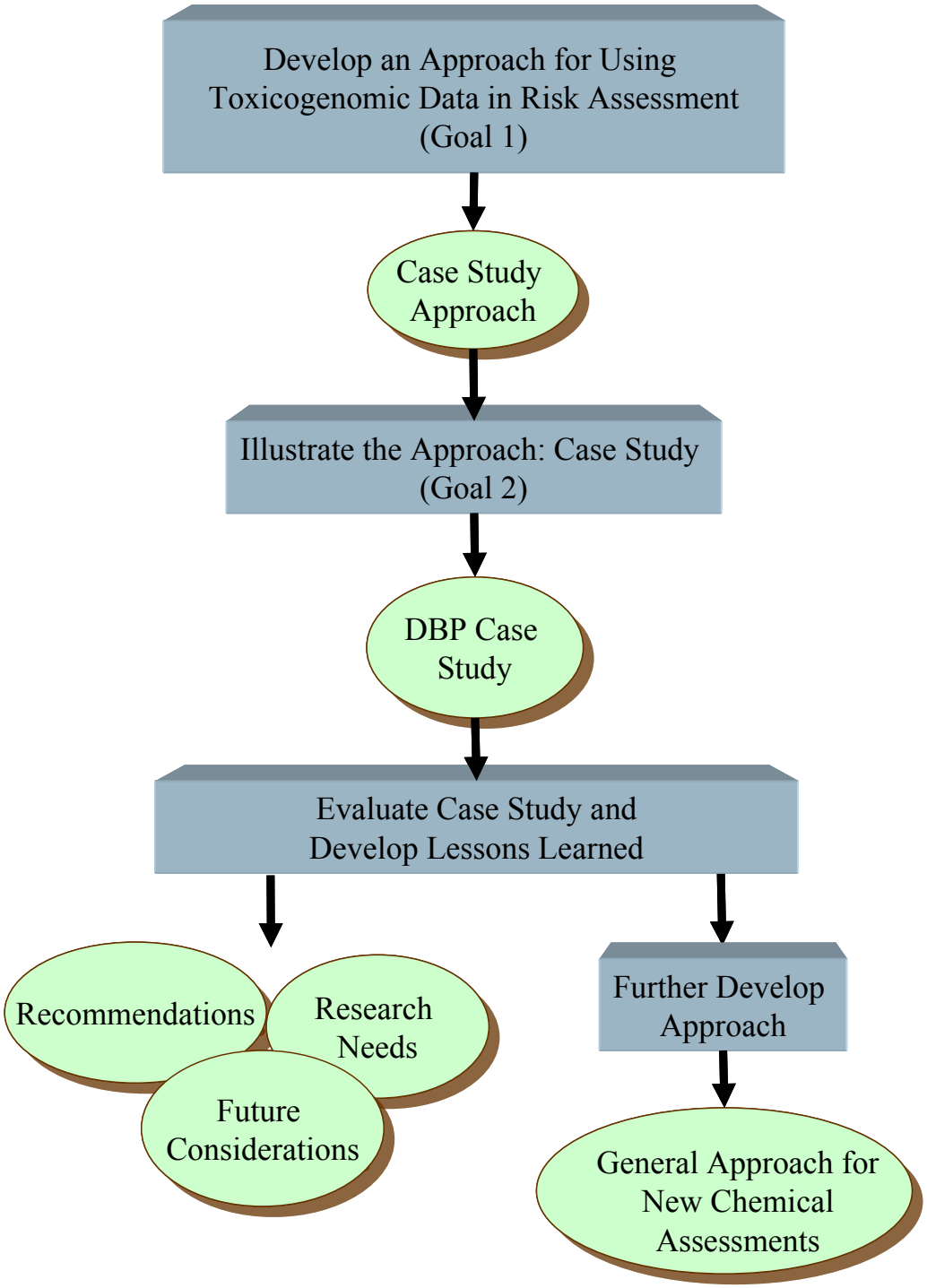

Figure 2-1. The relationship between the project process, goals, and products for the development of an approach and case study for the use of toxicogenomic data in risk assessment. Rectangles indicate processes; Ovals indicate products or outcomes; the two project goals are shown in parentheses within two of the rectangles.

- *How can current issues (e.g., reproducibility, variability in response) with the use of genomic technologies, particularly microarrays, be taken into account in the analysis and evaluation of genomic data?*

- *How can toxicogenomic data be used in conjunction with other types of information?*

- *What are the central issues and obstacles when utilizing genomic data in human health risk assessment?*

The issues raised by these questions are discussed in this report.

2.2. REPORT OVERVIEW

This report describes an approach to evaluating toxicogenomic data for use in risk assessment and presents a case study. The approach principles include examining genomic and toxicity data sets, defining a set of questions to direct the evaluation, and performing new pathway or other analyses of genomic data, when available. The DBP case-study example focuses on male reproductive developmental effects and the qualitative use of genomic data in risk assessment. The report includes the development of exploratory methods and preliminary results from genomic data analysis. In addition, recommendations, research needs, and potential future directions are identified.

Chapter 2 includes a focused review of the history and current use of genomic data in risk assessment and an introduction to the case-study project. Chapter 3 presents the approach developed for the case study. Chapters 4−6 describe the DBP case-study data evaluations and analyses. Chapter 4 presents the toxicology data set evaluation; Chapter 5 presents the toxicogenomic data set evaluation as well as a pathway analysis of one of the DBP microarray studies; and Chapter 6 presents exploratory methods that were developed for analyzing genomic data for risk assessment purposes as well as preliminary results from three different analyses of DBP genomic data. Chapter 7 presents the case-study conclusions including a refined approach for evaluating genomic data for risk assessment, research needs, and future considerations.

Risk assessors may find the approach and case-study evaluations (see Chapters 2–5 and Chapter 7) useful. Bioinformaticians and risk assessors trained in analyzing microarray data may be interested in the descriptions of the pathway analysis methods and the development of new

methods (see Chapter 6). Scientists performing toxicology and toxicogenomic research may find the research needs helpful for designing experiments intended for use in risk assessment.

2.3. USE OF TOXICOGENOMICS IN RISK ASSESSMENT

A number of fields have applied toxicogenomic technologies and data to identify biomarkers, to predict toxicity as a screening tool, and for use in defining the modes and mechanisms of action. In addition, a small number of studies have explored the application of toxicogenomics to dose-response, interspecies extrapolation, intraspecies variability, and TK/TD linkages. In the following section, pertinent definitions and activities in the area of genomics and risk assessment are described.

2.3.1. Definitions

The scientific community has a range of definitions for the terms 'genomics' and 'toxicogenomics'. In this report, we use definitions that are consistent with the NRC report, *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment* (NRC, 2007a). Genomics is the study of the genome and includes genome sequencing and genotype analysis techniques (e.g., polymorphism identification). Toxicogenomics is defined as "the application of genomic technologies (e.g., genetics, genome sequencing analysis, gene expression profiling, proteomics, metabolomics, and related approaches) to study the adverse effects of environmental and pharmaceutical chemicals on human health and the environment" (NRC, 2007a). One goal of toxicogenomic studies is to link genomic changes with adverse phenotypic effects/outcomes determined histopathologically or clinically.

The term -omics (referring to terms ending with the suffix -omics) is a broad discipline of science and engineering for analyzing total or global interactions within a biological system by utilizing the various genomic techniques. The main focus of this discipline is (1) mapping information objects such as genes and proteins, (2) finding interaction relationships among the objects, and (3) engineering the networks and objects to understand and manipulate the regulatory mechanisms (for more information about -omics, see www.omics.org). Thus, toxicogenomics measures the genome-wide expression of genes at the mRNA, protein, or small molecular weight metabolite level in order to characterize the response to toxic agent exposure at the molecular level.

Some current toxicogenomic techniques include transcriptomics (mRNA), proteomics (protein), and metabolomics (small molecular weight metabolites), as well as genome sequencing (DNA) which includes the identification and study of genetic polymorphisms (DNA). These toxicogenomic techniques are "near-global" because there are limitations in the annotation of genes such that the entire genome may not be represented. In addition, there are methodological detection limitations. In most cases, it is expected that toxicogenomic responses will be detected earlier than *in vivo* responses because gene expression changes tend to be precursors to *in vivo* outcomes. Toxicogenomic responses could also be detected at lower doses than animal toxicology assays because the techniques measure molecular responses on a genome-wide scale.

Transcriptomics, using microarrays, is a powerful tool for investigating the expression levels of thousands of genes, or sometimes a complete genome, following exposure to toxicants. The use of microarrays to study gene expression profiles from tissues, organs, or cells began in 1995 (Lobenhofer et al., 2001). Microarray information is different from other types of data used in toxicology for a number of reasons. Unlike single gene expression data that use specific methods, such as northern blots and real-time RT-PCR to evaluate individual genes, microarrays provide a nearly genome-wide (i.e., not all genes are currently annotated and have expressed sequence tags [ESTs]) transcriptional profile of a cell or tissue. Thus, each experiment generates a large amount of dense and complex data. Analyzing and interpreting the quantity and complex patterns of data requires expertise in bioinformatics.

Genetic polymorphisms are included in the definition of genomic techniques. Some microarrays are designed to detect single nucleotide polymorphisms (SNPs) and copy-number polymorphisms (CNPs) (Buckley et al., 2005). Polymorphism data can be evaluated qualitatively and quantitatively to assess risks to various subpopulations as well as to provide insights into mechanistic pathways (Shastry, 2006; Guerreiro et al., 2003). Transcriptomics measures genome-wide mRNA expression (NRC, 2007a). The transcriptomic technology with the greatest history and validation is microarray. It is a tool used to measure gene expression patterns across the entire genome. Subsequent pathway analysis of gene expression data can identify the affected pathways and biological processes. Genes that are annotated, as well as those that are not (i.e., ESTs), are included in microarray analysis. Common technologies for genome-wide analysis of gene expression include microarrays (complementary DNA [cDNA]

and oligo microarrays), cDNA-amplified fragment length polymorphism, and serial analysis of gene expression.

Proteomics is the study of proteins in an organism (NRC, 2007a). It involves the study of the protein expression, structural status (e.g., phosphorylated/dephosphorylated), functional states (i.e., activity specificity and activity level), and protein interactions with other cellular components, as a function of time and in response to intrinsic and extrinsic factors (Pandey and Mann, 2000). Thus, proteomics has the ability to study changes in protein expression and protein modification after toxic agent exposure (Ekins et al., 2005; Kennedy, 2002; Anderson and Anderson, 1998), and ultimately, changes in cellular function. Broadly, proteomics may be defined as "expression" (or "differential") proteomics and "functional" proteomics (Wu et al., 2002); the former relates to a differential expression of proteins among treatments or disease states, and the latter relates to protein interactions and changes in function due to posttranslational modifications or other protein-protein interactions.

Metabolomics is the study of low molecular weight (LMW) metabolic products (NRC, 2007a). Since metabolites are typically the final, functional products of genes, a metabolomic profile is best at capturing the most functional assessment of toxicity, compared to other -omic technologies. Metabonomics is also the study of LMW protein. There is a subtle distinction between the two: metabolomics refers to the study of LMW molecules within cells, whereas metabonomics refers to a more systemic and complex change in tissues and body fluids (Ekins et al., 2005). For example, the toxicity of acetaminophen in rodents has been examined via metabonomics using nuclear magnetic resonance (NMR) spectroscopy to characterize changes in intact and solubilized liver tissue and blood plasma (Coen et al., 2003). Such approaches to examine toxicity can be used qualitatively to help define or refine the MOAs of an environmental toxic agent or to use as a biomarker of exposure. In some cases, these approaches can be used quantitatively to represent a toxic response amenable to dose-response analysis. Due to the large size and complexity of information generated by -omic technologies, bioinformatics methods for data analysis continue to be developed and refined.

In the case study, the toxicogenomic and all other gene expression data were evaluated as part of the larger gene expression data set. In addition to the microarray studies detecting genome-wide gene expression, we decided to include single-gene and protein expression from RT-PCR, northern blot, transgene expression, and immunostaining studies. All of the gene

expression data are useful to risk assessment because these techniques provide (1) a validation method for microarray studies; (2) a larger data set of gene expression information, as there are typically a very small number of available microarray studies for a specific chemical; and (3) additional semiquantitative information such as RT-PCR and protein-expression assays.

The mechanism of action is defined herein as the complete molecular sequence of events between the interaction of the chemical with the target site and observation of the outcome. Thus, the mechanism of action can include TK and TD steps. By contrast, the term MOA is defined as one or a sequence of key events that the outcome is dependent upon. A "key event" is defined as an empirically observable precursor step that is a necessary element of the MOA or is a biologically based marker for such an element (U.S. EPA, 2005a).

2.3.2. Current Efforts to Utilize Toxicogenomic Data in Risk Assessment

Many of the advances in toxicogenomic technology are a result of their application to pharmaceutical development (Boverhof and Zacharewski, 2006). In drug discovery, genomic methods are used for assessing and predicting toxicity with the goal of selecting a drug with relatively high efficacy and low toxicity. Research and regulatory agencies are evaluating the applications for –omics data and the regulatory implications of using these data. However, to date, their application has been somewhat limited due, in part, to a lack of available data as well as expertise required to analyze and interpret these data when available. Nevertheless, approaches and considerations for using toxicogenomic data sets in a risk assessment or other regulatory scenarios continue to be explored (Kienhuis et al., 2009; Zhou et al., 2009; Thybaud et al., 2007; Boverhof and Zacharewski, 2006; Chan and Theilade, 2005; Leighton, 2005; Reynolds, 2005; Oberemm et al., 2005; Frueh et al., 2004; Pennie et al., 2004; Waters and Fostel, 2004; Cunningham et al., 2003; Hackett and Lesko, 2003; Pettit et al., 2003; Robinson et al., 2003; Simmons and Portier, 2002). Finally, toxicogenomic data have also been applied to exposure assessment. For example, some studies have used a gene expression successfully to determine occupational exposure levels (NRC, 2007a).

2.3.2.1. *Toxicogenomics Informs TD*

A number of studies have used microarrays to identify patterns of gene expression following chemical exposures (Ellinger-Ziegelbauer et al., 2005; Moggs et al., 2004; Lobenhofer

et al., 2001) and thus can be useful to defining the TD steps of mechanisms of action and MOAs. Underlying the use and interpretation of these technologies is the assumption that genes exhibiting a similar expression pattern may be functionally related and under the same genetic control. Common patterns of gene expression for specific groups of chemicals have been identified (Naciff et al., 2005; Hamadeh et al., 2002a; Hamadeh et al., 2002b). Hamadeh et al. (2002a) performed microarray analysis of liver tissue from animals exposed to four different chemicals: three pharmaceutical peroxisome proliferators (clofibrate, Wyeth 14,643, and gemfibrozil) and the CYP2B inducer phenobarbital. The three peroxisome proliferators gave similar patterns of gene expression indicating a common MOA; whereas, the gene expression pattern for phenobarbital was distinct from the three peroxisome proliferators. Further, Hamadeh et al. (2002b) were able to predict the enzyme induction or peroxisome proliferation MOA from blinded microarray data for three chemicals (phenytoin, diethylhexyl phthalate, and hexobarbital). Naciff et al. (2005) studied the transcriptional profile in the testis following exposure to three estrogen agonists (17α-ethynyl estradiol, genistein, or bisphenol A [BPA]), which have been shown to bind to the estrogen receptor (ER) with different affinities (e.g., BPA having the weakest bond). A common group of 50 genes, whose expression was changed in the same direction, was identified after exposure to any of the three estrogen agonists. Dose-response studies were performed, and the gene expression changes were associated with dose (i.e., lower dose, lower gene expression) among the 50 genes identified for each of the three chemicals. Differences in gene expression patterns were observed to be dependent on the duration of exposure (Hamadeh et al., 2002a), the organ (Naciff et al., 2005, 2002), or the life stage of exposure (Naciff et al., 2003, 2002). Tilton et al. (2008) identified an alternative mechanism for hepatic tumor promotion by perfluorooctanoic acid (PFOA) in rainbow trout using gene expression profiles. Here, the authors demonstrated a novel mechanism involving estrogenic signaling for the tumor promotion activity of PFOA. In their study, tumor promotion was not related to peroxisome proliferator-activated receptor alpha (PPARα) agonism, but was phenotypically linked to estrogenic gene signatures in trout liver. Furthermore, the evaluation of genomic data has corroborated the MOA for some chemicals within a risk assessment (see Section 2.3.2.7).

The use of genomics data, particularly gene expression "signatures" or "fingerprints," to make predictions about the toxicity of a chemical based upon gene expression patterns for a

given MOA class, is not always straightforward. Although peroxisome proliferators may exhibit a similar gene expression signature, some chemicals (e.g., PFOA) may exert effects through multiple mechanisms. One can be misled by the presence or absence of certain gene expression changes within a signature because the gene expression changes may not be related to the toxicity endpoint. This could lead to focusing the evaluation on a subset of genes in the overall signature pattern that may not represent a mechanism of toxicity, and/or focusing on one MOA when the chemical affects multiple MOAs.

2.3.2.2. *Toxicogenomics Informs Dose-Response*

As noted previously, most examples of the use of toxicogenomic data have focused on informing hazard characterization, and in particular the use of genomic data to inform the TD steps of modes and mechanisms of action. However, it is also important to consider whether and how toxicogenomic data can inform dose-response analysis and TK. In regards to dose-response analysis, toxicity endpoints (e.g., hepatotoxicity) will likely have characteristic genomic profiles of associated gene expression changes that can serve as fingerprints for these toxicity mechanisms (Aardema and MacGregor, 2002). Importantly, gene expression changes related to a toxic response may be observable at earlier times and at doses lower than those required to elicit *in vivo* toxicity. Gene expression signatures could aid risk assessors in choosing the most appropriate animal model for conducting toxicity studies (Aardema and MacGregor, 2002), possibly reducing uncertainty. Establishing such fingerprints and validating their utility for quantitative dose-response analysis is necessary for their use in risk and safety assessment.

Recent studies lend support to the notion that gene changes may be able to serve as early indicators of longer-term *in vivo* outcomes (Thomas et al., 2007; Andersen et al., 2008). Yu et al. (2006) developed a gene ontology (GO) categorization method to interpret microarray data for risk assessment purposes. Thomas et al. (2007) and Andersen et al. (2008) applied the GO categorization approach to microarray data from exposure to chemicals that cause rodent tumor formation. Significant changes in gene expression in genes associated with cell proliferation and DNA repair GO categories were observed after exposure to chemicals that induce cell proliferation and DNA repair at approximately the same doses at which long-term exposure led to tumor formation in rodents. The authors concluded that relevant gene changes may serve to predict the long-term outcome of bioassays.

Studies on formaldehyde carcinogenicity carried out by the Hamner Institute evaluated approaches to apply toxicogenomics data quantitatively to screening (Thomas et al., 2007). In examining the dose-response for formaldehyde-induced gene changes in rat nasal tissue, a BMD analysis was used to identify sets of genes in GO categories often thought to be involved in the MOA of formaldehyde (Thomas et al., 2007). GO categories for DNA damage response and repair, response to unfolded proteins, and regulation of cell proliferation had BMD values ranging from 5.68–6.76 ppm formaldehyde. The authors noted the relatively close agreement between the BMD (5.68 ppm) for the cell proliferation GO category, a previously published BMD (4.91 ppm) for the cell-labeling index (Schlosser et al., 2003), the BMD (6.31 ppm) for the DNA damage response GO category, and the lowest-observed-adverse-effect level (LOAEL) (6 ppm) reported for DNA-protein crosslink formation (Casanova et al., 1994). Similar conclusions were drawn from a longer-term, 3-week, study by Andersen et al. (2008). Daston (2008) suggested that gene expression changes may not occur below a threshold dose for some toxic agents. Alternatively, it is possible that longer-term exposure to low doses could lead to gene expression changes that are linked to toxicity. However, such aspects may not be captured in the small treatment group sizes in this study or under shorter durations of exposure. Although the justification for comparing these values (i.e., use of a 10% increase in cell labeling vs. 1.349 × standard deviation for cell proliferation genes) can be debated, dose-response modeling methodologies can be developed that, upon further validation, could be applied to risk assessment, screening, and prioritization.

2.3.2.3. *Toxicogenomics Informs Interspecies Extrapolations*

Interspecies extrapolations are comprised of TK and TD aspects. Changes in genes, proteins, or LMW molecules likely involved in chemical disposition (e.g., transporters, enzymes, cofactors) have the potential to inform TK extrapolations for risk assessment. For example, changes in expression of genes or proteins related to glutathione (GSH) synthesis following exposure to an environmental toxic agent suggest that further consideration of GSH (including synthesis or resynthesis) may be necessary when considering dose adjustments or building physiologically based pharmacokinetic (PBPK) models. In principle, this approach has been demonstrated for the depletion and resynthesis of GSH following exposure to trichloroethylene and 1,1-dichloroethylene, albeit without toxicogenomic data (El-Masri et al., 1996). In this

study, the PBPK model suggests that it is important to consider GSH resynthesis when assessing the toxicity of these chemicals. Additionally, transcriptomic and proteomic data can also inform TD aspects of interspecies extrapolation. Often, chemical-specific data that account for TD differences across species are not available. Differences in gene expression profiles between species may help qualitatively and quantitatively address these issues. In the case of GSH gene expression, differential changes across species may have implications for TD in cases when redox status plays a role in a chemical's MOA.

2.3.2.4. *Toxicogenomics Informs Intraspecies Variability*

Perhaps the most straightforward quantitative application of toxicogenomic data in risk assessment involves genetic polymorphisms. This application is also the most amenable to current risk assessment practices, specifically, in handling interindividual variation in TK. Both SNPs and CNPs in genes that are important for the disposition of toxic agents have the potential to inform the intraspecies uncertainty factor (UF_H) applied in risk assessment. For cases with a known impact of a polymorphism on enzyme function, polymorphism information can be used to characterize the difference in dose metric for a subpopulation relative to the most common alleles, or, incorporate population variability in enzyme function and dose-metric predictions to probabilistic assessments using the Monte Carlo analysis. El-Masri et al. (1999) demonstrated this approach for polymorphisms in GSH transferase-1. Polymorphisms related to the TD of a chemical could be incorporated into risk assessment when more sophisticated biologically based models are developed.

2.3.2.5. *TK/TD Linkages Informed by Toxicogenomic Data*

Toxicogenomic data will likely play an increasing role in the modeling of systems biology for use in risk assessment (Daston, 2007; Andersen et al., 2005). In order to use these data in systems biology, it will be critical to understand the normal biological processes and compensatory mechanisms in biological systems. Ultimately, this information will improve our understanding of the shape of dose-response curves at environmentally relevant concentrations and for low-incidence adverse effects (Andersen et al., 2005).

Although we often rely on *in vivo* data for informing TK, *in vitro* tools provide a relatively abundant and useful source of information (Donato et al., 2008). These methods have

been used to assess the expression of drug metabolizing enzymes in treated and untreated, primary and immortalized cells in some cases (Geng and Strobel, 1995; Raunio et al., 1999; Swanson, 2004). Toxicogenomic technologies can be applied to assess metabolic capacity between cell types of normal and abnormal phenotypes (Vondracek et al., 2001, 2002; Hedberg et al., 2001; Staab et al., 2008). Recently, an *in vitro* model of buccal epithelial tissue was used to examine the expression of carbonyl metabolizing enzymes in normal human basal and differentiated keratinocytes, as well as in immortalized malignant human keratinocytes (Cedar et al., 2007; Staab et al., 2008). Such approaches can inform the metabolic capacity of cells at a given stage of development (e.g., proliferation vs. differentiation) and, perhaps, the differential metabolic capacities of normal, premalignant, and malignant cells.

2.3.2.6. *Toxicogenomic Activities at the U.S. Food and Drug Administration (FDA)*

The FDA has been incorporating toxicogenomic information into their drug evaluation process and regulatory decisions, following the voluntary submission of data by the pharmaceutical industry. In 2003, the FDA developed draft guidance for the submission of pharmacogenomic data to FDA (U.S. FDA, 2003). This guidance furthers scientific progress in the field of pharmacogenomics and facilitates the use of pharmacogenomic data in informing regulatory decisions. The draft guidance encourages, but does not require, voluntary submission of microarray data from exploratory studies. This guidance does not include use of genetic or genomic techniques for the purposes of biological product characterization or quality control (e.g., cell bank characterization, bioassays). It also does not pertain to data resulting from proteomic or metabolomic techniques.

The MicroArray Quality Control (MAQC) Consortium is a scientific community-wide effort, spearheaded by FDA scientists. The MAQC effort was initiated to collaboratively tackle issues of variability and standardization of microarray procedures across government, industry, and academia (Shi et al., 2006; Casciano and Woodcock, 2006; Frueh, 2006; Dix et al., 2006; Ji and Davis, 2006; Canales et al., 2006; Shippy et al., 2006; Tong et al., 2006; Patterson et al., 2006; Guo et al., 2006). The two main objectives of the 1st phase of the MAQC (MAQC-I) project are (1) to compare cross-platform and interlaboratory performance of currently available microarray technologies and (2) to identify potential sources of variability. Seven different microarray platforms (six commercially available platforms [Applied Biosystems, Affymetrix®,

Agilent Technologies, GE Healthcare, Ilumina, and Eppendorf] and one private platform [the National Cancer Institute]) were tested by three independent laboratories. Each laboratory used five sample replicates derived from four titration pools of two well-characterized unique RNA samples. The working list of genes was refined to include 12,091 reference genes that were detected on each of the six high-density platforms. The MAQC-I study demonstrates that there is good reproducibility within sites, between sites, and among the various platforms.

The performance of the microarray platforms was further evaluated in comparison to three distinct quantitative gene expression assays: Taqman, Standardized RT-PCR, and Quantigene. There was excellent correlation between microarray results and quantitative gene expression results. Several sources of limited incongruence were identified: a decreased sensitivity for low expression genes in the microarray platforms as compared to the gene expression technologies and some differences in probe location.

A toxicogenomic study in rats was used to validate the observed congruence of microarray platforms in a biologically relevant framework. Rat RNA samples were collected and processed following exposure to three chemicals (aristolochic acid, ridelline, or comfrey). Results from four of the microarray platforms indicated a high degree of conformity. Specifically, gene lists generated using fold-induction criterion showed much greater concordance across platforms as compared to those generated by t-test *p*-values alone.

The MAQC-I project observed high reproducibility of findings between different microarray platforms tested at multiple locations. Additionally, microarray results were well-correlated with other available gene expression technologies. Consistent results were also observed in the toxicogenomic study in rats. These studies provide the stepping-stones for decreasing variability in microarray data and standardized quality-control measures. Taken together, the findings are encouraging for the future incorporation of microarray data into risk assessments. Since these results were a comparison of the same sample in different laboratories, it is important to assess differences in sample preparation across sources (e.g., independent laboratories/institutions, commercial sources).

The second phase of the MAQC project (MAQC-II) was initiated with the goal of assessing the capabilities and limitations of various data analysis methods in developing and validating microarray-based predictive models. The ultimate goal of MAQC-II is to reach consensus among stakeholders on the best practices for the development and validation of

predictive models based on microarray gene expression as well as genotyping data for personalized medicine. The details of this project and results have been submitted to the journal, Nature Biotechnology (www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm).

The third phase of the MAQC project (MAQC-III), also called "Sequencing Quality Control," aims to evaluate the technical performance of next-generation sequencing platforms. Benchmark data sets with reference samples will be developed, and then, the advantages and limitations of different bioinformatics genomic methods will be evaluated (www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm).

2.3.2.7. *Toxicogenomic Activities at EPA*

EPA is another agency that has initiated the development of methods, research, and guidance for using toxicogenomic data for a number of purposes including risk assessment (U.S. EPA, 2002a, 2004b, 2006b, 2006c). This includes training EPA risk assessors in genomics (e.g., Risk Assessment Forum Genomics Training Courses), developing guidance and methodology documents, and supporting numerous research activities that are expected to support chemical-specific risk assessment activities.

As previously described, EPA's SPC developed the Interim Policy on Genomics. This policy states that, "genomics may be used in U.S. EPA risk assessments on a case-by-case basis in a WOE [weight-of-evidence] approach" (U.S. EPA, 2002a). Currently, there is no EPA guidance for how to incorporate toxicogenomic data into chemical assessments. The Genomics Task Force produced a white paper, *Potential Implications of Genomics for Regulatory and Risk Assessment Applications at EPA,* that identified four areas of oversight likely to be influenced by genomic data: the prioritization of contaminants and contaminated sites, environmental monitoring, reporting provisions, and risk assessment. The paper also identifies a critical need for (1) analysis and acceptance criteria for genomic information in scientific and regulatory applications, (2) methods for interpreting genomic information for risk assessment, and (3) methods for determining a relationship between genomic changes and adverse outcomes (U.S. EPA, 2004b). In response to these needs, the SPC's Genomics Technical Framework and

Training Workgroup was established. This workgroup developed the *Interim Guidance for Microarray-Based Assays: Data Submission, Quality, Analysis, Management, and Training Considerations* (U.S. EPA, 2006b). This guidance addresses genomic data submission, quality assurance, analysis, and management in the context of current possible applications by EPA and the broader academic and industrial community. The guidance also identifies future actions envisioned in order that genomic information are utilized more fully into EPA's risk assessments and regulatory decision making (Dix et al., 2006). Furthermore, EPA has institutionalized a national center, the National Center for Computational Toxicology (NCCT; www.epa.gov/NCCT; U.S. EPA, 2004a) with one of its goals being to analyze and integrate the -omics data and other mechanistic data into computational models using systems biology approaches. EPA has also initiated discussions to strategize and recommend next steps in methods development for the use of genomic data in risk assessment. These activities include the 2003 Office of Research and Development's Computational Toxicology Workshop: *Research Framework, Partnerships and Program Development* (Kavlock et al., 2005), and the National Center for Environmental Assessment (NCEA) colloquium entitled *Current Use and Future Needs of Genomics in Ecological and Human Health Risk Assessment* (U.S. EPA, 2006c; http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=149984), both of which identify the need to perform a case study integrating toxicogenomic data in a chemical assessment.

Currently, EPA has evaluated toxicogenomic data qualitatively in two final assessments of environmental chemicals, acetochlor and dimethylarsenic acid (DMA). First, the Office of Pesticide Program's (OPP) Cancer Assessment Review Committee (CARC) evaluated genomic data in the cancer assessment of acetochlor (U.S. EPA, 2004c). OPP considered acetochlor, alachlor, and butachlor as a group based on a common mechanism of action based on the observation that exposure to each of the three pesticides induced nasal turbinate tumors via cytotoxicity and cell proliferation. Genomic data from studies with olfactory mucosa from rats treated with alachlor, were used to support cytotoxicity with regenerative cell proliferation as the MOA for acetochlor in the assessment. The genomic data identified processes and pathways affected by alachlor exposure including oxidative damage and the generation of reactive oxygen species (ROS), as possible mechanisms underlying cytotoxicity. Second, a recent EPA assessment of DMA evaluated the available genomic and toxicity studies (U.S. EPA, 2006d). The DMA data suggest that urothelial cytotoxicity and regeneration, key events in bladder tumor

formation, are relevant to humans. Pathway-level analysis revealed that DMA affects both common and unique pathways in the bladder transitional cells of rats and humans. The finding of common pathways in the rat urothelium and human cells provides evidence of concordant biological processes affected by DMA, thereby supporting the conclusion of biological plausibility in humans. Thus, the genomic data, in conjunction with toxicology studies, provided both MOA and human relevance information to the assessment. In both cases, the toxicogenomic data informed the MOA in the WOE analysis. There are also many examples of genomic studies performed at EPA laboratories with the purpose of testing MOA hypotheses for various chemicals, such as propiconazole.

Although EPA has evaluated toxicogenomic data during the course of risk assessments, it has not developed a formalized approach for the incorporation of these data into risk assessment. Therefore, case studies, when performed in an iterative, collaborative fashion, could reveal practical issues for developing approaches for utilizing toxicogenomic data in risk assessment. Nevertheless, as the use of the technologies continue to advance, EPA must prepare for the future increase in genomic data availability and submission.

2.3.2.8. *Toxicogenomic Activities at Other Agencies and Institutions*

In addition to the FDA and EPA, a number of other federal agencies, nongovernmental organizations, nonprofit organizations, industry, and the larger scientific community, are involved in efforts to apply toxicogenomic data. One major undertaking by the scientific community was the development and agreement on minimum information standards for microarray experiments (MIAME) for publications and submission to public repositories (Ball et al., 2004; Brazma et al., 2001).

A National Institute of Environmental Health Sciences (NIEHS) Division of Extramural Research and Training (DERT) consortium initiated projects in the following areas, to impact risk assessment and public health:

- perform research in the broad area of environmental stress responses using microarray gene expression profiling
- develop standards and practices that will allow analysis of gene expression data across platforms and provide an understanding of intra- and interlaboratory variation

- contribute to the development of a robust relational database, combining toxicological endpoints with changes in gene expression profiles

- improve public health through better risk detection and earlier intervention in disease processes (http://www.niehs.nih.gov/research/supported/centers/trc/)

The International Programme on Chemical Safety (IPCS) is involved in projects to utilize genomic data. IPCS conducted a workshop on *Toxicogenomics and the Risk Assessment of Chemicals for the Protection of Human Health*. The specific objectives of the IPCS workshop were to

- establish a scientific forum for dialogue among experts;

- share information about ongoing scientific activities using toxicogenomics at the national, regional, and international levels;

- discuss the potential of toxicogenomics to improve the risk assessment process for the protection of health from environmental exposure to chemicals, understanding the MOAs of environmental toxicants, and the relevance and scope of gene environment interactions;

- identify the near-term needs and necessary steps for enhancing international cooperation in toxicogenomics research for improving chemical safety; and

- identify and discuss data gaps, issues, and challenges that may present obstacles to the use of toxicogenomics for the protection of human health from environmental exposures.

The IPCS workshop concluded that toxicogenomics has the potential to improve the specificity and range of methods used to predict chemical hazards and to inform a number of uncertainties involved in chemical-related risk assessment.

The International Life Science Institute's (ILSI) Health Environmental Science Institute (HESI) has several completed and ongoing activities on the use of toxicogenomics in risk assessment. In 2004, Environmental Health Perspectives published a mini monograph (Pennie et al., 2004) with several articles relating to the application of toxicogenomic data and their implications to risk assessment. In addition, ILSI/HESI has undertaken a major and ongoing effort to develop a toxicogenomic database (http://www.hesiglobal.org/Committees/TechnicalCommittees/Genomics/EBI+Toxicogenomics.

htm). Furthermore, ILSI has conducted workshops and training courses on the use of toxicogenomic data in risk assessment.

The National Academy of Sciences (NAS) is also committed to projects in the area of using genomic data in screening and risk assessment as evidenced by numerous NAS workshops and reports on the topic. A recent NRC report, *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment,* is an excellent source of information about genomic technologies and their application to risk assessment (NRC, 2007a). Further, the NRC report, *Toxicity Testing in the 21st Century: A Vision and a Strategy* (NRC, 2007b), outlines a pathway-based risk assessment approach.

2.3.3. Current Challenges and Limitations of Toxicogenomic Technologies

Genomic data will likely continue to have an impact in multiple areas of science, medicine, law, and policy. One example of the application of genomic data to decision making is the use of biomarkers of disease in medicine. Nevertheless, there are a number of technical and analytical methodology issues for the use of genomic data in risk assessment. These limitations include the paucity of toxicogenomic data for most chemicals due to the cost and technical difficulties of conducting the experiments and analyzing the data (Shi et al., 2004; Smith, 2001). Evaluation of the technologies themselves, as well as the data analysis methods, needs validation.

One of the major challenges in using microarray data is its interpretation. In particular, one does not always have information to determine the functional meaning of a change in gene expression. Determining whether there is an association or causal link between the alterations in gene expression and *in vivo* toxicological endpoints, also referred to as "phenotypic anchoring," may not be possible with available data. Another issue is reproducibility/variability (Moggs, 2005; Hamadeh et al., 2002a, b) in toxicogenomic response. While the MAQC-I project results demonstrate good reproducibility when using the same biological sample and platform, other issues leading to variability in response (e.g., biological sample preparation) need to be addressed. To resolve these issues, an iterative and collaborative research process between risk assessors and research scientists would be beneficial.

Despite limitations in utilizing toxicogenomics data in risk assessment, these data can currently be informative to risk assessment. Genomic data can provide information about the

mechanisms of action or MOAs for a given chemical or group of chemicals; provide a screening assay for a class of chemicals or toxicity; inform the dose-response evaluation for precursor events; and understand the variability of responses in different species, among species (individual susceptibility), or in different organs or tissues. In the future, the application of toxicogenomics data to risk assessment will undoubtedly increase as genomic technologies and bioinformatics methods are further developed and refined.

2.4. INTRODUCTION TO THE CASE STUDY

2.4.1. Project Team

The methods development and case-study portions of the project were performed collaboratively between EPA and outside partners. Team members included: EPA scientists at NCEA, the National Health and Environmental Effects Research Laboratory (NHEERL), IRIS, and regional offices; and outside partners at the NIEHS; the Hamner Institutes for Health Sciences; and the EPA National Center for Environmental Research Science to Achieve Results (STAR) Environmental Bioinformatics and Computational Toxicology at the University of Medicine and Dentistry of New Jersey (UMDNJ) and Rutgers. The team was multidisciplinary, including experts in developmental and reproductive biology and toxicology, human health risk assessment, toxicogenomic data study design, toxicogenomic data analysis, and modeling.

2.4.2. Chemical Selection

We conducted a literature review to identify candidate chemicals for the case study. Because of the expertise of the team members and the availability of microarray studies for a number of endocrine disrupting chemicals (EDCs), we focused on our review on EDCs. Among EDC classes of chemicals, the androgen-mediated male reproductive development toxicity pathway was identified as the best choice for the case study (see Figure 2-2) for several reasons.

1. Androgens are essential for a number of male developmental events and are required during gestation for the normal development of the male genital tract and sexual differentiation; thus, this toxicity pathway has relevance to *in vivo* outcomes.

2. There are published studies for chemicals that affect androgen action (i.e., androgen antagonists and agonists) that support a relatively strong linkage between the MOAs and the resulting toxicological outcomes after exposure.

3. There are some published toxicogenomic data, as well as ongoing research, for some of the EDCs that affect androgen action.

4. There are recent or ongoing EPA health or risk assessments for some chemicals that affect androgen action.

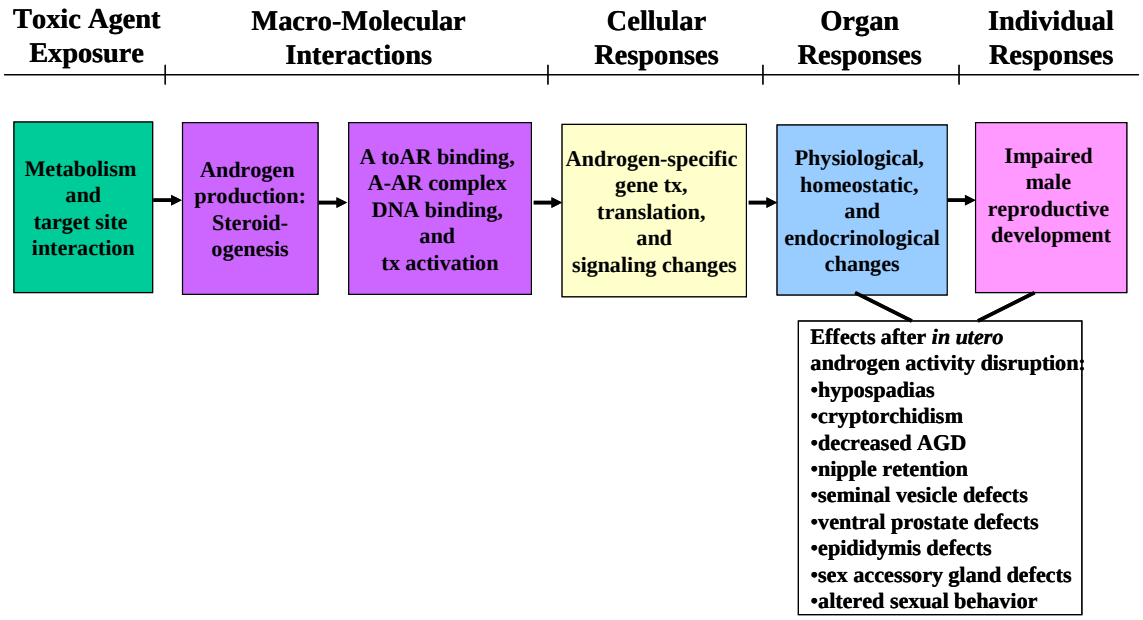

Figure 2-2. **Androgen-mediated male reproductive developmental toxicity pathway.** AGD, anogenital distance; tx, transcription; A, androgen; AR, androgen receptor.

2.4.2.1. *Six Candidate Chemicals*

Six candidate chemicals were identified and considered for the case study: linuron, procymidone, vinclozolin, di-(2-ethylhexyl) phthalate (DEHP), DBP, and prochloraz. The criteria for selecting a chemical for the case study were

1. relative abundance of available toxicogenomic data (preferably published data);
2. consistency of the toxicogenomic data set findings, as one indicator of high quality studies;
3. recent or ongoing EPA assessment; and
4. interest expressed by an EPA Program and/or Regional Office in performing a case study on this chemical.

We gathered information for each of the six candidate chemicals on these four criteria by consulting the toxicogenomic literature and the chemical managers of the EPA health or risk assessments (see Table 2-1). The summary of the information presented in the table is limited as it reflects the information available at the time of selection of the case-study chemical (July 2005).

2.4.2.2. *Selection of the Case-Study Chemical*

At the time of the decision, five of the candidate chemicals (all except prochloraz) met three of the four criteria for chemical selection: (1) a relative abundance of available toxicogenomic data, (2) a relatively consistent toxicogenomic data set, and (3) a recent (<5 years) or ongoing EPA assessment. Assessment of the fourth criteria was more subjective in nature because it was based on individual opinions. None of the chemicals was considered a poor choice; however, after discussion of the relative merits of each of the chemicals, we selected DBP for the case study for the following reasons:

1. Quantity and Quality of Toxicogenomic Data Set:
   DBP and DEHP both have a relatively large and high-quality (based on consistency of findings) toxicogenomic data set. The DBP data set includes gene expression changes in genes known to be involved in the androgen-mediated male reproductive toxicity pathway, providing phenotypic anchoring to a number of the male reproductive developmental effects following high dose DBP *in utero* exposure. Additionally, there is one dose-response RT-PCR study using low-to-high *in utero* DBP doses that observed alterations in nine genes involved in steroidogenesis as well as other pathways (Lehmann et al., 2004).

2. Application to Risk Assessment:
   The DBP assessment may allow the case study to address some interesting questions that may have broad application to the use of toxicogenomics in risk assessment. These questions include:

   - Do the toxicogenomic data provide information to identify or further determine the mechanisms of action and MOAs for DBP?

Table 2-1. Information available July 2005 on the selection criteria for the six candidate chemicals affecting the androgen-mediated male reproductive developmental toxicity pathway.[1]

Chemical	MOA(s)	EPA Assessments	Published TGx Data (amount)	Ongoing TGx Studies
Linuron	AR antagonist	IRIS Oral RfD, 1990[2]; IRIS Cancer, 1993[2]; OPPT RED (U.S. EPA, 1995); OPPT TRED (U.S. EPA, 2002b)	Yes (low)	Yes
Procymidone	AR antagonist	Discussed in REDs for vinclozolin (U.S. EPA, 2000) and iprodione (U.S. EPA, 1998); OPPT TRED (U.S. EPA, 2005b)	Yes (low)	Proposed[3]
Vinclozolin	AR antagonist	OPPT RED (U.S. EPA, 2000); IRIS Oral RfD, 1992[4]	Yes (low)	Yes
DEHP	Fetal testicular steroidogenesis inhibitor	Ongoing (IRIS)	Yes (high)	Yes
DBP	Fetal testicular steroidogenesis inhibitor	Ongoing; Internal review complete (IRIS)	Yes (high)	Yes
Prochloraz	Steroidogenesis inhibitor and AR antagonist	IRIS Oral RfD, 1989[5]; IRIS Cancer, 1997[5]	Yes (medium), but few studies focus on male reproductive tissues and/or endpoints	Proposed[3]

AR, androgen receptor; OPPT, Office of Prevention, Pesticides and Toxic Substances; RED, Reregistration Eligibility Decision; RfD, reference dose; TGx, toxicogenomic; TRED, Tolerance Reassessment Progress and Risk Management Decision.

[1]The information in this table reflects the available information at the time of the selection (July 2005).

[2]http://www.epa.gov/ncea/iris/subst/0170.htm

[3]Telephone conv. in July 2005 between L.E. Gray, Jr. (NHEERL]) and S. Euling (NCEA).

[4]http://www.epa.gov/ncea/iris/subst/0126.htm

[5]http://www.epa.gov/ncea/iris/subst/0378.htm

- Can toxicogenomic data be used to determine the adverse level for the reduction in fetal T, the MOA for a large number of the male reproductive developmental endpoints after *in utero* DBP exposure?

3. Availability of Draft Assessment:
   At the time of chemical selection for this case study, the external review draft IRIS Tox Review for DBP was being developed and, thus, available to utilize as a starting point for the case study. Risk assessment documents for the other candidate chemicals were either incomplete (i.e., too early in their development) or potentially missing new data (i.e., an assessment finalized over 5 years ago was our cut-off).

2.4.3. Case-Study Scope

The DBP case study is limited to effects on male reproductive development because (1) these endpoints are observed in the lower dose range; (2) the team members have expertise in reproductive and developmental biology and toxicology; and (3) there is evidence that some of the gene and pathway alterations after *in utero* DBP exposure are in the causal pathway for some of these endpoints, thus providing a high degree of phenotypic anchoring. After reviewing the data sets for DBP (see Chapter 3), the initial focus on androgen-mediated male reproductive developmental effects (see Section 2.3.2) was broadened to include all male reproductive developmental effects and not just those affecting androgen action, because DBP affects pathways (e.g., *Insl3*) other than the androgen pathway.

As a consequence of the data available for DBP, the approach focused on utilizing genomic data to inform the hazard characterization and dose-response steps of risk assessment because these are the steps that are included in an IRIS assessment. The case-study chemical selected, DBP, had an available draft IRIS assessment (the external peer review draft Tox Review). Thus, exposure assessment was not included in this approach. While there are many successes and ongoing efforts utilizing genomics in exposure assessment, both in ecological and human health risk assessment, such efforts will not be discussed in the case study.

The DBP case study is limited to the use of genomic data to inform the qualitative aspects of risk assessment because of the lack of available dose-response toxicogenomic data for DBP. Since the incorporation of toxicogenomic data into risk assessment includes both a quantitative and qualitative use of these data, the consideration of toxicogenomic data to quantitative aspects, such as TK modeling and dose-response assessment, is discussed in this report (see Chapters 3

and 7). These chapters include how to consider the available toxicogenomic data for quantitative application.

Finally, the case-study effort is limited to the review, evaluation, and analysis of existing data available in the published, peer-reviewed literature. For practical reasons, the DBP case-study exercise was purposely limited to publicly available published data prior to July 2008 (see Chapter 5).