) like comet with staining of fluorescent dyes such as ethidium bromide, acridine orange and propidium iodide etc. To harmonize comet assay, International Workshop on Genotoxicity Test Procedure (IWGTP) was held at Washington D.C. on March, 1999 by Environmental Mutagen Society supported with OECD. Our laboratory (Ryu *et al.*, 1997, 2001a, 2002c) also involved in this harmonization and recommended as OECD guideline with Tice *et al.* (2000). General reviews on this technique have been published by Tice *et al.* (2000) and Fairbairn *et al.* (1995, 1996).

Mouse lymphoma thymidine kinase (*tk*)+/- gene assay (MOLY) : MOLY with L5178Y *tk*+/- mouse lymphoma cells described by Clements (1994), with minor modification (Clive *et al.*, 1995, Garriott *et al.*, 1995; Oberly and Garriott, 1996; Ryu *et al.*, 1999b). The cytotoxicity of chemical was determined by relative survival (RS) after 3 hr treatment at concentrations up to 5,000 μg/ml in the presence and absence of S-9 mixture. The highest concentration chosen was one with a 10-20% RS. Cultures were exposed to the test chemical for 3 hr, then cultured for 2 days before plating in 96-well microtiter plates at 2000 cells/well with trifluorothymidine for mutant selection and at 1.6 cells/well for cell viability. The number of wells containing colonies was counted on day 12 after plating, and large and small colonies were scored. Mutation frequencies were analyzed by the statistical package, Mutant V2.31 program (Hazleton, England) in accordance with the UKEMS guidelines.

Supravital staining *in vivo* micronucleus assay with peripheral reticulocytes : The micronucleus (MN) assay *in vivo* is a method devised primarily for screening chemicals for chromosome-breaking effects. In the monitoring of chromosome breakage, the test is at least as sensitive as the metaphase method; in addition it includes effects on the spindle apparatus.

This MN assay using peripheral blood erythrocytes was introduced by MacGregor *et al.*, (1980) and developed by Hayashi *et al.* (1990, 1994) having more simple and convenient compared to conventional bone marrow assay by the introduction of supravital staining with acridine orange (Ryu *et al.*, 2001b). The conventional Giemsa staining method, however, has some disadvantages because not only MNs but also some cell inclusions containing RNA and other acidic materials are stained dark blue by Giemsa; it is occasionally difficult to identify MNs from these inclusions. Acridine orange metachromatic fluorochrome discriminates between DNA and RNA by green and red fluorescence, respectively.

***In vitro* cytokinesis-block micronucleus assay :** The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably (Heddle *et al.*, 1983). Because micronuclei can only be expressed in cells that complete nuclear division a special method was developed that identifies such cells by their binucleate appearance when blocked from performing cytokinesis by cytochalasin-B, a microfilament-assembly inhibitor. This assay allows better precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions (Fenech *et al.*, 1999).

Transgenic Mutagenesis assay system : The transgenic

mutagenesis system is a useful and powerful tool to evaluate the genotoxicity, and it also provides a window of carcinogenesis and mutagenesis mechanisms of chemicals based on information such as mutation pattern, frequency, and location in sequence context of the *lac I* target gene (Gorelick, 1995). The *lac I* transgenic Big Blue Rat2 fibroblast cell line carries over 40 copies of lambda shuttle vector (Dycaico *et al.*, 1994) containing *lac I* gene as a target (Heddle and Tao, 1995; Summers *et al.*, 1989). The *lac I* gene, as a mutational target, is very useful for the study of the mutational characteristics of a carcinogen for several reasons. First, the relatively small size (1,080 bp of coding region) of *lac I* gene facilitates sequence analysis. Second, the expression of repressor protein permits a rapid colorimetric assay to screen for mutations. The mutations induced in the *lac I* gene can easily be quantified by mutant frequency (MF), and the precise mutation type and distribution can quickly be identified by direct sequencing. Moreover, considering that mutations in *lac I* gene induced by chemicals reflect the effects of mutagens on other endogenous genes such as proto-oncogenes and tumor suppressor genes, and that mutations occurred in these genes are the most common events in many types of human cancer (Kohler *et al.*, 1991; Gossen *et al.*, 1989), this assay may provide a powerful tool to predict the mutation spectrum induced in cancer-related genes more accurately (Ryu *et al.*, 1998a,b, 1999a, 2000).

Toxicogenomics

The recent completion of the human genome sequencing project and the push to finish the mouse genome have raised the stakes in science with predictions of disease cures, more effective and safer pharmaceuticals, and a greater understanding of environmental effects on human health. The impact of human genome projects on toxicological research is high, heralding the emerging technologies of toxicogenomics, proteomics, and bioinformatics (Lovett, 2000; Pollack, 2000) for the future use of these technologies and their impact on drug discovery, safety evaluation, elucidation of pathways of toxicity, and risk assessment. The basis of toxicogenomics is summarized in Fig. 1. The NIEHS established the National Center for Toxicogenomics(NCT) in September 2000. According to the center's mission statement, its goal is "to use the methodologies and information of genomics science to significantly improve our understanding of basic biological responses to environmental stressors/ toxicants." Paul Gilman, who is an assistant administrator for EPA, states that toxicogenomics is a powerful tool with great promise for risk assessment (Bergeson *et al.*, 2002). And also, he stressed that EPA encourages and supports continued genomics research as a powerful tool for understanding the molecular basis of toxicity and developing biomarkers of exposure, effects, and susceptibility. He also mentioned that genomics information is useful in a weight-of-the-evidence approach for human health and ecological risk assessments on a case-by-case basis at this time. By combining these new "omics" approaches with classical and/or conventional toxicological methods, it is possible to develope the experimental models and strategies to evaluate 1) the diverse structure and properties of various chemicals; 2) the relationship between the time of exposure, dose, and health outcomes; 3) the influence of genetics and behavioral factors; 4) interaction between mutiple components of biological systems in development of toxic responses; 5) intrinsic biological health responses with extremely low concentration for long time exposure, and 6) toxicological responses of compounds at an early stage of the drug discovery process and health risk assessment.

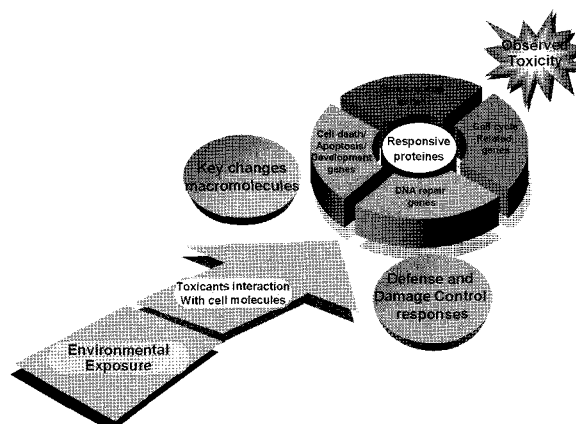

Fig. 1. The basis of Toxicogenomics

What is Toxicogenomics? : Toxicogenomics, in a broader sense, is defined as a study of the response of a genome to hazardous substances, using: i) Genomic-scale mRNA expression (transcriptomics), ii) Cell and tissue wide protein expression (proteomics), and iii) Metabolite profiling (metabon/lomics) in combination with bioinformatic methods and conventional toxicology (In a narrow sense, it refers to the use of transcriptomics). In relation to chemical hazard/risk assessment, this emerging science could provide tools for improving the understanding of mechanism of toxicity, identification of biomarkers for prediction of toxicity and exposure, and possibly alternative methods for chemical screening, hazard and toxicity identification, characterization, and classification.

Approaches and Application of Toxicogenomics : The utilization of these new technologies along with more established genetic approaches such as quantitative real time polymerase chain reaction (QRT-PCR), and the use of genetically altered animals will dramatically move the field of toxicology forward. As you know well, the recent remarkable advances in genomics, proteomics, and metabon/lomics, the interactions between multiple genes, proteins, and pathways can now be investigated with more easy and time-saving ways (Irwin *et al.*, 2004). cDNA and oligonucleotide microarrays and high-throughput 2-D electrophoresis systems have quickly emerged as the premier tools to enable genomewide analysis of gene expression at the RNA and protein level. These new technologies are heavily influencing drug discovery and preclinical safety in the biotechnology and pharmaceutical industry (Freeman, 2000, 2004). Toxicologists are also promoting genomic expression technologies as a superior alternative to traditional rodent bioassays to identify and assess the safety of chemicals and drug candidates for human safety (Afshari *et al.*, 1999; Nuwaysir *et al.*, 1999; Pennie *et al.*, 2000, 2002; Ryu *et al.,* 2002d). It is expected that gene expression profiling will identify mechanisms of action that underlie the potential toxicity of chemicals and drug candidates. Ultimately, toxicogenomics (the integration of genomics, bioinformatics, and toxicology) is expected to accelerate drug development (Suter, 2004; Yang *et al.*, 2004), and aid environment ecological (Neumann and Galvez, 2002) and health risk assessment.

In the laboratory, these are some examples of questions that researchers will be able to address by toxicogenomics as follows; Which genes are regulated upwards or downwards (apoptosis, cellular proliferation, metabolism, communication and cell adhesion, etc.) following an exposure to mercury, PCBs or triazines pesticides? Is the response similar in the liver, lung, testes, ovaries and brain? Are the responses of animal and cell models comparable to humans? The network is also particularly interested in the genetic factors responsible for environmental contaminant-induced breast cancers, unfavorable pregnancy outcomes, and children's health.

The approaches in the commercial basis, one of examples, TNO pharma (2004) has taken up the challenge of toxicogenomics. A research program is initiated with the following features;

Embedding in "classical" toxicology: comparison of multiple gene expression and proteome changes induced *in vivo* as a function of time and dose level.

Use of *in vitro* systems to underscore *in vivo* findings (but not the other way around!).

Integrated genomics and proteomics analysis of target organs.

Incorporation of kinetics and metabolism in target organ toxicity assessment.

Evaluation of possible polymorphisms in the mechanism of toxicity, and the consequences for extrapolation to the human situation.

Combination of tissue microdissection and gene expression analysis in order to relate pathology and genomics. Comparison of gene expression changes to a toxicogenomics database to allow for classification of the effects.

A proprietary pathway related bioinformatics system aiding the toxicologist in understanding the data.

Complex data analysis based on pattern recognition and proprietary principle component analysis.

First grade technology ("home-made" high density DNA arrays, 2D-proteomics with MALDI-TOF and nanospray MS).

Advantages and limitations : There are both advantages and limitations to the use of gene microarray and proteomics technologies in toxicological screening. The main advantage is a global approach to understanding the complex mechanisms involved in toxicology. Gene microarrays have been costly and limited in availability, but the past year has shown a commitment by the scientific community to the general use and availability of gene microarrays. Consequently, cost has been reduced by increased supply and demand. Furthermore, the availability and cost is substantially improving with many universities and research centers establishing genomic and proteomic facilities. Also recent experiments applied to cancer genetics have demonstrated the potential of gene expression profiling to accurately classify disease phenotypes (Alizadeh *et al.*, 2000; Bittner *et al.*, 2000), thus lending hope that expression profiling may classify and thus predict phenotypes of toxicity. Despite these expectations, it is still uncertain how gene expression profiling experiments will ultimately contribute to our understanding of toxicity and allow us to realize the full potential of this new technology. Pennie *et al.* (2000, 2002, 2004) have also discussed the possibilities and caveats of gene expression profiling in the context of mechanistic and predictive toxicology and have addressed the certainty, biological relevance. Therefore, toxicogenomics and proteomics will certainly become generally used technologies in the near future.

Health Risk Assessment and Biomarkers : Biomarkers will play an important role in early detection of environmentally induced disease, since routine surveillance programs in both human and animals in suspected environmentally hazardous areas could be instituted. Toxicogenomics and proteomics are also providing new biomarkers for

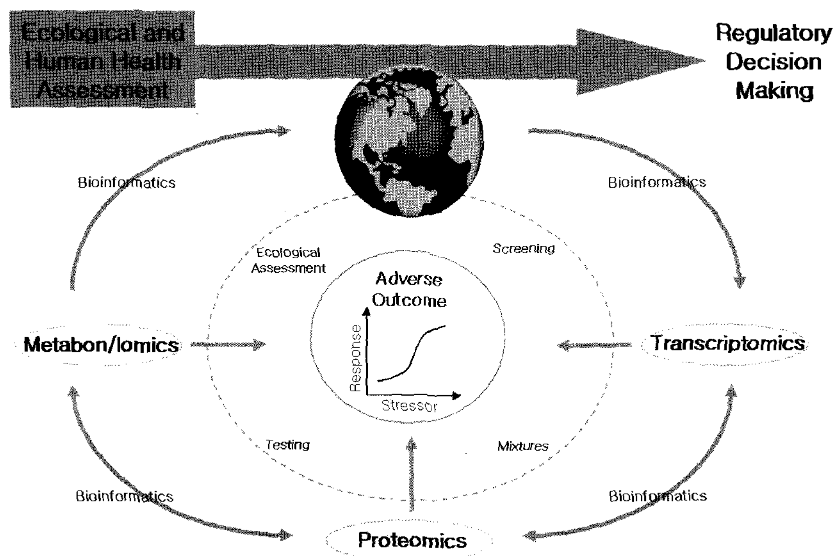

Fig. 2. Proposed Toxicogenomics Research with Coordinated International Research Program by OECD/IPCS workshop, Oct. 13-15, 2004, Kyoto, Japan

use in human studies. It is now possible to envision schemes for integrating the results of molecular epidemiological investigations into the general toxicological evaluations of environmental agents. These will allow intermediate endpoints to be used for making realistic human health assessments and for elucidating pathogenic mechanisms that identify targets for intervention, all with the goal of preventing environmentally mediated human disease. Also, select biomarker responses that predict the likelihood of disease occurrence will find application in the interpretation of individual medical diagnostic tests, with the goal of improving cancer detection and management. The objective is to determine whether gene, protein or metabolite expression profiles or "signatures" can serve as markers to predict toxicity. Current efforts are underway to establish "best choices and practices" and perform proof-of principle experiments to phenotypically anchor altered patterns of expression to conventional parameters of toxicity. These trials are more closely define the time and dose relationships to express "signatures" that develope tools of predictive toxicology and elucidate common mechanism of toxicity and drug action. Early detection of toxic exposures is a developing art, but many groups have already successfully classified chemical exposures based on profiling of mRNA from treated animals (Bartosiewicz *et al.*, 2001a,b; Hamadeh *et al.*, 2002a,b,c). This kind of information might be useful for risk assessment in that significant changes in expression in a small set of highly discriminatory genes can together act as a biomarker of toxic mechanisms or endpoints.

Toxicoinformatics : There is more specified fields such as toxicoinformatics. Toxicoinformatics is the important factor of toxicogenomics field. Toxicoinformatics is essential computational tools for the analysis of time- and dose-dependent changes in patterns of toxicant-induced gene expression. Development of these capabilities will require a database that complies high quality data from diverse sources involving different gene expression platforms, assay methods, validation results and diverse drugs, chemicals or environmental agents. Complete analysis will require linkage between and among additional databases that provide most current sequence identity can annotate gene identify and function, chemical structure, toxicity, pathology, pharmacokinetics/biodistribution, and genotoxicity (Mattes *et al.*, 2004; Mattingly *et al.*, 2004; Olden *et al.*, 2004; Tong *et al.*, 2004).

International Harmonization and QA/QC : To promote the further development and application of the "omic" technologies to toxicology and environmental health risk assessment, recently, on Oct 12-13, Toxicogenomics International Forum (2004) was held, and continuosly, OECD/IPCS organized a special workshop for toxicogenomics focusing eco-toxicogenomics at Kyoto, Japan on Oct 13-15, 2004. In this OECD/IPCS workshop (2004), 4 group sessions open to discuss the problems and future plan of toxicogenomics, especially focused on eco-toxicogenomics (Fig. 2).

One is biological breakout group and they summarized that toxicogenomic technologies have unique opportunities to address ecological and human health concerns, such as; i) offering possibilities to reduce, refine and replace

costly animal intensive methods for chemical screening and testing, ii) understanding how and why species and subgroups differ in sensitivity and response to chemical stress, and create a stronger scientific foundation for the safety factors. This will allow effective policies to be developed in order to protect endangered and importance species, iii) assessing the effects of chemical mixtures and combination of stressors. Previously, appropriate methods have been lacking. iv) reduced uncertainty in assessment of ecological conditions. These will allow effective policies to be developed to protect endangered and important species. For these reasons, it is important that these new tools are evaluated and implemented for chemical risk assessment.

In the technical group, several useful technical tools such as global oligo array, global cDNA array, targeted oligo array, targeted cDNA array, Q-PCR and SAGE for transcriptomics, and 2D-MALDI, 2D-MS/MS, Ciphergen-MALDI, Protein arrays, LC-MS/MS, ICAT-MALDI, ICAT-MS/MS and ELISA for proteomics, and NMR, targeted MS-based, LC-electrochemical for metabolomics were discussed.

The regulatory usefulness of toxicogenomic tools is very important (Frueh *et al.*, 2004). In this respect, in the regulatory group, expected outcomes are a road map for development, validation and regulatory use of genomic-based tools, proposals for further activities related to the use of genomic-based methods in chemical assessment and promotion of related research to be undertaken within the OECD Environment, Health and Safety Programme, and proposals for mechanism of international co-ordination for the development, validation and regulatory use of genome-based tools.

The bioinformatics group adopted general recommendations such as 1) stable funding and adequate funding must be provided to a national and internationals level for standardization initiatives, database and tool development and long-term maintenance, 2) provide training in data requirements and bioinformatics issues relating to ecotoxicogenomics for risk assessors, 3) governments should promote synergy between the toxicology and ecotoxicology communities, including a trialogue between scientists, regulators and bioinformaticians on an international scale, 4) in the broader context of ecotoxicogenomics studies, initial efforts should be centered on a few species representative of the ecological complexity, and 5) establish a task force/working group to implement these recommendations and coordinate future actions.

The ultimate promise of toxicogenomics lies in its potential ability; (i) to identify sources of interindividual variability in response to drugs and environmental xenobiotics, both in terms of efficacy and toxicity; This area of research requires genetics knowledge regarding individual variation which determines person's responses to drugs and xenobiotics; (ii) to provide a database for the development of high-throughput and low-cost platforms for screening substances for toxicity; and (iii) to improve the process of discovering new targets for drug action. However, the high degree of QA/QC on microarray chips to produce more reliable data will be remained to be solve at present.

As every human being on the earth are unique in their genetic make up, there are huge variations in responses against outer environmental factors and also in susceptibility to a disease. As the medical research is getting into the arena of personalized medicine which applies individual's genetic information in disease treatment and selection of drugs, toxicogenomic research also have to look into the individual genetic differences. A genetic variation which is involved in metabolism of toxicants and chemicals can answer the questions of why some people react more sensitively to same environments than others. Careful cataloging of association between genetic background and responses to toxic substances will serve as a powerful tool to understand toxicological phenomenon. This way, one can possibly assess each individual's risks in a given environment and design a plan to avoid any toxic effects. Consequently, toxicogenomics will be a great promising next generation technology (Waring and Halbert, 2002) in the fields of health risk assessment, drug safety, food safety and forensic toxicology etc.

Additionally, there will a new paradigm that is systems biology. The confluence of omics, bioinformatics, mechanistic studies at the molecular level, and computational biology has yielded a new discipline called systems biology (Oltvai and Barabasi, 2002). " Systems biology is a new field of biology that aims to develop a system-level understanding of biological systems(Kitano, 2002). It requires collective efforts from multiple research areas, such as molecular biology (genomics), high-precision measurement, computer science, control theory and other scientific and engineering fields.

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