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An Approach to Using Toxicogenomic Data in U.S. EPA Human Health Risk Assessments: A Dibutyl Phthalate Case Study

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National Center for Environmental Assessment
Office of Research and Development
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Washington, DC 20460

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LIST OF ABBREVIATIONS AND ACRONYMS

Please note that most gene and protein name abbreviations are not included in this list because of the large number of genes and proteins described in the report. The gene and protein names have been standardized using information from the Rat Genome Project.

ADH	alcohol dehydrogenase
ADME	absorption, distribution, metabolism, and excretion
AGD	anogenital distance
AMH	anti-mullerian hormone
ANOVA	analysis of variance
AR	androgen receptor
BBDR	biologically based dose-response
BBP	butyl benzyl phthalate
BMD	benchmark dose
BMDL	benchmark dose lower confidence limit
BPA	bisphenol A
cDNA	complementary DNA
CNPs	copy-number polymorphisms
DBP	dibutyl phthalate
DEG	differentially expressed gene
DEHP	di-(2-ethylhexyl) phthalate
DEP	diethyl phthalate
DMP	dimethyl phthalate
DOTP	diocytyl tere-phthalate
DPP	dipentyl phthalate
EDC	endocrine disrupting chemical
EPA	Environmental Protection Agency
ER	estrogen receptor
ESTs	expressed sequence tags
FDA	Food and Drug Administration
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GD	gestation day
GO	Gene Ontology
GSH	glutathione

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

HESI	Health and Environmental Sciences Institute
ILSI	International Life Sciences Institute
IPA	Ingenuity® Pathway Analysis
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Leydig cell
LMW	low molecular weight
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest-observed-effect level
MAPK/ERK	mitogen-activated protein kinase/extracellular signal-regulated kinase
MAQC	MicroArray Quality Control
MAS	microarray suite
MBP	monobutylphthalate
MMP	matrix metalloproteinase
MOA	mode of action
mRNA	messenger RNA
NCCT	National Center for Computational Toxicology
NCEA	National Center for Environmental Assessment
NIEHS	National Institute of Environmental Health Sciences
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NRC	National Research Council
NTP	National Toxicology Program
PA	pathway activity
PBPK	physiologically-based pharmacokinetic
PCA	principal component analysis
PCR	polymerase chain reaction
PFOA	perfluorooctanoic acid
PND	postnatal day
POD	point of departure
PPAR	peroxisome proliferator-activated receptor
PPS	preputial separation

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

RA	risk assessment
RACB	reproductive assessment by continuous breeding
RfD	reference dose
RT-PCR	reverse transcription-polymerase chain reaction
SD	Sprague-Dawley
SLR	signal log ratio
SNPs	single nucleotide polymorphisms
SNR	signal-to-noise ratio
SPC	Science Policy Council
STAR	Science to Achieve Results
T	testosterone
TD	toxicodynamics
TF	transcription factor
TK	toxicokinetics
Tox Review	Toxicological Review
UF_H	intraspecies uncertainty factor
UMDNJ	University of Medicine and Dentistry of New Jersey
VLI	valine, leucine, isoleucine
WD	Wolffian duct
WOE	weight-of-evidence

PREFACE

The U.S. Environmental Protection Agency (EPA) is interested in developing methods to use genomic data most effectively in risk assessments performed at EPA. The National Center for Environmental Assessment (NCEA) within the Office of Research and Development (ORD) prepared this document for the purpose of describing and illustrating an approach for using toxicogenomic data in risk assessment. The approach and dibutyl phthalate (DBP) case study described in this document were developed by a team of scientists at EPA laboratories and centers, and outside organizations including The Hamner Institutes for Health Sciences, the National Institute of Environmental Health Sciences (NIEHS), and the EPA National Center for Environmental Research (NCER) Science to Achieve Results (STAR) Environmental Bioinformatics and Computational Toxicology (Comp Tox) Center at the University of Medicine and Dentistry of New Jersey (UMDNJ) and Rutgers University. The intended audience for this document includes risk assessors as well as scientists with expertise in genomics, bioinformatics, toxicology, and statistics. The approach outlined in this document is expected to be useful to EPA risk assessors in the Integrated Risk Information System (IRIS) Program and other program offices and regions, as well as the scientific community at large. The review of the literature on the use of genomic data in risk assessment, as well as discussions of issues, recommendations, and methods for evaluating and analyzing toxicogenomic data, could be useful to scientists and risk assessors within and outside of EPA. The research needs identified in this document will be useful to scientists performing toxicology and toxicogenomic research studies for application to risk assessment. The DBP case study presented in this document is a separate activity from the IRIS DBP health assessment. The review of the literature included in this document was last updated in July 2007.

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1. EXECUTIVE SUMMARY

We developed a systematic approach for evaluating and utilizing toxicogenomic data in health assessment. This report describes this approach and a case study conducted for dibutyl phthalate (DBP) to illustrate the approach. As a result of the case-study exercise, we refined the initial case-study approach for general use in new chemical assessments. In this report, we reviewed some of the recent and ongoing activities regarding the use of genomic data in risk assessment, inside and outside of the U.S. Environmental Protection Agency (EPA). We also identified research needs, recommendations, and issues for future consideration when using genomic data in risk assessments.

Toxicogenomics is the application of genomic technologies (e.g., transcriptomics, proteomics, metabolomics, genome sequence analysis) to study the effects of environmental chemicals on human health and the environment. The EPA Interim Genomics Policy (U.S. EPA, 2002a) encourages the use of genomic data, on a case-by-case basis, in a weight-of-evidence (WOE) approach. Currently, EPA provides no guidance for incorporating genomic data into risk assessments of environmental agents. However, EPA's Science Policy Council (SPC) has developed interim guidance regarding other aspects of the use of microarray data at EPA, entitled *Interim Guidance for Microarray-Based Assays: Data Submission, Quality, Analysis, Management, and Training Considerations* (U.S. EPA, 2006b).

DBP was selected for the case study because it has a relatively large genomic data set and phenotypic anchoring of certain gene expression data to some male reproductive developmental outcomes. The scope of the case study was limited to the male reproductive developmental outcomes of DBP, and this effort was limited to evaluating the available published toxicity and toxicogenomic data for the DBP case study. The DBP case study is a separate endeavor with distinct goals from EPA's Integrated Risk Information System (IRIS) assessment of DBP.

1.1. APPROACH

Genomic data have the potential to inform toxicodynamics (TD), toxicokinetics (TK), inter- and intraspecies differences in TD and TK, exposure assessment, and dose-response assessment. Our strategy was to design an approach for evaluating genomic data for risk assessment that is both systematic and flexible enough to accommodate different health and risk

assessment practices. The first step of the approach is to evaluate the available genomic data set for its application to a broad range of information types (e.g., TD, TK, intra-and interspecies TD and TK differences) that are useful to risk assessment as well as the steps of health assessment (e.g., hazard characterization, dose-response assessment). Through this iterative process, the potential use of the available genomic data is determined. As part of the scoping step, the available human, toxicology, and genomics studies are reviewed to determine their use to the genomic data set evaluation. For instance, the toxicity, human, and toxicogenomic data sets are considered together to determine the relationship (i.e., degree of phenotypic anchoring) between gene and pathway changes to health or toxicity outcomes. As a result of the scoping step, questions are posed to direct and focus the evaluation of the genomic data set.

The next steps include detailed evaluations directed by the formulated questions of the toxicity and/or epidemiological data sets and the toxicogenomic data set. For example, when genomic data are available to inform mechanisms of action or modes of action (MOAs), the toxicogenomic and toxicity data sets can be evaluated together, relating the affected endpoints (identified in the toxicity data set evaluation) to the genes and/or pathways (identified in the toxicogenomic data set evaluation) to establish or formulate hypotheses about an MOA. In addition to informing the mechanisms of action and the MOAs (TD and TK steps), genomic data also have the potential to inform inter- and intraspecies TD differences, and dose-response assessment, depending on the genomic study design (e.g., species, organ, single dose vs. multiple doses, genomic method) of the available data. The approach also includes new analyses of the genomic data for the purpose of risk assessment when data are available and such new analyses may address questions that are relevant to the risk assessment.

1.2. DBP CASE STUDY

For the DBP case-study example, we utilized the data set summaries and data gaps identified in the external review draft IRIS Tox Review for DBP (U.S. EPA, 2006a) and asked whether the genomic data set could inform any of these data gaps. In parallel, the DBP genomic data set was considered, in light of all risk assessment aspects that these data might inform. As a result of following these two processes, we formulated two specific case-study questions that the available genomic data for DBP had the potential to inform:

- *Do the toxicogenomic data inform the mechanisms of action and/or MOAs for DBP?*
- *Do the toxicogenomic data inform interspecies differences in TD?*

The team considered it highly likely that the DBP toxicogenomic data set could inform the modes or mechanisms of action. The team considered it possible, but less certain, that the cross-species differences in one or more DBP MOAs could be informed by evaluating genomic data (e.g., DNA sequence data).

Additional questions were excluded because appropriate data were lacking. For example, one question of great interest is, *Do the toxicogenomic data inform dose-response?* However, this question could not be addressed in this case study because there were no dose-response genomic data for DBP. Few chemicals have available dose-response genomic data and DBP is not unusual in this respect. The evaluation of the one available DBP dose-response gene expression study, although not global, is discussed in the report. As a result of the DBP genomic data set limitations, the case study focuses on the qualitative application of genomic data to risk assessment. In addition, exposure assessment was not considered in this approach because the case study was performed using the IRIS chemical assessment model, which only includes hazard identification and dose-response steps of the risk assessment paradigm.

We found that the DBP toxicogenomic data did inform the mechanism of action, and generated hypotheses about possible additional MOAs, for DBP and male reproductive developmental outcomes. There is substantial evidence in the published literature that a number of the gene expression changes observed in genomic studies are phenotypically anchored for a number of the male reproductive developmental outcomes observed after *in utero* DBP exposure in the rat. The available genomic and other gene expression data, hormone level data, and toxicity data for DBP are instrumental in the establishment of two MOAs: (1) a decrease in fetal testicular testosterone (T); and (2) a decrease in Insulin-like 3 (*Insl3*) expression. A decrease in fetal testicular T is a well-established MOA for a number of the male reproductive developmental effects observed in the male rat after *in utero* DBP exposure. The genomic and single gene expression data, after *in utero* DBP exposure, identified changes in genes involved in steroidogenesis and cholesterol transport, consistent with the observed decrease in fetal testicular T. Decreased *Insl3* expression is a second well-established MOA responsible, in conjunction with reduced T, for the undescended testis effect observed following *in utero* DBP exposure.

Reverse transcription-polymerase chain reaction (RT-PCR) and *in vivo* toxicology study results support the role of *Insl3* in one of the two steps of testis descent.

Evaluating genomic and toxicity data together also provides information on putative novel MOAs. A number of the DBP toxicity and toxicogenomic studies were performed in the same strain of rat using similar doses and exposure intervals that allowed for comparisons across studies. In this case study, rodent reproductive developmental toxicity studies were evaluated for low incidence and low-dose findings and for the male reproductive developmental effects that currently do not have an explained MOA (termed "unexplained endpoints"). In the case study, we focused on the outcomes in the testes because all, but one, of the DBP toxicogenomic studies were performed on testes. We identified five testicular endpoints without a known MOA that were pursued further in the evaluation of the toxicogenomic data set.

The nine published RT-PCR and microarray studies in the rat were evaluated as part of the toxicogenomic and associated gene expression data set to identify genes and pathways affected after *in utero* DBP exposure. Both the microarray data set alone and the entire gene expression data set (including all gene expression studies including microarray studies) were evaluated for consistency of findings. At the gene level, the findings from the DBP genomic studies (i.e., microarray, RT-PCR, and protein expression) were relatively highly correlated with one another in both the identification of differentially expressed genes (DEGs) and their direction of effect. The evaluation of the published toxicity and toxicogenomic studies corroborates the two known MOAs for DBP.

The published microarray studies for DBP focused primarily on pathways related to the reduced fetal testicular T MOA, such as the steroidogenesis pathway. We performed new analyses of the data from one rat testes microarray study in order to identify all possible pathways significantly affected by *in utero* DBP exposure. Using two different analytical methods, pathways associated with the two well-established MOAs (decreased *Insl3* and fetal testicular T), as well as new processes (e.g., growth and differentiation, transcription, cell adhesion) and pathways (e.g., *Wnt* signaling, cytoskeleton remodeling) not associated with either *Insl3* or steroidogenesis pathways, were identified. The newly identified putative pathways may play a role in the regulation of steroidogenesis (i.e., related to a known MOA for DBP) or, alternatively, may inform additional MOAs for one or more unexplained outcomes in the testes.

The new analyses and the approach allowed us to develop hypotheses about possible DBP MOAs for some male reproductive developmental outcomes.

To address the question of whether the available genomic data for DBP could inform the interspecies TD part of the interspecies uncertainty factor, genomic data were evaluated to inform interspecies differences in the steroidogenesis pathway, relevant to the decreased fetal testicular T MOA. We explored the development of new methods to evaluate interspecies TD differences. To evaluate cross-species similarity metrics for the steroidogenesis pathway between rats and humans, we explored two approaches: protein sequence similarity and enzyme presence. Preliminary results from applying each method suggest that steroidogenesis genes are relatively highly conserved between rats and humans. However, we do not recommend utilizing these data to inform interspecies uncertainty for DBP because it is difficult to make unequivocal conclusions regarding a "high" versus "low" degree of conservation for the genes in this pathway based on these data alone. With further refinement and improved data sources, these methods could potentially be applied to other chemical assessments.

New methods for evaluating microarray data for the purposes of risk assessment were explored and developed during the DBP case study. A new pathway analysis method, the pathway activity level method, was developed and tested with two DBP study data sets. The pathway activity level method determines pathway level changes as the initial step as opposed to standard pathway analysis methods in which DEGs are first identified, followed by mapping of the DEGs to pathways, as a second step. Further, the pathway activity level method was used to evaluate time-course microarray data. A preliminary gene network model for DBP, based on the results from one time-course study, identified a temporal sequence of gene expression and pathway interactions that occur over an 18-hour interval within the critical window of exposure for DBP and testicular development effects.

1.3. RECOMMENDATIONS

In addition to following the principles of the approach (i.e., systematically consider all types of information with respect to the steps of risk assessment, identify questions to direct the evaluation, and evaluate genomic data and toxicity data together), several specific methodological recommendations arose from the DBP case-study experience. The first two recommendations are straightforward and could reasonably be performed by a risk assessor with

basic training in genomics data evaluation and interpretation. The third recommendation requires expertise in genomic data analysis methods for implementation. The recommendations are presented below:

1. *Evaluate the genomic and other gene expression data for consistency of findings across studies to provide a WOE evaluation of the affected gene expression and pathways.* Some simple methods, such as using Venn diagrams and gene expression compilation approaches, can be applied to risk assessment. When evaluating the consistency of toxicogenomic data findings, it is advantageous to include all available gene expression data (single gene, global gene expression, protein, RNA) because single gene expression techniques have been traditionally used to confirm the results of global gene expression studies and because single gene expression data add to the database.

2. *Perform benchmark dose (BMD) modeling on high-quality RT-PCR dose-response studies of genes known to be in the causal pathway of an MOA or outcome of interest.* Obtaining a BMD and benchmark dose lower confidence limit (BMDL) is a useful starting point for both linear low-dose extrapolation and reference value approaches. We are not indicating which approach is appropriate to take for making predictions about the potential risk below the BMD or BMDL. "High quality" is defined in this context as a well-conducted study that assessed enough animals and litters for sufficient statistical power for characterizing the mean responses and the variability (interlitter and intralitter).

3. *Perform new analysis of toxicogenomic data in cases when the new analysis is likely to yield new information that would be useful to the risk assessment. Examples include:*

    - Perform a new pathway analysis in order to identify all affected pathways or other risk assessment applications. When the available published microarray studies have been conducted for purposes (e.g., basic science, pharmaceutical development) other than risk assessment, it may be useful to reanalyze the raw data for risk assessment purposes. Information about all affected pathways may contribute to an understanding of the mechanisms and MOAs.

    - Identify the genes and pathways affected over a critical window of exposure if global gene expression time-course data are available. Specifically, by developing a gene network over time, it may be possible to identify the earliest affected genes and/or pathways, which in turn may represent the earlier or initiating events for the outcome of interest.

Based on these recommendations, we refined our initial case-study approach to produce a generalizable approach that can be used to evaluate genomic data in new chemical assessments.

1.4. RESEARCH NEEDS

The following research needs could potentially improve the utility of genomic data in risk assessment:

- Perform parallel toxicity and toxicogenomic studies with similar design characteristics (i.e., dose, timing of exposure, organ/tissue evaluated) in order to obtain comparable results which would aid our understanding of the relationship between gene expression changes and phenotypic outcomes.

- Test multiple doses, with increased numbers of animals, in microarray and toxicity studies (see bullet above) in order to relate the dose to the gene expression and pathway response, and to the *in vivo* response.

- Perform a time-course global gene expression study over a relevant exposure interval (e.g., critical window of development) in order to identify the earlier and possibly, initiating gene expression events.

- Generate TK data in an appropriate study (e.g., time, dose, tissue), and obtain a relevant internal dose measure to derive the best internal dose metric.

- Further develop bioinformatic methods for analyzing genomic data for the purpose of use in risk assessment.

As a result of considering how to best use genomic data in risk assessment, we identified a number of issues for future consideration. As more and various types of genomic studies are performed, genomic data will likely inform multiple steps of the risk assessment process beyond MOA. To facilitate the advancement of the use of genomics in risk assessment, first, we need approaches to utilize genomic data quantitatively, specifically, the application of genomic data to dose-response, intraspecies variability, and TK. Second, analytical methods tailored to use in risk assessment are needed. Bioinformatics methods development work, some initiated in this project, continues to evolve. The goal is to develop and/or adapt existing bioinformatic tools currently used for hypothesis generation to the express purpose of utilizing genomic data for risk assessment. The pathway activity level method presented in this report is a promising approach for application to risk assessment. However, continued efforts, with input from both statistical modeling and biology experts, is required to validate, test, and refine these methods. Third, training risk assessors in genomic data analysis methods would assist EPA in the evaluation and interpretion of complex, high-density data sets and in performing new analyses when necessary.

Finally, some of the issues in utilizing genomic data in health and risk assessment are not unique to genomic data but apply to precursor event information in general. Two of these issues are (1) defining adversity and (2) establishing biological significance of gene expression changes or pattern. The design and performance of appropriate studies, with both genomic and toxicity components, may help to address the scientific aspects of these two important issues (see research needs above).

To the best of our knowledge, this is the first systematic approach for using genomic data in health assessment at EPA. We believe that this report can be used by risk assessors when considering a large range of potential applications, issues, and methods to analyze genomic data for future assessments. This approach advances efforts in the regulatory and scientific communities to devise strategies for using genomic data in risk assessment, and it is consistent with the pathway-based risk assessment vision outlined in the National Research Council's (NRC's) report, *Toxicity Testing in the 21st Century*. We also anticipate that the research needs and future considerations described herein will advance the design of future toxicogenomic studies for application to risk assessment, and as a result, benefit the bioinformatic, toxicogenomic, and risk assessment communities.

3. DBP CASE-STUDY APPROACH AND EXERCISE

This chapter presents a description of the approach used to evaluate toxicogenomic data in risk assessment, and a description of the first three steps of the DBP case study. Our strategy for evaluating genomic data for risk assessment was to design a flexible yet systematic approach that would provide a thorough evaluation of the genomic data set for a particular chemical, while still accommodating different risk assessment practices. The discussion includes both (1) general (i.e., not chemical-specific) considerations for evaluating a genomic data set, and (2) consideration of the DBP genomic data set as part of the DBP case study.

3.1. EVALUATING THE EXTERNAL REVIEW DRAFT OF THE IRIS TOXICOLOGICAL REVIEW (TOX REVIEW) OF DBP

The case-study approach begins with an evaluation of the 2006 external review draft IRIS Tox Review for DBP (see Figure 3-1). Use of this draft assessment as the starting point allowed us to take advantage of (1) the compilation of the toxicity and human data sets, allowing us to focus on the toxicogenomic data set evaluation, and (2) data gaps that were identified, thus, providing possible questions that the toxicogenomic data may be able to address.

The IRIS assessment of DBP was in progress and the internal review draft was available when the DBP toxicogenomic case study project was initiated in 2005. The external review draft of the Tox Review for DBP and IRIS Summary were released for public comment and peer review on June 27, 2006 (U.S. EPA, 2006a; http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=155707). The External Review Peer Review Panel meeting was held July 28, 2006.

There are extensive studies documenting developmental toxicity of DBP and its primary metabolite, monobutyl phthalate, in rodents (Barlow et al., 2004; Barlow and Foster, 2003; Mylchreest et al., 2002, 2000, 1999, 1998; Ema and Miyawaki, 2001a, b; Ema et al., 2000a, b 1998, 1997, 1996, 1995, 1994, 1993; see Chapter 4 for further details). DBP exposure to the developing male rat fetus during a critical window of development in late gestation causes a variety of structural malformations of the reproductive tract (e.g., hypospadias); a decrease in anogenital distance (AGD); delayed preputial separation (PPS); agenesis of the prostate, epididymis, and vas deferens; degeneration of the seminiferous epithelium; interstitial cell hyperplasia of the testis; and retention of thoracic areolas and/or nipples (Bowman et al., 2005;

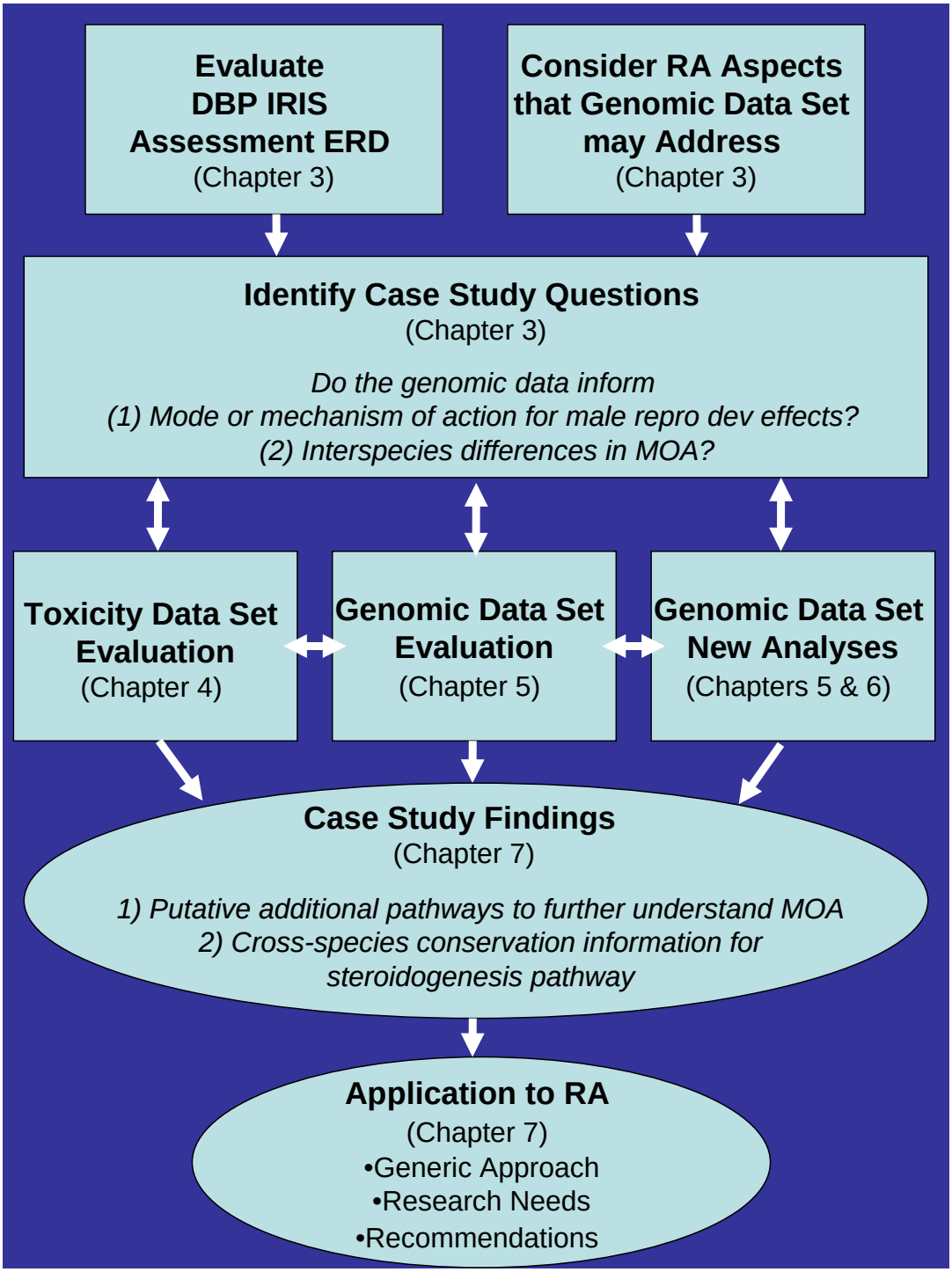

Figure 3-1. DBP case-study approach for evaluating toxicogenomic data for a health assessment. Evaluation steps in the case-study process are shown in rectangles. Findings or products of the case study are shown in ovals. ERD, external review draft. Numbers in parentheses indicate report chapters where the case-study step is described.

Kleymenova et al., 2005a; Barlow et al., 2004; Kim et al., 2004b; Barlow and Foster, 2003; Fisher et al., 2003; Higuchi et al., 2003; Mylchreest et al., 2002, 2000, 1999, 1998; Ema et al., 2000b, 1998, 1997, 1994; Saillenfait et al., 1998).

Figure 3-2 shows the studies that were candidates for the development of the reference dose (RfD) presented in the 2006 external review draft IRIS Tox Review for DBP (U.S. EPA, 2006a). The point of departure (POD) selected for derivation of the RfD for all exposure durations (acute, short-term, subchronic, and chronic) was the no-observed-adverse-effect level (NOAEL) of 30 mg/kg-d for reduced fetal testicular T (Lehmann et al., 2004). In this study, a statistically significant decrease in T concentration in the fetal testis was detected at 50 mg/kg-d. The reduction in fetal testicular T is one of the well-characterized MOAs for DBP that occurs after *in utero* DBP exposure (during the critical window), initiating the cascade of events for a number of malformations in the developing male reproductive tract. Studies using radioimmunoassay of T levels in fetal testes and studies using RT-PCR, microarrays, and/or immunochemical staining found a decrease in the expression of protein and mRNA for several enzymes in the biochemical pathways for cholesterol metabolism, cholesterol transport, and T biosynthesis (also called steroidogenesis more generally) in the fetus (Plummer et al., 2005; Thompson et al., 2004, 2005; Lehmann et al., 2004; Liu et al., 2005; Barlow et al., 2003; Fisher et al., 2003; Shultz et al., 2001). Collectively, these studies document that exposure to DBP disrupts T synthesis in the fetal testis. Thompson et al. (2004) established that following *in utero* exposure to 500 mg/kg-d, the T levels in the testes return to normal after the metabolites of DBP are cleared from the circulation. However, the malformations induced by 500 mg/kg-d exposure persist into adulthood (Barlow et al., 2004; Barlow and Foster, 2003). Thus, although the inhibition of T synthesis can be reversed, the biological effects resulting from the decrease in T during the critical developmental window are irreversible.

3.2. CONSIDERATION OF RISK ASSESSMENT ASPECTS THAT TOXICOGENOMIC DATA MAY ADDRESS

While microarray and RT-PCR data have been used to inform the MOAs of a chemical, the many types of -omic data have the potential to inform TK, dose-response, interspecies and intraspecies differences in TK or TD, and be utilized as biomarkers of exposure or effect (see Figure 3-3).

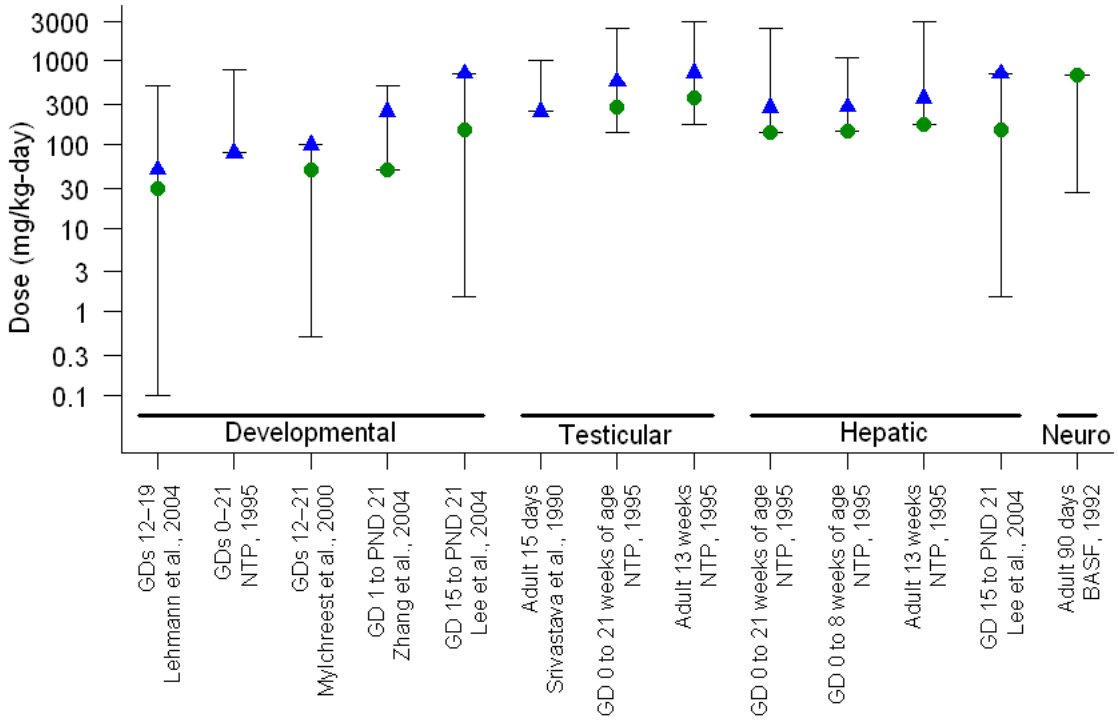

Figure 3-2. Exposure response array for candidate endpoints and PODs for RfD derivation presented in the external review draft IRIS Tox Review for DBP (U.S. EPA, 2006a). The studies are arrayed by toxicological endpoint category. All studies were performed in the rat using the oral route of exposure (although method of oral dosing varied). The study DBP exposure interval and reference are shown on the X axis. The dose (Y axis) is shown in a logarithmic scale. See the articles for additional study details. The vertical line indicates the dose range tested. The lowest and highest doses tested in the study are indicated by the horizontal lines. The green circle indicates the study NOAEL (no-observed-adverse-effect level) and the blue triangle indicates the study LOAEL (lowest-observed-adverse-effect level). Neuro, neurological; GD, gestation day; PND, postnatal day.

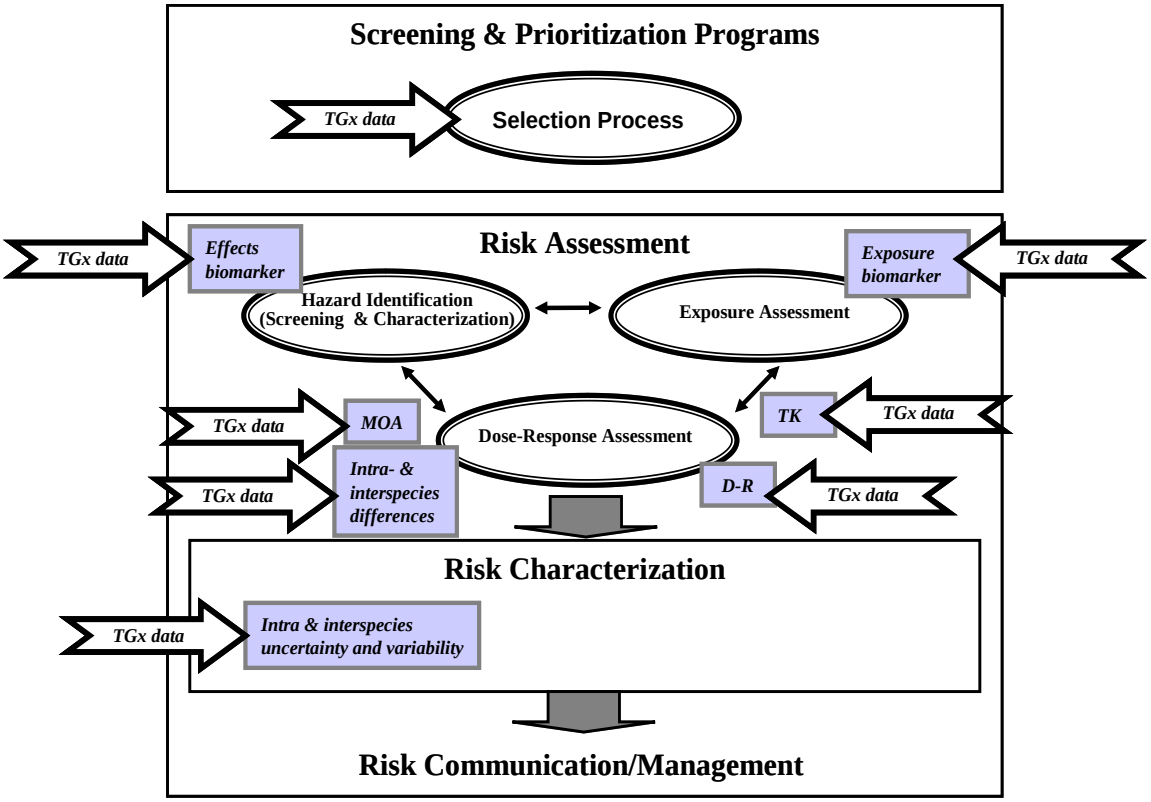

Figure 3-3. **Potential uses of toxicogenomic data in chemical screening and risk assessment.** -Omic data from appropriately designed studies have the ability to inform multiple types of information and in turn, steps in screening and prioritization, and risk assessment. Arrows with TGx data indicate the types of information these data can provide. Shaded boxes indicate some of the types of information that are useful in risk assessment. TGx, toxicogenomic.

However, in this case study, chemical screening and exposure assessment were not considered. Instead, we considered the many types of information useful to hazard characterization, dose-response, and risk characterization. Toxicogenomic data have been successful in providing information about the molecular events altered in the mechanisms of action, and, at times, information about the TD or TK key events of a MOA (see Figure 3-4; see Chapter 2). Further, data from appropriately designed toxicogenomic studies could be used to inform intra- and interspecies differences in molecular responses.

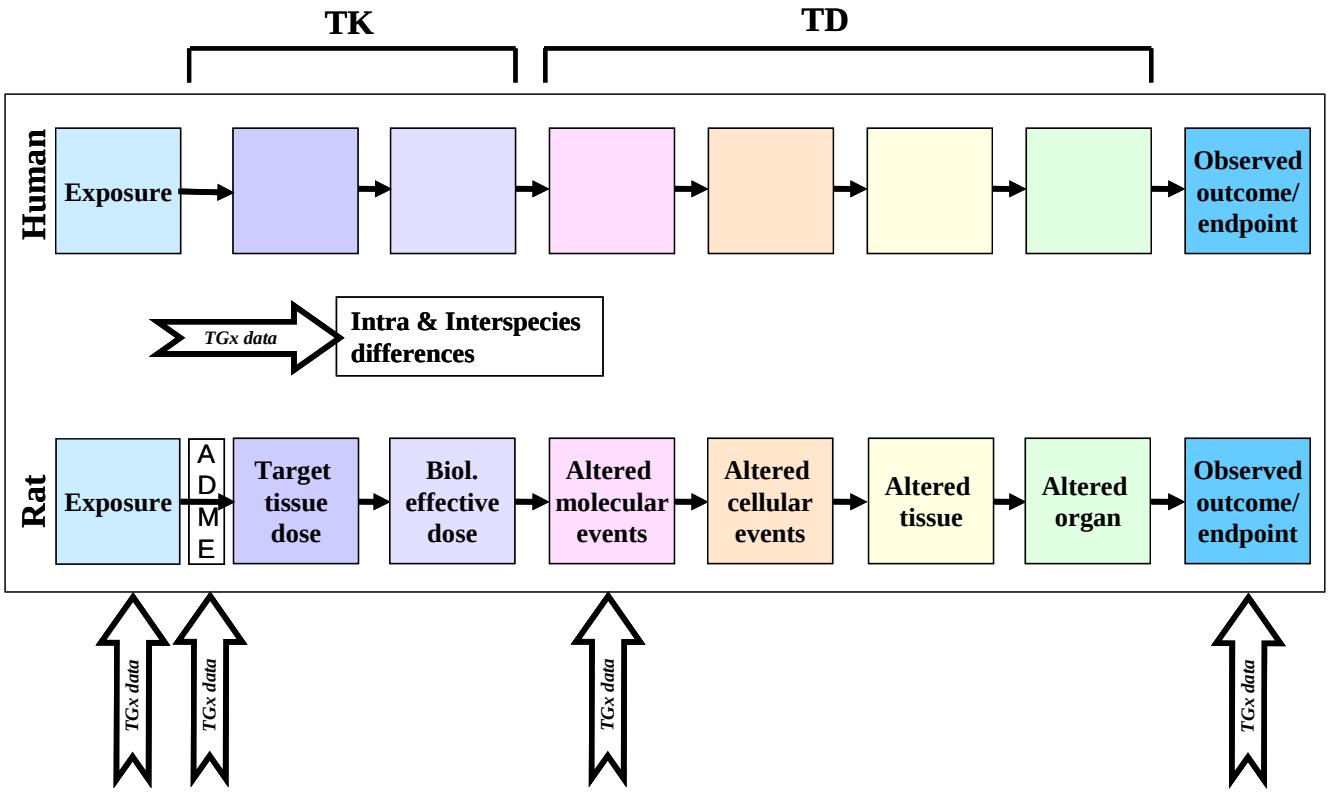

Figure 3-4. **Potential uses of toxicogenomic data in understanding mechanisms of action.** The process from exposure to outcome encompasses all of the steps of a mechanism of action, including both TK and TD steps. Available TGx data, such as microarray data and other gene expression data, can provide information about altered molecular events, at the gene expression level. In turn, appropriate TGx data can be used to inform intra- and interspecies differences in molecular responses. Appropriate TGx data could also inform internal dose and intra- and interspecies differences in internal dose. ADME, absorption, distribution, metabolism, and excretion.

3.2.1. Informing TK

Characterizing the absorption, distribution, metabolism, and excretion (ADME) of environmental toxicants is important for both the understanding and application of MOA information in predicting toxicity. Differences in TK across species, individuals, and exposure patterns (routes, level, duration, frequency) can lead to different biological effects for the same total exposure to a chemical. It is well-established that a quantitative understanding of chemical TK (e.g., using PBPK models) can be useful in analyzing dose-response data and extrapolating across species, individuals, and exposure patterns (U.S. EPA, 2006e). The principles of these uses for TK are the same regardless of the types of response data utilized (i.e., *in vivo* toxicity endpoints [e.g., pup weight] or molecular precursor events [e.g., toxicogenomic changes]), and will not be reviewed here. However, the inverse question of how toxicogenomic data can inform TK has not been fully explored. Here we consider whether toxicogenomic data could be useful for understanding four aspects of a chemical's TK: (1) identification of potential metabolic and clearance pathways; (2) selection of an appropriate dose metric; (3) intra- and interspecies differences in metabolism; and (4) TK/TD linkages and feedback. Each of these applications is discussed below. Finally, the available toxicogenomic data for DBP are evaluated for use in informing TK.

3.2.1.1. ***Identification of Potential Metabolic and Clearance Pathways***

While TK studies, themselves, are designed to help understand the pathways for metabolism and clearance of xenobiotics, toxicogenomic data may provide important complementary information as to what enzymes and tissues may be involved in metabolism. For example, many xenobiotics induce the expression of the Phase I and II enzymes that are responsible for their clearance. Thus, toxicogenomic data showing changes in the expression of genes, such as cytochrome P450s in a particular tissue, may implicate their involvement in metabolizing the compound. While such toxicogenomic data may confirm the major sources of metabolism or clearance, they may also identify minor TK pathways relevant for inducing toxicity. However, data indicating gene expression changes alone are insufficient to conclude that there is a corresponding increase in a protein levels or activity, or are relevant to the ADME of the chemical of interest. Ultimately, toxicogenomic data may be most useful for generating

hypotheses about metabolism and clearance pathways that can be tested with additional TK studies.

3.2.1.2. *Selection of Appropriate Dose Metrics*

Due to inherent differences in TK across species, individuals, and exposure patterns, dose-response relationships are best established based on an internal measure of a biologically effective dose as opposed to an external or applied dose. However, an understanding of TK alone may provide multiple options for the internal "dose metric," such as blood or tissue concentrations of the parent or metabolites, or rates of formation of reactive compounds. Thus, a key question in utilizing TK data for dose-response analyses and extrapolation is dose-metric selection, which depends on the determination of the active chemical species and the MOAs of toxicity. There often may be more than one biologically plausible choice of dose metric, which contributes to the uncertainty in the dose-response analysis. The potential utility of toxicogenomic data is that gene expression data may demonstrate earlier biological effects, and, thus, are closer both spatially and temporally to the interaction between the active chemical species and endogenous cellular molecules than traditional toxicological outcomes (see Figure 3-4). Thus, toxicogenomic data can, in principle, provide biological support for the choice dose metric. Different predictions for internal dose can be statistically analyzed along with toxicogenomic changes that inform TD to determine the dose metric that is best correlated.

3.2.1.3. *Intra- and Interspecies Differences in Metabolism*

Data from polymorphisms is one type of genomic data that can be extremely useful to informing intraspecies differences. Across species, data on differential expression of different isozyme genes may be indicative of differences in overall metabolizing capacity and affinity. In addition, toxicogenomic data may be informative about whether the tissue distribution of metabolizing enzymes may be different across species. Within species, interindividual variability in metabolizing capacity and/or affinity due to differences in enzyme expression or genetic polymorphism can greatly influence the overall TK of a chemical. For example, genetic polymorphisms in aldehyde dehydrogenase-2 (ALDH2) can result in an increase in blood acetaldehyde levels following alcohol consumption, thereby leading to overt health effects (Ginsberg et al., 2002). Similarly, data on CNPs can provide information (Buckley et al., 2005)

that directly informs TK. For example, some individuals possess different copy numbers of CYP2D6 that influence their response to pharmaceuticals (Bodin et al., 2005). When the impacts of gene expression levels and polymorphisms on enzyme levels and function have been established (i.e., preferably confirmed by measurement of enzyme level), this information can either be used to characterize the difference in a predicted dose metric for a subpopulation relative to the most common alleles, or it can be used in probabilistic (e.g., Monte Carlo) analyses to characterize the impact on population variability.

3.2.1.4. *TK/TD Linkages and Feedback*

Ultimately, toxicogenomic data may be useful for linking together TK and TD models into more comprehensive biologically based dose-response (BBDR) models (Daston, 2007). With an appropriate dose metric, one can link the TK predictions for a chemical (e.g., tissue concentration of a metabolite) with toxicogenomic changes (e.g., change in mRNA level) that, in turn, are linked through a TD model to alterations in cellular constituents and, ultimately, frank effects. Furthermore, toxicogenomic data may be useful in providing the link by which the TD feedback of gene and protein expression changes on TK (e.g., enzyme induction) can be modeled.

3.2.1.5. *Research Needs for Toxicogenomic Studies to Inform TK*

Changes in gene expression can be highly labile and vary as a function of dose and time. Thus, identification of appropriate dose metrics involves detection of relevant gene changes as well as the moiety that caused the changes. Therefore, simultaneous data collection of toxicogenomic data and tissue concentrations of the relevant chemical species would be beneficial. In order to inform interspecies extrapolation, it is important to mine toxicogenomic data for potential indicators of species differences in metabolism. For intraspecies variability, it is important to assess the potential impact of polymorphisms in Phase I and II enzymes. Microarray data may also be useful for identifying life stage and gender differences in relative expression of enzymes involved in the TK of the chemical of interest.

3.2.1.6. *DBP Case Study: Do the Available Toxicogenomic Data Inform TK?*

We considered whether the available toxicogenomic data set for DBP informs TK. A greater level of detail is presented for TK in this chapter than for TD because the latter subject is considered in greater detail in the subsequent chapters. This chapter also provides examples of considerations that may be helpful to risk assessors examining whether the available toxicogenomic data can inform TK for their chemical of interest.

The TK of DBP is reviewed in U.S. EPA, 2006a, and is summarized briefly here for context. Following ingestion, DBP is primarily hydrolyzed to monobutylphthalate (MBP) in the gastrointestinal tract and enters systemic circulation through the portal blood. MBP undergoes glucuronidation in the liver, and both free and glucuronidated MBP circulate in serum and are subsequently excreted in urine. While there are a number of TK studies in rats, little human TK data are available, particularly for known exposures to DBP. The available data suggest that free MBP is responsible for the effects on T biosynthesis. In terms of TK pathways, the data set did not lead to the identification of alternative metabolic pathways for DBP.

Toxicogenomic data could inform dose-metric selection in two broad ways: relating the metabolite to the gene expression or using gene expression as the dose metric. In a more traditional approach, changes in the expression for genes of interest could be related to a chemical moiety in a target tissue of relevance (or convenience). For example, Lehmann et al. (2004) provides a dose-response analysis of gene expression following DBP exposure. However, these data are limited for use in extrapolation without TK data (e.g., tissue concentrations of MBP). Ideally, TK data could be collected at various time points following various doses, but this would require a large number of fetuses. In the absence of such empirical data, analyses could be performed using physiologically based TK modeling, but none have yet been attempted. Such an approach might utilize the available published TK studies for DBP and attempt to reconstruct the exposure scenarios in the toxicogenomic studies with the intent to predict the MBP concentration in a target tissue (or blood) at the developmental time points where toxicogenomic samples were obtained.

A second and more complex approach might be to use a toxicogenomic change as a dosimeter (or "biomarker"), which may obviate the need for TK data altogether. For example, the microarray study of Wyde et al. (2005) reports changes in maternal liver *Cyp2b1* and *Cyp3a1*, and estrogen sulfotransferase mRNA levels following DBP exposure. Not only do

these gene expression changes serve as potential biomarkers, but they also suggest that there may be related changes in metabolic biomarkers (i.e., metabonomics) because these enzymes have roles in lipid and hormone synthesis, in addition to xenobiotic metabolism. Although it is not clear whether these changes have a relationship to a toxic endpoint of interest, it may be possible to establish, for instance, that an increase in a specific maternal liver mRNA is correlated with a decrease in a specific mRNA in the fetal testis. Indeed, Wyde et al. (2005) show that maternal liver estrogen sulfotransferase gene expression increases in a dose-dependent manner from 10−500 mg/kg-d, while Lehmann et al. (2004) observed a dose-dependent decrease in *Scarb1*, *Star*, *Cyp11a1*, *Hsd3b*, or *Cyp17a1* mRNA levels in fetal testes from 0.1–500 mg/kg-d.

With respect to interspecies extrapolation and interindividual variability, the lack of adequate human TK data precludes quantitative extrapolation, a situation that cannot be remedied with the available toxicogenomic data (unless, as discussed above, a toxicogenomic-based dosimeter/biomarker was developed). For instance, available blood measurements of MBP in humans were taken from spot samples in the general population where the individual exposure patterns were unknown. Although differences were observed in the ratio of free to conjugated MBP in human serum as compared to the rat, these data are insufficient for quantitative interspecies extrapolation, because in order to replace administered dose as a dose metric, it is necessary to determine the *absolute*, not the *relative*, level of free MBP in serum as a function of exposure. The Wyde et al. (2005) study suggests that DBP-induced enzyme induction occurred. Specifically, this study reported that exposure to 50 and 500 mg/kg-d DBP leads to an increase in rat liver UDP glucuronsyltransferase 2B1 (*Ugt2b1*) mRNA levels. More TK analysis would be required to ascertain whether this induction in rats occurs at levels that are relevant to low-dose exposures. This enzyme induction may occur in humans and such a response may increase interindividual sensitivity to DBP toxicity. With regard to human TK, none of the available toxicogenomic data on DBP were performed in humans and thus, do not provide any information on DBP interindividual TK variability. For example, there are no available data on polymorphisms in glucuronyltransferases responsible for metabolizing MBP. Finally, we considered the potential for TK/TD linkages with the available data and concluded that in order for TK and toxicogenomic data to be integrated for use in quantitative dose-response analysis, more sophisticated BBDR models are needed. Using such an approach,

it may be feasible to relate changes in expression of genes involved in T production to quantify testicular T levels (see Figure 3-5).

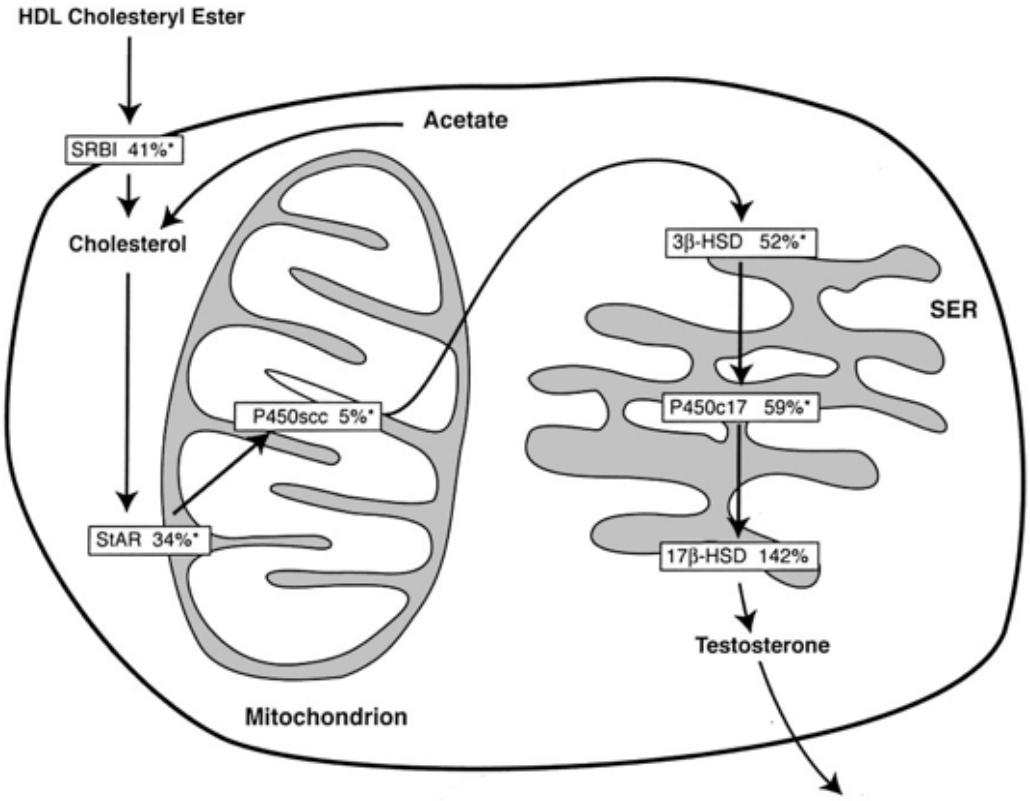

Figure 3-5. **The fetal Leydig cell in the fetal testis.** The boxes represent genes involved in the biosynthesis of T; the percentages (%) represent % control gene expression in fetal testis of dams treated with 500 mg/kg-d DBP.

Source: Adapted from Barlow et al. (2003).

The male reproductive developmental effects of DBP appear to be mediated by MBP, which causes a down-regulation of cholesterol transporters across the cell membrane (SCARB1) and mitochondrial inner membrane (STAR), as well as the down-regulation of two enzymes involved in converting cholesterol to T, CYP11A1, and CYP17A1 (Liu et al., 2005; Lehmann et al., 2004; Barlow et al., 2003; Shultz et al., 2001). Thus, it may be possible to relate DBP and/or MBP levels to reductions in cholesterol transporter (e.g., SCARB1 and STAR) and the levels of steroidogenic enzymes (e.g., CYP11A1 and CYP17A1) at the mRNA, protein, and/or activity

levels. Changes in these parameters may then be modeled to predict changes in testicular T levels, which may subsequently be correlated to developmental toxicity endpoints.

3.2.2. Informing Dose-Response

Toxicogenomic data that informs TK can be useful for informing or improving dose-response analysis because it may improve the dose metric selection among alternative dose metrics. However, use of toxicogenomic data as an endpoint in dose-response analysis has not been extensively explored. For example, BMD analysis of some dose-response studies determined PODs based on the GO categorization of gene expression changes (based on an approach of Yu et al., 2006) as a function of dose (Andersen et al., 2008; Thomas et al., 2007).

3.2.2.1. *DBP Case Study: Do the Toxicogenomic Data Inform Dose-Response?*

Unfortunately, there are currently no available dose-response microarray studies to assess the genome-wide gene expression over a dose range. However, there is one available dose-response gene expression study for DBP. Specifically, Lehmann et al. (2004) conducted a dose-response study evaluating testicular T, RT-PCR and protein expression for a subset of genes thought to underlie the male reproductive developmental outcomes. This study reported a significant reduction in fetal testicular T at 50 mg/kg-d DBP or higher. Western analysis found that steroidogenic acute regulatory protein (STAR) and scavenger receptor class B, member 1 (SCARB1) were significantly decreased at 50 mg/kg-d while cytochrome P450, family 11, subfamily a, polypeptide 1 (CYP11A1) was only reduced at 500 mg/kg-d. Further, RT-PCR analysis findings confirmed that the mRNA of these three genes was statistically significantly reduced at 50 mg/kg-d. The results of this study support the role of steroidogenesis enzymes and cholesterol transport proteins in the decreased testicular T MOA after *in utero* DBP exposure. However, without first establishing the biologically significant level of change in gene expression and the critical subset of genes that constitute a well-established precursor event, it is difficult to use these data in a dose-response assessment (see Chapter 7). It would be helpful to have dose-response microarray or proteomic studies to assess mRNA and protein expression on a genome-wide level.

3.2.3. Informing TD

There are numerous examples where toxicogenomic data have been used to inform the TD steps within mechanisms of action or MOAs for a chemical, and there are a small number of examples where such data have been used corroboratively for risk assessment decisions (see Chapter 2).

3.2.3.1. *General Considerations: TD Portion of Mechanisms of Action and MOAs*

One feature of the approach (see Figure 3-1) is the evaluation of the toxicity and toxicogenomic data sets in conjunction in order to consider the relevance of gene expression changes with respect to specific endpoints of interest identified in the toxicity data set. In this manner, data on affected pathways may generate hypotheses and inform the mechanisms of action for a chemical for specific endpoints. In addition, using this approach could provide connections between affected pathways (identified from the toxicogenomic data set) and endpoints (identified from the toxicity data set), which may, in turn, inform modes or the mechanisms of action, as illustrated by Figure 3-6. Chapter 2 and the glossary describe the distinction between the definitions for mechanisms of action and MOAs.

This approach is best suited to instances where comparable study designs between the toxicity/epidemiology and toxicogenomic data sets are available. For example, toxicogenomic and toxicity studies performed in the same species, using similar doses, similar exposure intervals, and assessing the same organ or tissue would be ideal for utilizing this approach. For the DBP toxicity (see Chapter 4) and toxicogenomic data sets (see Chapter 5), there is some comparability across some of the studies—i.e., some toxicity and toxicogenomic studies were performed at the same doses with similar exposure intervals, in the same species and strain, and assessed some of the same organs (e.g., testis). However, no two studies are the same for all study-design aspects, such as precise timing of exposure and time of assessment.

3.2.3.2. *DBP Case Study: MOAs for Male Reproductive Developmental Effects*

Developmental toxicity studies (reviewed in Chapter 4) and toxicogenomic studies (reviewed in Chapter 5) have contributed to a good understanding of DBP as a chemical that has multiple MOAs. Two well-characterized MOAs: a reduction in fetal testicular T and a reduction in *Insl3* signaling activity, explain a number of the observed male reproductive developmental

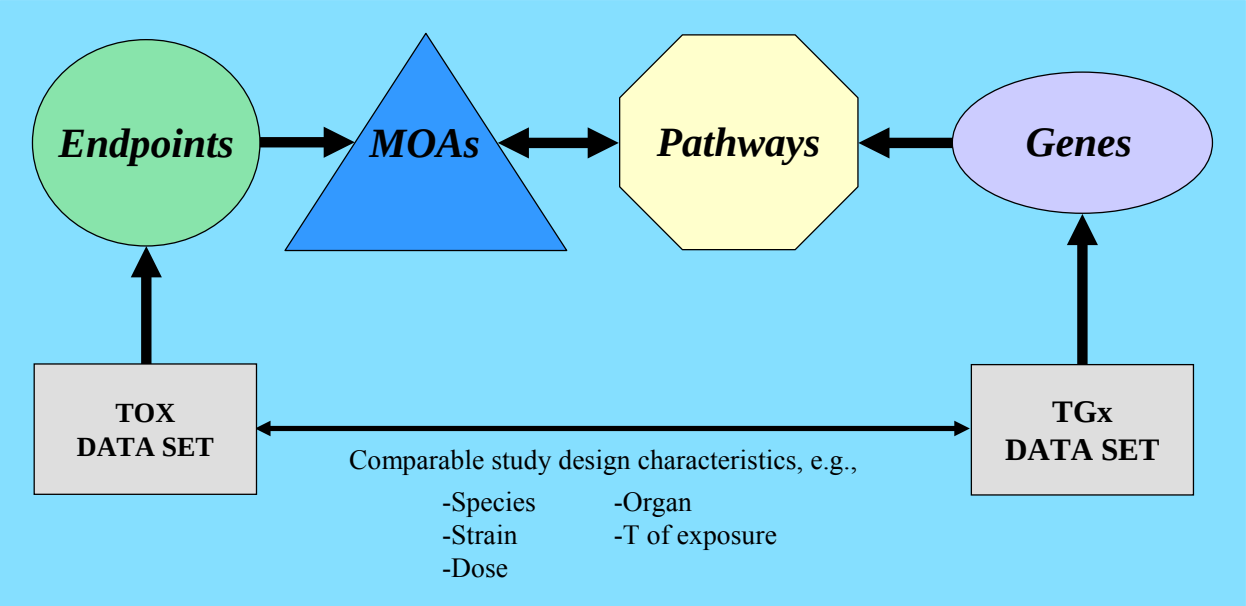

Figure 3-6. **Approach to utilizing toxicity and toxicogenomic data for identifying affected pathways and candidate modes and mechanisms of action.** Toxicogenomic data can be analyzed for differentially expressed genes (DEGs) and, in turn, grouped into affected pathways. Toxicity data can provide information about affected endpoints. Evaluating toxicogenomic and toxicity data together can provide a level of phenotypic anchoring between gene and pathway changes, and *in vivo* outcomes. The identification of affected pathways can inform mechanisms of action, including MOAs, for a chemical. Such an approach requires similar study parameters (e.g., dose, species, duration of exposure) for the toxicity and toxicogenomic studies. TGx, toxicogenomic.

abnormalities. Some other observed abnormalities are not explained by these two MOAs, suggesting that there are additional MOAs for DBP. Acknowledging that there are additional data not presented in Figure 3-7, this figure attempts to show where there is agreement in the scientific community, based on reproducibility of microarray and RT-PCR studies, about affected pathways and the well-characterized MOAs for DBP. There are some endpoints and pathways that need further characterization and, as a result, we were interested in determining whether the toxicogenomic data could be used to associate the DBP MOAs and endpoints, and/or form hypotheses about additional MOAs for DBP.

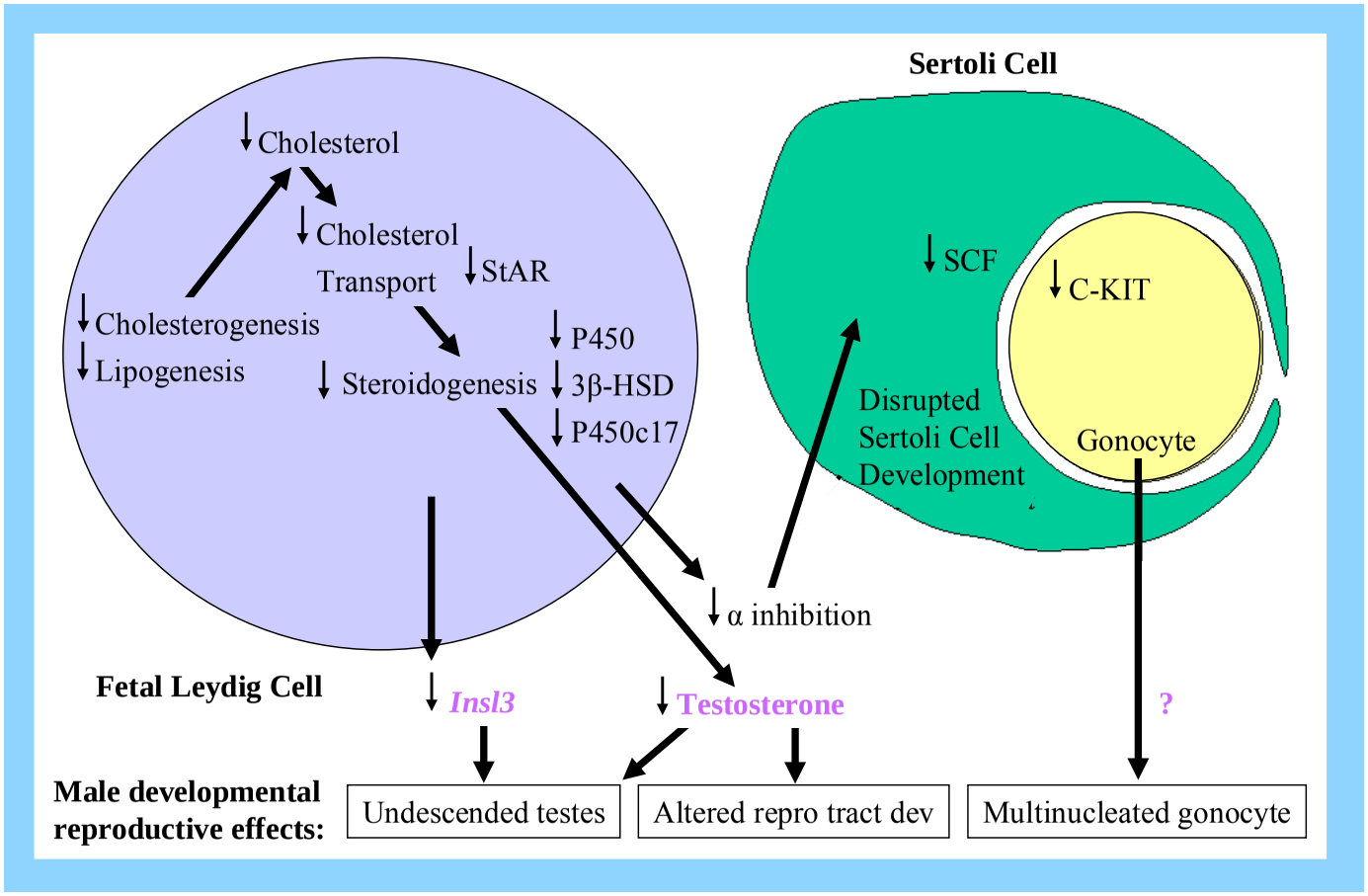

Figure 3-7. **The proposed DBP mechanism of action for the male reproductive developmental effects.** The mechanism of action is defined as all of the steps between chemical exposure at the target tissue to expression of the outcome. The steps shown are based on male reproductive developmental toxicity and toxicogenomic studies. Some of the affected pathways and individual genes whose expression was significantly affected after DBP exposure in multiple studies are included. By contrast, MOAs are shown in purple letters including two well-characterized MOAs and one example of an unidentified MOA.

Source: Figure adapted from Liu et al. (2005), Thompson et al. (2004), Wilson et al. (2004), Barlow et al. (2003), and Shultz et al. (2001).

3.3. IDENTIFYING AND SELECTING QUESTIONS TO FOCUS THE DBP CASE STUDY

In reviewing the external review draft IRIS Tox Review for DBP, data gaps in the assessment were noted. Then the DBP toxicogenomic data set was evaluated for these data could potentially address any of the gaps (see Figure 3-1). The identified data gaps led to formulation of questions of interest whose answers may be able to contribute valuable information to a risk assessment. The following questions were identified:

Can the DBP toxicogenomic data set inform the

- **biologically significant level of reduction in fetal T?** As the external review draft IRIS Tox Review for DBP used a reduction in fetal testicular T as the critical effect, we considered whether the toxicogenomic data set could aid in determining the biologically meaningful level of T reduction for the male reproductive developmental effects.
- **dose-response assessment in risk assessment?** The microarray and RT-PCR studies have identified genes and pathways associated with the reduced fetal testicular T. Thus, there is the potential for evaluating these genes and pathways in a dose-response assessment.
- **modes and mechanisms of action for male reproductive developmental outcomes?** Not all of the male reproductive developmental outcomes after *in utero* DBP exposure are a consequence of reduced fetal testicular T or reduced *Insl3* expression. Therefore, additional MOAs for these endpoints may be identified from pathway analysis of the microarray data.
- **interspecies (rat-to-human) differences in MOAs that could, in turn, inform the TD part of the UF_H?** There is evidence from toxicogenomic studies that a reduction in gene expression of some of the steroidogenesis genes underlies the reduction in fetal testicular T observed after *in utero* DBP exposure. Unfortunately, there are no genomic studies in appropriate human *in vitro* cell systems to make comparisons to *in vivo* rat MOA findings. Using available DNA sequence data and other methods, we would like to assess the rat-to-human conservation of the steroidogenesis pathway genes.

The existing genomic data for DBP had the potential to inform two of the questions: informing modes and mechanisms of action and interspecies differences for the reduced T MOA (see above). It was highly likely that the DBP toxicogenomic data set could aid in hypothesis generation of DBP modes or mechanisms of action. Using genomic data, such as DNA sequence data, it may be possible, but less likely, to inform cross-species differences in TD for the reduced T MOA. Although the other two questions (see list above) were of great interest, the available genomic data were not considered appropriate to address them.

The remaining steps of the DBP case study are presented in the subsequent chapters. The evaluations of the toxicity data set for the male reproductive developmental effects after developmental exposure to DBP (see Chapter 4) and the toxicogenomic data set including new analyses of one microarray study (see Chapter 5). Exploration of pathway analysis methods development for applying microarray data to risk assessment and the use of available methods to evaluate rat-to-human differences for the reduced T MOA are presented in Chapter 6. Chapter 4 follows with an in-depth evaluation of the DBP toxicity data set.

4. EVALUATION OF THE REPRODUCTIVE DEVELOPMENTAL TOXICITY DATA SET FOR DBP

This chapter presents the evaluation of the available toxicity data for the development of the male reproductive system following DBP exposure and the MOA(s) (see Chapter 2 and glossary for definition) that contribute to these outcomes. We used the compilation of the male reproductive toxicology literature cited in the 2006 external peer review draft IRIS Tox Review for DBP (U.S. EPA, 2006a) as a starting point for our toxicology literature review for this case study. Each toxicology study was examined for the lowest dose and low-incidence effects in order to identify the full spectrum of male reproductive developmental effects. In a second evaluation, we used available mechanistic information for each endpoint to identify potential MOAs. Endpoints with MOA information have support for phenotypic anchoring to some of the observed DBP gene expression changes (further discussed in Chapter 5). Endpoints with unexplained MOAs were used to identify and focus future research needs to study the mechanisms that underlie those endpoints using genomics and other techniques.

An extensive toxicological data set exists for DBP that includes acute and subchronic studies in multiple species, multigeneration reproduction studies in rodents, and studies that assess developmental outcomes following *in utero* or perinatal/postnatal exposures. Following DBP exposure during the critical stages of development, the male reproductive system development is perturbed in rodent studies (Gray et al., 1999, 2001; Mylchreest et al., 1998, 1999, 2000). Two MOAs of DBP, for a number of these outcomes, have been well established (David, 2006; Foster, 2005). The 2006 external draft IRIS Tox Review for DBP (U.S. EPA, 2006a) selected reduced fetal testicular T levels, observed in Lehmann et al. (2004), as the critical effect for the derivation of acute, short-term, subchronic, and chronic reference values for DBP. This case study evaluated information from genomic and other gene expression studies to target and further elucidate the molecular events underlying these developmental outcomes (see Chapter 5). The intent of performing this evaluation of the toxicology studies was to examine the usefulness of the toxicogenomic data in characterization of the MOA(s) that contribute to the adverse outcomes. We also examined the data for low-dose or low-incidence findings because such data may aid the interpretation of toxicological outcomes that can be misinterpreted as transient (e.g., AGD), or nonadverse due to low incidence or magnitude (e.g., not statistically

significant incidences of gross pathology findings in male offspring reproductive organs, or alteration of fetal T levels).

4.1. CRITERIA AND RATIONALE FOR INCLUSION OF TOXICOLOGY STUDIES IN THE EVALUATION

Figure 4-1 illustrates the process for evaluating the DBP toxicology data set for the case study (Section 4.2 discusses the later steps of the evaluation process in more detail). The first step in the process was the identification of studies to be included for consideration in the case study. We identified a number of study selection criteria in Step 1. One criterion of prime importance was that the studies should include exposures to DBP during sensitive periods of male reproductive system development. Secondly, a no-observed-effect level (NOEL), lowest-observed-effect level (LOEL), or BMDL would need to be identified for presumably adverse outcomes in the reproductive organs and/or function of male offspring. Additionally, the studies would need to be of adequate quality in order to establish confidence in the study conduct, methods, and results. These criteria, taken together, define a subset of the available toxicology studies that were considered possible candidates for determining the POD for derivation of reference values of various exposure durations in the 2006 external peer review draft Tox Review for DBP (see Tables 4-1, 4-2, and 4-3 in U.S. EPA, 2006a). These candidate study lists were considered during the external peer review of the IRIS document, conducted in July 2006, thereby providing a measure of confidence in their inclusiveness and veracity for the purpose of this case study. Though there are observable adverse effects on male reproductive system development in multiple species, the only available and relevant genomic studies with DBP (i.e., those that addressed effects on male reproductive system development following prenatal exposures) were conducted in rats. Table 4-1 lists the studies that were identified for inclusion as of July 2006. For each study, the following information was summarized: a description of the dose and exposure paradigm, the treatment-related outcomes observed at each dose level, and the experimentally derived reproductive NOEL and/or LOEL. The terms NOAEL and LOAEL are not used in this case-study report, although these terms are commonly used in risk assessment, because some study reports do not address the issue of adversity of observed study outcomes. In addition, some study reports do not specifically define NOELs or LOELs. For that reason, Table 4-1 presents those outcomes that could be considered biomarkers

of effects on the male reproductive system that were reported by the study authors, without specific consideration or judgment of adversity.

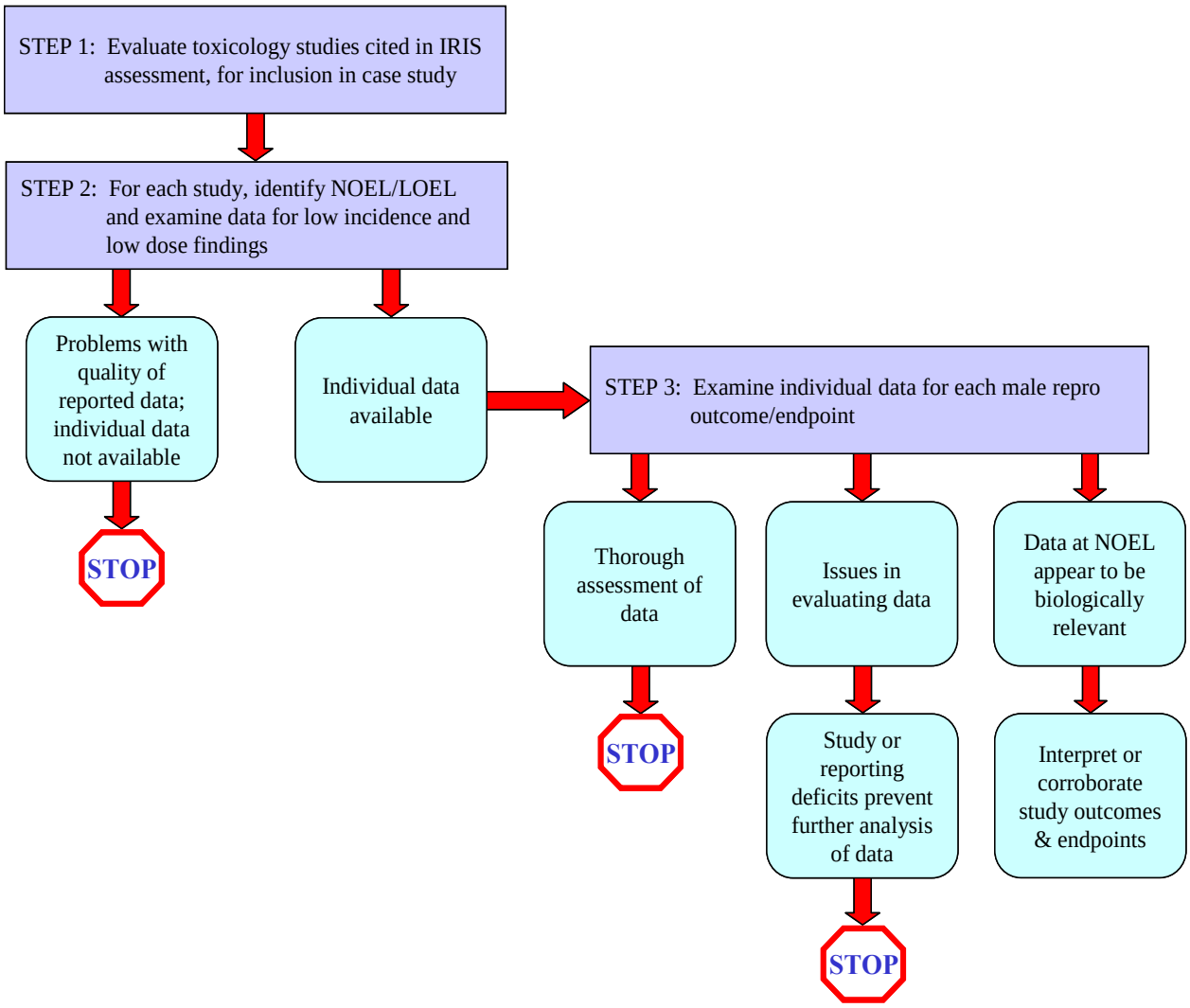

Figure 4-1. The process for evaluating the male reproductive developmental toxicity data set for low-dose and low-incidence findings. IRIS assessment, the 2006 external peer review draft IRIS Tox Review for DBP.

Table 4-1. Studies with exposures during development that have male reproductive outcomes (limited to reproductive organs and/or reproductive function) and were considered adequate for reference value determination

Study[a]	Species (strain), duration, and exposure	Reproductive system effects	NOEL mg/kg-d	LOEL mg/kg-d
Barlow and Foster, 2003	Rat (SD); GDs 12−21; 0 or 500 mg/kg-d	Large aggregates of Leydig cells, multinucleated gonocytes, and an increased number of gonocytes in fetal testes; a decreased number of spermatocytes on PNDs 16 and 21; epididymal lesions (decreased coiling of the epididymal duct, progressing to mild [PND 45], and then severe [PND 70] seminiferous epithelial degeneration).		500
Barlow et al., 2003	Rat (SD); GDs 12−19; 500 mg/kg-d	Large aggregates of Leydig cells with lipid vacuoles.		500
Barlow et al., 2004	Rat (SD); GDs 12−21; 0, 100, or 500 mg/kg-d	Testicular dysgenesis (proliferating Leydig cells and aberrant tubules); decreased AGD; areolae retention; small incidence of Leydig cell adenomas.	100	500
Bowman et al., 2005	Rat (SD); GDs 12−19 or 21; 0 or 500 mg/kg-d	Marked underdevelopment of the Wolffian ducts (characterized by decreased coiling).		500
Carruthers and Foster, 2005	Rat (SD); GDs 14−15, 15−16, 16−17, 17−18, 18−19, 19−20; 0 or 500 mg/kg-d	Decreased AGD; retained areolae and nipples; reduced epididymal weights, increased testes weight due to edema; malformations of the seminal vesicles, agenesis of various regions of the epididymis, small or flaccid testes; malformation of the coagulating gland.		500
Ema et al., 1998	Rat (Wistar); GDs 11−21; 0, 331, 555, or 661 mg/kg-d	At 555 and 661 mg/kg-d, increased incidences of cryptorchidism and decreased AGD.	331	555
Ema et al., 2000b	Rat (Wistar); GDs 15−17; 0, 500, 1,000, or 1,500 mg/kg-d	At 1,500 mg/kg-d, cryptorchidism observed in 80% of litters. At 500, 1,000, and 1,500 mg/kg-d, decreased AGD.		500
	Rat (Wistar); GDs 12−14, or GD 20; 0, 1,000, or 1,500 mg/kg-d	At 1,500 mg/kg-d (GDs 12−14), cryptorchidism observed in 50% of litters. At 1,000 and 1,500 mg/kg-d, decreased AGD.		1,000
Ferrara et al., 2006	Rat (Wistar); GDs 13.5−21.5; 0 or 500 mg/kg-d	Delayed entry of gonocytes into quiescence, increase in gonocyte apoptosis, and subsequent early postnatal decrease in gonocyte numbers (exposures: GDs 13.5−17.5); >10% increase in multinucleated gonocytes (exposures: GDs 19.5−21.5).		500

Table 4-1. (continued)

Study[a]	Species (strain), duration, and exposure	Reproductive system effects	NOEL mg/kg-d	LOEL mg/kg-d
Fisher et al., 2003	Rat (Wistar); GDs 13−21; 0 or 500 mg/kg-d	Cryptorchidism, hypospadias, infertility, and testis abnormalities similar to human testicular dysgenesis syndrome; abnormal Sertoli cell-gonocyte interaction.		500
Gray et al., 1999	Rat (Long-Evans) (P0); PND 21—adult; 0, 250, 500, or 1,000 mg/kg-d	At 250, 500, and 1,000 mg/kg-d, delayed puberty. At 500 and 1,000 mg/kg-d, reduced fertility related to testicular atrophy and reduced cauda epididymal sperm numbers.		250
	Rat (Long-Evans) (F1); GD 0−PND 21; 0, 250, 500, or 1,000 mg/kg-d	At 250 and 500 mg/kg-d, reproductive malformations (low incidences of hypospadias, testicular nondescent, and uterus unicornous); reduced fecundity.		250
	Rat (Long-Evans) (F1); GD 14 to PND 3; 0 or 500 mg/kg-d	Reduced AGD, retained nipples, permanently reduced androgen-dependent tissue weights.		500
Kim et al., 2004 **Ab**	Rat (SD); GDs 10−19; 0, 250, 500, or 700 mg/kg-d	Decreased testes and accessory sex organ weight; delayed testis descent; increased expression of estrogen receptor in testes.		250 (presumed)[b]
Kleymenova et al., 2004 **Ab**	Rat (strain not specified); GDs 12−17, 19, 20; 0 or 500 mg/kg-d	Altered proliferation of Sertoli and peritubular cells; multinucleated gonocytes; changes in Sertoli cell-gonocyte interactions.		500
Kleymenova et al., 2005a **Ab**	Rat (SD); GDs 12−20; 0, 0.1, 1, 10, 30, 100, or 500 mg/kg-d	At 30 and 50 mg/kg-d, disruption of Sertoli-germ cell contact. At 50 mg/kg-d, Sertoli cell hypertrophy, decreased total cell number and number of seminiferous tubules. At 100 mg/kg-d, increased multinucleated gonocytes.	10	30
Kleymenova et al., 2005b	Rat (SD); GDs 12−21; 0 or 500 mg/kg-d	Cytoplasmic changes in Sertoli cells with abnormal cell-cell contact with gonocytes, clustering of gonocytes in the middle of the tubules, altered morphometry of seminiferous tubules, clusters of interstitial cells, decreased number of tubular cross sections per testicular section; increased number of multinucleated gonocytes.		500

Table 4-1. (continued)

Study[a]	Species (strain), duration, and exposure	Reproductive system effects	NOEL mg/kg-d	LOEL mg/kg-d
Lee et al., 2004	Rat (SD); GD 15 to PND 21; 0, 1.5, 14.4, 148, or 712 mg/kg-d (converted from 0, 20, 200, 2,000, and 10,000 ppm DBP in diet)	At 712 mg/kg-d, decreased percent males; decreased AGD and retained nipples, decreased relative testis weight. At 1.5, 14.4, 148, and 712 mg/kg-d, on PND 21, reduction in spermatocyte development, increased foci of aggregated Leydig cells, and decreased epididymal ductular cross section. At 148 and 712 mg/kg-d, at wk 11, loss of germ cell development. At 1.5 mg/kg-d, degeneration and atrophy of mammary gland alveoli in males at 8−11 wks of age.		1.5
Lehmann et al., 2004	Rat (SD); GDs 12−19; 0, 0.1, 1, 10, 30, 50, 100, or 500 mg/kg-d	At ≥50 mg/kg-d, decreased fetal T concentration. At 500 mg/kg-d, a reduction in oil red O staining of lipids in fetal testes.	30	50
Liu et al., 2005	Rat (SD); GDs 12−19; 0, 500 mg/kg-d	Significant reduction in AGD at GD 19.		500
Mahood et al., 2005	Rat (Wistar); GDs 13.5−20.5; 0 or 500 mg/kg-d	Aggregation of fetal Leydig cells; reduced Leydig cell size; reduced T levels at GDs 19.5 and 21.5 (early event in testicular dysgenesis); cryptorchidism; partial absence of epididymis at PND 90.		500
Mylchreest et al., 1998	Rat (SD); GD 30 to PND 20; 0, 250, 500, or 750 mg/kg-d	At 500 and 750 mg/kg-d, decreased AGD. At 250, 500, and 750 mg/kg-d, absent or underdeveloped epididymis, associated with testicular atrophy and germ cell loss, hypospadias, ectopic or absent testes. At 500 and 750 mg/kg-d, absent prostate and seminal vesicles, small testes, and seminal vesicles.		250
Mylchreest et al., 1999	Rat (SD); GDs 12−21; 0, 100, 250, or 500 mg/kg-d	At 500 mg/kg-d, hypospadias; cryptorchidism; agenesis of the prostate, epididymis, and vas deferens; degeneration of the seminiferous epithelium; interstitial cell hyperplasia and adenoma; decreased weight of prostate, seminal vesicles, epididymis, and testes. At 250 and 500 mg/kg-d, retained areolae or thoracic nipples, decreased AGD. At 100 mg/kg-d, delayed preputial separation (attributed to highly affected litter, and not repeated in subsequent study).	100	250

Table 4-1. (continued)

Study[a]	Species (strain), duration, and exposure	Reproductive system effects	NOEL mg/kg-d	LOEL mg/kg-d
Mylchreest et al., 2000	Rat (SD); GDs 12−21; 0, 0.5, 5, 50, 100, or 500 mg/kg-d	At 500 mg/kg-d, decreased AGD, hypospadias, cryptorchidism, absent or partially developed epididymis, vas deferens, seminal vesicles, and ventral prostate; decreased weights of testes, epididymis, dorsolateral and ventral prostates, seminal vesicles, and levator anibulbocavernosus muscle; seminiferous tubule degeneration, focal Leydig cell hyperplasia, and Leydig cell adenoma. At 100 and 500 mg/kg-d, retained thoracic areolae or nipples in male pups.	50	100
Mylchreest et al., 2002	Rat (SD); GDs 12−21; 0 or 500 mg/kg-d	In GDs 18 and 21 fetuses, testicular atrophy, Leydig cell hyperplasia, enlarged seminiferous cords with multinucleated gonocytes; decreased testicular T; fewer epididymal ducts.		500
NTP, 1991	Rat (SD); continuous breeding (16 wks) (gestation and lactation); 0, 80, 385, or 794 mg/kg-d in dams (converted from 0.1, 0.5, and 1.0 % DBP in feed)	F1 adults: At 80, 385, and 794 mg/kg-d: Increased incidence of absent, poorly developed, or atrophic testis and underdeveloped or absent epididymis. At 385 and 794 mg/kg-d: Increased incidence of seminiferous tubule degeneration. At 794 mg/k-d: Decreased mating, pregnancy, and fertility indices; decreased epididymal, prostate, seminal vesicle and testis weights; decreased cauda epididymal sperm concentration; decreased average spermatid count, total spermatid heads/testis or total spermatid heads/g testis; increased incidence of absent, small/underdeveloped/poorly developed, or atrophic penis, seminal vesicles, epididymis, and prostate; interstitial/Leydig cell hyperplasia; delayed testicular descent or cryptorchidism.		80
NTP, 1995 (some of this is also reported in Wine et al., 1997)	Rat (SD); continuous breeding (16 wks) (gestation and lactation); 0, 80, 385, or 794 mg/kg-d in dams (converted from 0.1, 0.5, and 1.0% DBP in feed)	At 794 mg/k-d: Decreased mating, pregnancy, and fertility indices; decreased epididymal, prostate, seminal vesicle, and testis weights.	385	794

Table 4-1. (continued)

Study[a]	Species (strain), duration, and exposure	Reproductive system effects	NOEL mg/kg-d	LOEL mg/kg-d
NTP, 1995	Rat (Fischer 344); perinatal and lactation plus 17 wks; 0, 138, 279, 571, 1,262, or 2,495 mg/kg-d in dam (converted from 0 or 10,000 ppm during gestation and lactation; 0, 1,250, 2,500, 5,000, 7500, 10,000, 20,000 ppm for 4 wks PN; 0, 2,500, 5,000,10,000, 20,000, and 40,000 for last 13 wks PN)	At 571, 1,262, and 2,495 mg/kg-d: Degeneration of germinal epithelium. At 1,262 and 2,495 mg/kg-d: Decreased testes and epididymal weights, fewer sperm heads per testis, and decreased epididymal sperm concentration.	279	571[c]
NTP, 1995	Rat (Fischer 344); perinatal and lactation plus 4 wks; 0, 143, 284, 579, 879, or 1,115 mg/kg-d in dam (converted from 0, 1,250, 2,500, 5,000, 7,500, 10,000, and 20,000 ppm)	At 879 and 1,115 mg/kg-d: Moderate epididymal hypospermia in all males. At 579 mg/kg-d, mild epididymal hypospermia in 2 of 10 males.	284	579[d]
Plummer et al., 2005 **Ab**	Rat (strain not specified); gestation; 0 or 500 mg/kg-d	Decreased fetal T levels.		500
Shultz et al., 2001	Rat (SD), GDs 12−21; 0 or 500 mg/kg-d	Decreased fetal testicular T and androstenedione; increased progesterone.		500
Thompson et al., 2004a	Rat (SD); GDs 12−17, 12−18, or 12−19; 0 or 500 mg/kg-d	Decreased fetal T.		500
Thompson et al., 2005	Rat (SD); GD 19; 0 or 500 mg/kg-d	Decreased fetal T.		500
Wilson et al., 2004	Rat (SD); GDs 14−18; 0 or 1,000 mg/kg-d	Decreased fetal T, expression of *Insl3*.		1,000

Table 4-1. (continued)

Study[a]	Species (strain), duration, and exposure	Reproductive system effects	NOEL mg/kg-d	LOEL mg/kg-d
Zhang et al., 2004	Rat (SD); GD 1 to PND 21; 0, 50, 250, or 500 mg/kg-d	At 250 and 500 mg/kg-d, decreased AGD; underdeveloped epididymides; decreased epididymis or prostate weight at PND 70; decreased percent motile sperm and total sperm heads; degeneration of the seminiferous epithelium. At 500 mg/kg-d, cryptorchidism, absent epididymides, decreased total number of sperm.	50	250

Ab, Abstract only; AGD, anogenital distance; GD, gestation day; PND, postnatal day; LOEL, lowest-observed-effect level for male reproductive system outcomes found in the study; NOEL, no-observed-effect level for male reproductive system outcomes; SD, Sprague-Dawley; T, testosterone. Note: These terms are used solely in a descriptive manner in this table, they may not reflect the terminology of the source study, and they are not intended to convey any regulatory implication.

[a]All studies used an oral route of exposure. Lee et al. (2004) and NTP (1995, 1991) administered DBP in the diet. All other studies used oral gavage.
[b]The abstract states that the effects were "dose dependent," but a LOEL is not specifically indicated.
[c]Overall, the study NOEL and LOEL are lower based on liver peroxisome activity.
[d]Overall, the study NOEL and LOEL are lower based on increased liver weight.

It is also noted that although BMDL values were calculated for specific developmental endpoints identified by Lehmann et al. (2004), Mylchreest et al. (2000), and the NTP (1995) (see Table 4-4 of the 2006 external peer review draft Tox Review for DBP), these values were not used as a POD for reference value derivation.

4.2. REVIEW OF THE TOXICOLOGY DATA SET

Figure 4-1 illustrates the stepwise approach taken in the evaluation of the toxicity studies, focusing on low-dose and low-incidence outcomes. First, for each toxicology study, we examined the data at the lowest dose levels (as defined by the study NOELs and LOELs) (Step 2). If there was any indication of insurmountable problems with the quality of the reported data (e.g., excessive variability, critical methodological concerns, lack of peer review as with abstracts, etc.), or if there were no individual animal data reported (as is often the case for poster abstracts as well as for many published studies which only contain extracted summary data), the review of that study would be terminated. However, if individual data were available, the review could proceed (Step 3). The individual animal data were examined for evidence of reproductive system outcomes in the males. Although for most studies the exposures were only administered during the perinatal developmental period, we recognized that an adverse treatment-related outcome might be identified at any life stage that was assessed in the study. There were three possible courses that the data review could take from this point forward. In cases where problems were identified in the data, we attempted to analyze the extent of the issues and determine the ability to move forward with the study analysis. In some cases, the analysis stopped at this point, due to deficits in the study data or to inadequate reporting of individual animal data. However, if the data in the report appeared to be thoroughly assessed, then the study outcomes and endpoints were examined. Alternatively, in some cases where adequate individual study data were available for analysis (NTP, 1991, 1995), further examination of the study could identify effects at the lowest dose levels that had been considered biologically irrelevant in the original review, but it might require further consideration. At any point in this stepwise process where data were deemed insufficient to proceed further, we identified research needs (discussed in Chapter 7).

To begin the characterization and evaluation of the published studies according to this stepwise model, important aspects of each study protocol, conduct, and reporting were

summarized (see Table 4-2). Examination of this table demonstrates that approximately half of the studies that were selected for analysis (i.e., 14 of 29) were limited to a single dose group, which eliminated them from further examination for lower-dose level effects. It is also important to note that individual animal data were reported in only 2 of the 29 studies, thereby severely limiting, and in some cases even preventing, more rigorous evaluation of the study findings. These two characteristics alone tend to overshadow any of the other listed study attributes that might contribute to confidence in study findings (i.e., evidence that the study was conducted according to quality laboratory standards, description of statistical analysis of the data, and/or specific information regarding the number of litters and offspring assessed, which would provide an indicator of statistical power). Of the studies listed, only those conducted by NTP (1995, 1991) were considered suitable for extended examination.

In order to create a profile of outcomes to the male reproductive system following developmental exposures, which might then serve as a baseline for further comparison and analysis of toxicological findings across the studies, a list of observed effects was compiled (see Table 4-3). The content of this list is very clearly defined by the study protocols, both in terms of what endpoints were examined in each study and when (i.e., at what life stage) they were examined. For some endpoints, the precise GD or postnatal day (PND) of evaluation may even be critical. For example, fetal T should peak at approximately GD 18, so assessments made at earlier or later time points may be less sensitive in detecting adverse outcomes, and the effects will not be directly comparable across fetal ages. Decreases in T levels may not be observed postnatally unless treatment is continued or if testicular malformations disrupt T level (which is a different mechanism of perturbation than alterations to the steroidogenesis pathway). In neonates, examination for nipple retention is generally conducted at around PND 13 when the structure is readily visible but before it is obscured by hair growth. Cryptorchidism, even though present at birth, may not be readily observable in neonates until they reach the age of PND 16−21 (and of course, it should be detectable at postweaning ages and in adults). Preputial separation (PPS) delays can only be observed at the time of sexual maturation, which, in the male Sprague-Dawley (SD) rat, occurs at approximately PND 42; therefore, this effect cannot be detected at an earlier life stage, nor will it be observed in sexually mature adults. On the other hand, sperm alterations (count, morphology, or motility) and perturbations in male fertility can only be assessed in adult males, not in immature individuals at earlier life stages

Table 4-2. Reporting and study size characteristics of male reproductive studies following *in utero* exposure to DBP

Study	> One high dose	Individual data publicly available	Statistical analysis method reported	Study conduct level reported	Number evaluated/group	
					Litters	Offspring
Barlow and Foster, 2003			✓	✓	1−9[a]	7−60[a]
Barlow et al., 2003		✓subset[b]	✓	✓	NR	3
Barlow et al., 2004	✓		✓	✓	8−11[a]	35−74[a,c]
Bowman et al., 2005			✓	✓	18	All male fetuses
Carruthers and Foster, 2005			✓	✓	1−14[d]	1−91[d]
Ema et al., 1998	✓		✓		11 DBP treated	AGD: NR; crypt.: 144
Ema et al., 2000b	✓		✓		73 DBP treated	~770[e]
Ferrara et al., 2006			✓	✓	"in most instances" ~3−6	1−3/litter
Fisher et al., 2003			✓	✓	NR	Testis wt: 5−10 animals/age group (4); hyp. and crypt.: 10 adults
Gray et al., 1999	✓ PPS only		✓		4 (LE); 8 (SD)	LE: 30 male pups; 13 adult males SD: 48 male pups; 17 adult males[f]
Kim et al., 2004 **Ab**	✓				NR	NR
Kleymenova et al., 2004 **Ab**	✓				NR	NR
Kleymenova et al., 2005a **Ab**	✓				NR	NR
Kleymenova et al., 2005b			✓	✓	3	14−21 pups/evaluation

Table 4-2. (continued)

Study	> One high dose	Individual data publicly available	Stat analysis method reported	Study conduct level reported	Number evaluated/group	
					Litters	Offspring
Lee et al., 2004	✓		✓		6–8	11–20 adults
Lehmann et al., 2004	✓		✓	✓	1–4	3–4 fetuses/group
Liu et al., 2005			✓	✓	3	3 fetuses/litter
Mahood et al., 2005			✓	✓	2–7	NR
Mylchreest et al., 1998	✓		✓	✓	7–10	All males/litter
Mylchreest et al., 1999	✓		✓	✓	10	All males/litter
Mylchreest et al., 2000	✓		✓	✓	11–20	All males/litter
Mylchreest et al., 2002			✓	✓	5–6	23–49 fetuses
NTP, 1995, 1991	✓	✓	✓	✓	20	All pups/litter in-life thru necropsy; histopath: 10/selected groups
Plummer et al., 2005 **Ab**					NR	NR
Shultz et al., 2001			✓	✓	3	1 male/litter
Thompson et al., 2004a			✓	✓	4	1 male/litter
Thompson et al., 2005			✓	✓	4	3 fetuses/litter
Wilson et al., 2004			✓	✓	3	All males/litter
Zhang et al., 2004	✓		✓		14–16	20 pups/group

Table 4-2. (continued)

Ab, Abstract only; LE, Long Evans; NR, Not reported; PPS, preputial separation; ✓, present.

[a]Litters and pup numbers not reported for AGD and areolae retention.

[b]Data for three individual animals were reported for LC and Sertoli cell staining. The other results are not reported in this table because they were from toxicogenomic studies (see Chapter 5).

[c]57−100% of these pups survived to necropsy so for malformations that required necropsy, the number of pups is less than shown.

[d]Litters for AGD were the statistical unit; neither litter nor pup numbers for AGD were reported.

[e]Number derived from the mean number of live fetuses/litter.

[f]In some cases, data from two experiments were combined.

Table 4-3. Life stage at observation for various male reproductive system outcomes assessed in studies of developmental exposure to DBP

Findings	Life stage of animals (rats) at observation		
	Fetus	Neonate through puberty	Adult
Decreased T	✓	✓	✓
Malformations	✓	✓	✓
Decreased AGD		✓	✓
Hypospadias		✓	✓
Retained nipples/areolae		✓	✓
Cryptorchidism		✓	✓
Delayed PPS		✓	
Organ weights		✓	✓
Histopathology of male reproductive organs	✓	✓	✓
Abnormal sperm			✓
Decreased fertility			✓

T, Testosterone; AGD, Anogenital distance; PPS, Preputial separation.

Using the list in Table 4-3 as a guide, a more extended analysis was conducted for each of the selected studies. Table 4-4 presents the detailed results. In this table, the various observed outcomes are arrayed across three general life stage categories: prenatal (i.e., observations conducted in fetuses), neonatal through puberty (i.e., observations conducted in pups), and adult (i.e., observations conducted in young, sexually mature animals). These life stage categories do not represent the period of exposure for the study. While all studies include exposures during late gestation (i.e., during the critical window of male reproductive system development), some studies also maintained exposures during later life stages. For reference, Table 4-1 provides general descriptions of exposure durations.

Table 4-4. Age of assessment for individual endpoints across studies of the male reproductive system following developmental exposure to DBP

	Fetus		Neonate through puberty								Adult									
	↓ T[a]	Histo-path[b]	↓ AGD	Hyp	Ret. nip/ areolae	Crypt[c]	Del. PPS[d]	↓ Org wt	Histo-path[b]	↓ T[a]	Malf	↓ Org wt	Histo-path[b]	Ab. Sperm	↓ Fert	Hyp	Ret. nip/ areolae	Crypt	Δ AGD	↓ T[a]
Barlow and Foster, 2003		✓	✓	✓	✓	✓	✓—		✓		✓	✓[e]	✓	✓		✓		✓		
Barlow et al., 2003		✓																		
Barlow et al., 2004			✓		✓						✓	✓[f]	✓			✓[g]	✓	✓	✓↓	
Bowman et al., 2005		✓[h]																		
Carruthers and Foster, 2005			✓[i]		✓[j]						✓	✓	✓			—	✓[k]	—	✓↑	
Ema et al., 1998		✓	✓[l]			✓[l]														
Ema et al., 2000b			✓[l]			✓[l]														
Fisher et al., 2003	✓	✓						✓		✓/—[m]	✓	✓	✓	✓	✓	✓		✓		—[m]
Gray et al., 1999b			✓		✓		✓[n]				✓	✓	✓	✓[o]	✓[p]	✓	✓	✓		✓[p]
Kim et al., 2004 **Ab**				✓		✓		✓		✓[q]										
Kleymenova et al., 2004 **Ab**		✓																		
Kleymenova et al., 2005a **Ab**		✓																		
Kleymenova et al., 2005b		✓		—		—			✓											
Lee et al., 2004			✓	—[r]	✓	—[r]	—	—	✓		—[r]	✓	✓							
Lehmann et al., 2004	✓																			
Liu et al., 2005			✓[s]																	
Mahood et al., 2005	✓	✓				✓[t]		NR	✓		✓	NR	✓			NR		✓		
Mylchreest et al., 1998			✓	✓		✓	—				✓	✓		—[u]		✓		✓		

Table 4-4. (continued)

	Fetus		Neonate through puberty								Adult									
	↓ T[a]	Histo-path[b]	↓ AGD	Hyp	Ret. nip/ areolae	Crypt[c]	Del. PPS[d]	↓ Org wt	Histo-path[b]	↓ T[a]	Malf	↓ Org wt	Histo-path[b]	Ab. Sperm	↓ Fert	Hyp	Ret. nip/ areolae	Crypt	Δ AGD	↓ T[a]
Mylchreest et al., 1999			✓	✓	✓	✓	✓				✓	✓	✓			✓		✓		
Mylchreest et al., 2000			✓	✓	✓	✓	—				✓	✓	✓			✓		NR		
Mylchreest et al., 2002	✓	✓	NR[s]																	
NTP, 1991				✓							✓	✓	✓	✓	✓	✓		✓		
Plummer et al., 2005	✓					✓[v]														
Shultz et al., 2001	✓		NR[s]																	
Thompson et al., 2004a	✓																			
Thompson et al., 2005	✓																			
Wilson et al., 2004	✓																			
Zhang et al., 2004			✓	—		✓		—			✓	✓	✓	✓[u]		—		✓		

✓ Observed; —, Not observed; white box, Not evaluated; shaded box, Evaluated; NR, Not reported, although the study indicates that the endpoint was evaluated; **Ab**, Abstract only; PPS, preputial separation; AGD, anogenital distance; Hyp, hypospadias; Δ, change; Ret. nip/areolae, retained nipples and/or areolae; Cryp, cryptorchidism; Del, delay; Org wt, organ weight decrease (absolute or relative) in at least one reproductive organ; Malf, malformations including ventral/dorsal/lateral prostate, seminal vesicles, androgen dependent muscles, (accessory sex organs) epididymis, vas deferens external genitalia, cryptorchidism, small or flaccid testes; Fert, fertility; Ab. Sperm, abnormal changes in sperm count, motility, and/or morphology.

[a]Decreased testicular testosterone (T) was measured in the fetus; Decreased serum T was measured postnatally and in adults.
[b]Histological changes—Leydig cell hyperplasia (aggregation); multinucleated gonocytes; Wolffian duct increased coiling (can be measured in fetus, neonate through puberty, or adult).
[c]Cryptorchidism can be observed between PNDs 16−21 and older.
[d]Delayed preputial separation normally observed ~PND 42.
[e]Enlargement of the seminiferous cords was observed at PNDs 19−21.
[f]In addition to the observed decreases and absences of male reproductive organs, "occasional enlargement" of the testes was observed only in the 500 mg/kg-d group.

Table 4-4. (continued)

[g]Assessed in adult animals at PNDs 180, 370, and 540. Hypospadias only observed in the 500-mg/kg-d group.
[h]Wolffian ducts smaller, more fragile, adipose tissue surrounding duct was more gelatinous, and decreased coiling.
[i]Assessed at PNDs 1 and 13. Reduction in AGD observed in animals exposed to DBP on GDs 16 and 17, GDs 17 and 18, or GDs 19 and 20; no change in AGD in animals exposed GDs 14 and 15.
[j]Assessed on PND 13; assessed on an individual animal basis, significant increase in nipple retention was observed after dosing on GDs 15−16; 16−17; 17−18; or 19−20.
[k]Assessed at PND 90; significant increase in nipple retention only for males dosed GDs 16−17 (individual animal basis).
[l]AGD and cryptorchidism were assessed in fetuses on GD 21. Exposed pregnant dams were sacrificed on day 21, and live fetuses were removed.
[m] Blood plasma T levels significantly reduced on PND 25 but not on PNDs 4, 10, or in adult.
[n] Delayed PPS only reported for parental generation (P0) males exposed from weaning through to puberty.
[o] Reduced epididymal sperm numbers; not necessarily abnormal sperm.
[p] In P0 males.
[q] Evaluated T levels at 31 and 42 days (not fetus) and found decreased at 42 days.
[r]It is presumed that specific malformations would have been observed if present based on the study design and methods.
[s]Examined in GDs 19 or 21 fetuses.
[t] Observed at PNDs 25 and 90; nonscrotal testes were not evaluated histopathologically.
[u] Only motility was evaluated in Mylchreest et al. (1998); in Zhang et al. (2004), sperm number, motility, and morphology were evaluated, but only count was affected.
[v] Study mentions that adult cryptorchidism was observed, but study methods do not indicate that offspring were retained until adult age.

Table 4-4 summarizes the outcomes and presents a broad representation of positive and negative observations in a manner that demonstrates that not all relevant endpoints were evaluated at all life stages or even in each study. To facilitate summarization of the myriad individual study findings, information was often combined by category (e.g., "histopathology" includes a broad variety of outcomes in various reproductive organs), and for the sake of brevity, the minute details and nuances of the study design and observations, although quite interesting, are not typically presented. In a few cases, negative outcomes presented in the table are extrapolations based on the presumption that specific findings would have been observed if they were present. For example, with methods that include detailed external and internal (macropathology) examination of pups and/or adults, the absence of reported malformations at either of these life stages was presumed to indicate that no gross malformations were observed because they should have been readily detectable (Lee et al., 2004).

Tables 4-1, 4-3, and 4-4 clearly illustrate that the study protocols varied quite extensively. In general, with the exception of the NTP studies, the protocols were not designed to conform to a particular regulatory guideline. Rather, the majority of the studies were focused research efforts that were verifying and/or expanding upon previously observed outcomes; therefore, the differences across study methods are understandable. As a result, the apparent lack of consistency in male reproductive system observations across studies is generally attributable to differences in protocol design and implementation. Some examples are discussed in detail as follows:

- Although all of these studies used exposures during late gestation (i.e., a critical period of male reproductive system development in the rat), the specific endpoints that were assessed and/or the life stages at which endpoints were examined varied extensively across the studies. Obviously, treatment-related alterations of life-stage-specific events require examination during the most appropriate or optimal life stage (e.g., increased multinucleated gonocytes can only be observed in fetal testes, delays in PPS can only be observed in juvenile animals at the time of sexual maturation, and disturbances in reproductive function can only be observed in sexually mature adults). Other permanent structural abnormalities may be detected across multiple life stages (e.g., hypospadias or cryptorchidism could theoretically be observed in late gestation fetuses, in adolescents, and in adults). For some outcomes, it is difficult to predict *a priori* the optimal time point for evaluation. For example, DBP-related increases in the estrogen receptor (ER) were observed at 31 days but not at 42 days (Kim et al., 2004).

- It is important to realize that not all available offspring are evaluated in every study; therefore, identification of adverse outcomes may rely, in part, on sampling protocols and the statistical power of the sample size for detection of rare or low-incidence events. Calculations of statistical power are rarely provided in study reports.

- In some cases, apparent differences in studies may result because the report contains an insufficient level of detail on a particular endpoint or life stage—often because the emphasis of the scientific review lies in a slightly different direction. For example, if high doses of DBP are administered during sensitive periods of male reproductive system development, and the males are maintained and terminated as adults, at which time histopathological evaluation is performed, it might be assumed that various male reproductive system malformations and/or cryptorchidism would have been present in some of the males at necropsy. Yet, these findings may not be reported because the histopathological findings are the primary focus of the investigation and/or the publication (e.g., Lee et al., 2004).

- In other situations, the description of the findings at various life stages may vary. For example, evidence of cryptorchidism may be described as "testis located high in the abdomen" in a fetus, as "undescended testis(es)" in an adolescent rat, or as "unilateral testis" upon noninvasive clinical examination of an adult. To some extent, this lack of consistency in terminology may result from laboratory Standard Operating Procedures that direct technical staff to avoid the use of diagnostic terminology.

Overall, in spite of numerous differences in the study designs, the toxicity data set for DBP clearly demonstrates that exposure to DBP during critical stages of male reproductive system development can result in adverse structural and functional reproductive outcomes. When specific critical aspects of study design and implementation were similar, consistent outcomes were almost universally observed. The WOE embodied by the data described above is further supported by studies in rats that demonstrated similar incidences of cryptorchidism and decreased AGD in male pups of dams treated with either DBP or MBP, the metabolite of DBP (Ema and Miyawaki, 2001a). The ability of MBP to cross the placenta and reach the fetus has also been conclusively demonstrated (Fennell et al., 2004; Saillenfait et al., 1998), and these two TK events (metabolism and placental transport) are key to the MOA of reduced fetal testicular T (David, 2006). Available toxicogenomic data, described elsewhere in this case-study report, further elucidate the MOA(s) of DBP in producing adverse effects on male reproductive system development and are an important consideration in the WOE analysis of the toxicological data set.

In the selected DBP toxicology study data set, the presentation of extensive individual offspring data was limited to the NTP (1991) study conducted as a reproductive assessment by continuous breeding (RACB) in SD rats. The individual data from this study were carefully examined in order to confirm the NOEL and LOEL described in the study report. This analysis was conducted under the presumption that statistical and/or biological significance noted in the summary compilations of male reproductive system outcomes might not identify low incidence effects in individual offspring at lower dose levels. To further aid the identification of treatment-related outcomes, the male reproductive system outcomes were grouped by organ instead of individual animal. This analysis revealed apparently treatment-related findings in the testis and epididymis of F1 male offspring, as summarized in Table 4-5. At the highest dose tested (794 mg/kg-d, equivalent to 1.0% DBP in the diet), additional findings in the male reproductive organs of F1 offspring included single incidences of (1) underdeveloped prepuce; (2) mild secretion and severe vesiculitis of the prostate; (3) a mass on the testis; and (4) a focal granuloma with fluid and cellular degeneration in the epididymis; none of these findings were observed at the lower dose levels. Understandably, the findings at the low- and mid-dose groups were originally interpreted as not being treatment-related (Wine et al., 1997; NTP, 1991). However, consideration of MOA information for DBP, including toxicogenomic data, resulted in a more conservative interpretation of the toxicity data both by NTP researchers (conference call in 2008 between Paul Foster [NTP/NIEHS], Susan Makris [EPA/NCEA], and Susan Euling [EPA/NCEA]) and by the EPA IRIS program (U.S. EPA, 2006a). Consequently, further analysis of individual offspring data in the current case study did not identify any additional sensitive toxicological outcomes; the study LOEL was confirmed to be the lowest treatment level tested in the NTP RACB study (80 mg/kg-d).

4.3. UNEXPLAINED MOAs FOR DBP MALE REPRODUCTIVE TOXICITY OUTCOMES

Figure 3-6 illustrates the broad conceptual approach for consideration and interpretation of toxicogenomic and toxicology data to inform MOA. The toxicogenomic data can be evaluated to identify altered genes, gene products, and pathways; this information can lead to a more complete understanding of the mechanism of action or MOA(s) for the chemical toxicity. From the opposite perspective, the toxicity data can provide information critical to identifying

Table 4-5. Incidence of gross pathology in F1 male reproductive organs in one continuous breeding study with DBP[a]

Gross finding[b]	Dose (% in diet)			
	0	0.1	0.5	1.0
Testis: absent, poorly developed, atrophic, undescended	0/20	1/20	1/20	6/20
Penis: small/underdeveloped	0/20	0/20	0/20	4/20
Epididymis: underdeveloped/absent	0/20	1/20	1/20	12/20

[a]Incidences were compiled from reported individual animal macroscopic pathology data; statistical analysis was not performed.
[b]Some animals have more than one type of malformation, and these animals were counted separately for each of the three outcome categories.

Source: (NTP, 1991).

the relevant MOA(s) involved in the toxicological outcomes, and thereby inform the interpretation of gene alterations and relevant pathways.

Each male reproductive system outcome was evaluated for consistency with either or both of the two well-established MOAs using expert judgment based on the available published literature for DBP (see Figure 4-2). This exercise helped to identify the unexplained endpoints for which the evaluation of the toxicogenomic data set may suggest potential MOAs (see Chapter 5). For the DBP case study, Table 4-6 presents a compendium of the specific findings noted in the male reproductive system following exposures at critical windows of development. While reduced fetal testicular T and reduced *Insl3* signaling can be linked to some of the observed outcomes on the basis of available data, potential key events cannot specifically be identified for other outcomes. The unexplained MOAs are good candidates for further study, both in toxicology and toxicogenomic studies, to elucidate the underlying mechanism of action.

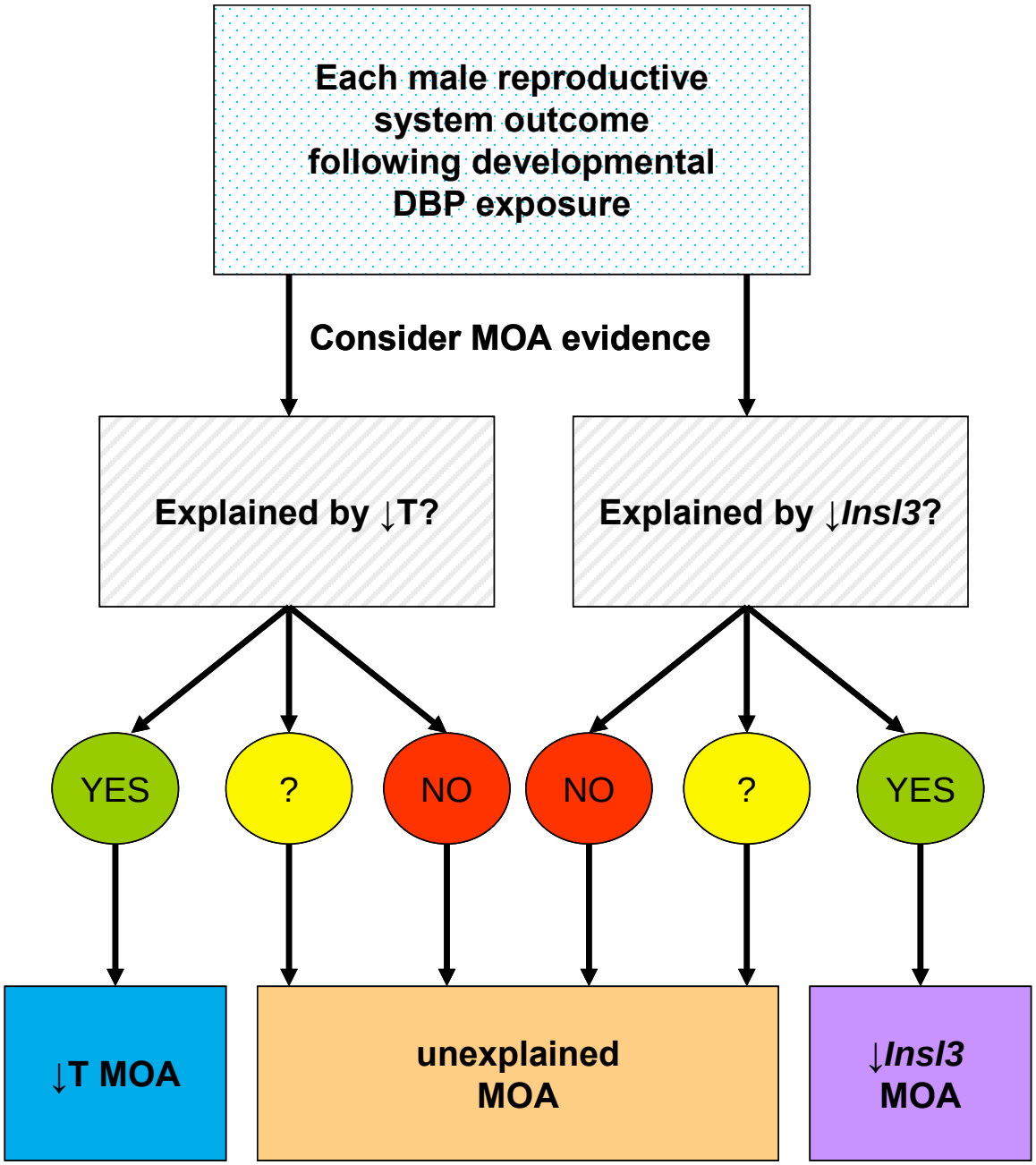

Figure 4-2. The process for evaluating the MOA for individual male reproductive system outcomes following developmental DBP exposure. The available data for MOA for each male reproductive outcome following developmental DBP exposure were evaluated by a team of experts. For each outcome, the current WOE of the data either support the MOA (“YES”), support that this is not the MOA ("NO"), or are inconclusive for the MOA, i.e., either unlikely or unclear (“?”). “Unexplained MOAs” include both "?" and "NO" conclusions.

Table 4-6. Evidence for MOAs for the observed effects in the male reproductive system after *in utero* DBP exposure

Organ/ Function	Effect	MOA	
		Reduced fetal testicular T	Reduced *Insl3* signaling
Testes	Multinucleated gonocytes; increased number of gonocytes in fetal testes	?[b]	?[c]
	Altered proliferation of Sertoli and peritubular cells; fewer Sertoli cells	?[b]	?[c]
	Gonocyte apoptosis increase; early postnatal decrease in gonocyte number	?[b]	?[c]
	Abnormal Sertoli cell-gonocyte interaction	?[b]	?[c]
	Small incidence of Leydig cell adenomas, aggregates, and hyperplasia	✓	?[c]
	Decreased number of spermatocytes or cauda epididymal sperm concentration.	✓	✓[d]
	Small or flaccid; other abnormalities; decreased weight	✓	✓
	Increased weight due to edema	?[e]	?
	Decreased number or degeneration of seminiferous cords/tubules; altered morphology; degeneration of the epithelium; enlarged cords/tubules	?[b]	?[c]
	Testes descent: none (cryptorchid) or delayed	✓[f]	✓[f]
Gubernacular ligament	Gubernacular ligament development effects: agenesis or elongation	X	✓
Epididymis	Lesions and agenesis; partial to complete absence; decreased epididymal ductular cross section	✓	X
	Reduced weights	✓	✓
Mammary gland	Nipple and/or areolae retention in males	✓	X
	Degeneration and atrophy of alveoli in males	?[b]	X

Table 4-6. (continued)

Organ/ Function	Effect	MOA	
		Reduced fetal testicular T	Reduced *Insl3* signaling
Wolffian ducts	Underdeveloped	✓	X
Seminal vesicles	Malformations or absent; decreased weight	✓	X
Coagulating gland	Malformations	✓	X
Penis	Small, underdeveloped	✓	X
	Hypospadias	✓	X
	Delayed preputial separation	✓	X
Accessory sex organ	Decreased weight	✓	X
Prostate	Decreased wt or absent	✓	X
Vas deferens	Agenesis	✓	X
Levator anibulbocavernosus muscle	Decreased weight	✓	?[c]
Male/female ratio	Decreased % male offspring as determined by AGD at birth	✓	X
Perineum	Decreased AGD	✓	X
Repro function	Infertility	✓	✓[d]

AGD, anogenital distance; ?, Current data indicate that it is unlikely the MOA; ✓, Current weight of evidence of the data support this MOA leading to the effect; X, Current weight of evidence of the data indicate that this MOA is not the MOA for this outcome.

[a]MOA is defined as one or a sequence of key events upon which the outcome is dependent (see glossary).
[b]Reduced fetal testicular T may play a role, but current data indicate that reduced T is not solely responsible for this outcome.
[c]The *Insl3* knockout mouse phenotype suggests that *Insl3* is specifically required for gubernacular ligament development and, therefore, testis descent in mice since these mice do not have other defects.
[d]Decreased fertility in males is a result of reduced *Insl3* signaling since reduced *Insl3* signaling leads to undescended testes, which, in turn, reduces sperm count (presumably by increasing the temperature) and can cause infertility.

Table 4-6. (continued)

[e]In some animals, increased weight, due to edema, can result in animals that have epididymal agenesis, which is a consequence of reduced testosterone (T).

[f]*Insl3* signaling is required for development of the gubernacular ligament and through this mechanism—the 1st stage of testis descent from the kidney region to the inguinal region. Testosterone is required for the 2nd stage of testis descent, from the inguinal region to the scrotum (reviewed in Klonisch et al., 2004). After *in utero* DBP exposure, the cryptorchid phenotype resembles the *Insl3* knockout. A delay in testis descent can result from reduced *Insl3* and T.

4.4. CONCLUSIONS ABOUT THE TOXICITY DATA SET EVALUATION: DECISIONS AND RATIONALE

The review of the toxicology data set identified a number of issues and limitations that are evident in the study descriptions and endpoint summaries presented in this chapter. These include the following:

- *Lack of dose-response information:* A number of studies conducted with DBP used a single high-dose treatment level (often at 500 mg/kg-d) in order to produce readily observable adverse outcomes to male reproductive system development that could be examined. In such studies, the absence of lower dose levels prevents the evaluation of dose-dependent responses and does not allow the identification of study-specific NOELs or LOELs. While this approach is useful for hazard characterization, it does not facilitate other aspects of risk assessment (e.g., dose-response assessment or risk characterization). Thus, studies utilizing a single high-dose level may provide important information for a WOE assessment of the toxicology profile, but they have diminished usefulness in identifying outcomes for use in risk calculations at environmentally relevant doses.

- *Insufficient information on study methods:* Even though every study report includes a section on study methods, there can be a great deal of unevenness in the amount of detailed information provided. Consequently, important questions may arise during study review that cannot be readily resolved. In some cases, this can have an impact on individual study interpretation or on conclusions that rely on a thorough WOE evaluation of the data set.

- *Unavailable individual outcome data*: A full range of individual animal data is seldom included in studies published in the open literature and is almost never available when the only available publication is a presentation abstract. Conversely, individual animal data are generally included in toxicology reports generated in response to a regulatory mandate or conducted by a federal agency (e.g., NTP). The availability of individual animal data can be quite important in interpreting the study findings, because it can reveal problems or inadequacies in the data, but it can also help identify low incidence adverse outcomes. In the case of DBP, the individual offspring data presented in the NTP study report (1991) include alterations in the reproductive system of F1 males that

had been exposed during development. These findings are similar to outcomes identified at higher dose levels, are consistent with the proposed MOA, and, consequently, are used to establish a LOEL for the study.

- *Protocol limitations:* Unless studies are designed to meet the recommendations of a standardized testing protocol (e.g., NTP or U.S. EPA/Office of Prevention, Pesticides and Toxic Substances reproductive toxicity study guidelines), there may be a high degree of variability among the protocols used for testing any one chemical. Between two studies, there can be differences in the treatment regimen or in the assessment of outcomes that render them incomparable. DBP provides a good example of a chemical that targets a very specific critical prenatal window of reproductive system development in males, and results in adverse outcomes that could go unidentified if the appropriate endpoint(s) are not assessed at the optimal life stage or time point.

- *Specific study's limitations:* Even when a study design optimizes the detection of adverse outcomes from chemical treatment, there may be challenges in study analysis and interpretation. Such is the case with the NTP study on DBP, which was conducted in several phases and reported both in the open literature (Wine et al., 1997) and by the Institute that conducted the experiments (NTP, 1995, 1991).

The analysis of the toxicology data in this chapter has provided a firm basis for expanded consideration of the toxicogenomic data for DBP as depicted in Figure 3-6. The extensive analysis of the toxicology data set and consideration of MOA(s) provide a source of information for use in phenotypic linking of known and potential MOAs. This chapter also provides an example of steps one can take to develop a toxicological data source, in particular, examining (1) the individual toxicity studies; (2) the WOE for the studies; (3) potential low incidence and low-dose effects; and (4) the MOA for the affected endpoints. All of these steps are useful exercises for evaluating toxicogenomic data in future risk assessments. The evaluations of both the toxicity and toxicogenomic data sets (detailed in Chapters 4 and 5) provide strong support for phenotypic anchoring for a number of gene expression changes occurring after *in utero* DBP exposure for several of the male reproductive outcomes. The available toxicogenomic studies for DBP are evaluated in Chapter 5.

5. EVALUATION OF THE DBP TOXICOGENOMIC DATA SET

This chapter presents an evaluation of the DBP toxicogenomic data set from the published literature and some new analyses of one of the microarray studies for DBP. The toxicogenomic studies include nine published RT-PCR and microarray studies in the rat after *in utero* DBP exposure. First, we evaluated the toxicogenomic data set from the published literature for the consistency of findings. Second, evaluating the published literature and performing new pathway analyses of the Liu et al. (2005) data set, we generated hypotheses about pathways/mechanism affected by DBP *in utero* exposure that may explain the testis endpoints for which there is no established MOA (these "unexplained" endpoints were identified in Chapter 4). The DBP genomic data set includes nine papers published through July 2007. The microarray studies all exposed animals to DBP doses of 500–1,000 mg/kg-d during the critical window for male reproductive development, which is during late gestation and correlates with the time of peak T production. The chapter first discusses the methodologies utilized in the nine studies and provides a brief overview of each study. The chapter then presents an evaluation of the consistency of the findings for the microarray, RT-PCR, and protein studies performed in the rat testes. The findings of the Lehmann et al. (2004) study, the one available dose-response RT-PCR study for DBP, are discussed. In addition, the pathway reanalysis of the Liu et al. (2005) study is presented, and data gaps and research needs are identified.

5.1. METHODS FOR ANALYSIS OF GENE EXPRESSION: DESCRIPTION OF MICROARRAY TECHNIQUES AND SEMI-QUANTITATIVE RT-PCR

5.1.1. Microarray Technology

Microarray technology allows for analysis of genome-wide expression of thousands of genes from the organ or tissue of interest. In principle, there are two main types of microarrays: the cDNA microarray and the oligonucleotide array. The cDNA microarray contains DNA from each open reading frame spotted onto glass microscope slides or nylon membranes. These probes are used to detect cDNA, which is DNA synthesized from a mature, fully spliced mRNA transcript. For example, Clontech's Atlas Arrays contain DNA sequences from thousands of genes immobilized on nylon membrane or glass slides. Each gene found on these arrays is well-characterized. These arrays use a radiolabelled detection system for analyzing the changes in

gene expression and have been optimized for high-quality expression profiling using a limited set of genes. Moreover, such arrays allow for the use of ^{32}P, and, therefore, offer a sensitive measure of gene expression. The second type of microarray is the oligonucleotide array. Here, short DNA sequences or oligonucleotides (oligos) are synthesized directly onto the glass slide via a number of different methods. For example, Affymetrix® uses 'Photolithographic' technology, where probes are directly synthesized onto the arrays. Briefly, the slide is coated with a light-sensitive chemical compound that prevents the formation of a bond between the slide and the first nucleotide of the DNA probe being created. Then, chromium masks are used to either block or transmit light onto specific locations on the surface of the slide. A solution containing thymine, adenine, cytosine, or guanine is poured over the slide, and a chemical bond is formed in areas of the array that are not protected by the mask (exposed to light). This process is repeated 100 times in order to synthesize probes that are 25 nucleotides long. This method allows for high-probe density on a slide.

Affymetrix uses an antibody detection system with horseradish peroxidase and streptavidin conjugates, and a 2-dye system (Cy3- and Cy5- labeled fluorescein dyes), which is unique to this platform. The Agilent scanner detects the relative intensities of the red and green labels and gives a relative measure of the gene expression changes between the control and treated samples. In the case of Affymetrix and Clontech, the detection system measures the absolute intensity of the individual probes of the treated and control samples. These values are then used to calculate the relative gene expression change between the treated and control samples.

5.1.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Polymerase Chain Reaction (PCR) is a method that allows exponential amplification of short DNA sequences within a longer double stranded DNA molecule using a thermo-stable DNA polymerase called Taq polymerase. RT-PCR is a semiquantitative technique for detection of expressed gene transcripts or mRNA. Over the last several years, the development of novel chemistries and instrumentation platforms enabling detection of PCR products on a real-time basis has led to widespread adoption of real-time RT-PCR as the method of choice for quantitating changes in gene expression. Real-time RT-PCR is a kinetic approach in which the reaction is observed in the early, linear stages. Furthermore, real-time RT-PCR has become the

preferred method for confirming results obtained from microarray analyses and other techniques that evaluate gene expression changes on a global scale.

5.2. REVIEW OF THE PUBLISHED DBP TOXICOGENOMIC STUDIES

5.2.1. Overview of the Toxicogenomic Studies

We evaluated nine studies published prior to July 2007 that characterized altered gene expression in rats following prenatal DBP exposure. Among these nine studies, four are based on the analysis of preselected genes by real-time RT-PCR, while the other five are based on the analysis of global gene expression by microarray technology. Table 5-1 summarizes general information (e.g., DBP dose, exposure route, exposure window, tissue type) for these nine studies, and each study is briefly reviewed. Section 5.2.3.2 presents information about the similarities and differences among these studies.

5.2.2. Microarray Studies

5.2.2.1. *Shultz et al. (2001)*

Six SD rats per group were treated by gavage with corn oil, DBP (500 mg/kg-d), or flutamide (reference antiandrogen, 50 mg/kg-d) from GDs 12–16, GDs 12–19, or GDs 12–21. Testes were then isolated on GD 16, 19, or 21. Global changes in gene expression were determined by Clontech cDNA expression array (588 genes). Shultz et al. (2001) isolated total RNA from testis of control and treated animals. Reverse transcription reactions were performed using total RNA, [^{32}P]-dATP, and superscript II MMLV-RT. Following purification, the probes were counted, and equal numbers of counts per minute were added to each rat gene cDNA expression array. The arrays were hybridized with cDNA using 1 fetus per dam. Hybridization and washing were performed according to manufacturer's instructions. Digital images were collected on a BioRad phosphorimager and analyzed using Clontech's Atlas Image software. Eight genes were further examined by real-time RT-PCR. Total RNA was isolated from both testes using RNA STAT60, and then the RNA was treated with DNase I in the presence of RNasin. cDNA was then synthesized using random primers and TaqMan reverse transcription reagents. Quality of RT reactions was confirmed by comparison of RT versus no enzyme control for each RNA sample using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer

Table 5-1. Study comparisons for the toxicogenomic data set from male tissues after *in utero* DBP exposure

Study[a]	Strain and species	DBP doses	Treatment interval[b]	Toxicogenomic method		Tissues collected
				Microarray (Platform)	RT-PCR	
Barlow et al., 2003	SD rat	500 mg/kg-d	GDs 12−19	No	Yes	Testis
Bowman et al., 2005	SD rat	500 mg/kg-d	GDs 12−19 or 19−21	Yes (Clontech cDNA arrays)	Yes	Wolffian ducts
Lehmann et al., 2004	SD rat	0.1, 1.0, 10, 50, 100, or 500 mg/kg-d	GDs 12−19	No	Yes	Testis
Liu et al., 2005[c]	SD rat	500 mg/kg-d	GDs 12−19	Yes (Affymetrix® GeneChip® oligo arrays)	Yes	Testis
Plummer et al., 2007[d]	Wistar rat	500 mg/kg-d	GDs 12.5−15.5; 12.5−17.5, or 12.5−19.5	Yes (Agilent 22K and 44K oligo arrays)	Yes	Testis: whole, seminiferous cord, and interstitial regions
Shultz et al., 2001	SD rat	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	Yes (Clontech cDNA arrays)	Yes	Testis
Thompson et al., 2004	SD rat	500 mg/kg-d	GDs 12−17, 18, or 19; 13−19, 14−19, 15−19, 16−19, 17−19, 18−19, or 19	No	Yes	Testis

Table 5-1. (continued)

Study[a]	Strain and species	DBP doses	Treatment interval[b]	Toxicogenomic method		Tissues collected
				Microarray (Platform)	RT-PCR	
Thompson et al., 2005	SD rat	500 mg/kg-d	0.5−24 hrs on GDs 18−19 or GD 19	Yes (Affymetrix GeneChip oligo arrays)	Yes	Testis
Wilson et al., 2004[e]	Rat, SD	1,000 mg/kg-d	GDs 13−17	No	Yes	Testis

[a]In all studies, oral gavage was the route of exposure.
[b]GD 0 = sperm positive.
[c]Study assessed 7 different phthalates.
[d]Plummer et al. (2007) reported dosing intervals spanning GDs 12.5−19.5, which is comparable to GDs 12−19 in the other studies due to differences in reporting of GD and sperm positive at GD 0.5.
[e]Wilson et al. (2004) reported a dosing interval of GDs 14−18, which is comparable to GDs 13−17 in the other studies due to differences in reporting of GD and sperm positive at GD 1.

set. Fourteen rat-specific primer sets were used for analyses. The ABI PRISM 7700 and the ABI PRISM 7900HT Sequence Detection System was used for RT-PCR with the SYBR Green PCR and TaqMan Universal PCR Master Mix reagents. GAPDH was used as an on-plate internal calibrator for all RT-PCR reactions.

Genes analyzed by real-time RT-PCR include clusterin (*Clu*), cytochrome P450, family 11, subfamily a, polypeptide 1 (*Cyp11a1*), myristoylated alanine-rich C-kinase substrate (*Marcks*), proliferating cell nuclear antigen (*Pcna*), cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*), steroidogenic acute regulatory protein (*Star*), scavenger receptor class B, member 1 (*Scarb1*), and v-kit Hardy Zuckerman 4 feline sarcoma viral oncogene homolog (*Kit*). Radioimmunoassay of steroid hormones and immunocytochemical analysis of certain proteins (i.e., CLU and b-cell leukemia/lymphoma 2 [BCL2]) in the fetal testes were also performed.

Of the 588 genes examined, ~45 genes had at least a 2-fold change in the average expression values in DBP-treated rats relative to the average values in control rats. DBP exposure led to a reduced expression of steroidogenic enzymes at GD 19, such as *Cyp11a1, Cyp17a1, Scarb1*, and *Star*. These genes were upregulated at GD 19 following flutamide exposure, suggesting that DBP does not act as an androgen antagonist at this time point. Flutamide and DBP demonstrate patterns of gene expression that overlap, though both have distinctly expressed genes. This suggests to Shultz et al. (2001) that there are both common and distinct molecular pathways within the developing fetal testes.

Other genes affected after DBP exposure were *Clu* (upregulated) and *Kit* (downregulated). Using immunocytochemical staining of CLU and BCL2 protein in the fetal testes, increased amounts of both proteins were observed in the Leydig and Sertoli cells of GD 21 testes. Decreases in testicular T and androstenedione in testes isolated on GDs 19 and 21 were observed, while increases in progesterone in testes isolated on GD 19 in DBP-exposed testis were observed.

Shultz et al. (2001) suggest that the antiandrogenic effects of DBP are due to decreased T synthesis. Furthermore, enhanced expression of cell survival proteins, such as CLU and BCL2, may be involved in DBP-induced Leydig cell (LC) hyperplasia, while downregulation of *Kit* may play a role in gonocyte degeneration.

5.2.2.2. *Bowman et al. (2005)*

Four to seven SD rats per group were treated by gavage with corn oil or DBP at 500 mg/kg-d from GDs 12–19 or GDs 12–21. The animals were sacrificed on GD 19 or 21, and Wolffian ducts (WD) were pooled from three to four fetuses (to obtain enough RNA for analysis) within the same litter for gene expression analysis. Global changes in gene expression were determined by Clontech Atlas Rat Toxicology 1.2 cDNA expression array (1,185 genes). Images were collected using a Phosphorimager and then imported into AtlasImage 2.01 and GeneSpring 4.2 for analysis. Selected genes were further examined by real-time quantitative RT-PCR using the GeneAmp 5700 Sequence Detection System. Total RNA was isolated, DNAse-treated, and reverse-transcribed using TaqMan reagents. Twenty-three primer sets were used for RT-PCR analysis. Reactions were standardized using GAPDH-specific primers. The genes analyzed by RT-PCR include those in the insulin-like growth factor (*Igf*) pathway, the matrix metalloproteinase (*Mmp*) family, the extracellular matrix, and other developmentally conserved signaling pathways: bone morphogenetic protein 4 (*Bmp4*), collagen, delta like (Dlk), mitogen-activated protein kinase 12 (*Map3k12*), epidermal growth factor receptor (*Egfr*), fibroblast growth factor 10 (*Fgf10*), FGF receptor 2 (*Fgfr2*), fibronectin, insulin-like growth factor 1 (*Igf1*), insulin-like growth factor 2 (*Igf2*), insulin-like growth factor 1 receptor (*Igf1r*), insulin-like growth factor binding protein 5 (*Igfbp5*), integrinA5, integrinB1, matrix Gla protein (*Mgp*), matrix metallopeptidase 2 (*Mmp2*), matrix metallopeptidase 14 (*Mmp14*), matrix metallopeptidase 16 (*Mmp16*), Notch2 receptor (*Notch2*), and tissue inhibitors of MMPs *(Timp1, Timp2,* and *Timp3)*. Immunohistochemistry was also performed to evaluate changes in localization and/or intensity of IGFLRβ and androgen receptor (AR) protein expression.

Microarray data were not presented due to considerable variability in gene expression levels within the treatment group at each age. Based on real-time RT-PCR analysis, compared with controls, prenatal exposure to DBP from GDs 12–19 or GDs 12–21 increased mRNA expression of different members of the IGF family including *Igf1* (on GDs 19 and 21)*, Igf 2*(on GD 19)*, Igfr1r* (on GD 19)*,* and *Igfbp5* (on GD 21) in the developing WD, while *Egfr* was unchanged on GDs 19 and 21. Additionally, mRNA expression of *Ar, Bmp4, integrinA5, Mmp2,* and *Map3k12* was increased on GD 19; mRNA expression of *Fgf10, Fgfr2, Notch2, Mmp2, Timp1,* and *Mgp* was increased on GD 21. IGFLRβ immunostaining was higher in the cytoplasm of the ductal epithelial cells and increased in the cytoplasm of mesenchymal cells in

DBP-exposed fetuses compared with that in controls. In general, reduction of AR immunostaining in the nuclei of ductal epithelial cells of DBP-exposed WD was observed on GD 19. Compared with controls, WDs dissected from GD 19 DBP-exposed fetuses were slightly smaller in size (underdeveloped) and appeared to be more fragile. By GD 21, control fetus WDs were markedly coiled, while those from the exposed fetuses exhibited less coiling.

Prenatal DBP exposure appears to alter the mesenchyme-epithelial signaling of growth factors (e.g., IGFs) and other developmentally conserved pathways (e.g., BMP4) in WDs. Bowman et al. (2005) contend that the effect of DBP on WD differentiation is likely a consequence of decreased fetal testicular T, although direct effects of DBP on the developing WD independent of T are also possible.

5.2.2.3. *Liu et al. (2005)*

Five to ten SD rats per group were treated by gavage with corn oil, DBP (500 mg/kg-d), or one of six other phthalate esters (500 mg/kg-d) daily from GDs 12–19. The six other phthalate esters include diethyl phthalate (DEP), dimethyl phthalate (DMP), diocytyl tere-phthalate (DOTP), diethylhexyl phthalate (DEHP), dipentyl phthalate (DPP), and butyl benzyl phthalate (BBP). Testes were collected on GD 19, homogenized, and then total RNA was isolated. RNA integrity was assessed using an Agilent 2100 Bioanalyzer. cDNA was synthesized from 2.5 μg total RNA and purified using RiboAmp OA. The BioArray High-Yield RNA Transcript Labeling Kit was used for cRNA amplification and biotin labeling. Affymetrix GeneChip Sample Cleanup Module was used for purifying and fragmenting the cRNA. The Complete GeneChip® Instrument System was then used to hybridize, wash, stain, and scan the GeneChip arrays (RAE230A and RAE230B; ~30,000 genes). The data were analyzed using analysis of variance (ANOVA [one-way, two-way, nested one-way]), Dunnett test (post hoc), Tukey test, and Bonferroni adjustment.

Image files obtained from the scanner were analyzed with the Affymetrix Microarray Suite (MAS) 5.0 software and normalized by global scaling. Absolute analysis was performed for each array prior to comparative analysis. To identify differentially expressed transcripts, pair-wise comparison analyses were carried out with MAS 5.0 (Affymetrix). The *p*-values were determined by the Wilcoxon's signed rank test and denoted as "increase," "decrease," or "no change." A transcript is considered significantly altered in relative abundance when $p < 0.05$. Analysis using MAS 5.0 provides a signal log ratio (SLR), which estimates the magnitude and

direction of change of a transcript when two arrays are compared (experimental versus control). The SLR output was converted into "fold-change" as recommended by Affymetrix. Furthermore, stringent criteria were used to identify robust signals as follows: (1) software call of "present," and (2) ≥ 2-fold change or SLR 1.0, in both replicates. Average and standard deviations were calculated for all the fold-change values. In general, only transcripts induced or suppressed by ≥ 2-fold were considered as differentially expressed.

Selected genes were further examined by real-time quantitative RT-PCR using 18 primer sets. The genes analyzed by RT-PCR include epididymal secretory protein 1 (*re1*), low-density lipoprotein receptor (*Ldlr*), 17β-hydroxysteroid dehydrogenase 3 (*Hsd17b3*), 17β-hydroxysteroid dehydrogenase 7 (*Hsd17b7*), luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), CCAAT/enhancer-binding protein (C/EBP), beta (*Cebpb*), early growth response 1 (*Egr1*), nuclear receptor subfamily 4, group A, member 1 (*Nr4a1*), nuclear factor, interleukin 3, regulated (*Nfil3*), nuclear receptor subfamily 0, group B, member 1 (*Nr0b1*), transcription factor 1 (*Tcf1*), insulin-induced gene 1 (*Insig1*), protein kinase C-binding protein (*Prkcbp1*), decay-accelerating factor (*Daf*), dopa decarboxylase (*Ddc*), seminal vesicle secretion 5 (*Svs5*), and testis-derived transcript (*Testin*). Anogenital distance (AGD) was measured and immunohistochemistry was performed for NR0B1, TESTIN, GEB14, DDC, and CEBPB proteins.

Of ~30,000 genes examined, 391 were statistically significantly altered following exposure to the four developmentally toxic phthalates (DBP, BBP, DPP, and DEHP) relative to the controls. While the four developmentally toxic phthalates were indistinguishable in their effects on global gene expression, no significant changes in gene expression were detected in the phthalates that do not lead to developmental effects (DMP, DEP, and DOTP). Of the 391 genes altered by the developmentally toxic phthalates, 225 were unknown and uncharacterized transcribed sequences. Of the remaining 166 genes, the largest GO classification (31 genes) was of genes related to lipid, sterol, and cholesterol homeostasis. Additional GO classification groups include genes involved in lipid, sterol, and cholesterol transport (10 genes); steroidogenesis (12 genes); transcription factors (9 genes); signal transduction (22 genes); oxidative stress (11 genes); and cytoskeleton-related (13 genes). RT-PCR results indicated that the developmentally toxic phthalates reduced the mRNA levels of *Hsd17b7, Lhcgr, Ldlr, re1, Svs5, Insig1,* and *Ddc.* Additionally, the RT-PCR results indicated that the developmentally

toxic phthalates induced the mRNA levels of *Grb14, Prkcbp1*, and *Testin*. RT-PCR results also indicated that gene expression of several transcription factors including *Dax-1, Cebpb, Nfil3, Nr4a1,* and *Tcf1* were significantly changed by at least one of the toxic phthalates. Based on immunohistochemical analysis, DAX-1 expression was reduced in the gonocyte population of DBP-treated testis compared with that of controls. Additionally, the expression of nuclear CEBPB, GRB14, and DDC proteins was reduced in interstitial cells of DBP-treated testis, while TESTIN and GRB14 expression levels were increased in Sertoli cells of DBP-treated testis. An AGD reduction was observed in male fetuses exposed to any of the developmentally toxic phthalates.

This study showed that the four phthalates (DBP, DEHP, BBP, and DPP) that have similar effects on the developing male rat reproductive tract are indistinguishable in their genomic signature for the developing fetal testis. These phthalates targeted pathways in LC production of T and other pathways that are important for normal interaction and development between Sertoli cells and gonocytes. By contrast, a different genomic signature was observed in animals exposed to any of the four phthalates that do not exhibit developmental toxicity.

5.2.2.4. *Thompson et al. (2005)*

Four SD rats per group were gavaged with corn oil or DBP daily at 500 mg/kg-d. In the first study, DBP treatment was 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, 12 hours, 18 hours, or 24 hours before sacrifice on GD 19. Global changes in gene expression were determined by Affymetrix GeneChips (the specific GeneChips used in the study were not reported). The methods were similar to Liu et al. (2005)—with the exception of the statistical analysis. Thompson et al. (2005) used JMP statistical software to perform Student t-tests or one-way ANOVAs with Tukey post hoc analysis. Selected genes were further examined by real-time quantitative RT-PCR. An ABI Prism 7900HT Detection System, the SYBR Green PCR Master Mix, and 30 primer pairs were used for analysis of DBP-induced changes in gene expression. The genes analyzed by RT-PCR included *Cyp11a1, Scarb1, Star, Cyp17a1, Egr1, Egr2, Nr4a1, Nfil3, Tcf1,* serum/glucocorticoid regulated kinase (*Sgk*), tumor necrosis factor receptor superfamily, member 12a (*Tnfrsf12a*), sclerostin domain containing 1 (*Sostdc1*), Wnt oncogene homolog 4 (*Wnt4*), B-cell translocation gene 2, antiproliferative (*Btg2*), C/EBP, delta (*Cebpd*), FBJ murine osteosarcoma viral oncogene homolog (*Fos*), dual specificity phosphatase 6

(*Dusp6*), *Hes6_predicted*, interferon-regulated developmental regulator (*Ifrd1*), *Ldlr*, nuclear receptor subfamily 4, group A, member 3 (*Nr4a3*), *Pawr*, *Nr0b1*, Jun-B oncogene (*Junb*), endothelial differentiation sphingolipid G-protein-coupled receptor 3 (*Edg3*), thrombospondin 1 (*Tsp1*), and stanniocalcin 1 (*Stc1*). Immunoblotting by SDS-PAGE was performed for SCARB1, CYP11a1, STAR, and CYP17a1. Fetal testicular T concentration was measured by radioimmunoassay.

Based on microarray analysis, 106 genes in the DBP-treated groups were significantly different from time-matched controls. Six genes were significantly elevated within 1 hour after DBP exposure. An additional 43 genes were upregulated, and five genes were downregulated 3 hours after DBP exposure. The rapid induction of these genes at 1 hour was a transient effect; none of the genes that were upregulated after 1 hour of DBP treatment remained significantly different than the controls 6 hours after treatment. Only nine genes showed significant changes from the control group between the 3- and 6-hour time points. After 1 and 3 hour DBP exposures, the majority of the changes in expression had reflected increased transcription. At 6 hours after exposure, 19 genes were downregulated and 17 were upregulated. Based on RT-PCR analysis, the immediate early gene, *Fos,* and the putative mRNA destabilizing gene, zinc finger protein 36 (*Zfp36*), were at peak expression level 1 hour after DBP exposure. Other immediate early genes were at peak expression at 2 hours after DBP exposure. At 3 hours after exposure, the expression of *Cebpd*, *Cxcl1*, and *Nr4a3* increased rapidly, while other genes showed a more gradual increase. *Tsp1* expression was increased 25-fold at 3 hours after exposure and returned to baseline at 6 hours after exposure. Genes involved in testicular steroidogenesis were first noticeably affected 2 hours after DBP exposure. Inhibition of *Star* transcription was detected ~2 hours after DBP exposure. *Scarb1, Cyp11a1,* and *Cyp17a1* showed a significant decrease in expression at about 6 hours after DBP exposure. At 6 hours after exposure, the T concentration dropped to approximately the level observed after long-term DBP treatment. At 12 hours after exposure, steroidogenesis-associated genes, *Nr0b1* and *Nr4a1,* were elevated. *Tcf1* and *Sgk* were downregulated soon after DBP exposure, but values returned to control levels by 3 hours after DBP exposure. *Sostdc1* and *Hes6_predicted* returned to control levels at 6 hours after exposure. Based on radioimmunoassay, a decrease in fetal testicular T up to 50% was observed within an hour after DBP exposure.

In a second experiment to compare the effect of DBP on steroidogenesis in the fetal adrenal gland, DBP treatment at GDs 12−19 was followed by analysis of gene expression in this tissue. A decrease (but not statistically significant) of corticosterone after GDs 12−19 DBP exposure was observed in the fetal adrenal. The expression of genes involved in steroidogenesis was less affected in the adrenal (males and females) than in the testes. This study indicates that the effect of DBP exposure on steroidogenesis gene expression is specific to the fetal testis and not in other steroidogenic organs.

Rapid transcriptional changes after DBP exposure in a number of genes could be responsible for the reduction in steroidogenesis. Peroxisome proliferator-activated receptors (PPAR) activation is ruled out since changes in expression of genes targeted by PPAR α and γ are not observed until 3 hours after DBP treatment. Many of the genes whose upregulation was detected within the first hour after treatment were "immediate early genes," meaning genes involved in cell growth and differentiation. One possible mechanism for DBP's repression of steroidogenesis is that DBP may initially stimulate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway in the fetal testis. Increased expression of *Egr1* and *Zfp36* could, in turn, lead to degradation of the transcripts involved in testicular steroidogenesis. Consistent with this possibility, the *Star* mRNA contains the AU-rich element, which are regions with many A and U bases that target the RNA for degradation, in target transcripts of *Zfp36*.

5.2.2.5. Plummer et al. (2007)

Five Wistar rats per group were gavaged with corn oil or DBP at 500 mg/kg-d from GD 12 until the day prior to sacrifice. Animals were sacrificed on GD 15, 17, or 19 and used for immunolocalization, Western analysis, or RNA quantification (of whole testes, seminiferous cord, or interstitial regions using laser capture microdissection). Samples for laser capture microdetection were collected from sections of single testes from GD 19 animals. RNA samples from three treated litters were compared to a pool of RNA samples from control animals to lessen errors due to biological variation. The Agilent 22K rat and 44K whole-rat oligonucleotide arrays were used for analysis of the whole-fetal testes and microdissected tissue, respectively. RNA was isolated from the homogenized whole-fetal testes using the RNeasy mini kit (Qiagen) and from laser capture microdissected samples using RNeasy micro kit (Qiagen). Isolated RNA

was labeled using the Agilent Low Input Linear Amplification Labeling kit according to the manufacturer's instructions. Specific activity of the labeled cRNA was measured using the microarray analysis program on a NanoDrop ND1000 spectrophotometer (Montchanin, USA). Microarray analysis with whole-fetal testis RNA was performed using Agilent 22K rat oligonucleotide arrays (Agilent #G4110A). Regional microarray analysis on RNA isolated from laser capture microdissected fetal testis tissue was performed using Agilent 44K whole-rat genome oligonucleotide microarrays (Agilent #G4131A). Microarray data analysis was conducted using Agilent feature extraction (v7.1) and Rosetta Luminator software (Rosetta Biosoftware, Kirkland, USA) to generate "signature" lists, defined as significantly ($p < 0.01$) different. The compare biosets function in Luminator was used to compare signature lists from different fetal testis regions. Pathway analysis used Ingenuity Pathways Analysis software.

DBP induced statistically significant changes in gene expression at all three time points. At GD 15 in whole testes, expression of genes regulating lipid metabolism, redox homeostasis, cell proliferation, and apoptosis were altered. At GDs 17 and 19, these four main gene clusters were altered: steroidogenesis (e.g., *Cyp17a1, Cyp11a1*), lipid metabolism, cholesterol (e.g., *Star, Scarb1*), and redox homeostasis. In laser capture microdissection studies of GD 19 tissue, both regions demonstrated altered expression of genes associated with steroidogenesis (e.g., *Cyp17a1*), cholesterol transport (e.g., *Scarb1*), cell/tissue assembly, and cellular metabolism. In the interstitial regions only, genes involved in fatty acid oxidation, testes morphogenesis, and descent (e.g., *Insl3*) were altered. In the cord samples, genes associated with stress responses, chromatin bending, and phagocytosis were altered.

RT-PCR analysis was performed on RNA from GD 19 testes from five rats/group using sequence-specific primers for the orphan nuclear receptor, nuclear receptor subfamily 5, group A, member 1 (*Nr5a1*; also known as steroidogenic factor 1 *[Sf1]*), *Star, Cyp11a,* and *Insl3*. The data were analyzed using a one-way ANOVA, followed by the Bonferroni post-test using GraphPad Prism. These studies showed a statistically significant reduction in the expression of *Star, Cyp11a1,* and *Insl3* but not *Nr5a1*.

Analysis of protein expression at GD 19 showed DBP-induced reduction in levels of CYP11A, inhibin-α, cellular retinoic acid binding protein 2 (CRABP2), and phosphatidylethanolamine binding protein (PEBP) in LCs, and no change in Sertoli cells/seminiferous cords. These data correlated with microarray data for the genes coding for

these proteins. Immunoreactivity for antimullerian hormone (AMH) was slightly increased in Sertoli cells following DBP treatment. Western blot analysis and immunolocalization of NR5A1 demonstrated no effects of DBP on protein expression in Sertoli or LCs. Using time plots to assess time-dependent changes in gene expression, a coordinate down-regulation of *Inhibin-α*, *Scarb1*, *Star, and Cyp11a1* was observed between GDs 15 and 19.

This study confirms other study results, showing down-regulation of *Scarb1, Star, Cyp11a1,* and *Cyp17a1*. The authors suggest that DBP induces LC dysfunction indirectly through sequestration of cofactors used in key signaling pathways and not through decreases in NR5A1 protein expression. They further state that the use of Wistar rats could be important, as Wistar rats may be more susceptible than SD rats to testicular effects of DBP.

5.2.3. RT-PCR Studies

5.2.3.1. *Barlow et al. (2003)*

Six to seven SD rats per group were gavaged with corn oil or DBP at 500 mg/kg-d from GDs 12–19. Testicular RNA was then isolated from three randomly selected male fetuses per litter. RT-PCR studies were performed as described in Shultz et al. (2001).

The mRNA of 13 preselected genes in the steroid biosynthetic pathway was analyzed by real-time RT-PCR; immunohistochemical and oil red O histochemical analyses were performed to further confirm mRNA changes. The 13 genes analyzed were *Scarb1, Star, Cyp11a1,* hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 *(Hsd3b), Cyp17a1,* hydroxysteroid (17-beta) dehydrogenase 3 *(Hsd17b3), Ar,* luteinizing hormone receptor *(Lhr),* follicle-stimulating hormone receptor *(Fshr), Kit,* stem cell factor *(Scf), Pcna,* and *Clu.*

Compared with controls, mRNA expression was downregulated for *Scarb1, Star, Cyp11a1, Hsd3b, Cyp17a1,* and *Kit* in DBP-treated testes; mRNA expression was upregulated for *Clu* following DBP exposure. These changes in mRNA expression were supported by immunohistochemical localization of selected proteins and by staining for lipids.

The results in the study of Barlow et al. (2003) confirm the gene expression changes observed in a previous study (Shultz et al., 2001). Furthermore, the data support alterations in cholesterol synthesis, transport, and storage that likely play a role in decreased T production by fetal LCs. The decreased level of mRNA expression for *P450scc* indicates another possible contributor, as P450scc conversion of cholesterol to pregnenolone is the rate-limiting enzymatic step in T biosynthesis.

5.2.3.2. *Lehmann et al. (2004)*

To date, Lehmann et al. (2004) is the only dose-response gene expression study on the testis performed with DBP. The other studies used a single high dose shown to affect male reproductive system development.

Five to seven SD rats per group were treated by gavage with corn oil or DBP at 0.1, 1.0, 10, 50, 100, or 500 mg/kg-d from GDs 12−19. Testes were then isolated on GD 19, and changes in gene and protein expression were measured by real-time RT-PCR (as described in Shultz et al., 2001) and Western analysis. Ten preselected genes in the steroid biosynthetic pathway were analyzed by RT-PCR: *Scarb, Star, Cyp11a1, Hsd3b1, Cyp17a1, Kit,* benzodiazepine receptor, peripheral *(Bzrp), Insl3, Clu,* and sterol regulatory element binding factor 1 (*Srebf1*). Fetal testicular T concentration was determined by radioimmunoassay in a separate group of animals using doses of 0.1, 1.0, 10, 30, 50, 100, or 500 mg/kg-d.

The aim of this study was to determine the DBP doses at which statistically significant alterations in the expression of a subset of genes and a reduction in fetal testicular T occur. As summarized in Table 5-2, Lehmann et al. (2004) established 50 mg /kg-d as an LOEL and 10 mg/kg-d as an NOEL for reductions in genes and proteins associated with T production as well as genes associated with other MOAs (e.g., *Kit, Insl3*) together with reductions in intratesticular T. The Lehmann et al. (2004) study demonstrated a decrease in *Hsd3b (*also called *3β-HSD)* gene expression involved in T synthesis was detected at levels as low as 0.1 mg/kg-d.

DBP exposure resulted in a dose-dependent decline in expression of the genes involved in cholesterol transport and steroidogenesis: *Scarb1, Star, Cyp11a1, Hsd3b, Cyp17a1,* and *Insl3*. Expression of *Bzrp* and *Clu* were increased in response to DBP. Furthermore, fetal testicular T was significantly reduced at DBP doses ≥ 50 mg/kg-d and reduced by 26% at 30 mg/kg-d. This study reported a LOEL of 50 mg DBP/kg-d and a NOEL of 10 mg DBP/kg-d for reductions in genes and proteins associated with T production together with reductions in intratesticular T. It demonstrates the coordinated reduction in genes and corresponding proteins involved in steroidogenesis and cholesterol transport, concurrent with a decrease in testicular T. Importantly, the study results identify changes in T concentration and gene expression at DBP doses lower than the observed effects on male reproductive development in toxicology studies reviewed in this report (see Chapter 4).

Table 5-2. Lehmann et al. (2004) DBP dose-response gene expression data measured by RT-PCR showing statistically significant changes from control

Gene Symbol (reported gene name)	Dose (mg/kg-d)					
	0.1	**1**	**10**	**50**	**100**	**500**
Scarb1 (Sr-B1)	NC	↓0.6	NC	↓0.5	↓0.3	↓0.2
Star	NC	NC	NC	↓0.4	↓0.3	↓0.1
Cyp11a1 (P450ssc)	NC	NC	NC	↓0.6	↓0.7	↓0.2
Cyp17a1	NC	NC	NC	NC	NC	↓0.3
Hsd3b (3β-HSD)	↓0.3	↓0.4	NC	↓0.5	↓0.3	↓0.5
Bzrp (PBR)	NC	NC	NC	NC	NC	↑2.0
Trpm2	NC	NC	NC	NC	NC	↑1.6
Kit (c-Kit)	↓0.3	↓0.5	NC	↓0.3	↓0.5	↓0.1
Insl3	NC	NC	NC	NC	NC	↓0.3

NC, no statistically significant change. Gene expression values are from DBP-exposed testes expressed relative to control values and are the statistically significant ($p < 0.05$)averages from five separate rat fetuses from different dams per treatment group.

For *Scarb1, Hsd3b,* and *Kit,* significant reductions in mRNA levels were observed at DBP doses that approach 0.1 mg/kg-d. Thus, alterations in the expression of *Scarb1, Hsd3b,* and *Kit* are at least sensitive indicators of DBP exposure. However, it is not clear whether alterations in any one of these three genes alone or together can cause one or more reproductive developmental effects of DBP.

5.2.3.3. *Thompson et al. (2004)*

Four to five SD rats per group were gavaged with corn oil or DBP at 500 mg/kg-d from GDs 12−19. Testes were isolated on GD 17, 18, or 19. Testes mRNA was isolated, and four preselected genes (*Scarbl, Star, Cyp11a1,* and *Cyp17a1*) in the cholesterol and steroidogenesis pathways were analyzed by real-time RT-PCR as described by Shultz et al. (2001). Immunoblotting was performed using the total protein extracted from paired testis, and the

expressed protein levels were quantified using FluorChem. Fetal testicular T concentration was determined by radioimmunoassay, and whole-cell cholesterol uptake assessment was performed on overnight cultures.

A significant decrease in fetal testicular T concentration was observed as early as GD 17 after *in utero* exposure to DBP. On GD 18, the decrease in T levels, as measured by the percent difference in testicular T between treated and control testes, was much higher (17.8% of control T levels) than on GD 17 (46.6% of control T levels). Furthermore, significant decreases in mRNA expression of *Scarbl, Star, Cyp11a1,* and *Cyp17a1* were observed as early as GD 17. In agreement with T levels, the percentage difference of gene expression between control and treated testes was higher on GD 18 than on GD 17. The suppression of the transcription by DBP was a reversible effect, as the mRNA levels for all genes returned to control levels 48 hours after DBP withdrawal. When protein expression was analyzed, results similar to the gene expression data were obtained (i.e., strong expression in controls, decreased expression in treated animals with 24-hour DBP withdrawal, and rising expression after the 48-hr DBP withdrawal). Additionally, there was a significant decrease in the amount of cholesterol transported across the mitochondrial membrane in the testes from DBP-treated fetuses as assayed in overnight cultures of testis explants. This observation indicates that the decrease in *Star* mRNA correlated with diminished protein function as transport of cholesterol from the outer to the inner mitochondrial membrane by the STAR protein is a rate-limiting steps of steroidogenesis (Miller, 2008).

The results of this study demonstrate that DBP-induced suppression of T production in the fetal testis correlates with diminished transcription of several genes in the cholesterol transport and steroidogenesis pathways as early as GD 17. This diminished effect was reversible, suggesting that DBP directly interferes with the signaling processes necessary for maintenance of steroidogenesis or with the transcriptional regulators required to maintain coordinate expression of the genes involved in cholesterol transport and T biosynthesis.

5.2.3.4. *Wilson et al. (2004)*

In the study by Wilson et al. (2004), SD rats were treated by gavage with corn oil or a developmental toxicant daily from GDs 14−18 in two separate experiments. In the first experiment, five rats were treated with DEHP at 750 mg/kg-d and five rats were treated with vehicle. In the second experiment, three rats were treated with one of six chemicals, each known

to induce male reproductive malformations and three rats were treated with vehicle. The chemicals used for the second study were three AR antagonists (vinclozolin [200 mg/kg-d], linuron [100 mg/kg-d], and prochloraz [250 mg/kg-d]) and three phthalate esters (DEHP [1 g/kg-d], DBP [1 g/kg-d], and BBP [1 g/kg-d]). Dams were sacrificed on GD 18, and testes were removed and pooled by litter. In the first study, RNA was prepared to quantify expression of one preselected gene, *Insl3*, by real-time RT-PCR. In the second study, both steroid hormone production (*ex vivo* incubation) and *Insl3* expression were assessed. Total RNA was isolated using Trizol, digested using Dnase I, and quantitated with RiboGreen. ImProm-II Reverse Transcriptase was used for RT, followed by amplification using Taq1. They completed RT-PCR for *Insl3* using a Bio-Rad iCycler.

In the first study, the mRNA expression of *Insl3* was reduced by ~80% in DEHP litters compared with that in control litters. In the second study, among the six chemicals tested, only phthalate esters (DEHP, DBP, or BBP) reduced mRNA levels in the fetal testis, with DBP and BBP being more effective than DEHP. In contrast, prochloraz, linuron, DEHP, DBP, or BBP significantly reduced *ex vivo* T production.

In a previous study with antiandrogenic chemicals that alter male sexual differentiation (Gray et al., 2000), phthalate esters were the only class that produced agenesis of the gubernacular ligaments; some of the phthalate ester-exposed rats had a phenotype similar to that seen in the *Insl3* knock-out mouse. The study of Wilson et al. (2004) confirms this hypothesis since only the three phthalates reduced *Insl3* gene expression. The authors proposed that the effects of DEHP, DBP, or BBP on *Insl3* mRNA and T production result from a delay in maturation of fetal LCs, resulting in hyperplasia as they continue to proliferate rather than differentiate.

5.2.4. Study Comparisons

5.2.4.1. *Microarray Study Methods Comparison*

Table 5-3 compares the study design and method of determining statistical significance across the five microarray studies. Because the Bowman et al. (2005) paper assessed changes in gene expression in WD rather than testis, and because the microarray data were not presented in the paper, the discussions will focus on the four other microarray studies. The Plummer et al. (2007) study pooled control tissue and used the Agilent platform, which differed from the

platforms used in the other studies. Liu et al. (2005), Schutz et al. (2001), and Thompson et al. (2005) all assessed mRNA levels in rat testis—but with somewhat differing significance criteria. All studies included vehicle-treated controls.

Table 5-3. Method comparisons for DBP microarray studies

Study	Tissue collected	Significance criteria	Individual animals (*n*) used?
Bowman et al., 2005	Wolffian ducts	ND (microarray data not presented)	No, pooled (3−4 fetuses/litter; 67 dams/treatment group)
Liu et al., 2005	Testis	$p < 0.05$ compared to control by either 1-way ANOVA, post hoc Dunnett test, or Tukey test	Yes, (6 fetuses/litter; 6 dams/treatment group)
Plummer et al., 2007	Testis (whole, laser captured interstitial tissue, or laser captured seminiferous cord tissue)	$p < 0.01$ using Agilent feature extraction software and then Rosetta Luminator software by performing one-way ANOVA on log fold change in the replicates	Yes for DBP-treated (3 pups from 3 different dams); Control RNAs were pooled
Shultz et al., 2001	Testis	2-fold change in average expression value compared to control	GDs 19 and 21 time points: Yes, 1 fetus/litter; 3 dams/treatment group. GD 16 time point: pooled RNA from 5 fetuses/1 litter; 3 arrays hybridized/treatment group.
Thompson et al., 2005	Testis	$p < 0.05$ multiple comparison using Bonferroni correction	Yes (NR)

ANOVA, analysis of variance; NR, not detected.

5.2.4.2. *RT-PCR Study Methods Comparison*

Table 5-4 compares the RT-PCR methods across the nine toxicogenomic published studies. There were many similarities among the studies. With the exception of Bowman et al. (2005), all groups extracted RNA from testis. All studies used a vehicle-treated control.

Table 5-4. Method comparisons among the RT-PCR DBP studies

Study	Tissue collected	Significance criteria (*p* values)	Individual animals (*n*) used?
Barlow et al., 2003	Testis	$p < 0.05$ compared to control	Yes (3 fetuses/litter; 5 dams/treatment group)
Bowman et al., 2005	Wolffian ducts	$p < 0.05$ compared to control	No, pooled (3−4 fetuses/litter; 6−7 dams/treatment group)
Lehmann et al., 2004	Testis	$p < 0.05$ compared to control	Yes (5 fetuses/litter; 4−5 litters/treatment group)
Liu et al., 2005	Testis	$p < 0.05$ compared to control by either 2-way nested ANOVA or Dunnett	Yes (control: 6 fetuses/dam; 6 dams for control. Treated: 3 fetuses/dam; 3 dams)
Plummer et al., 2007	Testis (whole, laser-captured interstitial tissue, or laser-captured seminiferous cord tissue)	$p < 0.05$ compared to control, normalized to 1.0. Expressed as mean +/– SEM; one-way ANOVA followed by Bonferroni post test using GraphPad Prism software	ND[a]; assessed GD 19.5 fetal testes
Shultz et al., 2001	Testis	$p < 0.05$ compared to control	GDs 19 and 21 time points: Yes, 1 fetus/litter; 3 dams/treatment group. GD 16 time point: pooled RNA from 5 fetuses/1 litter; 3 arrays hybridized/treatment group.
Thompson et al., 2004	Testis	$p < 0.05$ compared to control (Student's t-test or 1-way ANOVA)	ND
Thompson et al., 2005	Testis	$p < 0.05$ normalized mean of 3−5 fetuses/treatment group relative to control	Yes, 3−5 fetuses/litter
Wilson et al., 2004	Testis	$p < 0.01$ compared to control (means on a litter basis)	No, pooled for each litter (3 dams/treatment group)

[a]Not clear from the Materials and Methods.
ANOVA, analysis of variance; ND, not detected.

Most of the studies used the same significance criteria ($p < 0.05$). There were some differences in the number of fetuses used per experiment while some studies pooled tissues.

There were also important similarities among the nine toxicogenomic studies. Eight of the studies used the same strain of rat (SD), all purchased from the same vendor (Charles River, Raleigh, NC). All studies described dissolving the DBP in corn oil, using a corn oil vehicle control, and using oral gavage as the route of exposure. Six of the studies (Plummer et al., 2007; Bowman et al., 2005; Liu et al., 2005; Thompson et al., 2004; Barlow et al., 2003; Shultz et al., 2001) treated the animals by gavage with 500 mg/kg-d from GDs 12−19. This dose has been shown to adversely affect male reproductive development without causing maternal toxicity or fetal death. Lehmann et al. (2004) completed a dose-response during the GDs 12−19 period, using 0, 0.1, 1.0, 10, 50, 100, or 500 mg/kg-d. Bowman et al. (2005) and Shultz et al. (2001) included an additional exposure duration of GDs 12−21. Wilson et al. (2004) exposed for a slightly shorter duration (GDs 13−17) and at a higher dose (1,000 mg/kg-d). This paper reports exposures on GDs 14−18; however, these authors consider GD 1 as the day a sperm-positive smear was identified in dams, whereas the other studies consider the sperm-positive day as GD 0. Therefore, to be comparable with the other reports, we are reporting the exposure period as GDs 13−17. Similarly, Plummer et al. (2007) reports exposures ranging from GDs 12.5–19.5, which are equivalent to GDs 12−19 as the authors consider GD 0.5 to be the sperm-positive day, adjusted to facilitate comparison.

All of the other selected studies collected testes for RNA extraction, with the exception of Bowman et al. (2005), which collected WDs. Bowman et al. (2005) focused on the WD because they were interested in characterizing the mechanisms responsible for prenatal DBP-induced epididymal malformations. WD tissue from three to four fetuses was obtained to ensure enough RNA for analyses (see Table 5-3). Since WDs are the precursor of the vas deferens, epididymis, and seminal vesicles, the tissue assayed by Bowman et al. (2005) is different from the tissue evaluated in the other seven studies (RNA from the testes of 1−3 fetuses). The studies used a variety of toxicogenomic methodologies to assess changes in gene expression. General descriptions of these methods utilized by the studies were presented in Section 5.1.

An important consideration is the reliability of the data being generated and compared in these nine DBP studies. As discussed, the MAQC project (Shi et al., 2006) has recently completed a large study evaluating inter- and intraplatform reproducibility of gene expression

measurements (see Chapter 2). Six commercially available microarray platforms and three alternative gene expression platforms were tested. Both Affymetrix microarrays and RT-PCR assays were included in the MAQC testing. Affymetrix and the other one-color platforms showed similar coefficients of variation of quantitative signal values (5−15%) when used to detect 8,000 to 12,000 genes. When comparing variation within and between test sites, the one-color assays demonstrated 80−95% agreement.

Although it is difficult to compare expression values generated on different platforms because of differences in labeling methods and probe sequences, MAQC was able to show good agreement between the Affymetrix platform and the other platforms. This was particularly true when using the same biological sample (and, thus, removing variability introduced by the sample or sample preparation method). It is worth noting that Affymetrix displayed high correlation values with RT-PCR based on comparisons of ~500 genes. The results of the MAQC report suggest that the comparisons made in this case study are valid due to the reliability of the data. Additionally, since seven out of the nine experiments in the case study were performed in the same laboratory, interlaboratory variability is not an issue with these studies.

5.3. CONSISTENCY OF FINDINGS

In the assessment of consistency of findings, a potential source of incongruence is the decreased sensitivity for low-expression genes in the microarray platforms as compared to the gene expression technologies and differences in probe location.

5.3.1. Microarray Study Findings

An evaluation of the consistency across the four microarray studies of the testis was performed. Bowman et al. (2005) is not included because the microarray study results were not reported. In order to enhance comparability, the data from the whole testis microarray study of Plummer et al. (2007) are included in the evaluation, but the data from the microdissected regions of the testis are excluded because the lack of comparison to any other study.

Three of the four microarray studies used the same strain, SD, and all nine used the same species (rat). Plummer et al. (2007) was the only study to use the Wistar rat strain because it is considered more susceptible to effects on the testis than SD. Table A-1 in Appendix A includes those genes whose expression was reported to be significantly altered, as reported by Shultz et al. (2001), Thompson et al. (2005), Plummer et al. (2007) (for the whole testis only), or Liu et al.

(2005). Also presented in Table A-1 are the official gene names, exposure times, and directional response changes. It should be noted that some differences are to be expected in these comparisons because no two studies had identical study designs or platforms, or applied the same statistical cut-offs. For example, Thompson et al. (2005) used a very short duration of exposure, whereas the other three studies had longer exposure durations. In addition, the Affymetrix microarray platform was used only by Thompson et al. (2005) and Liu et al. (2005).

The three testis microarray studies (Plummer et al., 2007; Liu et al., 2005; Thompson et al., 2005) that used the "second generation chips" containing a much larger number of probes (therefore, covering many more genes) than the Clontech platform were compared. The Venn diagram, developed for these three studies, shows some unique gene expression changes for each study as well as a number of common gene expression changes (see Figure 5-1). Nevertheless, significant corroboration in the direction of effect among the common genes was observed in these three studies (see Appendix A). Additionally, most of the common genes were downregulated after *in utero* DBP exposure. Further, two genes in the steroidogenesis pathway, *Cyp11a1*, and *Scarb1*, are common among all four microarray studies. These findings indicate that the microarray data set for DBP is relatively consistent and findings are reproducible.

A number of genes involved in steroidogenesis (*Cyp11a1, Scarb1, Star,* and *Cyp17a1*) were found to be downregulated by DBP in all three studies (see Figure 5-1). Other genes significantly altered include a downregulation of the serotonin and catecholamine pathway enzyme, *Ddc*, and the myosin, heavy polypeptide 6, cardiac muscle, alpha (*Myh6*), and the androgen-regulated structural protein, *Svs5*.

Other genes were significantly altered in two of the three studies. For example, in comparing the results of the two studies that utilized the same platform (Affymetrix), the Liu et al. (2005) and Thompson et al. (2005) studies observed a downregulation of the steroidogenesis genes *Sqle* and *Hsd3b1_predicted,* cyclin-dependent protein kinase inhibitor *(Cdkn1c),* the cellular retinoic acid binding protein 2 *(Crabp2*), the FGF receptor activating protein 1 *(Frag1),* and the fatty acid binding protein *(Fabp3)*. These same two studies found upregulation of the steroidogenesis gene *Nr4a1*.

There are a number of genes for which the different studies found a similar significant alteration but the direction of effect varied. For example, GSH S-transferase, mu 2 (*Gstm2*), a gene involved in xenobiotic metabolism, was found to be significantly downregulated by Liu et

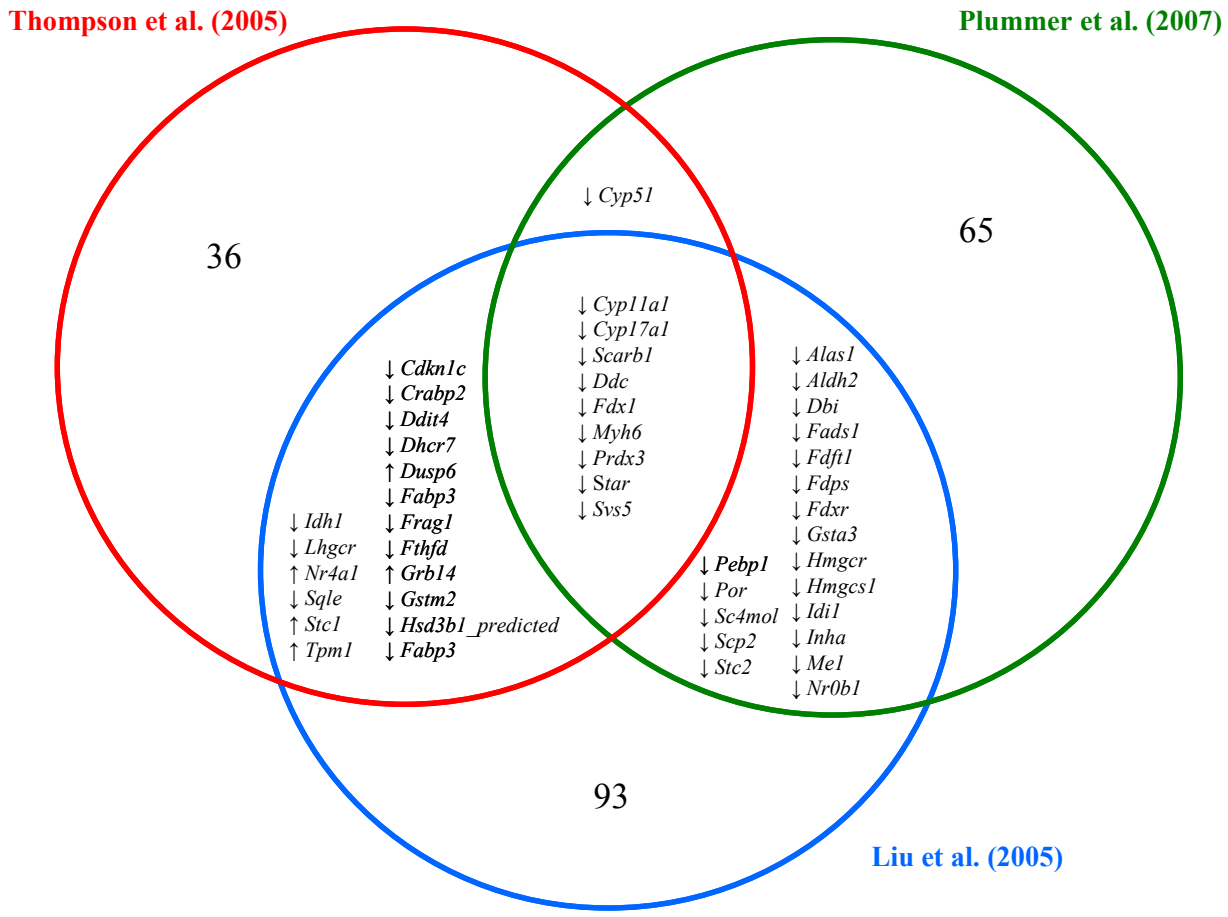

Figure 5-1. Venn diagram illustrating similarities and differences in significant gene expression changes observed in three recent microarray studies of the testes: Thompson et al. (2005), Plummer et al. (2007), and Liu et al. (2005). Numbers within each circle indicate genes whose expression was statistically significantly altered and that are unique to the study (i.e., not replicated by either of the other two studies). Gene numbers do not include expressed sequence tags (ESTs). The red circle indicates the Thompson et al. (2005) study; the green circle indicates the Plummer et al. (2007) study; and the blue circle indicates the Liu et al. (2005) study; Black arrows indicate the direction of effect, which was the same for all three of these studies.

al. (2005) and Thompson et al. (2005) and significantly upregulated by Shultz et al. (2001). The microsomal GSH S-transferase 1 gene (*Mgst1*) was downregulated in Liu et al. (2005) and upregulated in Shultz et al. (2001). Appendix A presents a table of the statistically significant gene expression changes in the Thompson et al. (2005), Shultz et al. (2001), Liu et al. (2005),

and Plummer et al. (2007) studies. These differences in microarray results can be explained by a number of factors including study differences (e.g., duration of exposure, platform, and rat strain) and/or variability of microarray study results.

Overall, the data indicate that there are some unique gene expression changes for each study as well as a number of common gene expression changes. Significant corroboration in the direction of effect among the common genes was observed in at least three studies. In addition, most of the common genes among these three studies were downregulated after *in utero* DBP exposure. These findings indicate that the microarray data set for DBP is very consistent and reliable although certain uncertainties remain when comparing data from different platforms with different study design.

5.3.2. RT-PCR Gene Expression Findings

Comparisons were also made of RT-PCR data (see Table A-2; Appendix A). All nine studies performed RT-PCR, and in the case of Liu et al. (2005), Shultz et al. (2001), Plummer et al. (2007), and Thompson et al. (2005), RT-PCR was performed following identification of the genes of interest from microarray studies. A number of genes were found to be similarly up- or downregulated by *in utero* DBP exposure. In the steroidogenesis pathway, five genes (*Cyp11a1, Cyp17a1, Hsd17b3, Scarb1,* and *Star*) were found to be downregulated by more than one laboratory. Some commonalities were also observed in altered gene regulation of transcription factors. *Egr1, Nfil3,* and *Nr4a1* were shown in two different studies to be upregulated. Two studies reported similar downregulation of *Nr0b1* and *Tcf1*.

Three studies (Plummer et al., 2007; Lehmann et al., 2004; Wilson et al., 2004) observed reduced *Insl3* gene expression. As discussed, *Insl3* has a role in sexual differentiation and testis descent. Reduced fetal *Insl3* has been shown to produce agenesis of the gubernacular ligaments. Two other genes have been shown to have DBP-induced altered expressions as assessed by RT-PCR in two laboratories: *Clu* (upregulated) and *Kit* (downregulated).

5.3.3. Protein Study Findings

All nine studies completed either Western analysis (immunoblotting) or immunohistochemistry to characterize fetal DBP-induced changes in protein expression. Usually, protein analysis was conducted for proteins that had demonstrated changes in mRNA

expression. However, up- or downregulation of genes and proteins does not always occur simultaneously, so a disparity between these two experimental results is quite common. Table 5-5 presents the protein-expression data from these studies.

Four proteins in the steroidogenesis pathway were shown to be downregulated by DBP exposure. These findings are fairly consistent with the gene expression data presented earlier. STAR was shown to be downregulated by Western blotting in three separate experiments, and by immunolocalization in another experiment. STAR expression was found only in LCs in both the control and DBP-treated testes, with the DBP-treated testes having decreased staining intensity (Barlow et al., 2003). Quantitatively, three experiments demonstrated reduced SCARB1 protein levels in DBP-treated fetal testes; however, immunolocalization showed DBP-induced increased staining of Sertoli cells and decreased staining of LCs. Both CYP11a1 and CYP17a1 protein levels were shown in several separate experiments to be reduced following DBP exposure, which correlated with microarray and PCR findings. Immunolocalization was completed for CYP11a1 and found to be downregulated in LCs (Plummer et al., 2007). Using immunolocalization, CLU was found to be increased in Sertoli cells and LCs. One study has shown that DBP lowers INSL3 protein immunoexpression levels in the fetal testis (McKinnell et al., 2005). The expression of NR5A1/SF1 was unchanged in Wistar rats, however, four proteins regulated by NR5A1 (CYP11a1, INHA, CRABP2, and PEBP) and AMH were reduced in LCs following DBP exposure (Plummer et al., 2007).

5.3.4. DBP Toxicogenomic Data Set Evaluation: Consistency of Findings Summary

A comprehensive summary of the DBP toxicogenomic data set assessed in this case study, including all microarray, RT-PCR, and protein data from the nine studies, is presented in Figure 5-2. The genes and protein included in the figure are limited to those for which two or more studies detected statistically significant results. In many cases, when comparing across RT-PCR and microarray studies, a DEG is found in one or even several studies that is not identified in another study. For example, *Kit* was downregulated in the Barlow et al. (2003),

Table 5-5. Evaluation of the published protein studies after DBP *in utero* exposure (testes only)

Official Gene Symbol	Protein Name Reported in Paper	Exposure Interval	Dose (mg/kg-d)	Method Used	Change in Protein Expression Compared to Controls	Reference
Amh	AMH	GDs 12−19	500	Immunolocalization	↑ slightly in Sertoli cells	Plummer et al., 2007
Bcl2	bcl-2	GDs 12−21	500	Immunolocalization	↑ in Sertoli and Leydig cells	Shultz et al., 2001
Bzrp	PBR	GDs 12−19	500	Immunolocalization	↓ in interstitial cells	Lehmann et al., 2004
Cebpb	CEBPB	GDs 12−19	500	Immunolocalization	↓ in interstitial cells	Liu et al., 2005
Crabp2	CRABP2 PEBP	GDs 12−19	500	Immunolocalization	↓ in Leydig cells	Plummer et al., 2007
Clu	TRPM-2	GDs 12−19 GDs 12−21	500	Immunolocalization	↑ in Sertoli and Leydig cells	Shultz et al., 2001
Clu	TRPM-2	GDs 12−19	500	Immunolocalization	↑ in Sertoli cells	Barlow et al., 2003
Cyp11a1	CYP11a1	GD 18 for 18 hrs	500	Western analysis	↓ (0.6 of control)	Thompson et al., 2005
Cyp11a1	P450ssc	GDs 12−19	500	Western analysis	↓ (0.5 of control)	Lehmann et al., 2004
Cyp11a1	P450ssc	GDs 12−17 or 18	500	Western analysis	↓ (0.15 at 24 hrs; 0.5 at 48 hrs)	Thompson et al., 2004
Cyp17a1	CYP17a1	GD 18 for 18 hrs	500	Western analysis	↓ (0.6 of control)	Thompson et al., 2005
Cyp17a1	CYP17	GDs 12−17 or 18	500	Western analysis	↓ (ND at 24 hrs; 0.4 of control at 48 hrs)	Thompson et al., 2004
Cyp17a1	cyp17	GDs 12−19	500	Western analysis	↓ (0.2 of control)	Lehmann et al., 2004
Ddc	Dopa decarboxylase	GDs 12−19	500	Immunolocalization	↓ in interstitial cells	Liu et al., 2005
Grb14	GRB14	GDs 12−19	500	Immunolocalization	↓ in interstitial cells and ↑ in Sertoli cells	Liu et al., 2005
Inha	INHA	GDs 12−19	500	Immunolocalization	↓ in Leydig cells	Plummer et al., 2007
Insl3	Insl3	GDs 12−19	500	Immunolocalization	↓ in interstitial cells	Lehmann et al., 2004
Kit	c-kit	GDs12−19	500	Immunolocalization	↓ in gonocytes	Barlow et al., 2003
Kitl	SCF	GDs 12−19	500	Immunolocalization	↑ in Sertoli cells	Barlow et al., 2003

Table 5-5. (continued)

Official Gene Symbol	Protein Name Reported in Paper	Exposure Interval	Dose (mg/kg-d)	Method Used	Change in Protein Expression Compared to Controls	Reference
Nr0b1	DAX-1	GDs 12−19	500	Immunolocalization	↓ in gonocytes	Liu et al., 2005
Pebp	PEBP	GDs 12−19	500	Immunolocalization	↓ in Leydig cells	Plummer et al., 2007
Scarb1	SCARB1	GD 19 for 6 hrs or GD 18 for 18 hrs	500	Western analysis	↓ (0.3 of control)	Thompson et al., 2005
Scarb1	SR-B1	GDs 12−17 or 18	500	Western analysis	↓ (0.15 at 24 hrs; (0.7 of control at 48 hrs)	Thompson et al., 2004
Scarb1	SR-B1	GDs 12−19	50, 100, 500	Western analysis	↓ (0.6, 0.5, and 0.1 of control)	Lehmann et al., 2004
Scarb1	SRB1	GDs 12−19	500	Immunolocalization	↓ in Leydig; ↑ in Sertoli cells	Barlow et al., 2003
Star	STAR	GD 18 for 18 hrs	500	Western analysis	↓ (0.4 of control)	Thompson et al., 2005
Star	STAR	GDs 12−17 or 18	500	Western analysis	↓ (ND at 24 hrs; 0.4 of control at 48 hrs	Thompson et al., 2004
Star	STAR	GDs 12−19	50, 100, 500	Western analysis	↓ (0.1, 0.2, 0.1 of control)	Lehmann et al., 2004
Star	STAR	GDs 12−19	500	Immunolocalization	↓ in Leydig cells	Barlow et al., 2003
Testin	testin	GDs 12−19	500	Immunolocalization	↑ in Sertoli cells and gonocytes	Liu et al., 2005

ND, not detected.

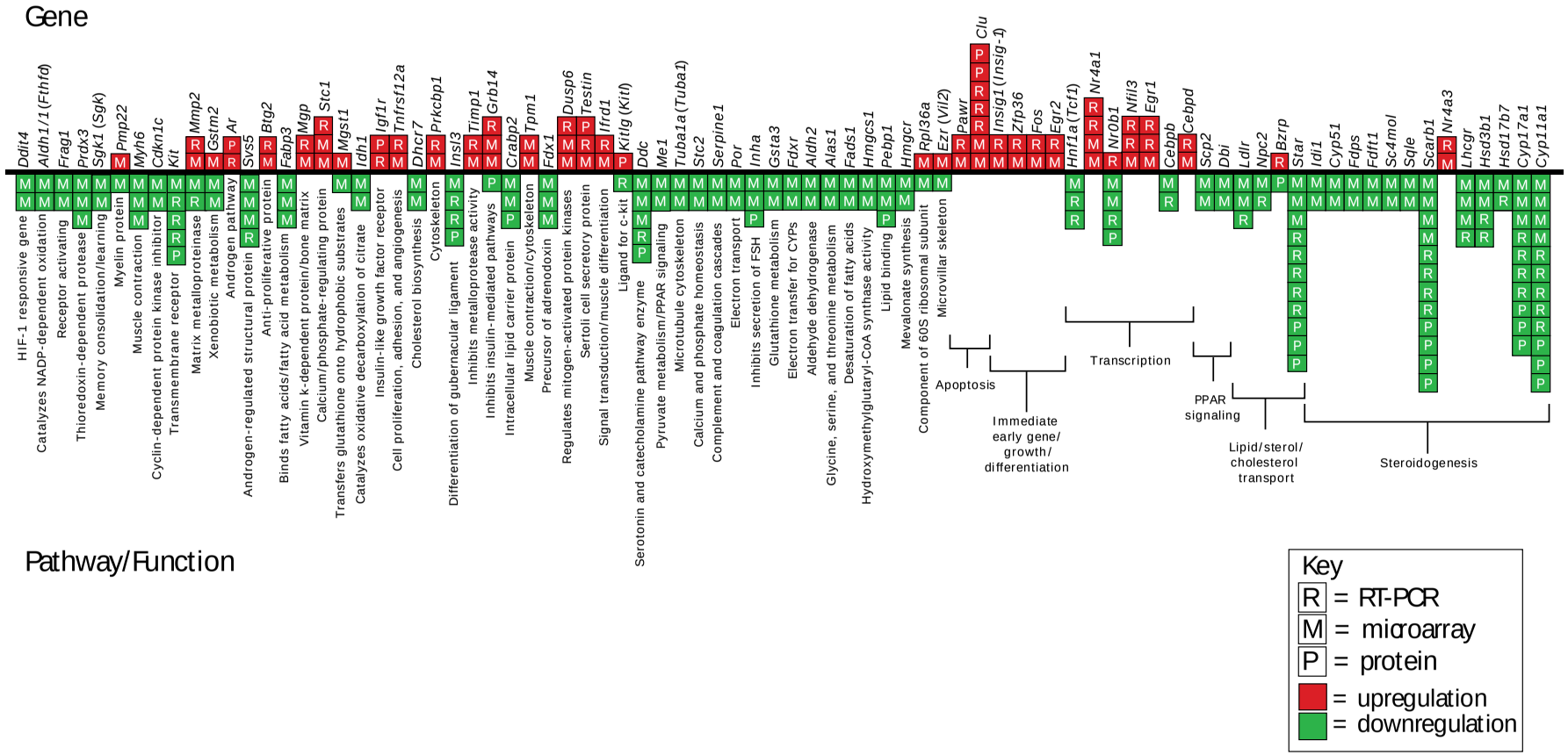

Figure 5-2. Summary of DBP-induced changes in fetal gene and protein expression. M = microarray; R = RT-PCR; P = protein. Red indicates upregulation; green indicates downregulation. Genes and protein included in the figure are limited to genes that were statistically significantly altered in two or more studies. Gene symbols are indicated at the top of the figure. The pathway or function of each gene is listed on the bottom of the figure. This information has been taken from the case-study articles or from the DAVID (Database for Annotation, Visualization and Integrated Discovery http://david.abcc.ncifcrf.gov/list.jsp) entry for that gene.

Lehmann et al. (2004), and Schultz et al. (2001) studies; by contrast, it was not altered significantly in the Liu et al. (2005) study even though it is represented on the Affymetrix array.

Data from the Bowman et al. (2005) paper were not included because it evaluated changes in DBP-induced gene expression in the WD rather than testes. There are no other WD studies for comparisons. If an increase or decrease was reported at any time point, it was included. Multiple time points from the Thompson et al. (2005) study were not all included; if several time points showed a change, then it was recorded as one study showing a change. For protein data, descriptions of immunohistochemical studies suggesting an increase, though without real quantitation, were still counted. For the dose-response study (Lehmann et al., 2004), data from only the 500 mg/kg-d dosing were used to allow better comparisons with the other studies.

Figure 5-2 presents a summary of the changes in gene and protein expression following *in utero* DBP exposure across studies. What is most striking is the consistency of evidence for the DBP-induced downregulation of the steroidogenesis pathway. Both microarray and RT-PCR analysis show consistent downregulation of *Cyp11a1, Cyp17a1, Star*, and *Scarb1* mRNA expression. Protein expression of *Cyp11a1, Cyp17a1, Star*, and *Scarb1* is concurrently downregulated. Downregulation of both *Hsd3b* and *Lhcgr* mRNA expression is demonstrated consistently. Significantly, two genes involved in lipid/sterol/cholesterol transport, *Npc2* and *Ldlr*, also show downregulation. Three transcription factors (*Nfil3, Egr1,* and *Nr4a1*) demonstrate DBP-induced upregulation, while two genes (*Nr0b1* and *Tcf1*) show downregulation in a number of experiments. Three immediate early genes (*Fos, Egr2*, and *Zfp36*) are upregulated by DBP exposure. Interestingly, *Clu* (also known as T repressed prostate message-2) is upregulated, as shown by two microarray, two RT-PCR, and two protein assays.

5.4. DATA GAPS AND RESEARCH NEEDS

Based on the evaluation of the nine toxicogenomic studies, a number of research needs became apparent. There are genomic data gaps for many environmental chemicals. For DBP, confirmatory RT-PCR studies for all of the genes identified from microarray studies, would give additional credence to the microarray results. Similarly, additional protein analysis, with quantitation by Western blotting and with immunolocalization, could further characterize DBP-induced effects on the male reproductive system. Looking at DBP-induced changes in

gene expression in additional reproductive and nonreproductive (Thompson et al., 2005) tissues could also add information about mechanism(s) of action and tissue specificity. As testes are comprised of a number of cell types, evaluating additional homogeneous cell populations within the testes, as Plummer et al. (2007) reported, could be useful.

In order to fully consider the question about informing the modes or mechanism of action (see Chapters 1 and 3), using the toxicogenomic data to determine whether there are other MOAs responsible for some of the male reproductive developmental effects, we decided that it would be helpful to analyze the raw data to assess all affected pathways. The published studies, while all of excellent quality, focused their pathway analyses and descriptions on particular pathways of interest to basic science. The following section describes efforts to reanalyze some of the DBP microarray studies with this goal in mind.

5.5. PATHWAY ANALYSIS OF DBP MICROARRAY DATA

We determined that it would be advantageous to reanalyze the raw data utilizing multiple analytical approaches (see Figure 3-1) because most of the DBP microarray studies in the published literature were focused on further delineation of the mechanism of action relevant to one MOA, the reduction in fetal testicular T. In fact, it was the microarray and RT-PCR study results that identified the modulation of the steroidogenesis pathway as leading to reduced fetal testicular T, one of the DBP MOAs, and then, leading to a number of the male reproductive developmental effects. Further, a second DBP MOA of reduced *Insl3* gene expression has also been identified (Wilson et al., 2004; see Chapter 3) leading to testis descent defects. Not all pathways for the identified DEGs were discussed (or presented) in detail in the published studies because of this focus. Therefore, a reanalysis that looks more broadly to define all pathways affected by DBP may inform additional pathways related to MOAs that could be linked to the unexplained male reproductive developmental outcomes identified in Chapter 4. Thus, the purpose of this reanalysis of the existing data set was to identify and characterize additional molecular pathways affected by DBP, beyond a reduction in fetal T and *Insl3* gene expression.

5.5.1. Objective of the Reanalysis of the Liu et al. (2005) Study

The goal was to reanalyze DBP microarray data to address the Case Study Question: *Do the genomic data inform DBP additional MOAs and the mechanism of action for the male*

reproductive developmental effects? The purpose for the reanalysis of the existing data sets is to identify and characterize additional molecular pathways affected by DBP, beyond the effects on the androgen-mediated male reproductive developmental toxicity pathways. This exercise was designed to generate hypotheses about mechanisms/pathways that could underlie the unexplained testicular endpoints after *in utero* DBP exposure (see Chapter 4).

The Liu et al. (2005) study was selected for reanalysis because the data set had a comprehensive exposure scenario that covered the critical window for developmental exposure to DBP (GDs 12−19). The Affymetrix chip was used (compatible with the proprietary and free software programs used for pathway-level analysis), and the data were provided by Dr. Kevin Gaido, a collaborator on this project. Some limitations of the Liu et al. (2005) data set are the small number of samples (i.e., 3 controls and 3 DBP-treated) and the lack of characterization of variance for treated and control. This study was a comparative analysis of six phthalate esters. However, only the DBP treatment and vehicle control data were used for this analysis. The Liu et al. (2005) study investigated global gene expression in the fetal testis following *in utero* exposure to a series of phthalate esters, including both developmentally toxic phthalates (DBP, BBP, DPP, and DEHP) and nondevelopmentally toxic phthalates (DMP, DEP, and DOTP) (Liu et al., 2005). The original analysis was based on a two-way nested ANOVA model using Bonferroni correction that identified 391 significantly expressed genes from the control out of the approximately 30,000 genes queried. In their analysis, two classes of phthalate esters were distinguished based on the gene expression profiles. The authors also showed that developmentally toxic phthalates targeted gene pathways associated with steroidogenesis, lipid and cholesterol homeostasis, insulin signaling, transcriptional regulation, and oxidative stress. We can assume that the differentially expressed genes in common among the "developmental phthalates" assessed in the Liu et al. (2005) study are due to phthalate exposure and not general toxicity, providing internal positive controls.

5.5.2. Pathway Analysis of Liu et al. (2005) Utilizing Two Different Methods to Generate Hypotheses for MOAs Underlying the Unexplained Testes Endpoints

Pathway analysis methods and software have been previously developed for analysis of microarray data for basic and applied research. Pathway-level analysis mainly depends on the definition of the pathways (database) and significance level uses to measure the differential expressions. Using these validated methods, a pathway analysis was performed. Differentially

expressed genes that were input into the pathway analysis (GeneGo) were identified by two different methods, Signal-to-Noise Ratio (SNR) and Rosetta Error Model (REM). By assessing the intersection of the pathways identified by each approach provides a more conservative list of pathways than using one approach. The overall process for generating hypotheses about pathways that may be relevant to the testis endpoints using pathway analysis is illustrated in Figure 5-3.

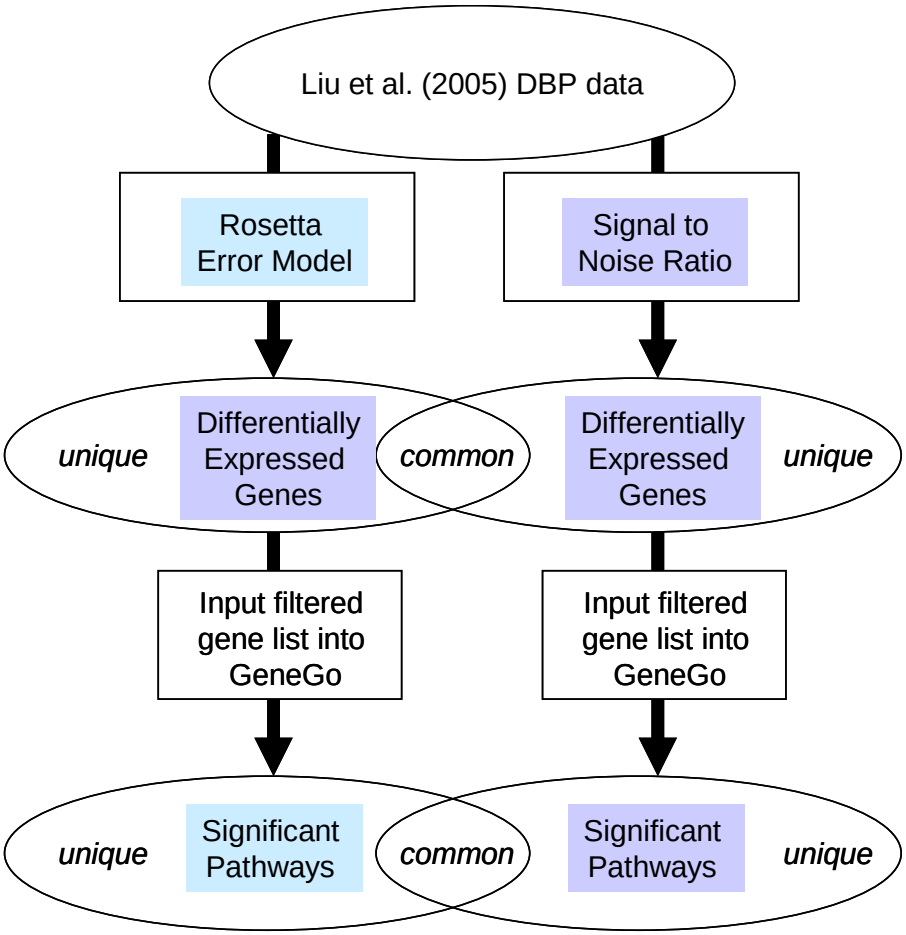

Figure 5-3. Schematic of the two analysis methods (REM and SNR) for identifying differentially expressed genes and subsequent pathway analysis using GeneGo. Two separate analyses, REM and SNR statistical filters, were performed to identify common and unique genes from the Liu et al. (2005) data. The two separate filtered gene lists were input into GeneGo to identify statistically significantly affected pathways. Common and unique pathway lists were generated.

5.5.2.1. *Two Methods for Identifying Differentially Expressed Genes (DEGs)*

5.5.2.1.1. *Rosetta error model (REM)*

The data set for the vehicle-treated and DBP-treated samples were input into the proprietary software, Rosetta Resolver. A principal component analysis (PCA) of the entire data set shows a distinct treatment response (i.e., the control and treated samples are clearly separated into two distinct groups) but also demonstrates the variance in the data set between similarly treated samples.

Next, the gene expression data were normalized using error-model algorithm in Rosetta Resolver®, in part, because this software was available, but more importantly, because we performed an internal evaluation of this algorithm compared to four other normalization methods. The REM is a method for identifying DEGs that takes into account the variance of the color intensity outputs from microarray studies. The error model conservatively estimates intensity error and uses this approach to decrease the likelihood of identifying a change in gene expression that is the result of intensity variance. When the results of REM were compared to t-test and fold-change methods, the REM provided higher detection power (Weng et al., 2006).

The Rosetta Resolver system is a comprehensive gene expression analysis solution that incorporates analysis tools with a robust, scalable database. Using the reference microarray data set, Choe et al. (2005) compared a number of normalization methods including the quantile, constant, invariant set, Loess, and error models. Receiver-operator characteristic curves were generated to evaluate the sensitivity and specificity. Results showed that the REM identified 40-50% more true positives compared to the other four methods (personal communication on June 2009 between Bill Ward [EPA/NHEERL] and Susan Hester [EPA/NHEERL]).

The annotated genes of the rat genome on the Affymetrix gene chip, ~30,000 genes, were input into the significance analysis using the Benjamini and Hochberg false discovery rate (FDR) for multiple testing correction applied at $p < 0.01$, a relatively stringent statistical cut-off. Of the ~30,000 genes, the analysis passed 118 genes as being significantly altered following DBP exposure. Of these, 17,496 genes did not pass the statistical filter and 13,428 genes were not affected by the treatment. One possible reason that only 118 genes passed the multiple-testing correction filter is that there is a high variance between individual samples, as demonstrated by the PCA.

Using the error-model algorithm analysis, the filtering criterion was changed to $p < 0.05$ without applying an FDR because so few genes passed the $p < 0.01$ plus FDR filter which would be limiting for pathway-analysis purposes. It is often the case that after correcting for multiple hypothesis testing, few or no genes pass the threshold of statistical significance because the biological variances are modest relative to the noise inherent in a microarray experiment (Tomfohr et al., 2005). In performing DEG and pathway analysis, professional judgment is required to determine when to use a highly stringent statistical significance filter and when to focus on the available information regarding the biological significance of gene expression changes. We considered it appriopriate to use a $p < 0.05$ without applying an FDR in order obtain a greater number of genes because the objective was to perform a pathway analysis in order to gain new information about DBP toxicity. The DEGs identified using the REM are shown in Table A-3 in Appendix A.

The set of 1,977 genes was deemed suitable to perform a comprehensive pathway-level analysis because about one third of the DEGs (999) did not meet the statistical cut-off criteria (a p-value ≤ 0.05). The list of 1,977 genes was input into the data analysis software program, GeneGo, for pathway analysis. MetaCore's™ analytical tools enable the identification and prioritization of the most relevant pathways, networks, and cellular processes affected by a given treatment.

5.5.2.1.2. *Signal-to-noise ratio (SNR)*

We also identified DEGs by analyzing the Liu et al. (2005) data via SNR (Golub et al., 1999), a method that differentiates between gene expression levels of two sample groups relative to the standard deviation within each group. Consequently, a high SNR indicates that the two sample groups are statistically more distinct whereas a low SNR indicates that the two sample groups are less statistically distinct.

For a given gene, g_i, SNR_{g_i} is evaluated as in Eq. 5-1

$$SNR_{g_i} = \frac{\left|\mu\left(g_{i,1}\right) - \mu\left(g_{i,2}\right)\right|}{\sigma\left(g_{i,1}\right) + \sigma\left(g_{i,2}\right)} \tag{5-1}$$

where the means and standard deviations of the expression levels of gene g_i are evaluated for the samples in group 1, $g_{i,1}$ (control), and group 2, $g_{i,2}$ (DBP treated).

SNR is used in quantitative noise analysis for microarray experiments (Tu et al., 2002) and feature selection in classification gene expression studies (Goh et al., 2004; Shipp et al., 2002). Here, SNR enables us to rank genes based on the assumption that genes whose expression is related to DBP treatment should exhibit higher SNR values than genes whose expression is unaffected by DBP. In order to identify DEGs, we evaluated a permutation test. The multiple testing of ~30,000 gene expressions poses a problem as the probability of Type I errors increases with the number of hypotheses (Dudoit et al., 2003). To address this issue, we executed thousands of comparisons by randomly permuting the gene expression levels from the chip for each gene expression. Following this randomization process, *p*-values were obtained as the fraction of the randomized SNR values that are higher than the actual SNR. The genes that were assigned a p-value < 0.05 were characterized as DEGs (see Appendix A; the algorithm for selecting DEGs [Figure A-1] and the list of identified DEGs [Table A-4]). 1,559 probe sets were identified as DEGs. The heat map (see Figure 5-4) illustrates the distinction between the control and DBP treated samples.

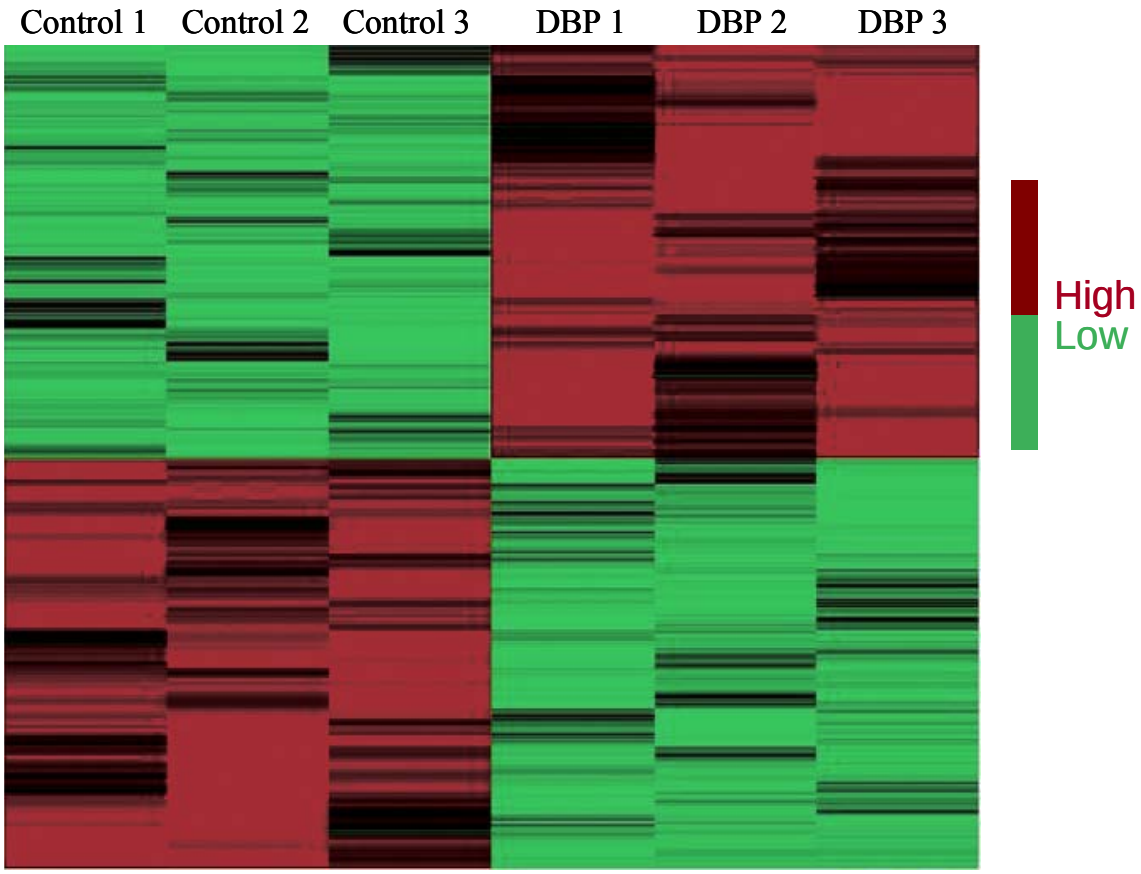

Figure 5-4. Heat map of 1,577 DEGs from SNR analysis method. The three lanes on the left are vehicle treated and the three lanes on the right are DBP treated. Data used for analysis from Liu et al. (2005). Control 1-3 lanes correspond to three replicate control samples. DBP 1-3 lanes correspond to three replicate DBP-treated samples. Rows represent the different 1,577 DEGs. The color red represents upregulation of gene expression, and green represents downregulation of gene expression.

5.5.2.2. *Pathway Analysis*

Analysis of DBP toxicogenomic studies was carried out using many proprietary databases and software packages with enhanced bioinformatic capabilities for pathway and functional level analysis (Rosetta Resolver, MetaCore GeneGo, Ingenuity® Pathway Knowledgebase). These software tools accept lists of genes of interest and then, using their database of knowledge about these gene elements, map them to cellular pathways known to exist from experimental literature. The advantage of trying to understand groups of genes acting in the same cellular process, such as the cell cycle, is that effects on a pathway or biological process likely provide meaningful biological information. In contrast, information about effects on expression of one gene does not necessarily capture the relationship of the exposure to a

chemical on a biological process or pathway. The rationale behind the exercise was that interrogation of multiple databases would result in a more complete mining of the microarray data sets, which may provide an understanding of all of the potential DBP MOAs underlying the testes reproductive developmental effects. Analysis using different statistical tools provides information about the similarities and differences in results.

The GeneGo analysis normalized data set revealed that 131 biological processes ($p < 0.05$) were associated with the 1,977 DEGs. The pathways with a $p < 0.05$ using the Rosetta Error Model (REM) are listed in Appendix A (see Table A-5). Comparisons made on the level of gene lists obtained by different statistical methods often do not converge (Manoli et al., 2006). We decided to perform a comparison of methods based on the assumption that biologically related groups of genes, such as metabolic or signaling pathways, may be more valid if identified using different microarray analysis methods. Towards this effort, we input the gene list (1,559 genes) using SNR to a pathway-level analysis using GeneGo, similar to the analysis performed on the REM results. The pathway-analysis results of significant genes identified by SNR are listed in Table A-6 of Appendix A. Table 5-6 lists the common pathways when two different statistical filters for DEGs were conducted using the GeneGo pathway analysis (i.e., the union of the two separate pathway lists; see Tables A-5 and A-6). In addition to the already established changes in the steroidogenesis pathway, this analysis highlights biological processes and pathways that are affected by DBP exposure to fetal testis. An assessment of linkages between the unique pathways and processes identified to the DBP-induced male reproductive toxicity outcomes can be made by querying the published literature.

Table 5-6. Common pathways between the REM and SNR analyses of differentially expressed genes (DEGs) after *in utero* DBP exposure from the Liu et al. (2005) data[a,b,c]

Biological Process	Pathways
Cell adhesion	Cytoskeleton remodeling
	ECM remodeling
	Endothelial cell contacts by junctional mechanisms
	Ephrins signaling
	Integrin inside-out signaling
	Integrin outside-in signaling
	Integrin-mediated cell adhesion
	Reverse signaling by ephrin B
Cell signaling*	Activation of PKC via G-Protein coupled receptor
	CCR3 signaling in eosinophils
	ChREBP regulation pathway
	G-Protein beta/gamma signaling cascades
	G-Proteins mediated regulation p. 38 and JNK signaling
	Leptin signaling via JAK/STAT and MAPK cascades[2]
	Regulation of actin cytoskeleton by Rho GTPases
	Role PKA in cytoskeleton reorganization
Disease	NF-AT signaling in cardiac hypertrophy
	NTS activation of IL-8 in colonocytes
Growth and differentiation	Angiotensin activation of ERK
	Angiotensin signaling via STATs
	EPO-induced Jak-STAT pathway
	MAG-dependent inhibition of neurite outgrowth
	Regulation of acetyl-CoA carboxylase 2 activity in muscle
	WNT signaling pathway
Hormones	Ligand-dependent activation of the ESR1/SP pathway
Immune response	MIF - the neuroendocrine-macrophage connector
	CXCR4 signaling pathway

Table 5-6. (continued)

Biological Process	Pathways
Metabolism*	***Androstenedione and testosterone biosynthesis and metabolism p.1***[2]
	Cholesterol biosynthesis[2]
	Cholesterol metabolism[2]
	dATP/dITP metabolism
	dGTP metabolism
	Estrone metabolism
	Fructose metabolism
	G-alpha(q) regulation of lipid metabolism
	Gamma-aminobutyrate (GABA) biosynthesis and metabolism
	Glutathione metabolism
	Glycolysis and gluconeogenesis (short map)
	Glycolysis and gluconeogenesis p. 1
	Glycolysis and gluconeogenesis p. 2
	Histamine metabolism
	Histidine-glutamate-glutamine and proline metabolism
	Leucine, isoleucine and valine metabolism p. 2
	Lysine metabolism
	Mitochondrial ketone bodies biosynthesis and metabolism
	Mitochondrial long chain fatty acid beta-oxidation
	Mitochondrial unsaturated fatty acid beta-oxidation
	Peroxisomal branched chain fatty acid oxidation
Metabolism*	Phenylalanine metabolism
	PPAR regulation of lipid metabolism[2]
	Propionate metabolism p.1[2]
	Propionate metabolism p.2[2]
	Regulation of fatty acid synthesis: NLTP and EHHADH
	Regulation of lipid metabolism by niacin and isoprenaline
	Regulation of lipid metabolism via LXR, NF-Y, and SREBP[2]
	Regulation of lipid metabolism via PPAR, RXR, and VDR[2]
	Serotonin—melatonin biosynthesis and metabolism
	TCA
	Triacylglycerol metabolism p.1
	Tryptophan metabolism

Table 5-6. (continued)

Biological Process	Pathways
Transcription*	Brca1 as transcription regulator
	Role of VDR in regulation of genes involved in osteoporosis
	Transcription factor Tubby signaling pathways

[a]Statistically significant gene lists from SNR and REM methods were input into the GeneGo pathway analysis program (www.genego.com). The Gene ontology process/pathway list was generated using a cut-off of $p < 0.05$ for each analysis. From those lists, the common pathway list was generated.
[b]Pathways that are part of, or overlap with, the testosterone synthesis pathways are indicated by bold italics. These pathways were identified by performing a PubMed literature search (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed) for "testosterone" and the name of each pathway (listed in the table).
[c]Entrez Gene indicates that *Insl3* is the ligand for the LGR8 receptor, but the *Insl3* pathway is not fully defined (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=114215&ordinalpos=3&itool=EntrezSystem2.PEntrez.Gene.Gene_ResultsPanel.Gene_RVDocSum).
*Biological processes identified in Liu et al. (2005).
Functions shown to be related to the *Insl3* pathway are G-protein-coupled receptor binding and hormone activity. Processes identified are G-protein signaling, adenylate cyclase inhibiting pathway, gonad development, *in utero* embryonic development, male gonad development, negative regulation of apoptosis, negative regulation of cell proliferation, oocyte maturation, positive regulation of cAMP biosynthetic process, and positive regulation of cell proliferation. While a number of G-protein pathways were identified in this analysis, none are considered exclusive to *Insl3* and are, therefore, not listed in bold italics.

Cholesterol biosynthesis/metabolism and associated pathways underlie one of the MOAs of DBP. To determine a metric for statistical analysis protocols of toxicogenomic data, we chose to compare the genes that are involved in the cholesterol biosynthesis/metabolism as identified by the three independent analysis methods (described herein) and the published data set from Liu et al. (2005) (see Table 5-7). These results show that there is a high degree of overlap in the most biologically relevant pathway/process involved in DBP toxicity, even when different statistical procedures are used for analysis of the same data set. These are in agreement with the published literature, giving the approaches used in this exercise biological confidence.

By utilizing databases such as GeneGo, additional canonical pathways and biological processes were identified that may play an important role in DBP male reproductive developmental toxicity. Regulation of steroidogenesis requires multiple signaling pathways and growth factors (Stocco et al., 2005). Signaling pathways, like the protein kinase C pathway, arachidonic acid metabolism, growth factors, chloride ion, and the calcium messenger system are

Table 5-7. Genes involved in cholesterol biosynthesis/metabolism that were identified by both the REM and SNR analyses of Liu et al. (2005)

REM (GeneGo)	SNR (GeneGo)	SNR (KEGG)
	Acat1	*Acat1*
Cyp27a1		
Cyp51a1	*Cyp51a1*	
Cyp7b1		
Dhcr7	*Dhcr7*	*Dhcr7*
	Dhcr24	
	Ebp	*Ebp*
	Fdft1	*Fdft1*
	Fdps	*Fdps*
Hmgcr	*Hmgcr*	*Hmgcr*
Hmgcs1	*Hmgcs1*	*Hmgcs1*
Hsd11b1		
Hsd3b1		
Idi1	*Idi1*	*Idi1*
	Mvd	*Mvd*
	Nsdhl	
Sqle	*Sqle*	*Sqle*
Sc4mol	*Sc4mol*	
Soat1		
	Tm7sf2	

capable of regulating/modulating steroid hormone biosynthesis. It is possible that some of the pathways and processes identified by the two methods may play a role in the regulation of steroidogenesis, a pathway that underlies one of the well-established MOAs by DBP. Another scenario could be that these pathways and processes have yet to be associated with DBP-induced toxicity. The androstenedione and T biosynthesis and metabolism pathway was one of the common pathways in the GeneGo analysis of the two different methods gene list (see Figure 5-5).

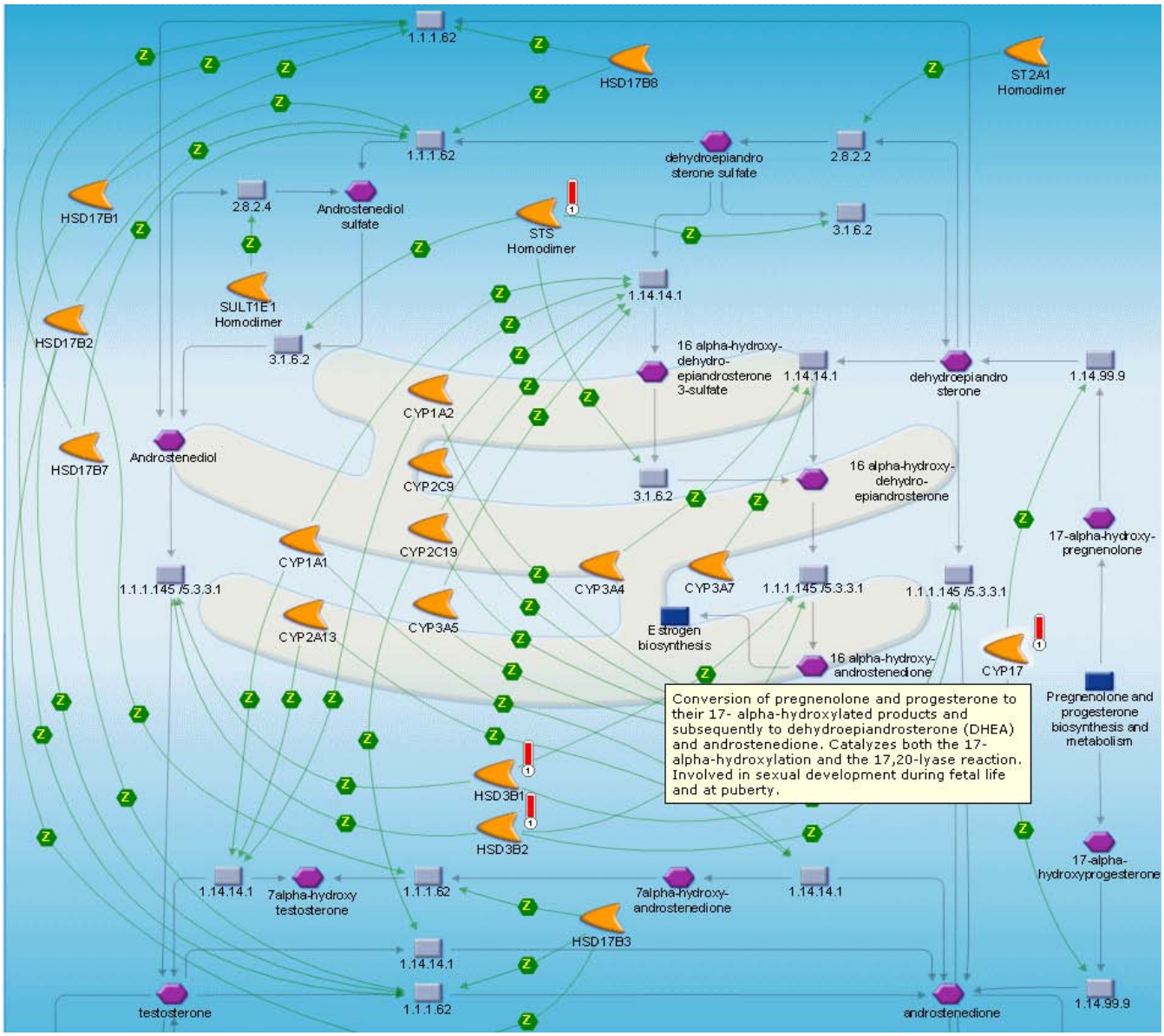

Figure 5-5. Mapping the Liu et al. (2005) data set onto the canonical androstenedione and testosterone (T) biosynthesis and metabolism pathway in MetaCore (GeneGo). Key enzymes activated by DBP are identified by red thermometers.

It has been reported in the literature (MAQC-I, see Chapter 2) that the results of microarray experiments often depend on the data analysis protocol and the biological pathway-analysis tools available to interpret the list of statistically significant genes. Dissimilar sets of gene expression signatures with distinct biological contexts can be generated from the same raw data by different data analysis protocols. Distinct biological contexts can also be generated from

the same gene expression signatures by different biological pathway protocols. Therefore, it becomes important to determine and understand the relationship between the gene expression and pathway changes and a biological outcome of interest.

In order to do a thorough investigation, it is necessary to use many sources of gene and pathway annotation. The intent of using multiple sources is to gain an enriched analysis. In practice, analysis is carried out with the suite of tools available to the analyst. In this case, the STAR Center primarily used KEGG (a resource rich in enzymatic and metabolic reactions but weak in signaling pathways); whereas the EPA used Rosetta Resolver, GeneGo, and Ingenuity Pathway Analysis, resources that are populated with signaling as well as metabolic pathways.

This exercise demonstrates that multiple approaches to microarray data analysis can yield similar biologically relevant outcomes and some differences. The differences observed in the results could be due to a number of factors including (1) the different data normalization procedures used in the two separate analyses; and (2) different data interpretation tools such as the software for pathway analyses. However, it cannot be ruled out that the differences may reflect differences in biological significance (i.e., one approach is superior).

We performed a number of reanalyses of the Liu et al. (2005) data because the pathway analysis presented in the article was not performed for risk assessment purposes. While the authors of this and other microarray studies support two MOAs for DBP, a reduction of fetal testicular T via affects on steroidogenesis and cholesterol transport genes, not all pathways associated with the differentially expressed genes were discussed in detail.

Two different bioinformatics tools to analyze the same data were utilized. Each analysis used multiple statistical filters to parse the noise from the signal in the microarray data set and to assess the quality of the data set. Ideally, for a high-quality study data set, there would be a minimum of variance between similarly treated samples, and the variance would lie between the control and treated sample data. PCA shows the quality of the Liu et al. (2005) data set to be of moderate quality based on the observed variance among similarly treated data sets (control and treated groups). One analysis utilized multiple proprietary software packages (GeneGo, Rosetta Resolver). The rationale for looking at the effect of DBP on the pathway level, as opposed to a cluster of genes, is that DBP is most likely affecting multiple pathways within a cellular environment. This exercise allowed us to generate a list of affected common pathways between the two methods, and in this way, provided more confidence about these pathways.

The results of the new pathway analyses both corroborate the previously identified two MOAs for DBP male reproductive development toxicity, and provide putative novel pathways affected by *in utero* DBP exposure that may play a role in DBP-mediated toxicity. The results of the new pathway analyses provide hypotheses for MOA that could be tested in new experimental studies. Future research could investigate the role of these pathways in DBP-induced toxicity. In addition, a gene network was developed for DBP based on the Liu et al. (2005) data. The GeneGo analysis corroborated prior findings for the role of the steroidogenesis pathway and identified the modulation in *Cyp17* and *Ar* that are involved in the androgen biosynthetic process. This is a new hypothesis that requires follow-up with new studies to confirm this observation. Performing new analyses was useful for the purposes to further our understanding of the DBP mechanism of action.

Analyzing any given data set multiple ways and arriving at the same conclusion provides confidence in the analytical approach; however, there is no "gold standard" analytical method. Applying stringent statistical filters in pathway analysis (e.g., $p < 0.05$, Benjamini Hochberg multiple testing correction) can limit the number of genes that are identified. Interpretation of the biology of the system using only a limited gene set is restrictive. It is important to remember that the genes that do not pass the statistical stringency cut-off that may be crucial for understanding the biology of the system, as statistical significance and biological significance are not necessarily the same. Therefore, it becomes incumbent upon the researcher to analyze the data in multiple ways in order to maximize the benefits of microarray data.

In summary, by identifying differentially expressed genes by two different approaches, performing pathway analysis, and compiling a list of common pathways between the two approaches, a list of corroborated pathways has been identified. The pathways (see Table 5-6) and processes identified have some overlap with those presented in the Liu et al. (2005) article as well as some differences. Comparisons of our results to those of Liu et al. (2005) are difficult because they presented differentially expressed genes and their associated process, not pathways. In Liu et al., 2005, oxidative stress and cytoskeleton processes were unique findings. Our results identified cell adhesion, disease, immune response, hormone, and growth and differentiation processes as unique findings. In addition, the reanalysis of the Liu et al. (2005) study identified common and unique pathways (see Table 5-6) with the tabulation of affected pathways from the published literature that we performed including all of the DBP gene expression studies (see

Table 5-2). This exercise has generated hypotheses about mechanisms/pathways that could underlie the unexplained testicular endpoints after *in utero* DBP exposure (see Chapter 4) that need to be tested in additional studies.

5.6. CONCLUSIONS

In this chapter, evaluations of the published studies and a reanalysis of pathways from one microarray study was performed. Nine toxicogenomic studies from the published literature were evaluated for study comparability and study result consistency. This was done by utilizing Venn diagrams and a visual method for looking at the consistency across all of the gene expression studies (see Figure 5-2). These methods could be applied in a new assessment for a chemical with genomic data.

The reanalysis of the Liu et al. (2005) data set provides some examples of methods for identifying differentially expressed genes and performing pathway analysis using either proprietary or publicly available methods and databases. In performing the reanalysis, hypotheses were generated about possible pathways underlying some of the known and unknown MOAs for the testes outcomes observed after *in utero* DBP exposure.

6. EXPLORATORY METHODS DEVELOPMENT FOR ANALYSIS OF GENOMIC DATA FOR APPLICATION TO RISK ASSESSMENT

6.1. OBJECTIVES AND INTRODUCTION

The overall goal of this chapter is to describe exploratory methods developed for analyzing and applying toxicogenomic data in risk assessment. The three objectives of the methods development projects were to

1. *Explore the development of new methods to analyze microarray data for application to risk assessment.*

   The motivation was to develop methods for performing gene expression analyses of microarray data for use in risk assessment. Microarray studies for basic research purposes do not necessarily require as high a level of stringency as for risk assessment purposes because the analyses are often performed to generate hypotheses (e.g., for developing MOA hypotheses) that are subsequently tested in additional studies.

2. *Utilize existing DBP genomic data to develop a temporal gene network model for use in risk assessment.*

   We asked whether there are data to understand gene expression changes over time. By modeling the gene and pathway interactions across the critical window of exposure to DBP, it may be possible to understand the relationships among genes and pathways over time, and possibly, to identify the initiating event(s) for the decreases in fetal testicular T or *Insl3* expression. Identifying the initiating event would be very useful to risk assessment, as this would provide a biologically significant gene whose expression is critical to the outcome.

3. *Utilize genomic and other molecular data to address the Case Study Question: Do the toxicogenomic data inform interspecies differences in TD?*

   We utilized the available gene sequence data, protein sequence, and pathway cross-species data to assess the rat-to-human conservation of the genes involved in the steroidogenesis pathway that underlie the reduced fetal testicular T MOA for DBP.

The work to address the objectives of this chapter is the result of a collaborative effort among scientists at the STAR Bioinformatics Center at UMDNJ and Rutgers, and the EPA. The analyses were performed at Rutgers University.

The work presented in this chapter is highly technical and thus, is intended to be beneficial to scientists with expertise in bioinformatics. The technical details of the analyses are

provided in order that scientists could apply these methods to their work. Such an approach will allow the risk assessor proficient in microarray analysis methodology an opportunity to apply these methods. The last section of this chapter (Section 6.4) summarizes the findings for a scientific audience that does not have a strong background in microarray analysis methods.

6.2. PATHWAY ANALYSIS AND GENE INTERACTIONS AFTER *IN UTERO* DBP EXPOSURE

6.2.1. Pathway Activity Approach

Usually, to identify significant biological pathways from transcriptional data, pathway analysis is performed after determining the DEGs using a statistical filter. Two examples of this approach are described in Chapter 5 (Section 5.5). An alternative approach is the use of "pathway scoring" methods, which begin with projecting gene expression changes onto pathways (Rahnenfuhrer et al., 2004; Moothaet al., 2003; Hanisch et al., 2002). The main advantage of applying pathway scoring methods to microarray data is that changes can be identified at the pathway level that may not be detected by first identifying individual DEGs. Most of these methods calculate the average correlation between pairs of genes within pathways (Rahnenfuhrer et al., 2004; Sohler et al., 2004; Hanisch et al., 2002; Zien et al., 2000). Another pathway scoring method tests for association between gene expression and a phenotype (e.g., Gene Set Enrichment Analysis [GSEA]; Mootha et al., 2003). In GSEA, all genes are ranked with respect to some measure that quantifies the gene expression associated with a phenotype (i.e., differentiation between healthy vs. disease samples). Tomfohr et al. (2005) introduced a pathway-based approach that is similar in spirit to GSEA. Their method translates the overall gene expression levels within a pathway to a "pathway activity level," which is derived from singular value decomposition (SVD), described below. Hence, pathway activity levels can be used in the same kinds of applications as gene expression levels (Tomfohr et al., 2005). Tomfohr et al. (2005) compared their pathway activity method to GSEA using expression data from two different studies, one that studied Type 2 diabetes and one that studied the influence of cigarette smoke on gene expression in airway epithelia. They found similar results to those obtained using GSEA in the diabetes set, and further, improved results for identifying differentially expressed pathways in the cigarette smoke data.

We applied a pathway activity level approach to DBP microarray data. Since pathway activity levels are a reduced form of the overall gene expression matrix (represented by the

largest deviation in the overall gene expressions within a pathway) Alter et al. (2000) and Cangelosi (2007) raised the critical issue that pathway activity levels (represented by the largest deviation in the overall gene expressions within a pathway) may be attributed to random deviations in the data. Therefore, we use a significance analysis to distinguish the information captured by pathway activity levels from random deviation.

6.2.1.1. *Significance Analysis of Pathway Activity Levels*

The procedure begins with mapping genes to the KEGG pathway database. The entire gene set represented by the Liu et al. (2005) data set (i.e., using the Affymetrix RAE230 A and B chips) maps to 199 pathways in the KEGG database with 4,772 associated genes.

Pathway activity formulation starts with SVD of the gene expression matrix of a given pathway. SVD involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables. It mathematically transforms the data to a new coordinate system such that the greatest variance by any projection of the data lies on the first coordinate (called the eigenvector), the second greatest variance on the second coordinate, and so on. Associated with each of these coordinate eigenvectors is a weight term (called the eigenvalue) that represents the variance in the data. The eigenvalues are normalized such that they express the fraction of the variance along their corresponding eigenvector. In this study, SVD is used to calculate pathway activity levels for each experimental condition where each pathway activity level represents the most significant gene expression pattern within each pathway. The details of SVD analysis are as follows:
Using Eq. 6-1, let $\Xi_p(k,t)$ be the gene expression data associated with a given pathway, p, composed of k genes measured at t different conditions (time, treatment, dose, etc.), normalized (i.e., to a mean of zero mean and unit standard deviation). The SVD of $\Xi_p(k,t)$ is given as follows:

$$\Xi_p(k,t) = U_p(k,k) \times S_p(k,t) \times V_p(t,t)^I \tag{6-1}$$

Eq. 6-1states that the columns of the matrix $U_p(k,k)$ are the orthonormal eigenvectors of $\Xi_p(k,t)$. $S_p(k,t)$ is a diagonal matrix containing the associated eigenvalues, and the columns of the matrix $V_p(t,t)$ are projections of the associated eigenvectors of $\Xi_p(k,t)$. As the elements of $S_p(k,t)$ are

sorted from the highest to the lowest, the first row of $V_p(t,t)$ represents the most significant pattern within a pathway across different samples. Hence, PAL_p is mathematically defined as the first vector of the $V_p(t,t)$ (given in Eq. 6-2).

$$PAL_p = V_p(n,1)^T \tag{6-2}$$

The fraction of the overall gene expression that is captured by PAL_p is evaluated through Eq. 6-3.

$$f_P = \frac{S_p(1,1)^2}{\sum_{g=1}^{L} S_p(L,L)^2} \tag{6-3}$$

An additional analysis is needed to evaluate whether PAL_p represents significant information about the pathway. As a standard procedure for evaluating significance of microarray data, random sampling is used. For each pathway, an equal number of gene expression values are permutated 1,000 times. The p-value is computed as the permutated f_p that exceeded the actual f_p (p-value < 0.05). Next, the pathways are filtered based on the associated p-value of their f_p value.

We illustrate the importance of the significance analysis of PAL_p in Figure 6-1 using the gene expression matrix for the tryptophan metabolism pathway. Panel A of Figure 6-1 depicts both the fraction of the overall gene expression captured by each eigenvector, f_p, and the average fraction of the overall gene expression captured by each eigenvector of the randomized data. We observe that the f_p value captured by the PAL_p of the tryptophan metabolism pathway can be retrieved with a randomly selected gene set and thus, the tryptophan metabolism pathway is not significantly affected by DBP exposure. We applied a significance analysis of PAL_p to improve the confidence of Tomfohr's pathway activity level formulation for further calculations.

6.2.1.2. *Pathway Activity Analysis*

The main goal of pathway analysis is to identify significantly affected pathways, based on gene expression data, due to DBP exposure. For this purpose, as described above,

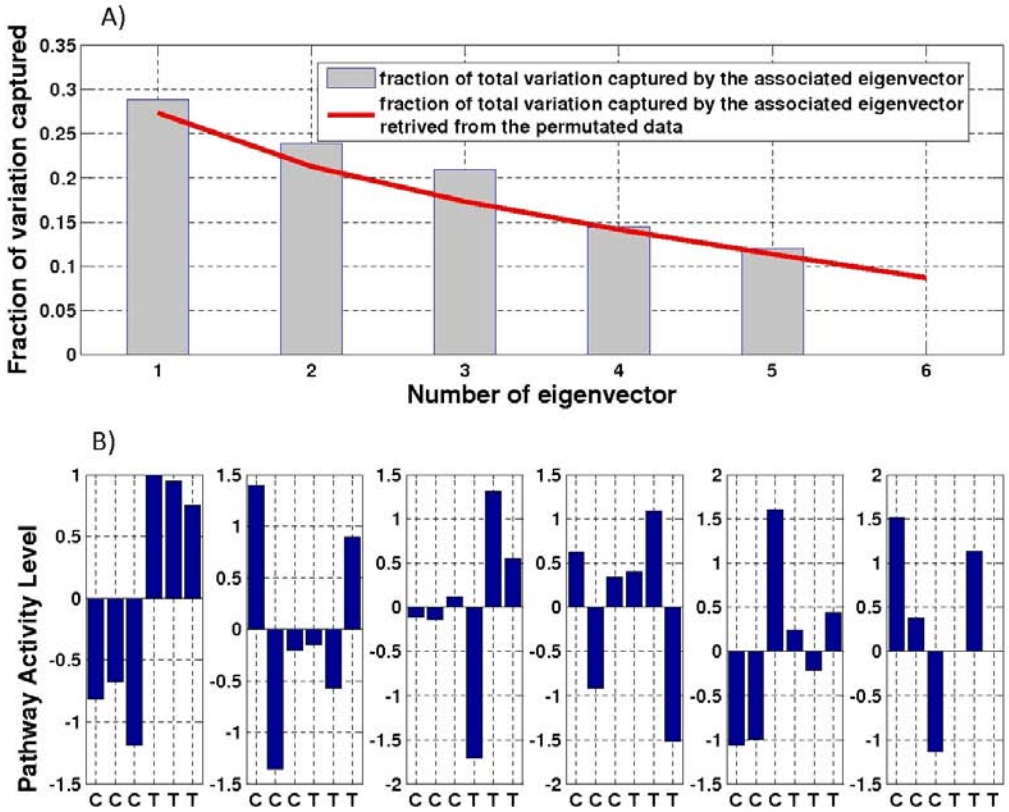

Figure 6-1. An illustration of the adapted version of pathway activity level analysis for the tryptophan metabolism pathway, a nonactive pathway for DBP. In panel A, the boxes indicate the variability in the actual gene expression data, associated with the tryptophan metabolism for each individual eigenvector. For comparison, the solid line represents the fraction of data variability captured by the corresponding eigenvectors when randomly generated data were used. No apparent distinction between the actual data and randomly generated data was identified, as quantified by the calculated *p*-value of 0.25. In panel B, the projection of the gene expression on each eigenvector is depicted for each sample of the control (C) and DBP-treated (T) groups. PAL_p is the first vector that corresponds to the largest variation in the data.

overall gene expressions within a pathway are reduced to PAL_p. The differentiation between PAL_p of different samples is denoted as pathway activity and is determined through a process analogous to SNR analysis.

If n_1 samples are associated with vehicle treatment (control) and n_2 samples with chemical treatment (DBP), then the activity levels associated with treatment groups are given in Eqs. 6-4 and 6-5, respectively.

$$PAL_p^1 = V_p(n_1, 1)^I \tag{6-4}$$

$$PAL_p^2 = V_p(n_2, 1)^I \tag{6-5}$$

Pathway activity is calculated using Eq. 6-6 where μ and σ represent the mean and standard deviation respectively.

$$PA_p = \frac{\left|\mu\left(PAL_p^1\right) - \mu\left(PAL_p^2\right)\right|}{\sigma\left(PAL_p^1\right) + \sigma\left(PAL_p^2\right)} \tag{6-6}$$

A high pathway activity represents a better differentiation between control and treated pathway activity levels. The statistical significance of pathway activity is determined using the randomization process. For each pathway, an equal number of genes within a given pathway are randomly assigned and gene expression changes are generated (from the chip) 10,000 times. The *p*-value of the pathway activity is computed as the fraction of the randomized pathway activity that exceeded the actual pathway activity. In this analysis, the pathways that have both statistically significant (*p*-value < 0.05) pathway activity and pathway activity level are defined as "active" pathways.

"Active" pathways are those for which the overall change in gene expression in a pathway of treated samples compared to control samples was statistically significant. For example, an active pathway could be one for which gene expression was downregulated or turned off after DBP exposure. Alternatively, a pathway that is not identified as active would still have gene expression occurring, but might not exhibit a significant difference in gene expression following DBP exposure compared to the control samples. Thus, the term active does not refer to gene expression from a particular pathway. The algorithm for selecting active pathways using the pathway activity method is shown in Appendix B, Figure B-1.

We identified 15 active pathways from querying the KEGG database (see Table 6-1). The pathway activity method identified pathways such as biosynthesis of steroids (C21 Steroid hormone metabolism pathways known to be biologically relevant to T levels) as well as other pathways including butanoate metabolism, pyruvate metabolism, and biosynthesis of unsaturated fatty acids (PPAR signaling pathway and fatty acid metabolism).

Table 6-1. The KEGG pathways ordered based on their *p*-value for pathway activity[a]

Pathway name	p-value of PA[b]	p-value of PAL[c]
Reductive carboxylate cycle (CO2 fixation)	<0.001	0.002
Valine, leucine and isoleucine degradation	<0.001	<0.001
Biosynthesis of steroids	0.001	<0.001
Citrate cycle (TCA cycle)	0.002	<0.001
Glutathione metabolism	0.002	0.006
Tryptophan metabolism†	0.002	0.250
Pentose phosphate pathway	0.002	<0.001
Glycolysis / Gluconeogenesis	0.003	<0.001
Butanoate metabolism	0.004	0.006
Pyruvate metabolism	0.004	<0.001
C21Steroid hormone metabolism	0.006	0.048
Glyoxylate and dicarboxylate metabolism†	0.012	0.480
Biosynthesis of unsaturated fatty acids	0.012	0.048
Fatty acid metabolism	0.020	0.030
Nicotinate and nicotinamide metabolism	0.028	0.068
Propanoate metabolism	0.030	0.018
Cyanoamino acid metabolism†	0.032	0.074
PPAR signaling pathway	0.042	<0.001

[a]Pathway activity quantifies the difference between control and DBP-treated samples from Liu et al. (2005) (see Eq. 6-6). PAL is the pathway activity level for both the control and treated samples (see Eq. 6-2). The statistical significance of PA and PAL values are evaluated through a randomization procedure. The *p*-value of PAL is used as an additional filtering process to eliminate potentially nonactive pathways.
[b]The *p*-value of the PA is computed as the fraction of the randomized PA that exceeded the actual PA. In the event that the PA of the randomly generated matrices exceeds the actual PA by more than 5 % of the randomization process, then the actual PA is attributed to a random variable (p-value < 0.05).
[c]The *p*-value of PAL quantifies the significance of fraction of the overall gene expression captured by PAL. It is computed as the fraction of the randomized f_p exceeding the actual f_p. In the event that the PA of the randomly generated matrices exceed the actual PA by more than 5 % of the randomization process, then the actual PA is attributed to a random variable (p-value < 0.05).
PA, pathway activity; PAL, pathway activity level.

To explore the biological significance of the active pathways, a metabolic pathway network of the active pathways illustrating their connections via metabolites was built (Figure 6-2). This process includes the integration of the statistical outcome of the pathway activity analysis and the relationships among these pathways by querying the KEGG database. After DBP *in utero* exposure, the pathways related to cholesterol biosynthesis exhibit more significant changes in their gene expression compared to the rest of the active pathways. This finding is consistent with the hypothesis that an early decrease in T level might be due to cholesterol unavailability (Thompson et al., 2005).

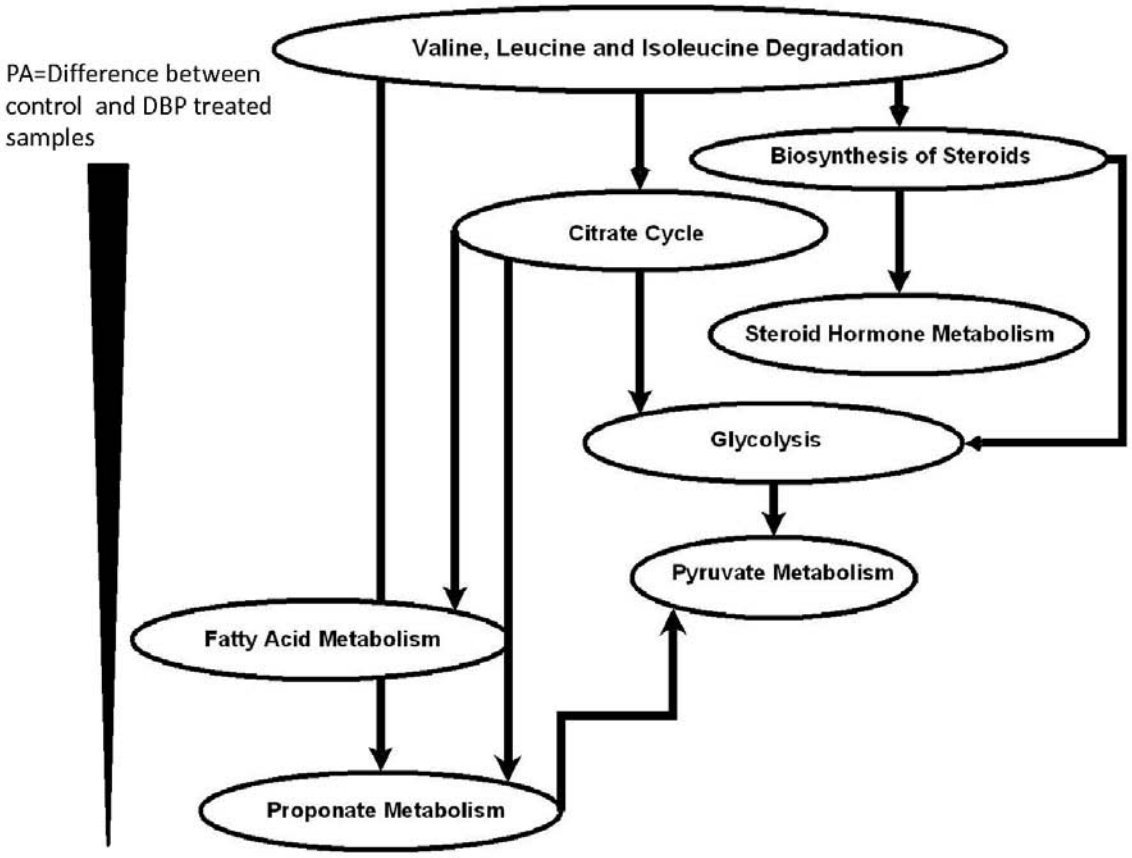

Figure 6-2. Metabolic pathway network for DBP (Liu et al., 2005 data) using the pathway activity method and the KEGG database. Active pathways connected to each other via metabolites are ordered from the most active pathway (top of the figure) to the less active pathways (bottom of the figure). The connections between the active pathways were retrieved from KEGG (Kanehisa and Goto, 2000).

We explored the contribution of DEGs to the pathway activity for a given pathway (Figure 6-3 A, B, C, and D). The pathway activity of each pathway is calculated by adding genes one-by-one starting with the gene with the highest SNR and adding genes sequentially in the order of their SNR until all genes in the pathway have been added. Figure 6-3 A and B illustrate examples of active pathways, whereas Figure 6-3 C and D are examples of pathways that were not identified as active in our analysis. For pathways that were identified as active or not active, the cumulative pathway activity value undergoes a decrease as genes of lower SNRs are added. Yet for the active pathways, the cumulative pathway activity remains high enough to be statistically significant. For pathways identified as not active, the cumulative pathway activity reaches a low level when all of the genes are added. Accordingly, their pathway activity value is not statistically significant. The four pathways are composed of a similar number of genes; therefore, the number of genes in the pathway is not an issue in this comparison. We hypothesize that there is a subset of genes that maintain the pathway activity value high enough within active pathways, even when all genes are added. The cumulative behavior of this subset enables us to differentiate the active and nonactive pathways. Differentially expressed genes in active pathways are defined as "informative genes" (see Table B-1). We identified a relatively small number of genes as informative, and these may represent genes that DBP has most greatly affected.

One of our goals was to utilize existing DBP genomic data to develop a gene network model useful to risk assessment. Gene network models illustrate interactions between genes and their products (e.g., mRNA, proteins). We used IPA software to construct a gene network model after DBP *in utero* exposure. IPA adds nodes (i.e., genes) to the input gene list (i.e., informative genes) and then, builds edges (i.e., relations) based on the literature. The interactions among the informative genes from the Liu et al. (2005) data were retrieved using IPA, and the resulting preliminary gene network model is shown in Figure 6-4.

6.2.2. Developing a Temporal Gene Network Model

The Thompson et al. (2005) study was selected to develop a temporal gene network because it was the only available time-course study. The study had the advantages of using the rat Affymetrix chip, which has ~30,000 gene transcripts represented, and availability of the data (i.e., kindly provided by Dr. Kevin Gaido). In the study, animals were exposed to DBP for 0.5,

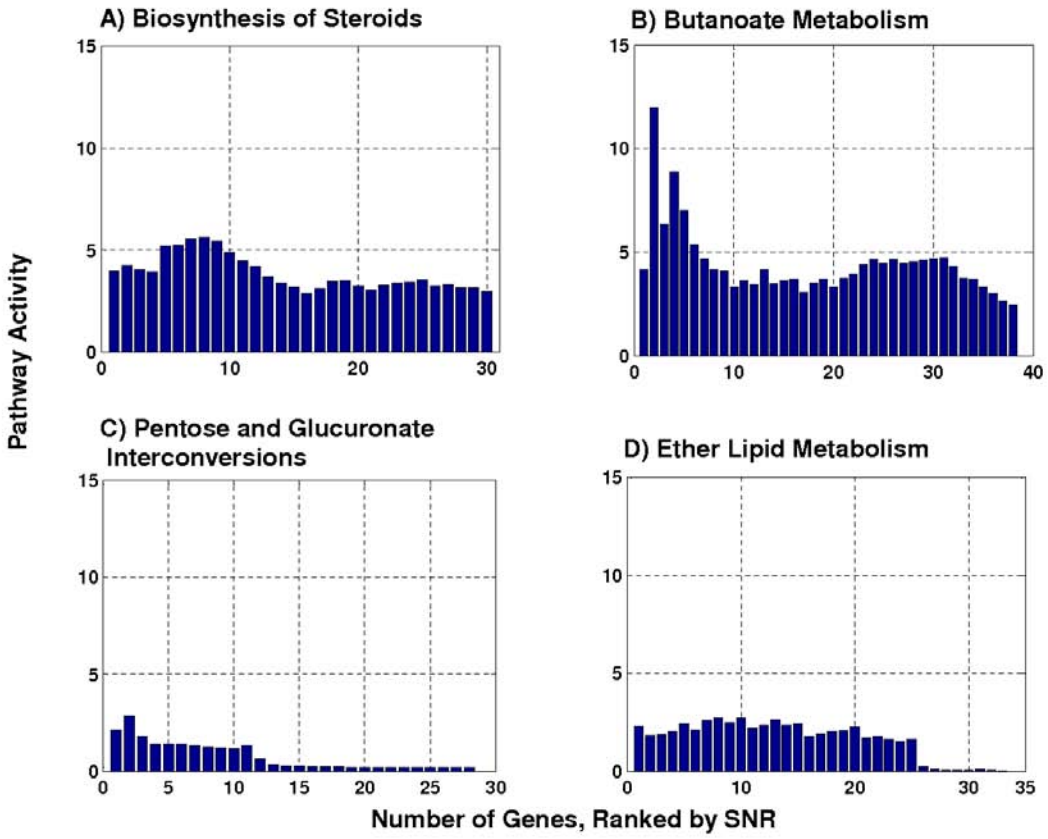

Figure 6-3. The relationship between differential expression of individual genes and pathway activity using the Liu et al. (2005) DBP data. The pathway activity of a given pathway is first evaluated using the gene that has the highest SNR. Subsequently, the genes are added in the order of their SNR, from highest to lowest. Pathways identified as active for DBP, such as biosynthethis of steroids (A) and butanoate metabolism (B), maintain high pathway activity values even when all genes in the pathway are added. Alternatively, pathways not identified as active for DBP such as pentose and glucuronate interconversions (C) and ether lipid metabolism (D), exhibit a decrease in pathway activity as the less discriminating genes (i.e., those with a lower SNR value) are added.

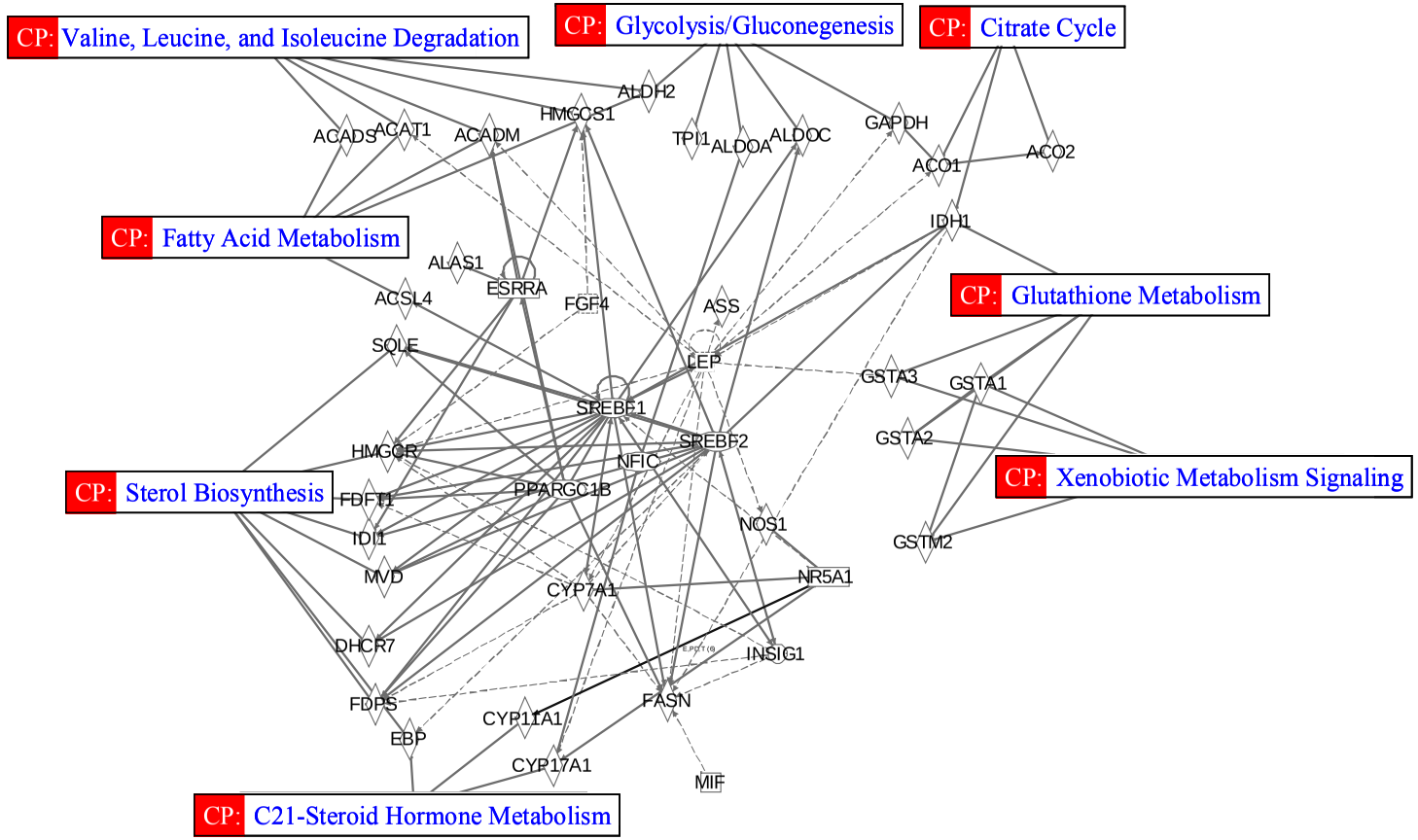

Figure 6-4. A gene network for DBP data of Liu et al. (2005) generated using Ingenuity Pathway Analysis (IPA). The figure illustrates the interactions among informative genes following *in utero* DBP exposure in the rat testis from Liu et al. (2005). Interactions among genes (shown in Appendix B, Table B-1) are derived from the Ingenuity database. Genes or gene products are represented as nodes. Diamonds, enzymes; Horizontal ovals, transcription regulators; Squares, cytokines; Rectangles, nuclear receptors. Solid lines represent direct relationships between nodes (i.e., molecules that make physical contact with each other, such as binding or phosphorylation). Dashed lines represent indirect interactions (i.e., not requiring physical contact between the two molecules, such as signaling events). CP, Canonical pathway.

1, 2, 3, 6, 12, 18, or 24 hours before sacrifice on GD 19. The limitations of the Thompson et al. (2005) study include (1) the dosing was initiated on GD 18, late in the critical window, and (2) the shortest duration exposure (30 minutes) began at the latest developmental time (i.e., GD 19); thus, developmental stage and duration of exposure do not coincide (see Chapter 5). Given this caveat, we utilized the available to test algorithms to build a prototype of a temporal gene network model.

We used the pathway activity level method described earlier to identify biologically active pathways at each time point. We evaluated the informative genes at each time point and the resulting preliminary temporal gene network, based on the Thompson et al. (2005) data, is shown in Figure 6-5. The analysis showed a preponderance of signaling pathways such as JAK/STAT, PPAR, and MAPK perturbed at the earlier exposure durations. After the longest DBP exposures (18 hours), the metabolic pathways, including amino acid metabolism, lipid metabolism, and carbohydrate metabolism, were affected. Thompson et al. (2005) hypothesized that the decrease in T level after a short duration of DBP exposure might be due to cholesterol unavailability and their findings support this hypothesis. To have a complete understanding of the temporal sequence of gene expression and pathway affect events after *in utero* DBP exposure, data from an exposure-duration series across the entire critical window of exposure are needed.

6.3. EXPLORATORY METHODS: MEASURES OF INTERSPECIES (RAT-TO-HUMAN) DIFFERENCES IN TOXICODYNAMICS

The goal of this section is to address whether genomic and mechanistic data could inform the interspecies (rat-to-human) differences TD for one of the DBP MOAs reduced fetal testicular T (one of the DBP case-study questions). Although progress has been made in understanding the MOAs of chemical toxicants, it is important to evaluate the mechanistic relevance of these MOAs to humans. The genomic data set for DBP does not include human genomic data of any type, including studies from *in vitro* cell lines. Even if such data were available, extrapolation of *in vivo* data (rat genomic) to *in vitro* data (human genomic) may confound the ability to generate accurate interspecies comparison. In the absence of DBP genomic data in human cell lines, we considered genetic sequence data and other data from rats and humans for making species comparisons. It is significant that the role of T in male reproductive development during sexual

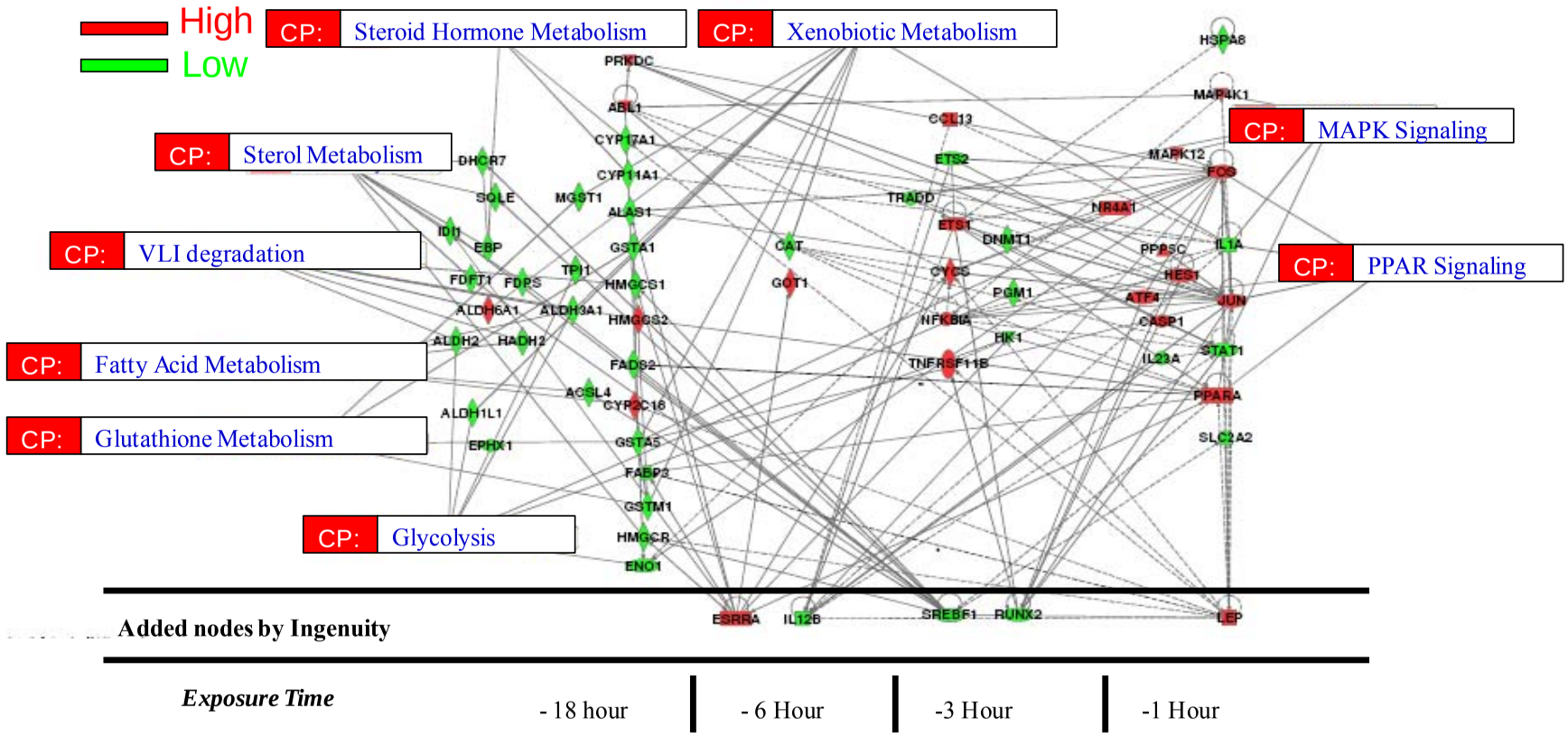

Figure 6-5. A temporal gene network model created by IPA from the informative gene list based on time-course data after *in utero* DBP exposure (Thompson et al., 2005). The informative genes were evaluated at each time point and mapped onto a global molecular network using the Ingenuity Pathways Knowledge Base. Diamonds, enzymes; Horizontal ovals, transcription regulators; Squares, cytokines; Rectangles, nuclear receptors. Solid lines represent direct relationships (also called edges) between nodes (i.e., molecules that make physical contact with each other, such as binding or phosphorylation). Dashed lines represent indirect interactions (i.e., not requiring physical contact between the two molecules, such as signaling events). CP, Canonical pathway. Low (green), downregulated expression with respect to control. High (red), upregulated expression with respect to control.

differentiation is conserved among vertebrates, thus providing a measure of human relevance of the reduced fetal testicular T observed in the rat after *in utero* DBP exposure.

Phylogenetic analysis, the reconstruction of evolutionary relations, is based on shared, derived characters presumed to have a common origin. Taxonomy of organisms is one method for determining species relatedness. However, since DBP perturbs the activity of the steroidogenesis pathway and leads to the decreased T MOA for DBP, we were interested in developing metrics by comparing this pathway between the rat (for which we have data) and human. Previous phylogenetic analyses of individual pathways have included assessing: the number of common enzymes and their conservation across different species (Forst, 2002; Forst and Schulten, 1999); the topology of the underlying enzyme-enzyme relational graphs including their sequence conservation (Heymans and Singh, 2003); and the intersection of compounds, reactions, and enzymes across species (Clemente et al., 2005).

We reconstructed the phylogenetic relationships for biosynthesis of steroids among eight species based on enzyme presence (Forst and Schulten, 1999; see Figure 6-6). The enzyme presence method is based on information available in the KEGG database about the presence of an enzyme (defined as catalyzing a specific reaction) in the pathway for a given species. As a result, a pathway topology can be represented and compared across species. In this representation of pathways, a vector containing binary information (where "1" is for presence, "0" is for absence of the enzyme) is created for a given pathway. Then, the similarity between pathways for two different species is defined as the ratio of the number of common enzymes to the number of unique enzymes. The results suggest that the steroidogenesis pathway is quite similar between rat and human. Further, we found that the species differences based on enzyme presence were different from those based on the NCBI taxonomy (Sayers et al., 2008) of the organisms, which is not surprising based on previous findings (Searls, 2003). In order to utilize more complete information about a pathway, cross-species pathway comparisons should include other biologically relevant information such as gene regulatory information and pathway interactions.

Sequencing of the human, mouse, and rat genomes and their comparison has increased our understanding of cross-species similarities and differences in genes and proteins. Co-expressed genes across multiple species are most likely to have a conserved function. The rat genome project reported that almost all human genes known to be associated with disease have

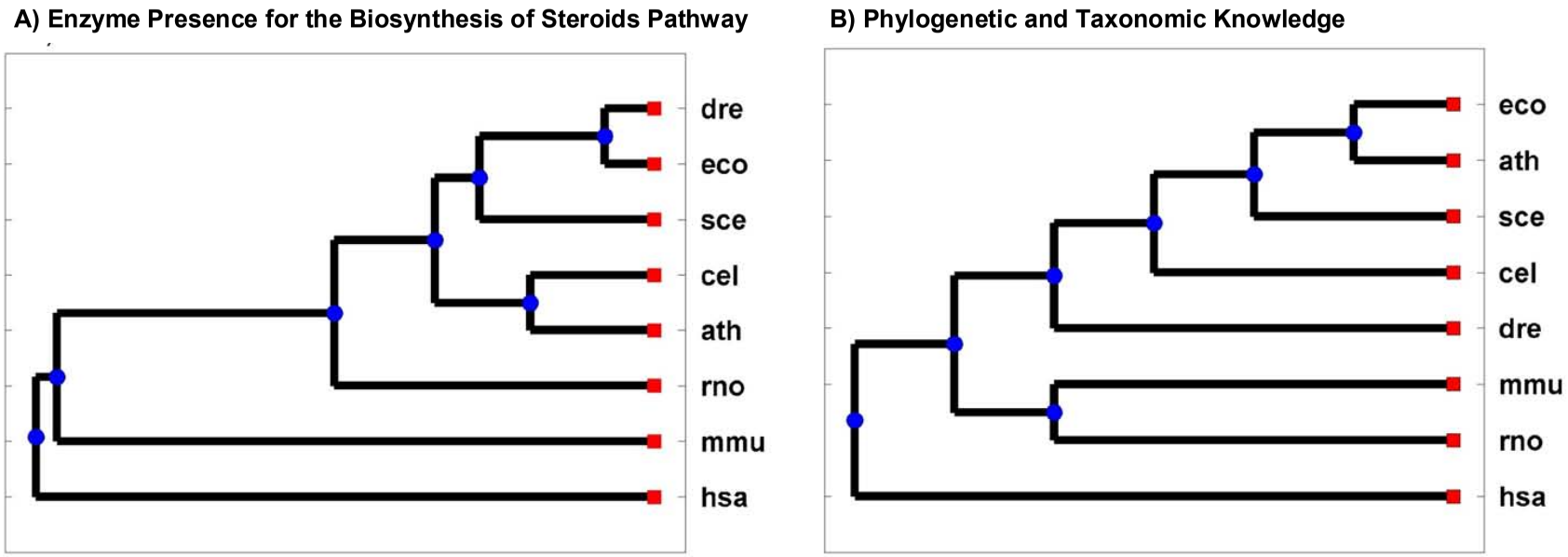

Figure 6-6. The phylogenetic relations among eight organisms based on enzyme presence, for the biosynthesis of steroids pathway, and based on information available on the NCBI taxonomy website (Sayers et al., 2008). Panel A shows the results of evaluating the phylogenetic relations for the biosynthesis of steroids pathway, based on enzyme presence (KEGG database), among eight model species (hsa, *Homo sapiens*; mmu, Mouse; rno, Rat; dre, Zebra fish; ath, Arabidopsis; cel, *C. elegans*, sce, Yeast; eco, *E. coli*). Panel B shows the phylogenetic relations among the same eight organisms based on taxonomic and phylogenetic information retrieved from the NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi).

orthologous genes in the rat genome, and that the human, mouse, and rat genomes are approximately 90% homologous (Gibbs et al., 2004). While the function of certain genes and their involvement in disease might not be conserved across species, the function of a pathway is likely to be more highly conserved among species that perform similar functions (Fang et al., 2005). Thus, cross-species pathway conservation metrics may be more useful.

Similarity among species can be investigated by phylogenomics analysis that involves a comparison of genes and gene products across a number of species, characterizing homologues and seeking further insights about evolutionary relationships. Analyzing the similarities between phylogenetic gene trees and their associated protein trees can reveal additional information. For example, a reconstruction of the CYP2A family of cytochrome P450 enzymes indicates that the rat liver isoform (CYP2A1) has diverged significantly from the human (CYP2A6) and mouse (CYP2A4) enzymes, having a distinct branch of the tree rooted outside the rest of the family (Searls, 2003). This considerable deviation is associated with a well-known functional shift that the rat enzyme causes the coumarin to be metabolized to a hepatotoxic epoxide, whereas the human and mouse enzymes act on the same substrate by way of a more harmless hydroxylation.

The same principles can be extended to amino acid sequence comparisons for the genes that make up a pathway. Utilizing the predicted amino acid sequence information for genes in the steroidogenesis pathway from rats and humans, we evaluated the similarity among this set of genes. Preliminary results suggest that proteins involved in the biosynthesis of steroids are highly conserved across rats and humans, with the average sequence similarity of enzymes between human and rat being ~87% as presented in Table 6-2. However, it is difficult to unequivocally determine a “high” versus “low” degree of conservation for the genes in this pathway—especially in light of the fact that events important to the effect of DBP on steroidogenesis are not well-understood. For example, initiating event after DBP exposure is not known. Additionally, there are likely differences between identifying a gene that is statistically highly conserved versus understanding whether or not the biologically meaningful regions of the predicted protein sequence, active sites, are conserved. However, endocrinological, developmental, and genetic studies in many vertebrate species indicate that the role of androgens is highly conserved across vertebrates, as androgens are critical for sexual differentiation in the male. Thus, taken together, this information suggests a high conservation of steroidogenesis and androgen synthesis in rats and humans.

Table 6-2. The amino acid sequence similarity of the enzymes in the steroidogenesis pathway between rat and human.

Gene symbol	Entrez gene ID	mRNA and protein IDs	Human homolog IDs	Identities[a]	Positives[b]	Gaps[c]
Dhcr7	64191	NM_022389.2→NP_071784.1	Q9UBM7	412/475 (86%)	443/475 (93%)	4/475 (0%)
Idi1	89784	NM_053539.1→NP_445991.1	AF003835	196/227 (86%)	215/227 (94%)	0/227 (0%)
Fdps	83791	NM_031840.1→NP_114028.1	M34477	301/353 (85%)	326/353 (92%)	0/353 (0%)
Fdft1	29580	NM_019238.2→NP_062111.1	AAP36671	356/413 (86%)	393/413 (95%)	0/413 (0%)
Hmgcr	25675	NM_013134.2→NP_037266.2	AAH33692	738/890 (82%)	768/890 (86%)	58/890 (6%)
Mvd	81726	NM_031062.1→NP_112324.1	AAP36301	338/398 (84%)	357/398 (89%)	1/398 (0%)
Sqle	29230	NM_017136.1→NP_058832.1	NP_003120	481/574 (83%)	528/574 (91%)	1/574 (0%)
Ebp	117278	NM_057137.1→NP_476478.1	NP_002331	618/732 (84%)	673/732 (91%)	1/732 (0%)
Lss	81681	NM_031049.1→NP_112311.1	NP_002331	618/732 (84%)	673/732 (91%)	1/732 (0%)
Sc5d	114100	NM_053642.2→NP_446094.1	NP_008849	246/299 (82%)	275/299 (91%)	0/299 (0%)
Mvk	81727	NM_031063.1→NP_112325.1	BAD92959	323/393 (82%)	355/393 (90%)	0/393 (0%)
Cyp27b1	114700	NM_053763.1→NP_446215.1	NP_000776	413/508 (81%)	453/508 (89%)	7/508 (1%)
Nqo1	24314	NM_017000.2→NP_058696.2	NP_000894	234/274 (85%)	250/274 (91%)	0/274 (0%)
Vkorc1	309004	NM_203335.2→NP_976080.1	AAQ13668	83/94 (88%)	88/94 (93%)	0/94 (0%)
			Average similarity scores	84%	94.14%	

Table 6-2. (continued)

[a]Identities, The number and fraction of total residues in the HSP which are identical.
[b]Positives, The number and fraction of residues for which the alignment scores have positive values.
[c]Gap, A space introduced into an alignment to compensate for insertions and deletions in one sequence relative to another. To prevent the accumulation of too many gaps in an alignment, introduction of a gap causes the deduction of a fixed amount (the gap score) from the alignment score. Extension of the gap to encompass additional nucleotides or amino acid is also penalized in the scoring of an alignment.

The HSP (high-scoring segment pair) is the fundamental unit of BLAST algorithm output. Alignment: The process of lining up two or more sequences to achieve maximal levels of identity (and conservation, in the case of amino acid sequences) for the purpose of assessing the degree of similarity and the possibility of homology.

Source: http://searchlauncher.bcm.tmc.edu/help/BLASToutput.html#anchor14684156.

The same principles can be extended from amino acid sequence comparisons to structures, pathways, and expression patterns.

6.4. CONCLUSIONS

The exploratory projects presented in this chapter include efforts to develop methods for analyzing genomic data for use in risk assessment and examples of genomic data analyses available to the risk assessor with expertise in bioinformatics. These methods include pathway level analysis (including the newly described pathway activity method), gene network analysis, and tools to assess cross-species similarities in pathways. A summary for a less technical reader is presented below, grouped by the three objectives for the work.

1. *Explore the development of new methods for pathway analysis of microarray data for application to risk assessment.*

   Quality-control requirements for microarray study analysis for use in risk assessment are distinct from basic research. In traditional pathway level analysis, differentially expressed genes are first identified and then mapped to their respective pathways. Depending on the number of genes that map to a given pathway, the role of the pathway can be over- or underestimated. To overcome this problem, we used the pathway activity method. This method scores pathways based on the expression level of all genes in a given pathway.

   The pathway activity analysis identified valine, leucine, isoleucine (VL1) degradation, sterol biosynthesis, citrate cycle, and fatty acid metabolism as the most active pathways following DBP exposure. These findings support the hypothesis of Thompson et al. (2005), that an early decrease in T levels may be a result of cholesterol unavailability. However, for this approach to be useful, knowledge of tissue-specific pathways is required. For example, even though bile acid biosynthesis does not take place in the testis, a pathway related to bile acid biosynthesis was identified as statistically significant in this analysis. This method shows promise for use in risk assessment.

2. *Utilize existing DBP genomic data to develop a gene network model for use in risk assessment.*

   Determining a sequence of gene expression changes and pathway level effects over time can be very useful for understanding the temporal sequence of critical biological events perturbed after chemical exposure, and thus, useful to a risk assessment. We developed a method for developing a gene network model for DBP based on the available data. The availability of time-course data (Thompson et al., 2005) enabled our group to model the series of events that occurred between exposure to DBP and the onset of toxic reproductive outcomes. However, given the limitations of the Thompson et al. (2005) study design, we did not draw conclusions about genes and pathways affected over time

for DBP. Instead, the Thompson et al. (2005) data was used to build a prototype of a temporal gene network model and thus, the exercise allowed us to develop methods for analyzing time-course data.

3. *Utilize genomic and other molecular data to address the Case Study Question: Do the toxicogenomic data inform interspecies differences in TD?*

   Extrapolation from animal-to-human data is critical for establishing human relevance of MOA(s) in risk assessment. Co-expressed genes across multiple species could have a conserved function. The human, mouse, and rat genomes have been reported to be 90% homologous (Gibbs et al., 2004). However, because it is not certain whether the function of a specific gene is conserved across species, conservation of pathways across species can be one important factor in establishing cross species concordance of one or more MOAs. In addition, a common critical role of androgens in both rodent and human male development of reproductive organs has been well-established.

   Using the available DNA, sequence, and protein similarity data for the steroidogenesis pathway, we used three different methods to assess rat-to-human conservation as metrics that may inform the interspecies differences in TD for one MOA, the reduced fetal testicular T. The pathways for the biosynthesis of steroids have similarity between human and rat. Comparing the predicted amino acid sequences for the steroidogenesis pathway genes, we found that the average sequence similarity between rat and human is ~87%, and the average promoter region similarity of genes is 52%. Some of the challenges in using similarity scores to estimate the cross-species relevance of a MOA are described (see Section 6.3).

In summary, the preliminary analytical efforts described in this chapter address and raise a number of issues about the best approaches for analyzing microarray and other genomic data for risk assessment purposes. Traditional pathway analysis methods, while useful, also restrict the incorporation of all genes for determining relevant pathways that are affected by DBP. There is a substantial amount of background noise generated in a typical microarray experiment (i.e., gene expression variability even among the controls; see Smith, 2001). For use in risk assessment, it is important to be able to identify and separate the signal from the noise. Innovative approaches, such as the pathway activity method described in this chapter, may provide more confidence when evaluating microarray data for use in risk assessment. These efforts reveal some of the promises and challenges of analyzing and interpreting genomic data for application to risk assessment.

7. CONCLUSIONS

This chapter describes the general approach for systematically evaluating genomic data for risk assessment. This general approach is a result of refining the DBP case-study approach (see Figure 3-1). In addition, conclusions from the DBP case study, recommendations, research needs, and future considerations for applying genomic data to risk assessment are described.

7.1. APPROACH FOR EVALUATING TOXICOGENOMIC DATA IN CHEMICAL ASSESSMENTS

There were two goals of this project (see Chapter 2):

- *Develop a systematic approach that allows the risk assessor to utilize the available toxicogenomic data in chemical-specific health risk assessments performed.*
- *Perform a case study to illustrate the approach.*

The first goal was to develop an approach for evaluating toxicogenomic data in future assessments. In the DBP case study, we had the benefit of the 2006 external peer-review draft IRIS Tox Review of DBP, including data summaries and gaps. Additionally, DBP has a more extensive toxicological and toxicogenomic database than most chemicals. The DBP published literature and the draft Tox Review provided a focus to the case study on one set of endpoints (the male reproductive developmental endpoints), that occur in the lower dose range. The case-study approach (see Figure 3-1) needed refinement because the case-study chemical and process had some differences from that of a new assessment. A generalized approach (Figure 7-1) was developed for use in future chemical assessments.

The steps of the approach are

- *STEP 1: Compile the available epidemiologic, animal toxicology, toxicogenomic, and other studies.*

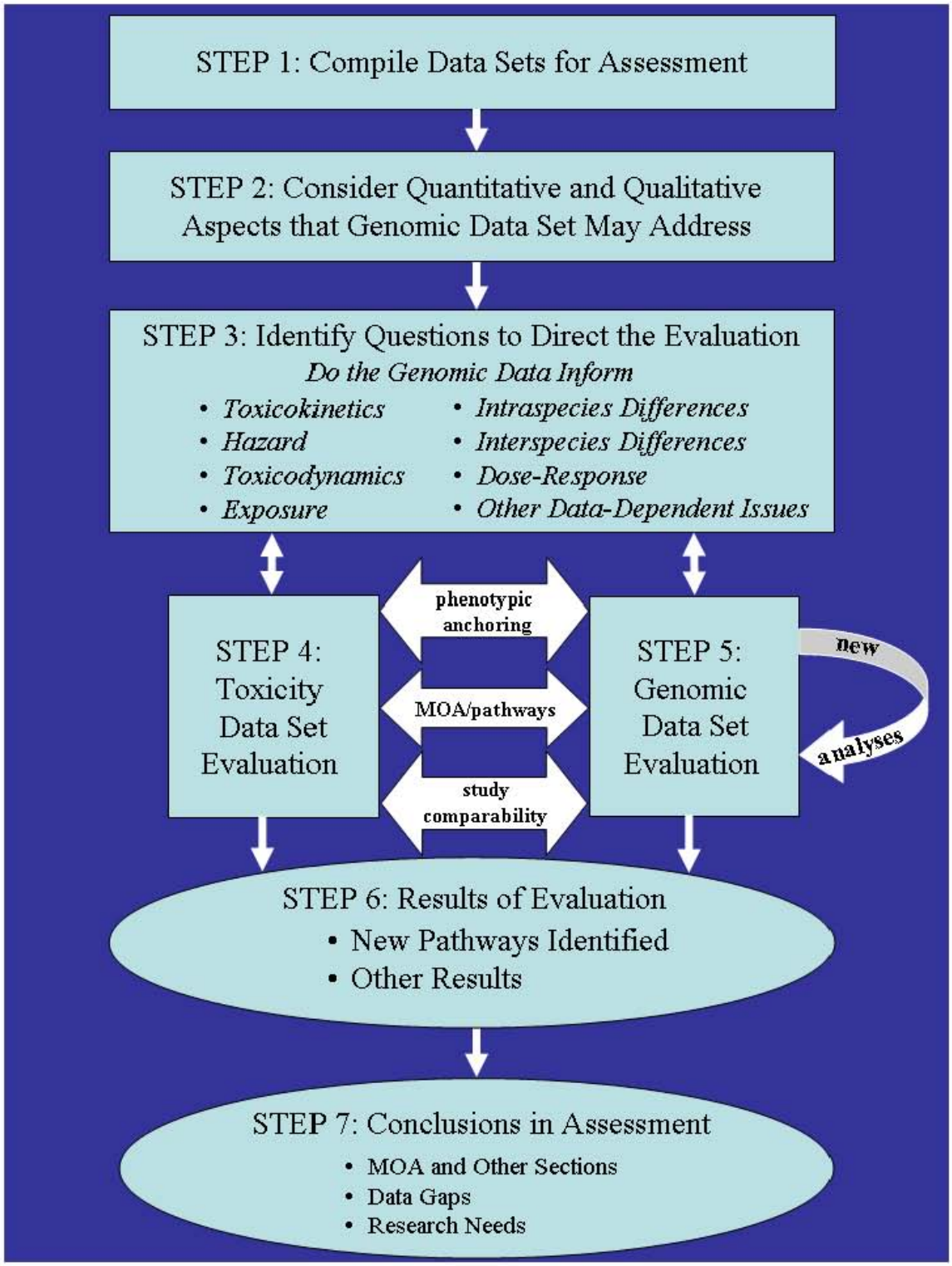

Figure 7-1. Approach for evaluating and incorporating genomic data into future chemical assessments. "Toxicity Data Set Evaluation" may include evaluation of animal toxicity data and/or human outcome data, depending on the available data for the chemical.

- *STEP 2: Consider the quantitative and qualitative aspects of the risk assessment that these data may address.*

  A thorough and systematic consideration of the types of information, in light of the available genomic data, will identify the potential utility of the genomic data and whether these data can be used quantitatively or qualitatively (see Section 3.2). The genomic data set is considered in light of whether these data could inform any risk assessment components (e.g., dose-response) and information (e.g., MOA information, interspecies TK differences) useful to risk assessment. The type of information that these data will provide to a risk assessment depends in part on the type of the available genomic studies (e.g., species, organ, design, and method). This step helps focus the genomic data evaluation and ensure that an important application is not overlooked.

- *STEP 3: Formulate questions to direct the toxicogenomic data set evaluation.*

  Questions are formulated that can direct and focus the genomic data evaluation, and thus, improve efficiency. This step is similar to a scoping exercise performed in ecological and cumulative risk assessment. Some examples of questions considered in the DBP case study were: Do the data inform the MOAs for multiple outcomes (e.g., male and female reproductive outcomes)? Do the data inform dose-response? For example, if microarray data are available, then one of the questions will likely include whether the genomic data can inform the mechanisms and/or MOAs for the chemical as microarray studies typically inform the mechanism of action of a chemical. The DBP case study describes some examples and considerations for determining the risk assessment components that may be informed by a particular genomic data set (see Section 3.3).

- *STEPS 4 and 5: Evaluate the toxicity and/or human outcome and genomic data sets.*

  The approach includes an integrated assessment of the toxicogenomic and toxicity data set to relate the affected endpoints (identified in the toxicity data set evaluation) to the pathways (identified in the toxicogenomic data set evaluation) as a method for

  <u>*Phenotypic Anchoring*</u>

  > Determining the level of support for phenotypic anchoring of genomic changes to *in vivo* outcomes is critical for appropriate interpretation of genomic data for use in risk assessment. In particular, determining whether gene expression changes are associated with or in the causal pathway for an outcome of interest.

  <u>*Informing the Mechanisms of Action/MOAs*</u>

  > Depending on the type of assessment performed, risk assessors may want to utilize aspects of the approach defined herein along with the MOA Framework in the EPA Cancer Guidelines (U.S. EPA, 2005a) and/or other risk assessment decision-logic frameworks for establishing MOAs.

Study Comparability

Another principle of the approach is comparing toxicity and toxicogenomic data study designs in order to identify a set of comparable studies. It is important to compare the study designs among studies. Study design aspects include the time of exposure (in light of critical windows), dose, species, strain, and time of assessment. As a result of assessing study comparability for a given data set, one can select studies for the best comparisons across the outcome and toxicogenomic genomic data sets. For example, in the DBP case study, all toxicogenomic studies were performed in the rat, and, in most cases, the testis. Therefore, the genomic data set was compared with the rat toxicity data and focused on effects in the testis. Broadening beyond the DBP example, the available toxicogenomic data are best considered in light of the toxicity or epidemiologic study data that share study design similarities with the toxicogenomic data. For example, if toxicogenomic data from human tissue or cells are available, then these data are best considered with the human epidemiologic outcome data for the chemical. However, even in the absence of comparable data in the same species, the genomic data may still be used, but with less confidence. See Chapters 4 and 5 for further details of the DBP case-study toxicity and toxicogenomic data set evaluations.

Chapter 5 includes a number of simple methods for assessing the consistency across the toxicogenomic studies. Venn diagrams have been used for illustrating the similarities and differences of DEG findings across genomic studies (see Figure 5-1). Figure 5-2 provides an excellent example of another method for assessing the consistency of findings across all types of gene expression data.

New Analyses

New analyses of toxicogenomic data may be valuable for the assessment depending on the questions asked and the nature of the analyses presented in the published studies. However, new analyses of the original data are not always needed. For instance, reanalysis may not be needed when available published data have been analyzed for application to risk assessment questions of interest. See Section 5.5 for more details of the new case study analyses methods and results, and Chapter 6 for exploratory methods development.

- *STEP 6: Describe results of evaluations and analyses to answer the questions posed in Step 3.*
- *STEP 7: Summarize the conclusions of the evaluation in the assessment.*

7.2. DBP CASE-STUDY FINDINGS

The second goal of the project was to develop a case study. The case-study findings are summarized below and the details of the case-study evaluation and analyses are presented in

Chapters 4−6 (with supplemental material in Appendices A and B). Three advantages to using DBP as the case-study chemical are as follows:

1. The temporal aspects (e.g., time of dosing and time of evaluation) could be considered because a number of well-designed studies exist.

2. A causal connection (i.e., a high degree of phenotypic anchoring) between gene expression changes for some of the steroidogenesis pathway genes with a number of the male reproductive developmental effects has been well-established.

3. Two well-established MOAs for DBP have been defined at the molecular level. A number of endpoints resulting from *in utero* DBP exposure have MOAs that have not been identified or established, thus allowing for a query of the genomic data for possible additional MOAs.

7.2.1. MOA Case Study Question: Do the DBP Genomic Data Inform Mechanism(s) of Action and MOA(s)?

In the DBP case study, we found that toxicogenomic data did inform the TD steps of the mechanisms of action and MOAs. The available genomic and other gene expression data, hormone measurement data, and toxicity data for DBP were instrumental in establishing two of its MOAs: (1) a decrease in fetal testicular T, and (2) a decrease in *Insl3* expression. A decrease in fetal testicular T is the MOA responsible for a number of the male reproductive developmental effects in the rat. The genomic and other gene expression data identified changes in genes involved in steroidogenesis and cholesterol transport, providing evidence for the underlying basis for the observed decrease in fetal testicular T. A decrease in *Insl3* expression is one of the two MOAs responsible for the undescended testis effect, and this MOA is well-established from the results of RT-PCR and *in vivo* toxicology studies. RT-PCR studies identified reduced *Insl3* expression after *in utero* DBP exposure (Wilson et al., 2004) as an MOA for agenesis or abnormalities in the gubernaculum, effects that are not seen after exposure to anti-androgens (i.e., chemicals that affect T synthesis or activity). These results provided support for the *Insl3* MOA as a second well-established MOA for the male reproductive developmental effects of DBP.

The rodent reproductive developmental toxicity data set is robust, having a high quantity and relatively high quality of studies. Additionally, there are a number of rodent toxicity studies that used similar study designs (e.g., dose, species, strain, timing of exposure) as some of the

toxicogenomic studies. This aspect of the DBP data set is exceptional for the case study, allowing for the establishment of the relationship between dose, pathways, and outcomes. We evaluated the rodent reproductive toxicity data set for low incidence and low-dose findings but due to data limitations (see Chapter 4), no new findings were identified. We also evaluated the male reproductive developmental toxicity data set for effects that currently do not have a well-established MOA (see Chapter 4). The testes outcomes were the focus of the case study because the DBP toxicogenomic studies were all performed on testicular tissue. Five effects in the testes effects associated with DBP exposure that do not have well-defined MOAs were identified in this evaluation.

The toxicogenomic and other gene expression studies, including nine published RT-PCR and microarray studies in the rat after *in utero* DBP exposure (Plummer et al., 2007; Bowman et al., 2005; Liu et al., 2005; Thompson et al., 2005; Lehmann et al., 2004; Thompson et al., 2004; Wilson et al., 2004; Barlow et al., 2003; Shultz et al., 2001), were evaluated. The review of the toxicogenomic data set focused on an evaluation of the consistency of findings from the published studies, both across microarray studies and all gene expression data, and on whether any additional pathways may illuminate the unexplained endpoints. The evaluation of the published literature found that the gene level findings from the DBP genomic studies (i.e., microarray, RT-PCR, and protein expression) were highly consistent in both the identification of DEGs and their direction of effect.

New analyses of the Liu et al. (2005) microarray study were performed because we were interested in performing a complete pathway analysis of these data (which had not been the purpose of the published study). These evaluations (see Chapter 5) indicate that there are a number of pathways affected after *in utero* DBP exposure; some of these pathways are related to new MOAs that are distinct from the reduced fetal testicular T or the *Insl3* signaling MOAs. The Liu et al. (2005) DBP data set was reanalyzed using two different methods, the SNR and REM, both using a statistical cut-off of $p < 0.05$. Each method identified the steroidogenesis and cholesterol transport pathways, corroborating prior study conclusions. Each analysis also identified putative new pathways and processes that are not associated with either *Insl3* or steroidogenesis pathways; some were similar across analytical methods and some were different. The common pathways identified between the two methods (see Table 6-3) fall into eight processes (characterized by Ingenuity): cell signaling, growth and differentiation, metabolism,

transcription, immune response, cell adhesion, hormones, and disease. Among these, 54 putative new pathways that are not related to the two known MOAs, reduced T or *Insl3* expression, were identified. Further, a subset of pathways (e.g., WNT signaling and cytoskeleton remodeling) was identified in our analysis that had not previously been identified in the published literature for DBP. One or more of these putative new pathways may be related to the toxicity endpoints without identified MOAs in the rat testes, but additional hypothesis testing studies are needed. Evaluating the genomic and toxicity data sets together provided information on potential, heretofore unexplored, MOAs.

There are a number of possible reasons for the differences in findings between our reanalysis and the published analysis of the Liu et al. (2005) data. These include but are not limited to

- The analyses had different purposes. Liu et al. (2005) was interested in determining whether there is a developmental phthalate genomic signature. The purpose of our analysis was to identify all affected pathways.
- In the four years since the study was published, gene and pathway annotation has increased.

Repeated identification of DEGs and pathways via different analysis methods provides an additional level of confidence regarding the importance of "common" DEGs and pathways. However, it is important to note that the lack of repeated identification of a gene or pathway does not necessarily indicate a lack of biological importance for these genes or pathways.

We also asked whether there were appropriate data to develop a temporal gene network model, a sequence of the gene and pathway interactions over time, for DBP. Using the data from Thompson et al. (2005), the only time-course study available at the time of the project, changes in gene expression and pathways were modeled (see Figure 6-5). Two limitations of these data are that (1) the exposure interval was at the tail end of the critical window of exposure, GD 18, a time that most consider too late to induce the full spectrum of male reproductive developmental effects; and (2) the duration of exposure and developmental time were not aligned because all animals were sacrificed on GD 19 (i.e., the 1 hr time point was the *latest* in development; see Sections 5.3.1 and 6.2.2 for more discussion). The more recent study of Plummer et al. (2007) could provide more appropriate data for building a temporal and spatial network model as both

time-course of exposure over the critical window of development and microdissection of the testis cell types were employed in their study.

7.2.2. Interspecies MOA Case Study Question: Do the DBP Genomic Data Inform Interspecies Differences in TD?

Human gene expression data are not available for DBP. Therefore, the case study used information on interspecies similarities of the affected pathways from other available data and methodologies. We explored the interspecies (rat-to-human) differences in the TD part of MOA, focusing on the steroidogenesis pathway underlying one of the DBP MOAs, the decrease in fetal testicular T MOA. Comparisons of the steroidogenesis genes and pathway were performed to evaluate cross-species similarity metrics using three approaches: (1) protein sequence similarity; (2) pathway network similarities; and (3) promoter-region conservation (see Chapter 6). Results from all three approaches indicate that steroidogenesis pathways are relatively highly conserved across rats and humans and, thus, qualitatively, the rat and human mechanisms for steroidogenesis are highly similar.

These results further corroborate what is known about the similar roles for androgens during normal male development in both rats and humans. However, the data sources used for all three approaches have gaps in the knowledge bases. The pathway network diagramming data source is not of high enough quality or comprehensive enough to utilize quantitatively. In fact, it is difficult to use any of the three new lines of evidence to quantitatively inform the relative sensitivity to DBP across species. It is possible that the small differences across species have a strong penetrance, leading to significant differences in the specific enzymes that may become more sensitive to DBP and thus, affecting T production. We further considered whether some steroidogenesis genes are of higher relative importance and, thus, should be weighted higher in a cross-species assessment of the steroidogenesis pathway. The initiating event for DBP action in the male reproductive developmental outcomes has not been established. However, some information about the rate-limiting steps for steroidogenesis, in the unperturbed scenario, is available (reviewed in Miller, 2008). Some studies have identified CYP11A1 (also called P450SCC) as a limiting enzymatic step for T production (Omura and Morohashi, 1995; Miller, 1988). However, the available information on kinetics reflects the unperturbed state because the rate-limiting step was defined in assays without DBP exposure. Additionally, the rate-limiting step information is limited in scope to steroidogenic enzymes and not all upstream activities

leading to T production, such as STAR, a protein that impacts the availability of cholesterol (by transporting cholesterol to the inner mitochondrial membrane for cleavage by P450SCC) for T production. Thus, there is no *a priori* knowledge to argue for placing more weight on a particular gene leading to T production.

Because there are some questions as to the reliability of the data used to generate the pathway comparisons used for each species and the relative importance of individual steroidogenesis enzymes, there is no basis on which to transform a measure of conservation to a quantitative measure of sensitivity. While the confidence in these cross-species comparisons of the steroidogenesis pathway were not high enough to use the findings quantitatively, for the DBP example, the findings do add to the WOE suggesting that the role of T in male fetal development in rats and humans is well-conserved. These methods, however, when based on high-quality data, could be applied quantitatively to future chemical assessments. Further, the exploratory methods for developing metrics for cross-species pathway similarities described in this document (see Chapter 6) could be developed further and validated in the future for quantitative use in risk assessment.

7.2.3. Application of Genomic Data to Risk Assessment: Exploratory Methods and Preliminary Results

Chapter 6 describes exploratory methods and preliminary results for analyzing genomic data for risk assessment application, developing a DBP gene network model, and measuring cross-species differences for a given pathway.

None of the DBP genomic studies were designed with the application to risk assessment in mind. Methods for analyzing microarray and other -omic data were originally developed for screening purposes (i.e., designed to err on the side of false positives over false negatives). For risk assessment application, genomic analytical tools are needed that are different from those used in screening that can reliably separate signal from noise. In traditional pathway level analysis, first, DEGs are identified by a statistical filter, and second, significant genes are mapped to their respective pathways. Typically, the presence of three affected genes (DEGs) within a pathway is the cut-off for identifying a particular pathway. Depending on the number of genes that map to any given pathway, the role of the pathway can be over- or underestimated. To overcome this problem, we explored using the pathway activity level method (calculating *PALp*) that identifies affected pathways in the single step. This method ranks pathways based on

the expression level of all genes in a given pathway and shows promise for use in risk assessment and further validation is underway.

Gene network models can be very useful for understanding the temporal sequence of critical biological events perturbed after chemical exposure, and thus, useful to a risk assessment. We developed a method for developing a gene network model for DBP based on the available data. The availability of one time-course study (Thompson et al., 2005) enabled our group to model the series of events that occurred between exposure to DBP and the onset of reproductive outcomes. However, given the limitations of the Thompson et al. (2005) study design, we could not determine the genes and pathways affected by DBP exposure earliest in the critical window from this study. However, the exercise allowed us to develop methods for analyzing time-course data for use in gene network modeling.

We also explored the use of three different methods to assess rat-to-human conservation as metrics that may inform the interspecies differences for one MOA, reduced fetal testicular T (Section 7.2.2). More work in the area of cross-species metrics is needed. Efforts to address the challenges in using similarity scores to quantitatively estimate the human relevance of an MOA are ongoing (Section 6.3).

7.2.4. Application of Genomic Data to Risk Assessment: Using Data Quantitatively

This case study was limited to qualitative uses of genomics in risk assessment due to the absence of dose-response, global gene expression studies (i.e., microarray studies) for DBP. EPA and the larger scientific community working with genomics are interested in methods to use genomic data quantitatively in risk assessment. There is one dose-response RT-PCR study that, although not a genomic (i.e., not global) study, was considered for use quantitatively in risk assessment (Lehmann et al., 2004; see Table 7-1). Some strengths of the Lehmann et al. (2004) study include the following:

- The study includes multiple doses ranging from low to high.
- Some of the genes assessed in this study were first identified in microarray studies, providing a relatively high level of confidence in the connection between the expression of some of the genes and particular outcomes, as well as demonstrating reproducibility across studies. For example, findings for *Star* gene expression are reproduced across protein expression, RT-PCR, and microarray studies.

Table 7-1. DBP dose-response progression of statistically significant events illustrated with a subset of precursor event data (steroidogenesis gene expression, T expression) and *in vivo* endpoints with the reduced T MOA

	0.1 mg/kg-d	1 mg/kg-d	10 mg/kg-d	30 mg/kg-d	50 mg/kg-d	80 mg/kg-d	100 mg/kg-d
Precursor event[a]	↓ *Hsd3b*	↓ *Hsd3b* ↓*Scarb1*	NC in gene exp. NC in [T]	ND for gene exp. NC in [T]	↓*Scarb1* ↓*Hsd3b* ↓*Star* ↓*Cyp11a1* ↓[T]	ND for gene exp.	↓*Scarb1* ↓*3β-HSD* ↓*StAR* ↓*P450scc* ↓[T]
In vivo endpoint						↑ incidence of absent, poorly developed, or atrophic testis and underdeveloped or absent epididymis[b]	Retained nipples and areolae[c]

NC, no statistically significant change; ND = not determined (Lehmann et al. [2004] did not test 80 mg/kg-d).

[a]Lehmann et al. (2004).
[b]NTP (1991).
[c]Mylchreest et al. (2000).

However, there are a number of issues in applying these dose-response RT-PCR data with confidence to BMD modeling. These limitations include the following:

- Some of the gene expression changes are not reproducible. For example, *Kit* was observed to be significantly altered in the Lehmann et al. (2004) study but was not observed to be significantly reduced after *in utero* DBP exposure in a microarray study (Liu et al., 2005) utilizing the Affymetrix gene chip, yet *Kit* is on the Affymetrix rat chip.

- The relationship between statistical and biological significance is not known for these gene expression data. For example, the expression of *Hsd3b* mRNA is statistically significantly altered at lower doses than a statistically significant T decrease was observed. Thus, Lehmann et al. (2004) argued that the changes in *Hsd3b* at 0.1 and 1.0 mg/kg-d were not biologically significant. Alternatively, *Hsd3b* gene expression changes could be a precursor to T level changes in time and thus, be a valid precursor event. It is also not known whether changes in the expression of a single or multiple steroidogenesis genes would lead to a significant alteration in T and the phenotype.

- Interlitter variability could not be characterized from the Lehmann et al. (2004) data because the RT-PCR data were collected on five individual pups representing four to five litters per treatment group (i.e., ~1 pup/litter). In order to have appropriate data for BMD modeling, litter mean values calculated from a study with a greater sample size and multiple litters are needed to allow characterization of intra- and interlitter variability. The use of the litter as the statistical unit is generally agreed upon because of the high variability in gene expression for pups within one litter (Barlow et al., 2003).

We concluded that the available dose-response RT-PCR data for DBP are not of sufficient quality due to the lack of information about interspecies variability. Additionally, there is not sufficient knowledge about the biological significance of a gene expression change (and the level of change that is biologically significant), for one or a subset of genes, that would invariably lead to a reduction in T and in turn, lead to the observed male reproductive developmental outcomes.

7.3. LESSONS LEARNED

The lessons learned from the case study are grouped by research needs that are useful to research scientists and recommendations that are useful to risk assessors.

7.3.1. Research Needs

7.3.1.1. *Data Gaps and Research Needs: DBP*

There are some research needs that would be very useful, specifically for a DBP risk assessment including the following:

1. *Develop a gene network model for DBP using the Plummer et al. (2007) data.* This data set would be an excellent source of temporal and spatial gene expression information because one of its studies includes three time intervals, thus covering the entire critical window for male reproductive outcomes, and a second study used microdissection of the cord and interstitial cells of the testis. This study was not modeled because it was not published until after the modeling work had been completed, and we had not obtained the data. By comparing gene expression, Plummer et al. (2007) hypothesized the MOA underlying the gonocyte and LC effects.

2. *Perform microarray studies in male reproductive tissues, other than the testis, affected by DBP in order to understand the similarities and differences in DBP-affected pathways across reproductive organs and tissues in the male rat.* Bowman et al. (2005) performed such a study in the WDs, but studies in other male tissues are needed.

3. *Perform microarray studies in human tissues (either cell lines or from aborted male fetal tissue), along with parallel in vitro and in vivo studies in rats for validation and comparison.* Such data would provide valuable information about interspecies differences in TD sensitivity. Some human studies found an association between *in utero* phthalate exposure and newborn male reproductive developmental measures (Main et al., 2006; Swan et al., 2005) that indicate human relevance for some of the DBP effects observed in male rat studies.

4. *Perform well-designed proteomic and metabolomic studies to understand the effects of in utero DBP exposure on the function of expressed proteins and on cellular metabolites.* These data may provide complementary data to the available transcriptomic data, which could yield some new insights.

5. *Perform genomic studies to identify early, critical, upstream events as a means to identify the initiating event for DBP's action in the testis.* This would require performing studies much earlier in gestation, at the beginning of sexual differentiation. In addition, such studies may require greater sensitivity regarding gene expression change identification because a statistically significant change may be greater than a biologically significant change. If identified, the initiating event could be utilized in the risk assessment, thereby reducing uncertainty.

6. *Perform genomic studies to understand whether the female reproductive tract malformations after DBP exposure have common or different MOAs with the male development reproductive effects.* This line of research would identify pathways affected in the developing female reproductive tracts after early gestational DBP exposure.

7. *Compare the affected DEGs and pathways between the phthalates with and without developmental effects could be useful for a cumulative risk assessment of the developmental phthalates.* All of the data from the Liu et al. (2005) data set could be utilized to evaluate this issue. Further, evaluating consistency of findings across chemicals in the same MOA class that do and do not produce the same set of effects could be useful for improving specificity of the pathway and MOA findings for DBP.

8. Studies to distinguish affected genes and pathways that may be compensatory vs. those in the causal pathway for DBP-toxicity.

7.3.1.2. *Research Needs for Toxicity and Toxicogenomic Studies for Use in Risk Assessment*

EPA and the larger scientific community are interested in methods to use genomic data quantitatively in risk assessment. This case study was limited to qualitative uses of genomics in risk assessment due to the absence of dose-response global gene expression studies for DBP. This is the case for many chemicals as multiple dose studies are very costly. However, multiple dose microarray or other global gene expression studies are needed (see Table 7-2). Such studies need to be designed properly such that the identification and interpretation of lower dose effects is possible. Gene expression changes at the lower dose may not be affected in every organ, tissue or cell sample assessed. High single dose microarray studies have been performed such that all organs are affected and one can assess a smaller sample size than for a dose-response study. In a dose-response study including low- to high doses, the sample size per dose group would need to be high enough to increase statistical power (i.e., the detection of gene expression changes when only a few animals are affected). For example, if an endpoint is affected in 20% of the animals at lower doses, then the sample size for microarray studies must be large enough to identify the affected animals (with affected gene expression). Perhaps the highest priority study is one that assesses global gene expression and toxicity endpoints of interest as components of the same experiment; the organ or tissue of interest would be collected at the appropriate age in one group of animals and a second group would be followed through to evaluation of the endpoint of interest. In this manner, such a study would generate data that could define the relationships between dose, time of exposure, gene expression level changes, pathway level changes, and *in vivo* changes.

Table 7-2 describes some of the priority research needs for toxicogenomic studies for developmentally toxic chemicals, including DBP. First, appropriate time-course gene expression data over the critical window, using a small subset of genes whose altered expression is linked to

Table 7-2. Research needs for toxicogenomic studies to be used in risk assessment

Purpose	Study needed
1) Develop a gene network model	Exposure time-course microarray data.
2) Improve pathway analysis statistical power	Number of replicates increased.
3) Use of toxicogenomic data to inform toxicokinetics in dose-response analysis	Genomic and toxicity studies with same study design: Generate TK data in relevant study (time, dose, tissue), and obtain relevant internal dose measure to derive best internal dose metric.
4) Use of toxicogenomic data in dose-response analysis	Multiple doses in microarray studies in parallel with toxicity studies for phenotypic anchoring.
5) Determining the degree of phenotypic anchoring; informing MOAs (see Figure 3-4)	Similar study design characteristics for genomic and toxicity studies (i.e., dose, timing of exposure, organ/tissue evaluated). This includes assessing whether genes and pathways are due to compensatory mechanisms and/or general toxic responses.
6) Assess intraspecies differences	A study assessing multiple doses across rat strains (e.g., Wistar vs. SD); endpoint and microarray component of the study.
7) Assess interspecies differences	A study to assess whether different species with similar pathways (genes and sequence of steps) have a similar sensitivity to a given chemical. The findings could potentially enhance the utility of toxicogenomic data to aid species extrapolation in risk assessments.
8) Appropriate statistical pathway analysis methods for use in risk assessment	Further comparisons and evaluations of different methods.
9) Screening and categorizing chemicals by MOA in risk assessment (e.g., cumulative risk assessment)	Genomic (transcriptomic, proteomic, and/or metabolomic) signatures can be particularly useful for screening and categorizing chemicals by MOA in risk assessment.

the outcome of interest, would be very relevant for developing a regulatory network model. These studies need to be carefully designed based on the information on the critical window of exposure and the relationship to the particular outcome of concern. Second, the statistical power of pathway-analysis methods for global expression techniques, including microarrays,

proteomics and metabolomics, could be improved by designing and performing studies with more replicates. Thus, variability would be better characterized. Third, it would be helpful to design genomic studies that could inform both TK and dose-response (see Table 7-2, #3 and #4).

Performing genomic and toxicity studies with similar designs would provide useful information. These studies would be designed at the most relevant time of exposure, include low to high doses, and assess the relevant tissues. Relevant internal dose measurements could be obtained on which to base the internal dose metric. These studies, employing genomic and toxicity studies of comparable designs, would provide information about the relationship of dose, gene expression, and outcome, and thus, could potentially be used in dose-response analysis. Studies with both a toxicity and toxicogenomic component would obviously require assessment of a large sample size to be informative. These same studies could be used to inform MOAs (Table 7-2, #5) and could be adapted to comparing species (Table 7-2, #6). Regarding quantitative measures of intraspecies and interspecies differences, it should be noted that the same information which is necessary for quantitative assessment of interspecies differences (Section 7.2.2) may be useful for characterizing intraspecies variability, and vice versa. In particular, factors that explain or predict interstrain differences in rodent sensitivity to DBP, such as those noted between Wistar and SD rats, may be hypothesized to contribute to human variability. Further, toxicologically important interstrain differences identified from the toxicogenomic data could be an excellent data source for investigating whether they are also important for modulating interspecies sensitivity.

Finally, further development and comparison studies to identify appropriate statistical pathway analysis methods for use in risk assessment are needed (Table 7-2, #8). It is important to note that such studies require research funding and laboratories with expertise in both genomics and toxicology. Research needs for toxicity studies that would improve the utility in risk assessment are also described in Table 7-3. As was noted for the DBP case (see Chapter 4), complete reporting is necessary for studies that are intended for use in risk assessment.

7.3.2. Recommendations

Based on the lessons learned from performing the DBP case-study exercise, we developed some recommendations or best practices for evaluating genomic data in new assessments. The approach includes systematic consideration of

Table 7-3. Research needs for toxicity studies for utilizing toxicogenomic and toxicity data together in risk assessment

Study aspect	Research need
Study design	Exposing animals during optimal developmental stage/time (i.e., for the critical window).
	Assessing outcome at optimum developmental stage/time for that outcome.
	Parallel study design characteristics with toxicogenomic studies (i.e., dose, timing of exposure, organ/tissue evaluated) to obtain comparable toxicity and toxicogenomic studies to aid in determining relationships between gene expression changes and outcomes.
Reporting	Individual animal data to aid identification of low incidence effects, correlate gene expression changes and outcomes, and characterize intraspecies variability.
	All endpoints that were evaluated (independent of whether the outcome was positive or negative).

the genomic data for whether they could inform risk assessment steps, identification of questions to direct the evaluation, and evaluation of the genomic data and toxicity data to assess phenotypic anchoring. In addition, we have some specific recommendations. The first two recommendations are straightforward and could reasonably be performed by a risk assessor with basic training in genomics data evaluation and interpretation while the third recommendation requires expertise in genomic data analysis methods for implementation. The recommendations are presented below:

1. *Evaluate the genomic and other gene expression data for consistency of findings across studies to provide a WOE evaluation of the affected gene expression and pathways.* Some simple methods, such as using Venn diagrams and gene expression compilation approaches, can be applied to risk assessment. When evaluating the consistency of toxicogenomic data findings, it is advantageous to include all available gene expression data (single gene, global gene expression, protein, RNA) because single gene expression

techniques have been traditionally used to confirm the results of global gene expression studies and because single gene expression data add to the database.

2. *Perform BMD modeling on high-quality RT-PCR dose-response studies of genes known to be in the causal pathway of an MOA or outcome of interest.* Obtaining a BMD and BMDL is a useful starting point for both linear low-dose extrapolation and reference value approaches. We are not indicating which approach is appropriate to take for making predictions about the potential risk below the BMD or BMDL. "High quality" is defined in this context as a well-conducted study that assessed enough animals and litters for sufficient statistical power for characterizing the mean responses and the variability (interlitter and intralitter).

3. *Perform new analysis of toxicogenomic data in cases when the new analysis is likely to yield new information that would be useful to the risk assessment. Examples include:*

    - Perform a new pathway analysis in order to identify all affected pathways or other risk assessment applications. When the available published microarray studies have been conducted for purposes (e.g., basic science, pharmaceutical development) other than risk assessment, it may be useful to reanalyze the data for risk assessment purposes. Information about all affected pathways may contribute to an understanding of the mechanisms and MOAs.

    - Identify the genes and pathways affected over a critical window of exposure if global gene expression time-course data are available. Specifically, by developing a gene network over time, it may be possible to identify the earliest affected genes and/or pathways, which in turn may represent the earlier or initiating events for the outcome of interest.

7.3.3. Application of Genomic Data to Risk Assessment: Future Considerations

A number of the issues that emerged in evaluating the DBP genomic data set are relevant to using genomic data in risk assessment in general. Some issues regarding the use of genomic data are to the same as for the use of precursor information in risk assessment, regardless of the technique used to gather the information. Two outstanding questions are

- *How is the biologically significant level of change in a precursor marker determined?* And, specifically for toxicogenomic data, *what are the key genes (i.e., a key gene, a handful of genes associated with the outcome of interest, a genomic signature) whose altered expression leads to an adverse outcome?* Currently, decisions about the degree of change of a precursor event tend to be based on statistical significance because data to address biological significance are typically lacking (as is the case for T levels and male development of the testis). Genes are identified as DEGs in microarray studies based on statistical-significance criteria that may not reflect biological significant changes (i.e., identified genes may not be biologically meaningful while unidentified genes may be

meaningful). This point is also relevant to the question: *What pathway analysis methods are most appropriate for risk assessment?* As noted in Section 5.5, it is difficult to know whether one has identified the biologically relevant DEGs and pathways. Statistically significant changes and repeated findings of the same genes and pathways across studies and using different analytical methods, while providing corroboration, do not necessarily provide a greater confidence regarding biological significance of these genes and pathways over other genes and pathways. Further, there is a bias towards the well-annotated genes as biologically significant when, in fact, the unannotated genes could be of greater importance.

- *What are the requirements for linkage of precursor events to in vivo endpoints?* Studies to assess the relationship between the gene expression and outcomes are needed to establish a causal connection. It is important to note that DBP has two well-established MOAs and strong phenotypic anchoring of some gene expression changes, which is not typical.

There are also a number of technical issues in utilizing microarray data in EPA risk assessments that have not fully been surmounted. The primary technical issue is the validation of the reproducibility of microarray study results. Reproducibility depends on biological sample preparation, interlaboratory (presumably related to operator and protocol differences), intralaboratory (presumably related to operator differences), and batch and platform variability. The results of the MAQC-I project (see Chapters 2 and 5) revealed that reproducibility was achieved when using the same biological sample. This is very encouraging for using microarray data in risk assessment. However, biological sample variability still needs to be addressed in order that protocols and details of the underlying reasons for the variability can be understood. MACQ-II and III projects are underway to address additional technical issues (see Chapter 2).

A number of the issues stem from the complexity of the data output from the global expression techniques (e.g., microarrays, proteomics, and metabolomics). This is in part a training issue. To address the training needs, the EPA Risk Assessment Forum held introductory and intermediate level training in genomics in 2007, and the FDA held genomics training (http://www.fda.gov/cder/genomics/Default.htm). However, it would be advantageous for organizations that perform risk assessments to embark on further training of risk assessors to enable them to perform analyses of microarray and other genomic data analysis techniques, and to understand the issues in applying traditional analytical methods to risk assessment.

If additional case studies are performed using the approach outlined in Figure 7-1, then we recommend a chemical whose exposure leads to both cancer and noncancer outcomes to

explore use of these data for multiple outcomes, as well as the impacts on the different risk assessment paradigms and processes (e.g., cancer vs. noncancer). Further, performing case studies on data-rich and data-poor chemicals would aid in further evaluating the approach described herein. For instance, performing a case study on a chemical with dose-response data and on a chemical with human polymorphism data would address issues in evaluating these types of data for risk assessment, allowing further refinement of the approach.

The approach for utilizing toxicogenomic data in risk assessment outlined in this document may be applied to other chemical assessments. This document advances the effort to devise strategies for using genomic data in risk assessment by defining an approach, performing a case study, and defining critical issues that need to be addressed to better utilize these data in risk assessment. This case study serves as an example of the considerations and methods for using genomic data in future risk assessments.

APPENDIX A.

SUPPORTING TABLES FOR CHAPTER 5

Appendix A contains additional tables that support the work shown in Chapter 5.

Table A-1. Weight of evidence (WOE) for statistically significant gene expression changes after in utero exposure to dibutyl phthalate (DBP) from the whole rat testis microarray studies[a] as reported in Thompson et al. (2005)[b], Shultz et al. (2001)[b], Liu et al. (2005)[c,d], and Plummer et al. (2007)[e]

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Aacs	Acetoacetyl-CoA synthetase	GDs 12−19	Down	−0.37 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Aadat	Aminoadipate aminotransferase	GDs 12−19	Down	−0.38 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	GDs 12−19	Up	0.38 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Acaa1	Acetyl-Coenzyme A acyltransferase 1	GDs 12−19	Down	−0.37 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Acaca	Acetyl-Coenzyme A carboxylase alpha	GDs 12−19	Down at GD 19	≥ 2	2-fold	Shultz et al., 2001
Acadl	Acetyl-Coenzyme A dehydrogenase, long-chain	GDs 12−19	Down at GD 19	≥ 2	2-fold	Shultz et al., 2001
Acads	Acyl-Coenzyme A dehydrogenase, short chain	GDs 12.5−15.5	Up	1.50	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Acsl4	Acyl-CoA synthetase long-chain family member 4	GDs 12−19	Down	−0.60 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Adam15	A disintegrin and metallopeptidase domain 15 (metargidin)	GDs 12.5−17.5	Up	1.20	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Adamts1	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	GDs 12.5−19.5	Down	−1.35	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Admr	Adrenomedullin receptor	GDs 12−19	Down	−0.90 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Adra1b	Adrenergic receptor, alpha 1b	GDs 12−19	Down	−0.30 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Akt2	Thymoma viral proto-oncogene 2	GDs 12−21	Down at GD 21	≥ 2	2-fold	Shultz et al., 2001
Alas1	Aminolevulinic acid synthase 1	GDs 12−19	Down	−1.01 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Alas1	Aminolevulinic acid synthase 1	GDs 12.5−17.5	Down	−1.33	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Alas1	Aminolevulinic acid synthase 1	GDs 12.5−19.5	Down	−1.44	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Aldh1a3	Aldehyde dehydrogenase family 1, subfamily A3	GDs 12−19	Down	−0.43 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Aldh2	Aldehyde dehydrogenase 2	GDs 12−19	Down	−0.82 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Aldh2	Aldehyde dehydrogenase 2	GDs 12.5−17.5	Down	−1.50	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Aldh2	Aldehyde dehydrogenase 2	GDs 12.5−19.5	Down	−1.91	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Aldoa	Aldolase A, fructose-bisphosphate	GDs 12.5−19.5	Down	−1.24	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Aldoc	Aldolase C	GDs 12−19	Down	−0.44 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Anxa5	Annexin A5	GDs 12.5−19.5	Down	−1.20	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Aox1	Aldehyde oxidase 1	GDs 12−19	Down	−0.50 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Aqp1	Aquaporin 1	GDs 12.5−15.5	Down	−1.29	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Arf3	ADP-ribosylation factor 3	GDs 12.5−17.5	Down	−1.23	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Arrb2	Arrestin, beta 2	GDs 12−21	Down at GD 21	≥ 2	2-fold	Shultz et al., 2001
Asns	Asparagine synthetase	GDs 12−19	Down	−0.24 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ass	Argininosuccinate synthetase	GDs 12−19	Down	−0.82 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Atf2	Activating transcription factor 2	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Atf4	Activating transcription factor 4	GD 19 for 3 hr	Up after 3 hr	0.67	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Atp1b1	ATPase, Na^{+}/K^{+} transporting, beta 1 polypeptide	GDs 12−19	Down	−0.24 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Atp4b	ATPase, H^{+}/K^{+} exchanging, beta polypeptide	GDs 12−19	Down	−0.60 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Atp5f1	ATP synthase, H^{+} transporting, mitochondrial F0 complex, subunit B1	GDs 12.5−15.5	Up	1.22	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Baiap2	Brain-specific angiogenesis inhibitor 1-associated protein 2	GDs 12−19	Down	−0.22 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Bhlhb2	Bhlhb2 basic helix-loop-helix domain containing, class B2	GD 19 for 3 hr	Up after 3 hr	0.88	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Bhmt	Betaine-homocysteine methyltransferase	GDs 12−19	Down	−0.24 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Birc5	Baculoviral IAP repeat-containing 5	GDs 12.5−15.5	Up	1.68	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Btg2	B-cell translocation gene 2, anti-proliferative	GD 19 for 1 hr	Up after1 hr	1.30	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Btg2	B-cell translocation gene 2, anti-proliferative	GD 19 for 3 hr	Up after 3 hr	1.88	$p < 0.05$ (ANOVA)	Thompson et al., 2005
C4a	Complement component 4a	GD 19 for 6 hr	Down after 6 hr	−0.77	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cadps	Ca^{2+}-dependent secretion activator	GDs 12−19	Up	0.31 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Calb2	Calbindin 2	GDs 12−19	Down	−0.77 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cd63	CD63 antigen	GDs 12.5−19.5	Down	−1.36	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	GDs 12−19	Down	−0.81 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	GD 19 for 6 hr	Down after 6 hr	−1.08	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	GDs 18−19 for 18 hr	Down after 18 hr	1.63	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	GDs 12−19	Down	−0.6 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	GD 19 for 3 hr	Up after 3 hr	1.62	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Clu	Clusterin	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Clu	Clusterin	GD 18 for 18 hr	Up after 18 hr	1.03	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cmklr1	Chemokine-like receptor 1	GDs 12.5−19.5	Down	−1.17	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cnr1	Cannabinoid receptor 1 (brain)	GD 19 for 3 hr	Up after 3 hr	0.99	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cnbp	Cellular nucleic acid binding protein	GDs 12.5−19.5	Down	−1.29	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cpa1	Carboxypeptidase A1	GDs 12.5−17.5	Down	−1.73	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cpa1	Carboxypeptidase A1	GDs 12.5−19.5	Down	−2.33	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cpd	Carboxypeptidase D	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Cpe	Carboxypeptidase E	GDs 12−19	Up	0.59 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cpt1a	Carnitine palmitoyltransferase 1a, liver	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Cpt1a	Carnitine palmitoyltransferase 1a, liver	GDs 12−21	Down at GD 21	−≥ 2	2-fold	Shultz et al., 2001
Cpt1b	Cpt1b carnitine palmitoyltrans-ferase1b, muscle	GDs 12−19	Up	0.23 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cpz	Carboxypeptidase Z	GDs 12−19	Up	0.21 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Crabp2	Cellular retinoic acid binding protein 2	GDs 12−19	Down	−0.31 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Crabp2	Cellular retinoic acid binding protein 2	GD 19 for 6 hr	Down after 6 hr	−1.24	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Crem	cAMP responsive element modulator	GD 19 for 3 hr	Up after 3 hr	0.58	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Crispld2	Cysteine-rich secretory protein LCCL domain containing 2	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cryab	Crystallin, alpha B	GDs 12−19	Up	0.22 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ctgf	Connective tissue growth factor	GD 19 for 3 hr	Up after 3 hr	2.10	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Ctgf	Connective tissue growth factor	GD 19 for 6 hr	Up after 6 hr	2.37	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Ctsb	Cathepsin B	GDs 12.5−15.5	Up	1.53	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Ctsd	Cathepsin D	GDs 12.5−19.5	Down	−1.22	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cxcl10	Chemokine (C-X-C motif) ligand 10	GD 19 for 3 hr	Up after 3 hr	2.07	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cyb5	Cytochrome b-5	GDs 12−19	Down	−0.30 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	GDs 12−19	Down	−1.07 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	GDs 12−19	Down at GD 19	−≥ 2	2-fold	Shultz et al., 2001
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	GD 18 for 18 hr	Down after 18 hr	−1.93	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	GDs 12.5−17.5	Down	−1.71	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	GDs 12.5−19.5	Down	−2.85	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cyp11b1	Cytochrome P450, subfamily 11B, polypeptide 1	GD 18 for 18 hr	Down after 18 hr	−1.63	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	GDs 12−19	Down	−1.76 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	GD 18 for 18 hr	Down after 18 hr	−2.1	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	GDs 12.5−17.5	Down	−2.15	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	GDs 12.5−19.5	Down	−3.08	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cyp51	Cytochrome P450, subfamily 51	GD 18 for 18 hr	Down after 18 hr	−1.06	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Cyp51	Cytochrome P450, subfamily 51	GDs 12.5−17.5	Down	−1.59	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Cyp51	Cytochrome P450, subfamily 51	GDs 12.5−19.5	Down	−1.81	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Dab2	Disabled homolog 2 (Drosophila)	GDs 12−19	Up	0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Daf1	Decay accelerating factor 1	GDs 12−19	Up	0.19 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Dbi	Diazepam binding inhibitor	GDs 12−19	Down	−0.38 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Dbi	Diazepam binding inhibitor	GDs 12.5−19.5	Down	−1.28	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Dcc	Deleted in colorectal carcinoma	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Ddc	Dopa decarboxylase	GDs 12−19	Down	−1.14 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ddc	Dopa decarboxylase	GD 18 for 18 hr	Down after 18 hr	−1.38	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Ddc	Dopa decarboxylase	GDs 12.5−19.5	Down	−1.44	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Ddit4	DNA-damage-inducible transcript 4	GDs 12−19	Down	−1.02 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ddit4	DNA-damage-inducible transcript 4	GD 18 for 18 hr	Down after 18 hr	−1.57	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Ddt	D-dopachrome tautomerase	GDs 12.5−19.5	Down	−1.22	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Decr1	2,4-dienoyl CoA reductase 1, mitochondrial	GDs 12−19	Down	−0.21 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Dhcr7	7-dehydrocholesterol reductase	GDs 12−19	Down	−0.73 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Dhcr7	7-dehydrocholesterol reductase	GD 19 for 6 hr	Down after 6 hr	−1.34	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Dhcr7	7-dehydrocholesterol reductase	GDs 18−19 for 18 hr	Down after 18 hr	−1.18	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Dnm3	Dynamin 3	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Dusp1	Dual specificity phosphatase 1	GD 19 for 3 hr	Up after 3 hr	0.91	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Dusp6	Dual specificity phosphatase 6	GDs 12−19	Up	0.39 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Dusp6	Dual specificity phosphatase 6	GD 19 for 3 hr	Up after 3 hr	1.28	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Ebp	Phenylalkylamine Ca^{2+} antagonist (emopamil) binding protein	GDs 12−19	Down	−0.64 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Echs1	Enoyl Coenzyme A hydratase, short chain 1, mitochondrial	GDs 12−19	Down	−0.18 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Egr1	Early growth response 1	GDs 12−19	Up	0.77 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Egr2	Early growth response 2	GD 19 for 1 hr	Up after 1 hr	1.93	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Egr2	Early growth response 2	GD 19 for 3 hr	Up after 3 hr	1.53	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Elovl5	ELOVL family member 5, elongation of long chain fatty acids (yeast)	GDs 12−19	Down	−0.17 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	GDs 12−19	Down	−0.40 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Emp3	Epithelial membrane protein 3	GDs 12.5−19.5	Down	−1.24	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Eno1	Enolase 1, alpha non-neuron	GDs 12.5−19.5	Down	−1.63	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Enpep	Glutamyl aminopeptidase	GDs 12−19	Up	0.48 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Entpd5	Ectonucleoside triphosphate diphosphohydrolase 5	GDs 12−19	Down	−0.52 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Epas1	Endothelial PAS domain protein 1	GDs 12−19	Down	−0.21 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ephx1	Epoxide hydrolase 1, microsomal	GDs 12−19	Down	−0.57 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Erbb2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	GDs 12.5−17.5	Up	1.26	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Etfdh	Electron-transferring-flavoprotein dehydrogenase	GDs 12−19	Down	−0.39 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ezr	Ezrin	GDs 12−19	Up	0.20 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ezr	Ezrin	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
F10	Coagulation factor X	GDs 12−19	Down	−0.51 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fabp3	Fatty acid binding protein 3	GDs 12−19	Down	−0.49 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fabp3	Fatty acid binding protein 3	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Fabp3	Fatty acid binding protein 3	GD 19 for 3 hr	Down after 3 hr	−0.78	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fabp3	Fatty acid binding protein 3	GD 19 for 6 hr	Down after 6 hr	−1.68	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fabp3	Fatty acid binding protein 3	GDs 18−19 for 18 hr	Down after 18 hr	−1.09	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fabp5	Fatty acid binding protein 5, epidermal	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Fabp6	Fatty acid binding protein 6, ileal (gastrotropin)	GDs 12−19	Down	−0.23 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fads1	Fatty acid desaturase 1	GDs 12−19	Down	−0.80 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fads1	Fatty acid desaturase 1	GDs 12.5−15.5	Up	1.42	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fads1	Fatty acid desaturase 1	GDs 12.5−19.5	Down	1.47	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fads2	Fatty acid desaturase 2	GDs 12−19	Down	−0.42 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fat1	FAT tumor suppressor homolog 1 (Drosophila)	GDs 12.5−15.5	Down	−1.32	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Fbp2	Fructose-1,6-bisphosphatase 2	GDs 12−19	Up	0.28 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fdft1	Farnesyl diphosphate farnesyl transferase 1	GDs 12−19	Down	−0.58 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fdft1	Farnesyl diphosphate farnesyl transferase 1	GDs 12.5−19.5	Down	−1.40	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fdps	Farensyl diphosphate synthase	GDs 12−19	Down	−0.73 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fdps	Farensyl diphosphate synthase	GDs 12.5−17.5	Down	−1.49	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fdps	Farensyl diphosphate synthase	GDs 12.5−19.5	Down	−1.41	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fdx1	Ferredoxin 1	GDs 12−19	Down	−1.65 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fdx1	Ferredoxin 1	GD 18 for 18 hr	Down after 18 hr	−2.53	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fdx1	Ferredoxin 1	GDs 12.5−17.5	Down	−2.06	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fdx1	Ferredoxin 1	GDs 12.5−19.5	Down	−2.97	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fdxr	Ferredoxin reductase	GDs 12−19	Down	−0.37 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fdxr	Ferredoxin reductase	GDs 12.5−17.5	Down	−1.41	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Fgfr4	Fibroblast growth factor receptor 4	GDs 12−19	Down	−0.19 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Folr1	Folate receptor 1 (adult)	GDs 12−19	Down	−0.48 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fos	FBJ murine osteosarcoma viral oncogene homolog	GD 19 for 1 hr	Up after 1 hr	3.28	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fos	FBJ murine osteosarcoma viral oncogene homolog	GD 19 for 3 hr	Up after 3 hr	2.70	$p < 0.05$ (ANOVA)	Thompson et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Frag1	FGF receptor activating protein 1	GDs 12−19	Down	−0.48 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Frag1	FGF receptor activating protein 1	GD 18 for 18 hr	Down after 18 hr	−0.65	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fthfd	Formyltetrahydro-folate dehydrogenase	GDs 12−19	Down	−1.03 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Fthfd	Formyltetrahydro-folate dehydrogenase	GD 19 for 6 hr	Down after 6 hr	−0.98	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fthfd	Formyltetrahydro-folate dehydrogenase	GDs 18−19 for 18 hr	Down after 18 hr	−0.83	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Fzd2	Frizzled homolog 2 (Drosophila)	GD 19 for 3 hr	Down after 3 hr	−0.7	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Gaa	Glucosidase, alpha, acid	GDs 12−19	Down	−0.30 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ggtl3	Gamma-glutamyltransferase-like 3	GDs 12−19	Down	−0.32 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Gja1	Gap junction membrane channel protein alpha 1	GDs 12−19	Down	−0.36 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Glrx1	Glutaredoxin 1 (thioltransferase)	GDs 12−19	Down	−0.20 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Gnrhr	Gonadotropin releasing hormone receptor	GD 19 for 3 hr	Up after 3 hr	1.38	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Gnrhr	Gonadotropin releasing hormone receptor	GD 19 for 6 hr	Up after 6 hr	2.03	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Gpsn2	Glycoprotein, synaptic 2	GDs 12−19	Down	−0.42 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Grb14	Growth factor receptor bound protein 14	GDs 12−19	Up	0.68 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Grb14	Growth factor receptor bound protein 14	GD 19 for 6 hr	Up after 6 hr	1.78	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Grb14	Growth factor receptor bound protein 14	GDs 18−19 for 18 hr	Up after 18 hr	0.93	$p < 0.05$ (ANOVA)	Thompson et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Grina	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	GDs 12.5−15.5	Up	1.59	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Gsta2	Glutathione-S-transferase, alpha type2	GDs 12.5−17.5	Down	−1.48	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Gsta2	Glutathione-S-transferase, alpha type2	GDs 12.5−19.5	Down	−2.23	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Gsta3	Glutathione S-transferase A3	GDs 12−19	Down	−0.96 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Gsta3	Glutathione S-transferase A3	GDs 12.5−17.5	Down	−1.75	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Gsta3	Glutathione S-transferase A3	GDs 12.5−19.5	Down	−2.63	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Gstm2	Glutathione S-transferase, mu 2	GDs 12−19	Down	−0.42 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Gstm2	Glutathione S-transferase, mu 2	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Gstm2	Glutathione S-transferase, mu 2	GDs 18−19 for 18 hr	Down after 18 hr	−0.47	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Gsto1	Glutathione S-transferase omega 1	GDs 12−19	Down	−0.42 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Gstp1	Glutathione-S-transferase, pi 1	GDs 12.5−15.5	Up	1.34	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Hao2	Hydroxyacid oxidase 2 (long chain)	GDs 12−19	Down	−0.58 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	GDs 12−19	Down	−0.47 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	GDs 12.5−19.5	Down	−1.83	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	GDs 12−19	Down	−1.03 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	GDs 12.5−17.5	Down	−1.72	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	GDs 12.5−19.5	Down	−1.87	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Hmox1	Heme oxygenase (decycling) 1	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hpgd	Hydroxyprostaglandin dehydrogenase 15 (NAD)	GDs 12−19	Down	−0.46 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hprt	Hypoxanthine guanine phosphoribosyl transferase	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Hrasls3	HRAS like suppressor 3	GDs 12−19	Down	−0.45 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hsd11b2	Hydroxysteroid (11-beta) dehydrogenase 2	GD 19 for 6 hr	Down after 6 hr	−1.16	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Hsd17b3	Hydroxysteroid (17-beta) dehydrogenase 3	GDs 12−19	Up	0.28 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hsd17b7	Hydroxysteroid (17-beta) dehydrogenase 7	GDs 12−19	Down	−0.32 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hsd3b1_predicted	Hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted)	GDs 12−19	Down	−0.50 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Hsd3b1_predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted)	GD 18 for 18 hr	Down after 18 hr	−0.7	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Hspb7	Heat shock 27kD protein family, member 7 (cardiovascular)	GDs 12−19	Up	0.41 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	GDs 12−19	Down	−0.52 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	GD 18 for 18 hr	Down after 18 hr	−0.67	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Idi1	Isopentenyl-diphosphate delta isomerase	GDs 12−19	Down	−0.85 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Idi1	Isopentenyl-diphosphate delta isomerase	GDs 12.5−17.5	Down	−1.57	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Igfbp2	Insulin-like growth factor binding protein 2	GDs 12−19	Down	−0.39 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Igfbp3	Insulin-like growth factor binding protein 3	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Il6st	Interleukin 6 signal transducer	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Ifitm2	Interferon induced transmembrane protein 2	GDs 12.5−17.5	Down	−1.11	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Inha	Inhibin alpha	GDs 12−19	Down	−1.00 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Inha	Inhibin alpha	GDs 12.5−19.5	Down	−1.64	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Insig1	Insulin induced gene 1	GDs 12−19	Down	−0.77 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Insl3	Insulin-like 3	GDs 12−19	Down	−1.56 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
interim symbol: *Loc314323*	Interim full name: transporter	GDs 12−19	Down	−0.35 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
interim symbol: *Ratsg2*	Interim name: *Ratsg2*	GDs 12−19	Down	−0.13 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Kcnj8	Potassium inwardly-rectifying channel, subfamily J, member 8	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Khk	Ketohexokinase	GDs 12.5−17.5	Up	1.30	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Kit	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Krt2-8	Keratin complex 2, basic, gene 8	GDs 12−19	Up	0.28 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Ldha	Lactate dehydro-genase A	GDs 12.5−19.5	Down	−1.30	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Ldlr	Low density lipoprotein receptor	GDs 12−19	Down	−0.79 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ldlr	Low density lipoprotein receptor	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	GD 19 for 6 hr	Down after 6 hr	−1.00	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	GDs 18−19 for 18 hr	Down after 18 hr	−1.51	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	GDs 12−19	Down	−1.39 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Limk1	LIM motif-containing protein kinase 1	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Lnk	Linker of T-cell receptor pathways	GD 19 for 3 hr	Up after 3 hr	1.17	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Lr8	LR8 protein	GDs 12−19	Down	−0.45 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Lss	Lanosterol synthase	GDs 12−19	Down	−0.48 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Mapk1	Mitogen activated protein kinase 1	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Marcks	Myristoylated alanine rich protein kinase C substrate	GDs 12−19	Up at GD 19	≥ 2	2-fold	Shultz et al., 2001
Mdk	Midkine	GDs 12−19	Up	0.20 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Me1	Malic enzyme 1, NADP($^+$)-dependent, cytosolic	GDs 12−19	Down	−0.67 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Me1	Malic enzyme 1, NADP(+)-dependent, cytosolic	GDs 12.5−17.5	Down	−1.36	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Men1	Multiple endocrine neoplasia 1	GDs 12.5−15.5	Down	−1.17	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Mgat1	Mannoside acetylglucosaminyltransferase 1	GDs 12−19	Up	0.28 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Mgp	Matrix Gla protein	GD 19 for 6 hr	Up after 6 hr	1.66	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Mgst1	Microsomal glutathione S-transferase 1	GDs 12−19	Down	−0.36 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Mgst1	Microsomal glutathione S-transferase 1	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Mir16	Membrane interacting protein of RGS16	GDs 12−19	Down	−0.56 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Mlxipl	MLX interacting protein-like	GDs 12−19	Down	−0.31 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Mmp2	Matrix metallopeptidase 2	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Mtus1	Mitochondrial tumor suppressor 1	GD 19 for 3 hr	Up after 3 hr	0.67	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Mtus1	Mitochondrial tumor suppressor 1	GD 19 for 6 hr	Up after 6 hr	0.55	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Mvd	Mevalonate (diphospho) decarboxylase	GDs 12−19	Down	−0.41 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Myd116	Myeloid differentiation primary response gene 116	GD 19 for 3 hr	Up after 3 hr	0.58	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha	GDs 12−19	Down	−0.72 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha	GDs 18−19 for 18 hr	Down after 18 hr	−1.52	$p < 0.05$ (ANOVA)	Thompson et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha	GDs 12.5−19.5	Down	−1.64	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Myom2	Myomesin 2	GDs 12−19	Up	0.64 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Myrip	Myosin VIIA and Rab interacting protein	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nalp6	NACHT, leucine rich repeat and PYD containing 6	GDs 12−19	Up	0.45 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nexn	Nexilin	GDs 12−19	Up	0.26 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nf1	Neurofibromatosis 1	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Nfil3	Nuclear factor, interleukin 3 regulated	GDs 12−19	Up	0.31 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	GD 19 for 3 hr	Up after 3 hr	0.79	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Npc2	Niemann pick type C2	GDs 12−19	Down	−0.26 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nppc	Natriuretic peptide precursor type C	GDs 12−19	Down	−0.56 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nr0b1	Nuclear receptor subfamily 0, group B, member 1	GDs 12−19	Down	−0.37 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nr0b1	Nuclear receptor subfamily 0, group B, member 1	GDs 12.5−19.5	Down	−1.15	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	GDs 12−19	Up	0.3 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	GD 19 for 3 hr	Up after 3 hr	1.83	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Nr4a3	Nuclear receptor subfamily 4, group A, member 3	GD 19 for 3 hr	Up after 3 hr	2.25	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Nr5a1	Nr5a1 nuclear receptor subfamily 5, group A, member 1	GDs 12.5−19.5	Down	−1.18	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Ntf3	Neurotrophin 3	GDs 12.5−17.5	Up	1.34	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Okl38	Pregnancy-induced growth inhibitor	GDs 12−19	Down	−0.33 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Olfm1	Olfactomedin 1	GDs 12−19	Down	−0.14 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
P2ry14	Purinergic receptor P2Y, G-protein coupled, 14	GDs 12−19	Down	−0.37 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Park7	Parkinson disease (autosomal recessive, early onset) 7	GDs 12.5−17.5	Down	−1.32	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Pawr	PRKC, apoptosis, WT1, regulator	GD 19 for 3 hr	Up after 3 hr	1.02	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Pcna	Proliferating cell nuclear antigen	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Pcyt2	Phosphate cytidylyltransferase 2, ethanolamine	GDs 12−19	Down	−0.20 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Pdap1	PDGFA associated protein 1	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
Pdyn	Prodynorphin	GDs 12−19	Down	−1.06 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Pebp1	Phosphatidylethanolamine binding protein 1	GDs 12−19	Down	−0.36 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Pebp1	Phosphatidylethanolamine binding protein 1	GDs 12.5−19.5	Down	−1.67	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Penk1	Proenkephalin 1	GDs 12.5−17.5	Down	−1.41	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Penk1	Proenkephalin 1	GDs 12.5−19.5	Down	−1.86	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Pfkp	Phosphofructokinase, platelet	GDs 12.5−19.5	Down	−1.41	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Pgam1	Phosphoglycerate mutase 1	GDs 12.5−19.5	Down	−1.26	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Pgk1	Phosphoglycerate kinase 1	GDs 12.5−19.5	Down	−1.25	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Phb	Prohibitin	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Phb	Prohibitin	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Phyh	Phytanoyl-CoA hydroxylase	GD 19 for 6 hr	Down after 6 hr	−1.02	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Plat	Plasminogen activator, tissue	GDs 12−19	Up at GD 19	≥ 2	2-fold	Shultz et al., 2001
Plaur	Plasminogen activator, urokinase receptor	GD 19 for 3 hr	Up after 3 hr	0.86	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Pmp22	Peripheral myelin protein 22	GDs 12−19	Up at GD 19	≥ 2	2-fold	Shultz et al., 2001
Pmp22	Peripheral myelin protein 22	GD 19 for 3 hr	Down after 3 hr	−0.75	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Pmp22	Peripheral myelin protein 22	GD 19 for 6 hr	Down after 6 hr	−0.59	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Pnliprp2	Pancreatic lipase-related protein 2	GDs 12−19	Down	−0.28 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Por	P450 (cytochrome) oxidoreductase	GDs 12−19	Down	−0.64 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Por	P450 (cytochrome) oxidoreductase	GDs 12.5−19.5	Down	−1.39	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Ppib	Peptidylprolyl isomerase B	GDs 12.5−17.5	Down	−1.21	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Ppp1cb	Protein phosphatase 1, catalytic subunit, beta isoform	GDs 12.5−17.5	Down	−1.37	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Prdx3	Peroxiredoxin 3	GDs 12−19	Down	−0.53 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Prdx3	Peroxiredoxin 3	GDs 18−19 for 18 hr	Down after 18 hr	−0.86	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Prdx3	Peroxiredoxin 3	GDs 12.5−19.5	Down	−1.63	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Prg1	Plasticity related gene 1	GDs 12−19	Down	−0.97 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Prkar2b	Protein kinase, cAMP dependent regulatory, type II beta	GDs 12−19	Down	−0.33 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Prkcbp1	Protein kinase C binding protein 1	GDs 12−19	Up	0.32 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Prlr	Prolactin receptor	GDs 12−19	Down	−1.02 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ptma	Prothymosin alpha	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Ptp4a1	Protein tyrosine phosphatase 4a1	GDs 12−21	Up at GD 21	≥ 2	2-fold	Shultz et al., 2001
PVR	Poliovirus receptor	GD 19 for 3 hr	Up after 3 hr	1.26	$p < 0.05$ (ANOVA)	Thompson et al., 2005
PVR	Poliovirus receptor	GD 19 for 6 hr	Up after 6 hr	0.92	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Rabep2	Rabaptin, RAB GTPase binding effector protein 2	GD 19 for 3 hr	Down after 3 hr	−0.48	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Rasd1	RAS, dexamethasone-induced 1	GDs 12−19	Down	−0.52 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Rln1	Relaxin 1	GDs 12−19	Down	−0.36 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Rnh1	Ribonuclease/angiogenin inhibitor 1	GDs 12.5−17.5	Down	−1.20	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rpa2	Replication protein A2	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Rpl13	Ribosomal protein L13	GDs 12.5−15.5	Up	1.17	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rpl32	Ribosomal protein L32	GDs 12.5−19.5	Up	1.13	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rpl37	Ribosomal protein L37	GDs 12.5−19.5	Up	1.13	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rpl36a	Large subunit ribosomal protein L36a	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Rpl36a	Large subunit ribosomal protein L36a	GDs 12.5−15.5	Up	1.22	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rpn2	Ribophorin II	GDs 12.5−19.5	Down	−1.19	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rps13	Ribosomal protein S13	GDs 12.5−15.5	Up	1.30	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rps17	Ribosomal protein S17	GDs 12.5−19.5	Up	1.25	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rps19	Ribosomal protein S19	GDs 12.5−17.5	Up	1.25	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Rps29	Ribosomal protein S29	GDs 12.5−19.5	Down	−1.13	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Sc4mol	Sterol-C4-methyl oxidase-like	GDs 12−19	Down	−1.02 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Sc4mol	Sterol-C4-methyl oxidase-like	GDs 12.5−17.5	Down	−1.82	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Sc4mol	Sterol-C4-methyl oxidase-like	GDs 12.5−19.5	Down	−2.36	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Sc5d	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (*S. cerevisae*)	GDs 12−19	Down	−0.32 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Scarb1	Scavenger receptor class B, member 1	GDs 12−19	Down	−1.91 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Scarb1	Scavenger receptor class B, member 1	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Scarb1	Scavenger receptor class B, member 1	GD 19 for 6 hr	Down after 6 hr	−1.60	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Scarb1	Scavenger receptor class B, member 1	GDs 18−19 for 18 hr	Down after 18 hr	−2.72	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Scarb1	Scavenger receptor class B, member 1	GDs 12.5−17.5	Down	−2.23	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Scarb1	Scavenger receptor class B, member 1	GDs 12.5−19.5	Down	−2.85	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Scd1	Stearoyl-Coenzyme A desaturase 1	GDs 12−19	Down	−0.58 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Scn3b	Sodium channel, voltage-gated, type III, beta	GD 19 for 6 hr	Up after 6 hr	1.49	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Scp2	Sterol carrier protein 2	GDs 12−19	Down	−0.17 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Scp2	Sterol carrier protein 2	GDs 12.5−19.5	Down	−1.24	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Sdf4	Stromal cell derived factor 4	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Sepp1	Selenoprotein P, plasma, 1	GDs 12−19	Down	−0.45 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	GD 19 for 3 hr	Up after 3 hr	1.56	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Serpinh1	Serine (or cysteine) peptidase inhibitor, clade H, member 1	GDs 12.5−15.5	Down	−1.32	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Sgk	Serum/glucocorticoid regulated kinase	GDs 12−19	Down	−0.45 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Slc3a2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	GDs 12−19	Down	−0.48 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Slc12a2	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	GDs 12.5−17.5	Down	−1.39	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Slc16a6	Solute carrier family 16 (monocarboxylic acid transporters), member 6	GDs 12−19	Down	−0.38 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Slc25a1	Solute carrier family 25, member 1	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Slc25a20	Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20	GDs 12−19	Down	−0.23 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Slc7a8	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	GDs 12.5−17.5	Down	−1.82	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Slc7a8	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	GDs 12.5−19.5	Down	−2.18	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Smpx	Small muscle protein, X-linked	GDs 12−19	Up	0.21 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Sod2	Superoxide dismutase 2, mitochondrial	GDs 12−19	Down	−0.51 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Sod3	Superoxide dismutase 3, extracellular	GDs 12−19	Down	−0.33 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Sqle	Squalene epoxidase	GDs 12−19	Down	−0.59 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Sqle	Squalene epoxidase	GD 18 for 18 hr	Down after 18 hr	−1.26	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Ssr4	Signal sequence receptor 4	GDs 12−19	Down	−0.23 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Ssrp1	Structure specific recognition protein 1	GDs 12−19	Down at GD 19	−≥ 2	2-fold	Shultz et al., 2001
Star	Steroidogenic acute regulatory protein	GDs 12−19	Down	−2.45 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Star	Steroidogenic acute regulatory protein	GDs 18−19 for 18 hr	Down after 18 hr	−2.33	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Star	Steroidogenic acute regulatory protein	GDs 12.5−17.5	Down	−2.19	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Star	Steroidogenic acute regulatory protein	GDs 12.5−19.5	Down	−2.53	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Stc1	Stanniocalcin 1	GDs 12−19	Up	0.98 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Stc1	Stanniocalcin 1	GD 19 for 6 hr	Up after 6 hr	1.61	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Stc2	Stanniocalcin 2	GDs 12−19	Down	−1.18 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Stc2	Stanniocalcin 2	GDs 12.5−19.5	Down	−1.59	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Sts	Steroid sulfatase	GDs 12−19	Down at GD 19	−≥ 2	2-fold	Shultz et al., 2001
Suclg1	Succinate-CoA ligase, GDP-forming, alpha subunit	GDs 12.5−19.5	Down	−1.21	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Svs5	Seminal vesicle secretion 5	GDs 12−19	Down	−3.75 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Svs5	Seminal vesicle secretion 5	GDs 18−19 for 18 hr	Down after 18 hr	−3.36	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Svs5	Seminal vesicle secretion 5	GDs 12.5−17.5	Down	−5.89	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Svs5	Seminal vesicle secretion 5	GDs 12.5−19.5	Down	−3.75	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Syngr1	Synaptogyrin 1	GDs 12−19	Down	−0.16 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tcf1	Transcription factor 1	GDs 12−19	Down	−0.14 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tcf21	Transcription factor 21	GDs 12−19	Up	0.17 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tec	Tec protein tyrosine kinase	GD 19 for 3 hr	Up after 3 hr	0.69	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Testin	Testin gene	GDs 12−19	Up	0.59 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tfrc	Transferrin receptor	GDs 12−19	Down	−0.23 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tgfb3	Transforming growth factor, beta 3	GDs 12−19	Down at GD 19	$-\geq 2$	2-fold	Shultz et al., 2001
Timp1	Tissue inhibitor of metallopeptidase 1	GD 19 for 6 hr	Up after 6 hr	1.04	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Timp3	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Tkt	Transketolase	GDs 12.5−17.5	Down	−1.19	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Tkt	Transketolase	GDs 12.5−19.5	Down	−1.28	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Tmed10	Transmembrane emp24-like trafficking protein 10 (yeast)	GDs 12.5−19.5	Down	−1.20	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	GD 19 for 6 hr	Up after 6 hr	1.34	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Tnni1	Troponin I, skeletal, slow 1	GDs 12−19	Up	0.33 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tnni3	Troponin I type 3 (cardiac)	GDs 12−19	Up	0.26 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tnnt2	Troponin T2, cardiac	GDs 12−19	Up	0.77 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Tpi1	Triosephosphate isomerase 1	GDs 12−19	Down	−0.24 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tpm1	Tropomyosin 1, alpha	GDs 12−19	Up	0.36 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tpm1	Tropomyosin 1, alpha	GD 19 for 6 hr	Up after 6 hr	1.04	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Tsc22d1	TSC22 domain family, member 1	GDs 12.5−19.5	Down	−1.34	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Tsn	Translin	GDs 12.5−17.5	Up	1.54	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Tst	Thiosulfate sulfurtransferase	GDs 12−19	Down	−0.33 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Tuba1	Tubulin, alpha 1	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Tuba1	Tubulin, alpha 1	GDs 12.5−19.5	Down	−1.26	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Txn1	Thioredoxin 1	GD 18 for 18 hr	Down after 18 hr	−0.62	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Txnl1	Thioredoxin-like 1	GDs 12.5−15.5	Up	1.20	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Uba52	Ubiquitin A-52 residue ribosomal protein fusion product 1	GDs 12.5−19.5	Up	1.10	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Unc5b	Unc-5 homolog B (*C. elegans*)	GDs 12−21	Down at GD 21	$-\geq 2$	2-fold	Shultz et al., 2001
Vapa	VAMP (vesicle-associated membrane protein)-associated protein A	GDs 12.5−19.5	Down	−1.37	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Vcam1	Vascular cell adhesion molecule 1	GDs 12−19	Down	−0.63 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Vdac1	Voltage-dependent anion channel 1	GDs 12.5−19.5	Down	−1.13	$p < 0.01$ (ANOVA)	Plummer et al., 2007

Table A-1 (continued)

Official gene symbol	Official gene name[f]	Exposure window	Up or down	Fold change	Cutoff used (method)	Reference
Vim	Vimentin	GDs 12.5−19.5	Down	−1.60	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Vnn11	Vanin 1	GDs 12−19	Down	−0.32 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Vsnl1	Visinin-like 1	GDs 12−19	Down	−0.62 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Ywhae	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	GDs 12.5−19.5	Down	−1.37	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Zfp36	Zinc finger protein 36	GD 19 for 1 hr	Up after 1 hr	1.79	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Zyx	Zyxin	GD 19 for 3 hr	Up after 3 hr	1.03	$p < 0.05$ (ANOVA)	Thompson et al., 2005
Not found	Listed as "Tppc" and 289920_Rn in article, and Genbank #BF400584 (Plummer, personal communication) does not match a gene name.	GDs 12.5−17.5	Down	−1.39	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Not found	Listed as "Similar to mouse IAP-binding protein" and 205510_Rn in article, and Genbank #:BG378907 (Plummer, personal communication) does not match a gene name.	GDs 12.5−15.5	Up	1.26	$p < 0.01$ (ANOVA)	Plummer et al., 2007
Not found	LOC499942 similar to WAP four-disulfide core domain protein 8 precursor (Putative protease inhibitor WAP8) (*Rattus norvegicus*).	GDs 12−19	Down	−0.25 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005
Not found	LOC497726 hypothetical gene supported by NM_138518 (*Rattus norvegicus*). This record was discontinued.	GDs 12−19	Down	−0.27 log2	$p < 0.05$ (ANOVA)	Liu et al., 2005

[a]The four studies dosed at 500 mg/kg-d DBP in the Sprague-Dawley (SD) rat.
[b]Thompson et al. (2005) and Shultz et al. (2001) dosed with DBP alone; gene expression changes for DBP were relative to vehicle control expression.

Table A-1. (continued)

[c]Liu et al. (2005) presented microarray data for all five developmental phthalates, including DBP, since they did not find any differences in statistical significance among the five phthalates. Thus, we present the data for all five phthalates, which should be the same as for DBP.

[d]The Affy ID 1387057_at was found to be significantly down-regulated by Liu et al. (2005). This Affy ID was listed as the gene *Slc7a8* (solute carrier family 7 [cationic amino acid transporter, y+ system], member 8) at the time of their publication. As of January 2007, Affy now lists both *Slc7a8* and *Syngap1*. This probeset is apparently capable of hybridizing with two different genes. Thus, this Affy ID was not incorporated in the case study evaluation since it is not clear which gene was altered after DBP in utero exposure.

[e]The Plummer et al. (2007) data from the whole testis are included in this table. The data from microdissection of testicular regions are not presented since no other studies were comparable. Plummer et al. (2007) performed their study in the Wistar rat whereas the other three microarray studies were performed in the SD rat.

[f]Gene function and pathway information was gathered from GeneGo (www.genego.com).

ANOVA, analysis of variance; GD, gestation day; hr, hour.

Table A-2. WOE for statistically significant gene expression changes after in utero exposure to DBP from whole-rat testis reverse transcription-polymerase chain reaction (RT-PCR) studies

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Ar	Androgen receptor	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Bmp4	Bone morphogenetic protein 4	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Btg2	B-cell translocation gene 2, anti-proliferative	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−6 hr (peak ~2 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Bzrp	Benzodiazepine receptor, peripheral	500 mg/kg-d	GDs 12−19	Up	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA; $p < 0.05$	Liu et al., 2005
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−6 hr (peak ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Clu	Clusterin	500 mg/kg-d	GDs 12−19	Up	ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Clu	Clusterin	500 mg/kg-d	GDs 12−19	Up	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Clu	Clusterin	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	Up	$p < 0.05$	Shultz et al., 2001
Cxcl1	Chemokine (C-X-C motif) ligand 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−12 hr (peak at ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−19	Down	ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	50 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	100 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	Down	$p < 0.05$	Shultz et al., 2001
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−17 and 12−18	Down at GD 18	t-test, ANOVA (one-way) with Tukey post hoc analysis; $p < 0.05$	Thompson et al., 2004
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12.5−19.5	Down	One-way ANOVA followed by Bonferroni post test using GraphPad Prism; $p < 0.05$	Plummer et al., 2007
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−19	Down	Repeated measure ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	Down at GD 19	$p < 0.05$	Shultz et al., 2001
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	500 mg/kg-d	GDs 12−17 and 12−18	Down at GD 17 and 18	t-test, ANOVA (one-way) with Tukey post hoc analysis; $p < 0.05$	Thompson et al., 2004
Daf1	Decay accelerating factor 1	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Ddc	Dopa decarboxylase	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Dusp6	Dual specificity phosphatase 6	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−12 hr (peak at ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Edg3	Endothelial differentiation sphingolipid G-protein-coupled receptor 3	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−6 and 18 hr (peak ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Egfr	Epidermal growth factor receptor	500 mg/kg-d	GDs 12−19 and 12−21	Un-changed	t-test, $p < 0.05$	Bowman et al., 2005
Egr1	Early growth response 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−7 hr (peak ~2 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Egr1	Early growth response 1	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA; $p < 0.05$	Liu et al., 2005

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Egr2	Early growth response 2	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−12 hr (peak ~2 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Fgf10	Fibroblast growth factor 10	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005
Fgfr2	Fibroblast growth factor receptor 2	500 mg/kg-d	GDs 12−19 and 12−21	No stat. change	t-test, $p < 0.05$	Bowman et al., 2005
Fos	FBJ murine osteosarcoma viral oncogene homolog	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after 30 min and 6 hr (peak at 1 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Grb14	Growth factor receptor bound protein 14	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Hes6	Hairy and enhancer of split 6 (Drosophila)	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Down after 1−3 hr (peak at 3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Hsd17b3	Hydroxysteroid (17-beta) dehydrogenase 3	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Hsd17b7	Hydroxysteroid (17-beta) dehydrogenase 7	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Hsd3b1_predicted	Hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted)	500 mg/kg-d	GDs 12−19	Down	ANOVA, nested design, $p < 0.05$	Barlow et al., 2003

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Hsd3b1_ predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted) or Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	0.1 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Hsd3b1_ predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted) or Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	1 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Hsd3b1_ predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted) or Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	10 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Hsd3b1_ predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted) or Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	50 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Hsd3b1_predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted) or Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	100 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Hsd3b1_predicted	Hsd3b1_predicted hydroxysteroid dehydrogenase-1, delta< 5 >-3-beta (predicted) or Hsd3b1 hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Ier3	Immediate early response 3	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after 1−12 hr (peak ~2 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Ifrd1	Interferon-related developmental regulator 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−6 and 18 hr (peak ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Igf1	Insulin-like growth factor 1	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005
Igf1	Insulin-like growth factor 1	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Igf1r	Insulin-like growth factor 1 receptor	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Igf2	Insulin-like growth factor 2	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Igfbp5	Insulin-like growth factor binding protein 5	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005
Insig1	Insulin induced gene 1	500 mg/kg-d	GDs 12−19	Down	One way; and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Insl3	Insulin-like 3	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Insl3	Insulin-like 3	1000 mg/kg-d	GDs 13−17 (GDs 14−18 in Wilson et al., 2004 was changed to GDs 13−17 to make the GD comparable to the other 7 studies)	Down	ANOVA followed by LSMEANS, $p < 0.01$ or less	Wilson et al., 2004
Insl3	Insulin-like 3	500 mg/kg-d	GDs 12.5−19.5	Down	One-way ANOVA followed by Bonferroni post test using GraphPad Prism; $p < 0.05$	Plummer et al., 2007
Itgav	Integrin alpha V	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Junb	Jun-B oncogene	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	UP after ~1−12 hr (peak ~2−3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	500 mg/kg-d	GDs 12−19	Down	ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.1 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	1 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	50 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	100 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	500 mg/kg-d	GDs 12-19	Down at GD 19	$p < 0.05$	Shultz et al., 2001
Kitl	Kit ligand	500 mg/kg-d	GDs 12−19	Down	ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Ldlr	Low density lipoprotein receptor	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Map3k12	Mitogen activated protein kinase kinase kinase 12	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Marcks	Myristoylated alanine rich protein kinase C substrate	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	No stat. Change	$p < 0.05$	Shultz et al., 2001
Mgp	Matrix Gla protein	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Mmp2	Matrix metallopeptidase 2	500 mg/kg-d	GDs 12−19	Up	t-test, $p < 0.05$	Bowman et al., 2005
Mmp2	Matrix metallopeptidase 2	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005
Nfil3	Nuclear factor, interleukin 3 regulated	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Nfil3	Nuclear factor, interleukin 3 regulated	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~2−24 hr (peak ~6 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Notch2	Notch gene homolog 2 (Drosophila)	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005
Npc2	Niemann Pick type C2	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Nr0b1	Nuclear receptor subfamily 0, group B, member 1	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Nr0b1	Nuclear receptor subfamily 0, group B, member 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Down at 2 hr, Up 12 hr (peak at 12 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~6 and 18 hr (peak at 12 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Nr4a3	Nuclear receptor subfamily 4, group A, member 3	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−12 hr (peak at ~3−6 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Pawr	PRKC, apoptosis, WT1, regulator	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~2−24 hr (peak ~6 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Pcna	Proliferating cell nuclear antigen	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	No stat. change	$p < 0.05$	Shultz et al., 2001
Prkcbp1	Protein kinase C binding protein 1	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Scarb1	Scavenger receptor class B, member 1	500 mg/kg-d	GDs 12−19	Down	ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Scarb1	Scavenger receptor class B, member 1	1 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Scarb1	Scavenger receptor class B, member 1	50 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Scarb1	Scavenger receptor class B, member 1	100 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Scarb1	Scavenger receptor class B, member 1	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Scarb1	Scavenger receptor class B, member 1	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	Down	$p < 0.05$	Shultz et al., 2001
Scarb1	Scavenger receptor class B, member 1	500 mg/kg-d	GDs 12−17 and 12−18	Down at GDs 17 and 18	t-test, ANOVA (one-way) with Tukey post hoc analysis, $p < 0.05$	Thompson et al., 2004

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Sgk	Serum/glucocorticoid regulated kinase	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Down and Up; Down after 2 hr; Up after 4 and 10 hr (peak at 6 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Sostdc1	Sclerostin domain containing 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Down after 2−6 hr; Up at 18 hr (peak)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Star	Steroidogenic acute regulatory protein	500 mg/kg-d	GDs 12−19	Down	Repeated measure ANOVA, nested design, $p < 0.05$	Barlow et al., 2003
Star	Steroidogenic acute regulatory protein	50 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Star	Steroidogenic acute regulatory protein	100 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Star	Steroidogenic acute regulatory protein	500 mg/kg-d	GDs 12−19	Down	Dunnett's test, ANOVA (one way), $p < 0.05$	Lehmann et al., 2004
Star	Steroidogenic acute regulatory protein	500 mg/kg-d	GDs 12−16, 12−19, or 12−21	Down at GDs 16, 19, and 21	$p < 0.05$	Shultz et al., 2001
Star	Steroidogenic acute regulatory protein	500 mg/kg-d	GDs 12−17 and 12−18	Down at GDs 17 and 18	t-test, ANOVA (one-way) with Tukey post hoc analysis; $p < 0.05$	Thompson et al., 2004
Star	Steroidogenic acute regulatory protein	500 mg/kg-d	GDs 12.5−19.5	Down	One-way ANOVA followed by Bonferroni post test using GraphPad Prism; $p < 0.05$	Plummer et al., 2007

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Stc1	Stanniocalcin 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~3−24 hr (peak ~6 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Svs5	Seminal vesicle secretion 5	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Tcf1	Transcription factor 1	500 mg/kg-d	GDs 12−19	Down	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Tcf1	Transcription factor 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Down after 1−3 hr (peak at 1 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Testin	Testin gene	500 mg/kg-d	GDs 12−19	Up	One way and two-way nested ANOVA, $p < 0.05$	Liu et al., 2005
Thbs1	Thrombospondin 1	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after 2−4 hr (peak ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Timp1	Tissue inhibitor of metalloproteinase 1	500 mg/kg-d	GDs 12−21	Up	t-test, $p < 0.05$	Bowman et al., 2005
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~1−12 hr (peak at ~3 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005
Wnt4	Wingless-related MMTV integration site 4	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after ~12 and 18 hr (peak 12 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005

Table A-2. (continued)

Official gene symbol	Official gene name*	Dose	Exposure window	Up or down	Statistical analysis method	Reference
Zfp36	Zinc finger protein 36	500 mg/kg-d	GD 19 for 30 min to 6 hr timepoints and GD 18 for 12, 18, and 24 hr time points	Up after 30 min and 6 hr and 15 and 20 hr (peak at 1 hr)	Relative expression determined using mean Ct; triplicate samples; GADPH control; SE; but *p* value not calculated	Thompson et al., 2005

*Gene function and pathway information was gathered from GeneGo (www.genego.com).

Table A-3. Genes identified using the Rosetta Error Model statistical filter and mapping to the five most significant biochemical functions and /or pathways using Ingenuity

Gene symbol	Gene name
Genes mapped to integrin pathway	
F2r	Coagulation factor II (thrombin) receptor
Src	Rous sarcoma oncogene
Gng5	Guanine nucleotide binding protein (G protein), gamma 5 subunit
Gnai3	Guanine nucleotide binding protein, alpha inhibiting 3
Gng7	Guanine nucleotide binding protein, gamma 7
Mapk3	Mitogen activated protein kinase 3
Gnao1	Guanine nucleotide binding protein, alpha o
Actc1	Actin alpha cardiac 1
Camk2d	Calcium/calmodulin-dependent protein kinase II, delta
Gnaq	Guanine nucleotide binding protein
Cxcl12	Chemokine (C-X-C motif) ligand 12
Prkce	Protein kinase C, epsilon
Genes mapped to cholesterol biosynthesis/metabolism	
Hmgcs1	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Hsd3b1	Hydroxyl-delta-5-steroid dehydrogenase
Dhcr7	7-Dehydrocholesterol reductase
Sqle	Squalene epoxidase
Soat1	Sterol O-acyltransferase 1
Cyp51a1	Cytochrome P450, family 51, subfamily a, polypeptide 1
Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1
Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1
Hmgcr	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase
Idi1	Osopentenyl-diphosphate delta isomerase
Sc4mol	Sterol-C4-methyl oxidase-like
Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1

Table A-3. (continued)

Gene symbol	Gene name
Genes mapped to chemokine mediated signaling	
Src	Rous sarcoma oncogene
Gng5	Guanine nucleotide binding protein (G protein), gamma 5 subunit
Hmgcs1	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Serpine2	Serine (or cysteine) proteinase inhibitor, clade E, member 2
Itgb5	Integrin, beta 5
Dhcr7	7-Dehydrocholesterol reductase
Gnai3	Guanine nucleotide binding protein, alpha inhibiting 3
Gng7	Guanine nucleotide binding protein, gamma 7
Sqle	Squalene epoxidase
Mapk3	Mitogen activated protein kinase 3
Gnao1	Guanine nucleotide binding protein, alpha o
Actn1	Actinin, alpha 1
Actc1	Actin alpha cardiac 1
Cav2	Caveolin 2
Cyp51a1	Cytochrome P450, family 51, subfamily a, polypeptide 1
Genes mapped to chemokine mediated signaling	
Col1a2	Procollagen, type I, alpha 2
Cfl1	Cofilin 1, non-muscle
Cav1	Caveolin 2
Hmgcr	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase
Mmp2	Matrix metallopeptidase 2
Msn	Moesin
Gsk3b	Glycogen synthase kinase 3 beta
Idi1	Isopentenyl-diphosphate delta isomerase
Plat	Plasminogen activator, tissue
Sdc2	Syndecan 2
Sc4mol	Sterol-C4-methyl oxidase-like
Lef1	Lymphoid enhancer binding factor 1

Table A-3. (continued)

Gene symbol	Gene name
Vegf	Vascular endothelial growth factor
Genes mapped to glycolysis/gluconeogenesis	
Pgk1	Phosphoglycerate kinase 1
Hmgcs1	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Tpi1	Triosephosphate isomerase 1
Fbp2	Fructose-1,6-bisphosphatase 2
Dhcr7	7-Dehydrocholesterol reductase
Pfkm	Phosphofructokinase, muscle
Pfkp	Phosphofructokinase, platelet
Mdh1	Malate dehydrogenase 1, NAD (soluble)
Sqle	Squalene epoxidase
Pgam1	Phosphoglycerate mutase 1
Aldoa	Aldolase A
Cyp51a1	Cytochrome P450, family 51, subfamily a, polypeptide 1
Hmgcr	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase
Hk1	Hexokinase 1
Gpi	Glucose phosphate isomerase
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Idi1	Isopentenyl-diphosphate delta isomerase
Sc4mol	Sterol-C4-methyl oxidase-like
Pfkl	Phosphofructokinase, liver

Table A-4. Differentially expressed genes that mapped to statistically significant pathways identified using the Signal to Noise Ratio (SNR) statistical filter

Gene symbol	Entrez gene ID	Gene name
Aadat	29416	Aminoadipate aminotransferase
Acadm	24158	Acetyl-Coenzyme A dehydrogenase, medium chain
Acads	64304	Acyl-Coenzyme A dehydrogenase, short chain
Acat1	25014	Acetyl-Coenzyme A acetyltransferase 1
Aco2	79250	Aconitase 2, mitochondrial
Acsl4	113976	Acyl-CoA synthetase long-chain family member 4
Akr1b4	24192	Aldo-keto reductase family 1, member B4 (aldose reductase)
Alas1	65155	Aminolevulinic acid synthase 1
Aldh1a4	29651	Aldehyde dehydrogenase family 1, subfamily A4
Aldh2	29539	Aldehyde dehydrogenase 2
Aldh6a1	81708	Aldehyde dehydrogenase family 6, subfamily A1
Aldoa	24189	Aldolase A
Aldoc	24191	Aldolase C, fructose-biphosphate
Ass	25698	Arginosuccinate synthetase
Bhmt	81508	Betaine-homocysteine methyltransferase
Chkb	29367	Choline kinase beta
Cyp11a1	29680	Cytochrome P450, family 11, subfamily a, polypeptide 1
Cyp17a1	25146	Cytochrome P450, family 17, subfamily a, polypeptide 1
Dcxr	171408	Dicarbonyl L-xylulose reductase
Ddc	24311	Dopa decarboxylase
Dhcr7	64191	7-dehydrocholesterol reductase
Ebp	117278	Phenylalkylamine Ca^{2+} antagonist (emopamil) binding protein
Ephx1	25315	Epoxide hydrolase 1
Fbp2	114508	Fructose-1,6-bisphosphatase 2
Fdft1	29580	Farnesyl diphosphate farnesyl transferase 1
Fdps	83791	Farnesyl diphosphate synthase
Fh1	24368	Fumarate hydratase 1

Table A-4. (continued)

Gene symbol	Entrez gene ID	Gene name
G6pdx	24377	Glucose-6-phosphate dehydrogenase
Gad2	24380	Glutamate decarboxylase 2
Gapdh	24383	Glyceraldehyde-3-phosphate dehydrogenase
Gatm	81660	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)
Ggtl3	156275	Gamma-glutamyltransferase-like 3
Gsta2	24422	Glutathione-S-transferase, alpha type2
Gsta3	24421	Glutathione S-transferase A5
Gstm2	24424	Glutathione S-transferase, mu 2
Gstm3	81869	Glutathione S-transferase, mu type 3
Hmgcr	25675	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
Hmgcs1	29637	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Idh1	24479	Isocitrate dehydrogenase 1 ($NADP^+$), soluble
Me1	24552	Malic enzyme 1
Mgst1	171341	Microsomal glutathione S-transferase 1
Mif	81683	Macrophage migration inhibitory factor
Mvd	81726	Mevalonate (diphospho) decarboxylase
Nos1	24598	Nitric oxide synthase 1, neuronal
Pycr2	364064	Pyrroline-5-carboxylate reductase family, member 2 (predicted)
Sqle	29230	Squalene epoxidase
Suclg1	114597	Succinate-CoA ligase, GDP-forming, alpha subunit
Tpi1	24849	Tpi1 protein

Table A-5. GeneGo pathway analysis of significant genes identified by REM

Pathway	Biological process	p-Value[a]	No. of genes[b,c]
NF-AT signaling in cardiac hypertrophy	Disease	2.23E−04	19/90
MIF—the neuroendocrine-macrophage connector	Immune response	3.00E−04	19/92
Lysine metabolism	Amino acid metabolism	3.05E−04	9/27
Cholesterol metabolism	Steroid metabolism	6.95E−04	6/14
Glycolysis and gluconeogenesis (short map)	Carbohydrates metabolism	7.40E−04	10/36
Integrin-mediated cell adhesion	Cell adhesion	8.44E−04	18/92
Tryptophan metabolism	Amino acid metabolism	9.56E−04	9/31
Cholesterol biosynthesis	Steroid metabolism	1.44E−03	7/21
ECM remodeling	Cell adhesion	1.64E−03	13/60
Regulation of lipid metabolism via PPAR, RXR, and VDR	Transcription	1.96E−03	7/22
Propionate metabolism p.2	Carbohydrates metabolism	1.96E−03	7/22
PPAR regulation of lipid metabolism	Regulation of lipid metabolism	2.04E−03	8/28
Mitochondrial long chain fatty acid beta-oxidation	Lipid metabolism	2.28E−03	6/17
Role of VDR in regulation of genes involved in osteoporosis	Transcription	3.16E−03	12/57
ChREBP regulation pathway	G-protein coupled receptor signaling	3.82E−03	10/44
Androstenedione and testosterone biosynthesis and metabolism p.1	Steroid metabolism	4.30E−03	6/19
Arginine metabolism	Amino acid metabolism	4.45E−03	9/38
Regulation of fatty acid synthesis: NLTP and EHHADH	Regulation of lipid metabolism	5.02E−03	4/9
Angiotensin signaling via STATs	Growth and differentiation	5.18E−03	11/53
Cytoskeleton remodeling	Cell adhesion	5.19E−03	26/176
dGTP metabolism	Nucleotide metabolism	5.34E−03	9/39
TCA	Amino acid metabolism	5.70E−03	6/20
Glycolysis and gluconeogenesis p. 1	Carbohydrates metabolism	5.70E−03	6/20
Peroxisomal branched chain fatty acid oxidation	Lipid metabolism	5.70E−03	6/20

Table A-5. (continued)

Pathway	Biological process	p-value[a]	No. of genes[bc]
Gamma-aminobutyrate (GABA) biosynthesis and metabolism	Metabolism of mediators	5.70E−03	6/20
Ligand-dependent activation of the ESR1/SP pathway	Response to hormone stimulus	6.38E−03	9/40
Integrin inside-out signaling	Cell adhesion	6.85E−03	14/78
Reverse signaling by ephrin B	Cell adhesion	6.86E−03	15/86
G-protein beta/gamma signaling cascades	G-protein coupled receptor protein signaling pathway	6.94E−03	11/55
Activation of PKC via G-Protein coupled receptor	G-protein coupled receptor protein signaling pathway	7.65E−03	15/87
Gap junctions	Cell adhesion	8.51E−03	10/49
WNT signaling pathway	Proteolysis	8.59E−03	7/28
Angiotensin activation of ERK	G-protein coupled receptor protein signaling pathway	9.12E−03	11/57
Role of Akt in hypoxia induced HIF1 activation	Proteolysis	9.83E−03	10/50
Regulation of actin cytoskeleton by Rho GTPases	Small GTPase mediated signal transduction	1.18E−02	11/59
CCR3 signaling in eosinophils	Immune response	1.22E−02	18/117
MAG-dependent inhibition of neurite outgrowth	Response to extracellular stimulus	1.47E−02	10/53
Endothelial cell contacts by junctional mechanisms	Cell adhesion	1.80E−02	7/32
Fructose metabolism	Carbohydrates metabolism	1.80E−02	7/32
Regulation of lipid metabolism via LXR, NF-Y and SREBP	Transcription	1.80E−02	7/32
CXCR4 signaling pathway	Cytokine and chemokine mediated signaling pathway	1.89E−02	10/55
Serotonin-melatonin biosynthesis and metabolism	Metabolism of mediators	2.04E−02	5/19
Glycolysis and gluconeogenesis p. 2	Carbohydrates metabolism	2.15E−02	4/13
Oxidative phosphorylation	Energy metabolism	2.37E−02	15/99
Urea cycle	Amino acid metabolism	2.58E−02	6/27
G-proteins mediated regulation p.38 and JNK signaling	G-protein coupled receptor protein signaling pathway	2.60E−02	11/66

Table A-5. (continued)

Pathway	Biological process	p-value[a]	No. of genes[bc]
Transcription factor tubby signaling pathways	Transcription	2.63E−02	8/42
Role PKA in cytoskeleton reorganization	Protein kinase cascade	2.64E−02	13/83
Ephrins signaling	Cell adhesion	2.66E−02	10/58
Propionate metabolism p.1	Carbohydrates metabolism	2.81E−02	4/14
Estrone metabolism	Steroid metabolism	2.81E−02	4/14
Regulation of acetyl-CoA carboxylase 2 activity in muscle	Response to extracellular stimulus	2.81E−02	4/14
Chemokines and adhesion	Cytokine and chemokine mediated signaling pathway	2.82E−02	23/174
Arachidonic acid production	Lipid metabolism	2.87E−02	7/35
dCTP/dUTP metabolism	Nucleotide metabolism	2.99E−02	8/43
Regulation of lipid metabolism by niacin and isoprenaline	Regulation of lipid metabolism	3.01E−02	9/51
Ubiquinone metabolism	Vitamin and cofactor metabolism	3.01E−02	9/51
Phenylalanine metabolism	Amino acid metabolism	3.05E−02	6/28
Leptin signaling via JAK/STAT and MAPK cascades	Response to hormone stimulus	3.57E−02	6/29
IMP biosynthesis	Nucleotide metabolism	3.70E−02	3/9
EPO-induced Jak-STAT pathway	Response to extracellular stimulus	3.78E−02	7/37
Integrin outside-in signaling	Cell adhesion	3.95E−02	12/79
Brca1 as transcription regulator	Cell cycle	4.15E−02	6/30
P53 signaling pathway	Transcription regulation	4.28E−02	8/46
Bile acid biosynthesis	Steroid metabolism	4.43E−02	5/23
Histidine-glutamate-glutamine and proline metabolism	Amino acid metabolism	4.79E−02	8/47
NTS activation of IL-8 in colonocytes	Immune response	4.85E−02	10/64

[a]Ordered from most significant (lowest *p*-value) to less significant.
[b]Number of genes from the DBP-exposed gene list mapping to the GeneGo pathway.
[c]Total number of genes in the GeneGo pathway.

Table A-6. Significant biological pathways corresponding to differentially expressed genes (DEGs) obtained from SNR analysis input into GeneGo

Pathway	Biological Process	p-Value[a]	No. of genes[bc]
Cholesterol Biosynthesis	Steroid metabolism	1.81E−09	15/21
Propionate metabolism p.2	Carbohydrates metabolism	5.54E−06	12/22
MIF—the neuroendocrine-macrophage connector	Immune response	3.22E−04	25/92
Tryptophan metabolism	Amino acid metabolism	3.78E−04	12/31
Lysine metabolism	Amino acid metabolism	3.93E−04	11/27
Cholesterol metabolism	Steroid metabolism	1.09E−03	7/14
NF-AT signaling in cardiac hypertrophy	Disease	1.38E−03	23/90
Glycolysis and gluconeogenesis (short map)	Carbohydrates metabolism	1.77E−03	12/36
G-alpha(q) regulation of lipid metabolism	Regulation of lipid metabolism	1.93E−03	13/41
Activation of PKC via G-protein coupled receptor	G-proteins/GPCR	2.00E−03	22/87
Fructose metabolism	Carbohydrates metabolism	2.06E−03	11/32
Regulation of lipid metabolism by niacin and isoprenaline	Regulation of lipid metabolism	2.08E−03	15/51
ATP metabolism	Nucleotide metabolism	2.09E−03	16/56
Angiotensin activation of ERK	Growth and differentiation	2.55E−03	16/57
NTS activation of IL-8 in colonocytes	Immune response	3.60E−03	17/64
Leucine, isoleucine, and valine metabolism.p.2	Amino acid metabolism	3.64E−03	9/25

Table A-6. (continued)

Pathway	Biological Process	p-Value[a]	No. of genes[bc]
Reverse signaling by ephrin B	Cell adhesion	3.92E−03	21/86
Cortisone biosynthesis and metabolism	Steroid metabolism	4.31E−03	7/17
CXCR4 signaling pathway	Immune response	4.63E−03	15/55
G-Protein beta/gamma signaling cascades	G-proteins/GPCR	4.63E−03	15/55
Glutathione metabolism	Vitamin and cofactor metabolism	5.77E−03	11/36
Mitochondrial ketone bodies biosynthesis and metabolism	Lipid metabolism	5.96E−03	5/10
Integrin inside-out signaling	Cell adhesion	6.07E−03	19/78
Propionate metabolism p.1	Carbohydrates metabolism	6.51E−03	6/14
Role of VDR in regulation of genes involved in osteoporosis	Transcription factors	6.63E−03	15/57
Endothelial cell contacts by junctional mechanisms	Cell adhesion	7.02E−03	10/32
EPO-induced Jak-STAT pathway	Cell survival	7.24E−03	11/37
A3 receptor signaling	G-proteins/GPCR	8.08E−03	19/80
Angiotensin signaling via STATs	Growth and differentiation	8.28E−03	14/53
MAG-dependent inhibition of neurite outgrowth	Growth and differentiation	8.28E−03	14/53
Phenylalanine metabolism	Amino acid metabolism	8.48E−03	9/28
Androstenedione and testosterone biosynthesis and metabolism p.1	Steroid metabolism	8.76E−03	7/19
Cytoskeleton remodeling	Cell adhesion	9.69E−03	35/176

Table A-6. (continued)

Pathway	Biological Process	p-Value[a]	No. of genes[bc]
ChREBP regulation pathway	Regulation of transcription	1.08E−02	12/44
Leptin signaling via JAK/STAT and MAPK cascades	Growth and differentiation	1.09E−02	9/29
dGTP metabolism	Nucleotide metabolism	1.10E−02	11/39
TCA	Amino acid metabolism	1.20E−02	7/20
Glycolysis and gluconeogenesis p. 1	Carbohydrates metabolism	1.20E−02	7/20
Gamma-aminobutyrate (GABA) biosynthesis and metabolism	Metabolism of mediators	1.20E−02	7/20
BAD phosphorylation	Apoptosis	1.21E−02	19/83
Ligand-dependent activation of the ESR1/SP pathway	Hormones	1.34E−02	11/40
RAB5A regulation pathway	G-proteins/RAS-group	1.49E−02	5/12
Integrin outside-in signaling	Cell adhesion	1.50E−02	18/79
Hedgehog and PTH signaling pathways participation in bone and cartilage development	Growth and differentiation	1.62E−02	11/41
G-Proteins mediated regulation MARK-ERK signaling	G-proteins/GPCR	1.64E−02	17/74
Integrin-mediated cell adhesion	Cell adhesion	1.78E−02	20/92
Mitochondrial long chain fatty acid beta-oxidation	Lipid metabolism	1.88E−02	6/17
CCR3 signaling in eosinophils	Immune response	2.02E−02	24/117
Regulation of lipid metabolism via PPAR, RXR, and VDR	Transcription factors	2.07E−02	7/22

Table A-6. (continued)

Pathway	Biological Process	p-Value[a]	No. of genes[bc]
Glycolysis and gluconeogenesis p. 2	Carbohydrates metabolism	2.16E−02	5/13
Regulation of fatty acid synthesis: NLTP and EHHADH	Regulation of lipid metabolism	2.30E−02	4/9
Role PKA in cytoskeleton reorganization	Kinases	2.43E−02	18/83
Arginine metabolism	Amino acid metabolism	2.44E−02	10/38
ECM remodeling	Cell adhesion	2.45E−02	14/60
Ca ($^{2+}$)-dependent NF-AT signaling in cardiac hypertrophy	Disease	2.55E−02	15/66
WNT signaling pathway	Growth and differentiation	2.64E−02	8/28
PPAR regulation of lipid metabolism	Regulation of lipid metabolism	2.64E−02	8/28
Insulin regulation of the protein synthesis	Translation regulation	2.67E−02	13/55
CXCR4 signaling via second messenger	Immune response	2.67E−02	13/55
Angiotensin signaling via beta-Arrestin	Growth and differentiation	2.71E−02	11/44
Estrone metabolism	Steroid metabolism	2.99E−02	5/14
Regulation of acetyl-CoA carboxylase 2 activity in muscle	Growth and differentiation	2.99E−02	5/14
Prolactin receptor signaling	Growth factors	3.19E−02	14/62
Triacylglycerol metabolism p.1	Lipid metabolism	3.23E−02	8/29
Serotonin-melatonin biosynthesis and metabolism	Metabolism of mediators	3.27E−02	6/19
Angiotensin signaling via PYK2	Growth and differentiation	3.32E−02	16/74

Table A-6. (continued)

Pathway	Biological Process	p-Value[a]	No. of genes[bc]
G-Protein alpha-i signaling cascades	G-proteins/GPCR	3.36E−02	12/51
dATP/dITP metabolism	Nucleotide metabolism	3.86E−02	12/52
Brca1 as transcription regulator	Cell-cycle control	3.90E−02	8/30
Ephrins signaling	Cell adhesion	3.99E−02	13/58
Mitochondrial unsaturated fatty acid beta-oxidation	Lipid metabolism	4.01E−02	5/15
GDNF signaling	Growth and differentiation	4.08E−02	7/25
Aspartate and asparagine metabolism	Amino acid metabolism	4.15E−02	6/20
Peroxisomal branched chain fatty acid oxidation	Lipid metabolism	4.15E−02	6/20
Histidine-glutamate-glutamine and proline metabolism	Amino acid metabolism	4.24E−02	11/47
TGF-beta receptor signaling	Growth and differentiation	4.51E−02	13/59
Regulation of actin cytoskeleton by Rho GTPases	G-proteins/RAS-group	4.51E−02	13/59
G-Protein alpha-s signaling cascades	G-proteins/GPCR	4.51E−02	13/59
A1 receptor signaling	G-proteins/GPCR	4.61E−02	16/77
Membrane-bound ESR1: interaction with growth factors signaling	Growth and differentiation	4.64E−02	10/42
Transcription factor Tubby signaling pathways	Regulation of transcription	4.64E−02	10/42
Histamine metabolism	Metabolism of mediators	4.83E−02	4/11
PPAR pathway	Transcription factors	4.86E−02	11/48

Table A-6. (continued)

Pathway	Biological Process	p-Value[a]	No. of genes[bc]
Cross-talk VEGF and angiopoietin 1 signaling	Growth and differentiation	5.08E−02	9/37
EPO-induced MAPK pathway	Growth and differentiation	5.08E−02	13/60

[a]Ordered from most significant (lowest *p*-value) to less significant.
[b]Number of genes from the DBP exposed gene list mapping to the GeneGo pathway.
[c]Total number of genes in the GeneGo pathway.

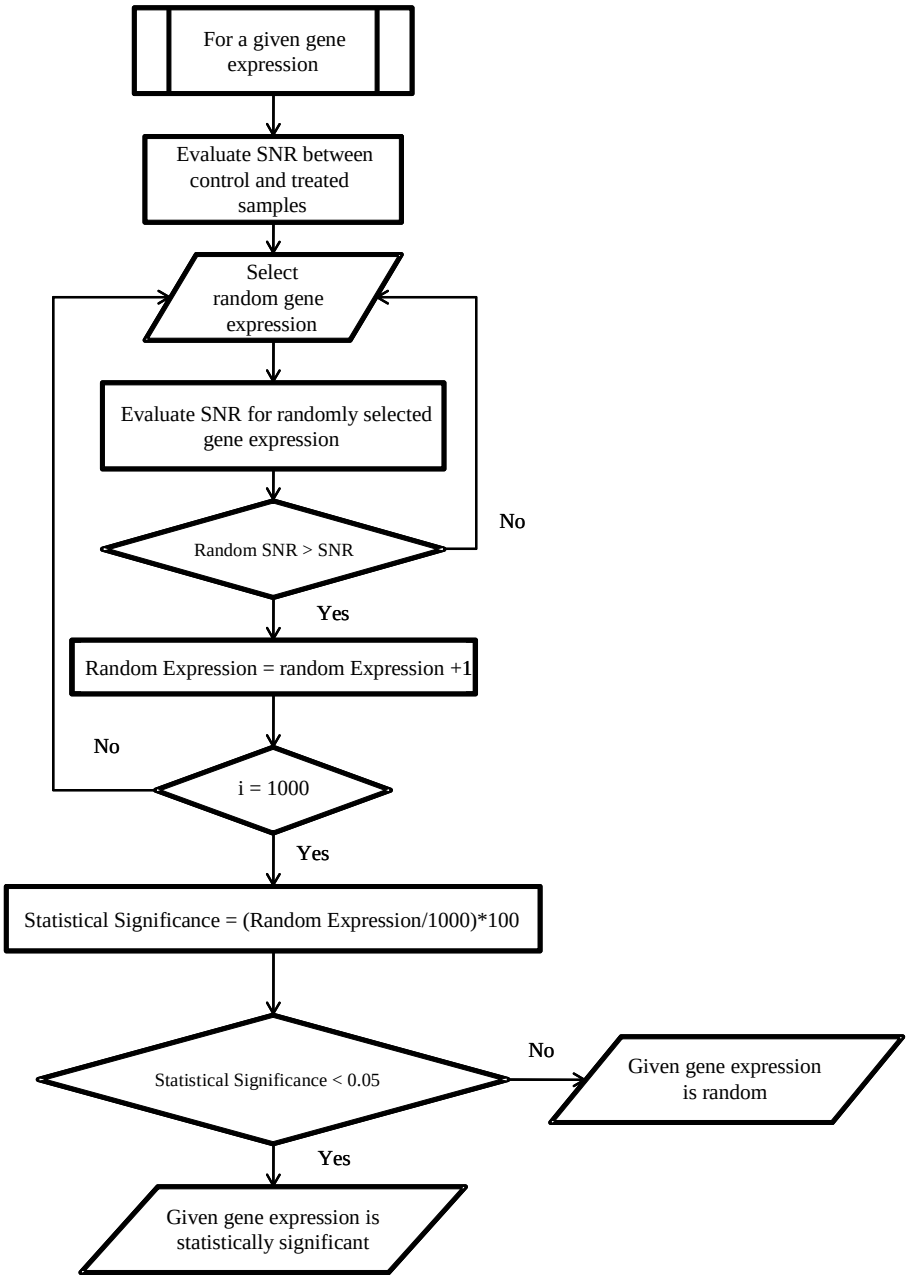

Figure A-1. Algorithm for selecting differentially expressed genes (DEGs) using signal-to-noise ration (SNR). 1,000 random gene expressions were generated for each probe set, and then, SNRs were calculated. The ratio of the randomly generated SNR that was higher than the actual SNR determined whether individual probe set's expression was discriminating or not.

APPENDIX B.

SUPPORTING TABLES AND FIGURES FOR CHAPTER 6

Appendix B contains additional tables and figures supportive of the work described in Chapter 6.

Table B-1. Nodes added by using Ingenuity® Pathway Analysis (IPA) software in developing the gene network model for DBP

Gene symbol	Gene name
Aco1	Aconitase 1, soluble
Esrra	Estrogen-related receptor alpha
Fgf4	Fibroblast growth factor 4
Insig1	Insulin induced gene 1
Kcnj11	Potassium inwardly-rectifying channel, subfamily J, member 11
Lep	Leptin
Lnpep	Leucyl/cystinyl aminopeptidase
Nfic	Nuclear factor I/C (CCAAT-binding transcription factor)
Nme1	Non-metastatic cells 1, protein (NM23A) expressed in
Nr2f1	Nuclear receptor subfamily 2, group F, member 1
Nr5a1	Nuclear receptor subfamily 5, group A, member 1
Pld2	Phospholipase D2
Ppargc1b	Peroxisome proliferative activated receptor, gamma, coactivator 1, beta
Srebf1	Sterol regulatory element binding transcription factor 1
Srebf2	Sterol regulatory element binding transcription factor 2
Zdhhc23	Zinc finger, DHHC-type containing 23

Figure B-1. Algorithm for selecting significant pathways using the pathway activity method. 1,000 random sets of gene expressions were generated for each pathway, then pathway activity, *PAp*, was evaluated. The *p*-value of each *PAp* is computed as the fraction of the randomized *PAp* that exceeded the actual *PAp*.

APPENDIX C.

QUALITY CONTROL AND ASSURANCE

Appendix C contains quality assurance/quality control (QA/QC) information for the work described in Chapters 5 and 6. The work described in this Appendix (C) is secondary data analysis. The studies include exploratory studies using new methods for analyzing genomic data for risk assessment purposes as well as some preliminary analyses using well-established of the raw data from two published studies.

Three projects were performed:

(1) A qualitative analysis and presentation of the 9 toxicogenomic DBP studies. No statistical analyses were performed by members of our team.

(2) In-house analysis of the raw data from Liu et al. (2005) study performed at both NHEERL, US EPA by Drs. Susan Hester and Banalata Sen, and by by collaborators, Dr. Ioannis Androulakis and Meric Ovacik, STAR Grantees at the STAR Bioinformatics Center at Rutgers/UMDNJ.

(3) New analyses of Thompson et al. (2005) data performed by collaborators, Dr. Ioannis Androulakis and Meric Ovacik, STAR Grantees at the STAR Bioinformatics Center at Rutgers/UMDNJ.

C.1. PROJECT 1

The data presented in 9 published toxicogenomic studies for DBP were compared. No additional analyses were performed. Data were entered directly into an excel spreadsheet from the published literature. Study descriptions in tables and figures were developed. The data entry process included team members entering in the data from the published articles into tables for differentially expressed genes and pathways affected. One person entered the data for a subset of genes. A second person checked the results in the table against the articles.

C.2. PROJECT 2

The data source was the DBP treatment only data from the Liu et al. (2005) study. The Liu et al. (2005) data were kindly provided by Dr. Kevin Gaido, a collaborator on this project. The study was performed in his laboratory at The Hamner Institutes for Health Sciences (formerly CIIT). His QA statement for the collection and analysis of the data is provided below.

C.3. PROJECT 3

The data source was the Thompson et al. (2005) study. The Thompson et al. (2005) data were kindly provided by Dr. Kevin Gaido, a collaborator on this project. The study was performed in his laboratory at The Hamner Institutes for Health Sciences (formerly CIIT). His QA statement for the collection and analysis of the data is provided below.

C.4. PROJECTS 2 AND 3: DATA SOURCES

The sources of the data used in the secondary analyses were the Liu et al. (2005) and Thompson et al. (2005) studies. Both of these studies were performed in the laboratory of Dr. Kevin Gaido. The QA details for the two studies are presented below. The Hamner Institute's Quality Assurance Director is Patricia O. Pomerleau, M.S., RQAP (pomerleau@thehamner.org).

C.4.1. Sample Handling Procedures

Virgin female SD outbred CD rats, 8 weeks old, were time mated. Dams were assigned to a treatment group by randomization using Provantis NT 2000 and subsequently be identified by an ear tag and cage card. Dams were kept in the Association for Assessment and Accreditation of Laboratory Animal Care International accredited animal facility at The Hamner Institute (at the time of the two studies, The Hmaner was named CIIT) in a humidity- and temperature-controlled, high-efficiency particulate-air-filtered, mass air-displacement room.

Dams were treated by gavage daily from gestation day (GD) 12−19 with corn oil (vehicle control) and dibutyl phthalate. Body weights were recorded daily before dosing (GDs 12−19). The oral treatments were administered on a mg/kg-body weight basis and adjusted daily for weight changes. Animal doses were calculated through Provantis NT 2000. All calculations were checked by a second individual and recorded in the investigators' The Hamner Institute notebooks. Analytical support staff confirmed appropriate dose solutions at the beginning of the dosing period. Body weights and doses administered were recorded each day in Provantis NT 2000. Pups and dams were euthanized by carbon dioxide asphyxiation.

Fetal tissues for RIA's and RNA isolation were snap frozen in liquid nitrogen and stored at −80°C. The remaining tissues were either be embedded in optical coherence tomography and frozen or fixed in formalin for 6 to 24 hours followed by 70% ethanol and then processed and embedded in paraffin for histological examination within 48 hours. The embedded tissues were

sectioned at approximately 5 microns and stained with hematoxylin and eosin. The study pathologist in consultation with the histology staff determined the gross trim, orientation, and embedding procedure for each tissue. RNA were isolated from the frozen male reproductive tract, and changes in gene expression were identified by real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis (following manufacturer's protocols P/N 402876 and P/N 4304965, Applied Biosystems, Foster City, CA) and in some cases, by complementary DNA (cDNA) microarray (following manufacturers protocol PT3140, Clontech, Palo Alto, CA).

Total RNA were treated with DNase I at 37°C for 30 minutes in the presence of RNasin to remove DNA contamination before cDNA synthesis, followed by heat inactivation at 75°C for 5 minutes. Primer pairs were selected using the program Primer Express and optimized for use prior to quantification. cDNA were synthesized using random hexamers and TaqMan Reverse Transcription Reagents according to the manufacturer's suggested protocol. Real-time PCR (TaqMan) were performed on a Perkin-Elmer/Applied Biosystems 7500 Prism using TaqMan probe chemistry according to the manufacturer's instructions for quantification of relative gene expression. Relative differences among treatment groups were determined using the CT method as outlined in the Applied Biosystems protocol for reverse transcriptase(RT)-PCR. A CT value was calculated for each sample using the CT value for glyceraldehyde-3-phosphate dehydrogenase (or an appropriate housekeeping gene) to account for loading differences in the RT-PCRs.

C.4.2. Microarray Hybridization

Testes from individual fetuses were homogenized in RNA Stat 60 reagent (Tel-Test, Inc., Friendswood, TX) and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer's protocol. RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and optical density was measured on a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from 2.5 or 3 µg total RNA and purified using the Affymetrix® One-Cycle Target Labeling and control reagents kit (Affymetrix, Santa Clara, CA) according to manufacturer's protocol. Equal amounts of purified cDNA per sample were used as the template for subsequent in vitro transcription reactions for complementary RNA (cRNA) amplification and biotin labeling using

the Affymetrix GeneChip® IVT labeling kit (Affymetrix) included in the One-Cycle Target Labeling kit (Affymetrix). cRNA was purified and fragmented according to the protocol provided with the GeneChip® Sample Cleanup module (Affymetrix). All GeneChip® arrays were hybridized, washed, stained, and scanned using the Complete GeneChip® Instrument System according to the Affymetrix Technical Manual.

For immunocytochemistry, tissues were rapidly removed, immersed in 10% (v/v) neutral-buffered formalin for 24−48 hours, and then stored in ethanol 70% (v/v) until processed. The reproductive tissues were embedded in paraffin, sectioned at 5 μ, and processed for immunohistochemistry or stained with hematoxylin and eosin.

Experimental notes and data were entered into uniquely numbered Hamner Institute laboratory notebooks and three-ring binders along with descriptions of procedures used, according to SOP# QUA-007. Specimens (RNA and frozen tissue) were retained until analysis or discarded after a maximum of 1 year after collection. Formalin-fixed tissues, blocks, and slides were archived at the end of the study. Retention of these materials will be reassessed after 5 years.

C.4.3. Quality Assurance

Both QA and QC procedures are integral parts of our research program. The research was conducted under the The Hamner Institute Research Quality Standards program. These standards include (1) scientifically reviewed protocols that are administratively approved for meeting requirements in data quality, animal care, and safety regulations; (2) standardized laboratory notebooks and data recording procedures; (3) documented methods or standard operating procedures for all experimental procedures—including calibration of instruments; (4) a central managed archive for specimens and documentation; and (5) internal peer review for scientific quality of abstracts and manuscripts. The Hamner Institute QA and QC processes assessing overall study performance and records ensure that conduct of the proposed research satisfies the intended project objectives.

C.4.4. Statistical Analysis

RT-PCR data were analyzed using JMP statistical analysis software (SAS Institute, Cary, NC). RNA were isolated from at least 3 pups from 3 different dams for each treatment group.

PCR reactions, radioimmunoassays, and protein analysis were repeated 3−5 times for each sample. Based on our experience, the number of animal replicates has the statistical power to detect a significant change in gene expression ≥20% at $p < 0.05$. The effect of treatment was analyzed using a general-linear model regression analysis. Posthoc tests were conducted when the overall analysis of variance is significant at the $p < 0.05$ level using the LS-means procedure and adjusted for multiple comparisons by Dunnett's method.

Microarray data were analyzed by a linear mixed model with SAS Microarray Solution software. Perfect-match only data were normalized to a common mean on a log2 scale, and a linear mixed model was then applied for each probe set. Restricted maximum likelihood was used for estimating the parameters for both the fixed and random effects. Significance was determined using mixed-model based F-tests ($p < 0.05$).

C.4.5. Procedures used to Evaluate Success

Uniquely numbered written protocols were prepared and reviewed internally prior to the start of this study. The content of a protocol includes study design, materials, laboratory methods, sample collection, handling and custody, record keeping, data analysis and statistical procedures, animal care requirements, and safety measures. Numbered standardized laboratory notebooks and guidelines for date recording ensures completeness of data and the ability to reconstruct the study. An independent QA department manages the overall research data quality. Manuscripts describing the results of our study were prepared at the completion of each stage of this study. All manuscripts undergo a rigorous internal peer review that includes review by all authors, at least two additional PhD- level scientists, the science editor, the division manager, and the vice president for research.

C.5. PROJECT 2: DATA REVIEW, VERIFICATION, AND VALIDATION

Banalata Sen received the Liu et al. (2005) raw data files from Dr. Kevin Gaido. Two team members, Dr. Banalata Sen (National Center for Environmental Assessment, Research Triangle Park [NCEA-RTP]) and Dr. Susan Hester (National Health and Environmental Effects Research Laboratory [NHEERL]) performed the data analysis at NHEERL, RTP. Barbara Collins (collins.barbara@epa.gov) at NHEERL-RTP has agreed to serve as the Quality Assurance Manager (QAM) for the project. Dr. Hester and Sen performed analyses of the “DBP

only" data that is a subset of the data presented in Liu et al. (2005). The analyses at NHEERL included statistical filtering to identify of differentially expressed genes and pathway analysis.

C.5.1. Verification of Data upon Receipt

Upon receiving data from Kevin Gaido at the Hamner Institute, EPA NHEERL scientisits conducted a QA review of the data by gross inspection of the cel files to confirm that the data had been transmitted successfully. The scientists at the STAR Bioinformatics Center/Rutgers received the data files from Susan Euling at EPA NCEA who had received the data from Kevin Gaido at the Hamner Institute. Kevin Gaido gave permission to Susan Euling to provide the data for these analyses. A review of the data was performed by inspection of the txt files and the published data to confirm that the data had been transmitted successfully.

C.5.2. Verification of Data Analysis Calculations

EPA NHEERL used a principal component analysis (PCA) to evaluate the within-group and across-group variance of the six samples. PCA elucidates the separation of different treatment groups and provides information about whether the data contain significant information. This was conducted using the raw data cel files in Rosetta Resolver Software. The analyses were in silico without functional validation (RT-PCR of individual genes).

The Star Bioinformatics Center also performed a principal component analysis (PCA) and displayed a 3-D plot to evaluate the within-group and across-group variance of the samples. This was conducted using the txt files in MATLAB® Software. This was an in silico analysis. The data were normalized to a zero mean and a unity standard deviation over samples. They assessed the degree of separation for Liu et al. (2005) data. A regular regular t-test and ANOVA analyses of the data were performed. The filtered data were visualized in a heatmap to determine the statistically significant subset of genes to provide a differentially expressed gene (DEG) list.

Drs. Susan Hester and Banalata Sen also performed some comparative analyses between the two outpus (above). The two independent analyses of the same dataset were contrasted with one another. Correlation plots comparing the Log10 average intensities of control samples vs. DBP treated samples was performed in order to determine the noise in both groups. Average background signal and scaling factors will be applied based on the vendor recommendations. QC plots will be made to determine the relationship between light intensity and each genechip.

C.6. PROJECT 3: DATA REVIEW, VERIFICATION, AND VALIDATION

This project analyzed the time-course data from Thompson et al. (2005) dataset to then build a regulatory network model. The STAR Center's internal QA/QC procedures are implemented and monitored by a QA official, Clifford Weisel (weisel@eohsi.rutgers.edu), at Rutgers University that reports to the National Center for Environmental Research (NCER), the granting organization for the STAR program.

C.6.1. Verification of Data upon Receipt

Data were received from Susan Euling at EPA who had received the data from Kevin Gaido at the Hamner Institute. Kevin Gaido gave permission to Susan Euling to provide the data for these analyses. A review of the data was performed by inspection of the txt files and the published data to confirm that the data had been transmitted successfully.

C.6.2. Verification of Data Analysis Calculations

A principal component analysis (PCA) was performed and a 3-D plot was displayed to evaluate the within-group and across-group variance of the samples. This was conducted using the txt files in MATLAB® Software. This was an in silico analysis. The data were normalized to a zero mean and a unity standard deviation over samples. They assessed the degree of separation for the Thompson et al. (2005) data. A regular regular t-test and ANOVA analyses of the data were performed. The filtered data will be visualized in a heatmap to determine the statistically significant subset of genes to provide a differentially expressed gene (DEG) list.

GLOSSARY

Amplified Fragment Length Polymorphism Polymerase Chain Reaction (AFLP-PCR or AFLP): A PCR-based DNA fingerprinting tool that is a highly sensitive method for detecting DNA polymorphisms.

Benchmark Dose (BMD) or Concentration (BMC): A dose or concentration that produces a predetermined change in response rate of an adverse effect (called the benchmark response or BMR) compared to background.

Complementary DNA (cDNA): DNA synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase.

Copy Number Polymorphism (CNP): The normal variation in the number of copies of a gene or of sequences of DNA in the genome of an individual.

Exposure: Contact made between a chemical, physical, or biological agent and the outer boundary of an organism. Exposure is quantified as the amount of an agent available at the exchange boundaries of the organism (e.g., skin, lungs, gut).

Exposure Assessment: An identification and evaluation of the human population exposed to a toxic agent, describing its composition and size, as well as the type, magnitude, frequency, route and duration of exposure.

Expressed Sequence Tag (EST): A short subsequence of a transcribed cDNA sequence, produced by sequencing of a cloned mRNA representing portions of expressed genes, which can be used to identify gene transcripts.

Gene Network: An illustration of the interactions between genes and gene products based on gene expression and other molecular information curated from the published literature.

Gene Ontology (GO): A bioinformatics initiative of the GO Consortium with the goal of standardizing terminology for describing gene and gene product characteristics across species and databases. The GO has developed three structured vocabularies (ontologies), independent of species, to describe gene products in terms of their associated: 1) biological processes; 2) cellular components; and 3) molecular functions. The GO also provides tools to access and process these data.

Genomics: The study of the structure and function of the whole genome. This term can also refer to "genomic technologies," defined as methods to study the genome at the level of DNA (including genome sequencing and genotype analysis). Sometimes this term refers more generally to all of the methods to study the genome (see –omics).

Genomic Technologies: Methods to study the genome including genome sequencing technologies and genotype analysis.

Hazard Assessment: The process of determining whether exposure to an agent can cause an increase in the incidence of a particular adverse health effect (e.g., cancer, birth defect) and whether the adverse health effect is likely to occur in humans.

Hazard Characterization: A description of the potential adverse health effects attributable to a specific environmental agent, the mechanisms by which agents exert their toxic effects, and the associated dose, route, duration, and timing of exposure.

Human Health Risk Assessment: The evaluation of scientific information on the hazardous properties of environmental agents (hazard characterization), the dose-response relationship (dose-response assessment), and the extent of human exposure to those agents (exposure assessment). The product of the risk assessment is a statement regarding the probability that populations or individuals so exposed will be harmed and to what degree (risk characterization).

Key Event: An empirically observable precursor step that is, itself, a necessary element of the mode of action or is a biologically based marker for such an element.

Lowest Observed Adverse Effect Level (LOAEL): The lowest exposure level at which there are biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control group.

Lowest Observed Effect Level (LOEL): In a study, the lowest dose or exposure level at which a statistically or biologically significant effect is observed in the exposed population compared with an appropriate unexposed control group.

Mechanism of Action: 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 toxicokinetic and/or toxicodynamic steps.

Metabolic Pathway Network: An illustration of interactions between metabolites derived from pathway information curated from the published literature.

Metabolomics: The analysis of collections of small molecule metabolic intermediates and products of diverse biologic processes.

Microarray: A transcriptomics tool for analyzing gene expression that consists of a small membrane or glass slide containing samples of many genes arranged in a regular pattern.

Microarray Quality Control (MAQC): An FDA project that was developed to provide quality-control tools, guidelines, and standard operating procedures (SOPs) to the microarray community in order to avoid procedural failures. To facilitate this effort, the MAQC has provided the public with large reference data sets and reference RNA samples.

Mode of Action (MOA): One or a sequence of key events, that a particular outcome is dependent upon (i.e., part of the causal pathway and not a coincident event).

No Observed Adverse Effect Level (NOAEL): The highest exposure level at which there are no biologically significant increases in the frequency or severity of adverse effect between the

exposed population and its appropriate control; some effects may be produced at this level, but they are not considered adverse or precursors of adverse effects.

No Observed Effect Level (NOEL): An exposure level at which there are no statistically or biologically significant increases in the frequency or severity of any effect between the exposed population and its appropriate control.

-omics: A suffix that is used as a general term for the genome-wide study of biological information objects (or "omes"), such as toxicogenome, proteome, and metabolome; a term referring to all of the methods for assessing the genome including genomics, metabolomics, proteomics, and transcriptomics.

Physiologically Based Pharmacokinetic (PBPK) Model: A model that estimates the dose to a target tissue or organ by taking into account the rate of absorption into the body, distribution among target organs and tissues, metabolism, and excretion.

Principal Component Analysis (PCA): A technique for analysis of multivariate data involving a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables, called principal components.

Proteomics: The study of the protein complement of the genome of an organism.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): A two-step process for converting mRNA to cDNA, using the enzyme reverse transcriptase, and the subsequent PCR amplification of the reversely transcribed DNA using the enzyme DNA polymerase.

Serial Analysis of Gene Expression (SAGE): A technique based on sequencing strings of short expressed sequence tags (ESTs) representing both the identity and the frequency of occurrence of specific sequences within the transcriptome. This method allows the entire collection of transcripts to be catalogued without assumptions about which transcripts are actually expressed.

Single-Nucleotide Polymorphism (SNP): A DNA sequence variation occurring when a single nucleotide in the genome (or other shared sequence) differs between members of a species or between paired chromosomes in an individual.

Singular Value Decomposition (SVD): A technique for the analysis of multivariate data where a rectangular, real or complex matrix, is factorized. SVD has been extensively used in microarray data analysis in order to achieve a linear projection of the data and represent these data in a reduced dimensionality space which further enables clustering and visualization of gene expression data patterns.

Toxicogenomics: The application of genomic technologies to study the adverse effects of environmental and pharmaceutical chemicals on human health and the environment.

Transcriptomics: A set of techniques to measure genome-wide mRNA expression that are used to understand the expression of genes and pathways involved in biological processes; also called "gene expression profiling."

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