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

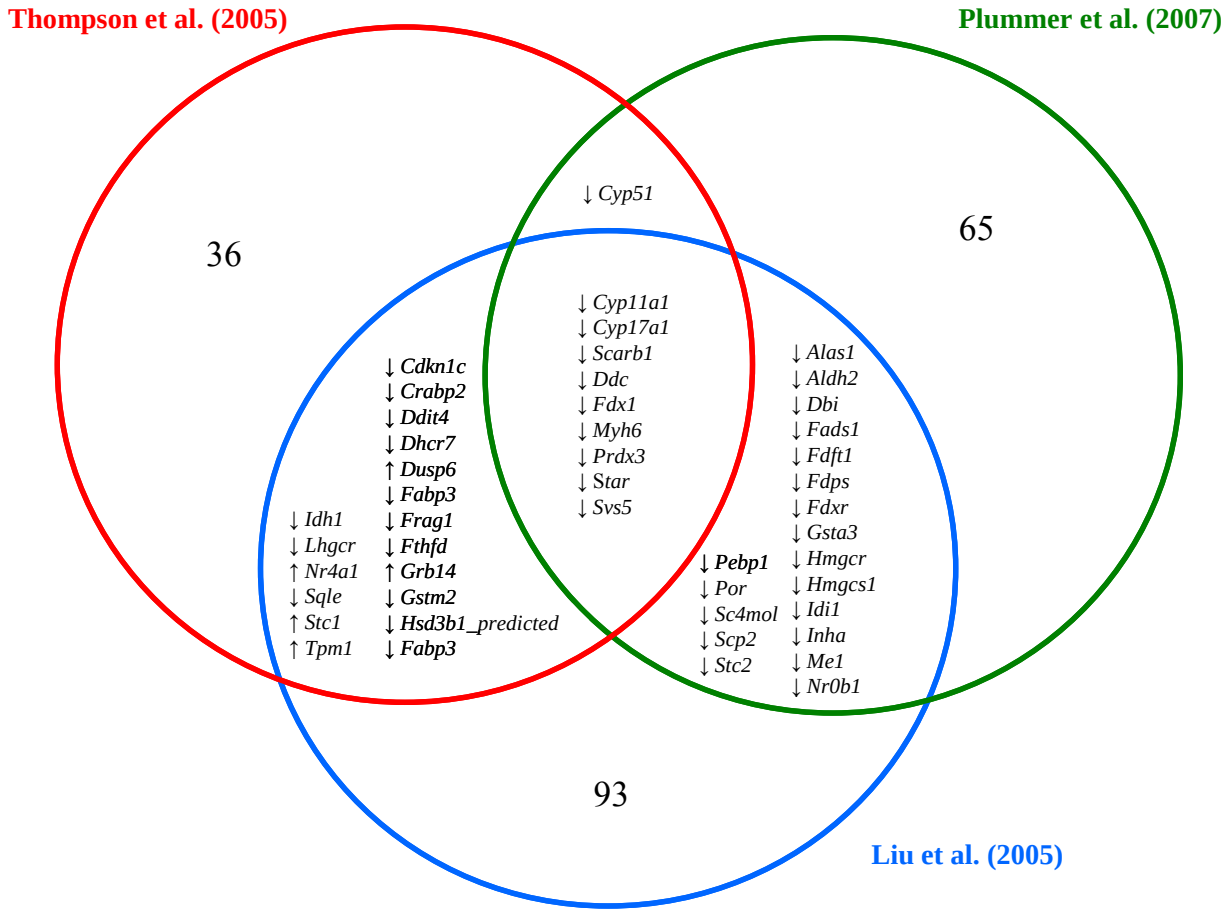

Figure 5-1. Venn diagram illustrating similarities and differences in significant gene expression changes observed in three recent microarray studies of the testes: Thompson et al. (2005), Plummer et al. (2007), and Liu et al. (2005). Numbers within each circle indicate genes whose expression was statistically 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.

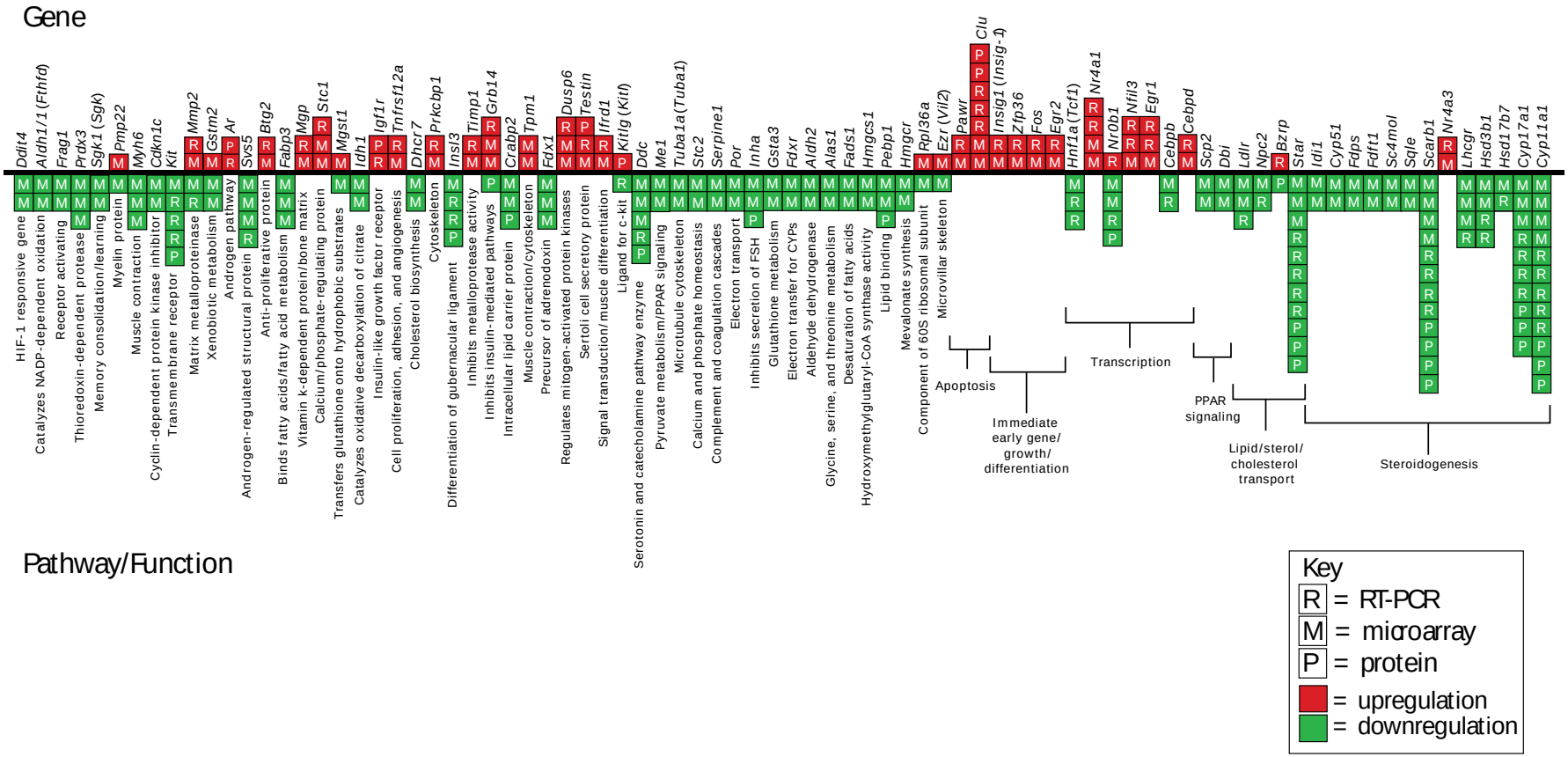

Figure 5-2. Summary of DBP-induced changes in fetal gene and protein expression. M = microarray; R = RT-PCR; P = protein. Red indicates upregulation; green indicates downregulation. Genes and protein included in the figure are limited to genes that were statistically significantly altered in two or more 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.

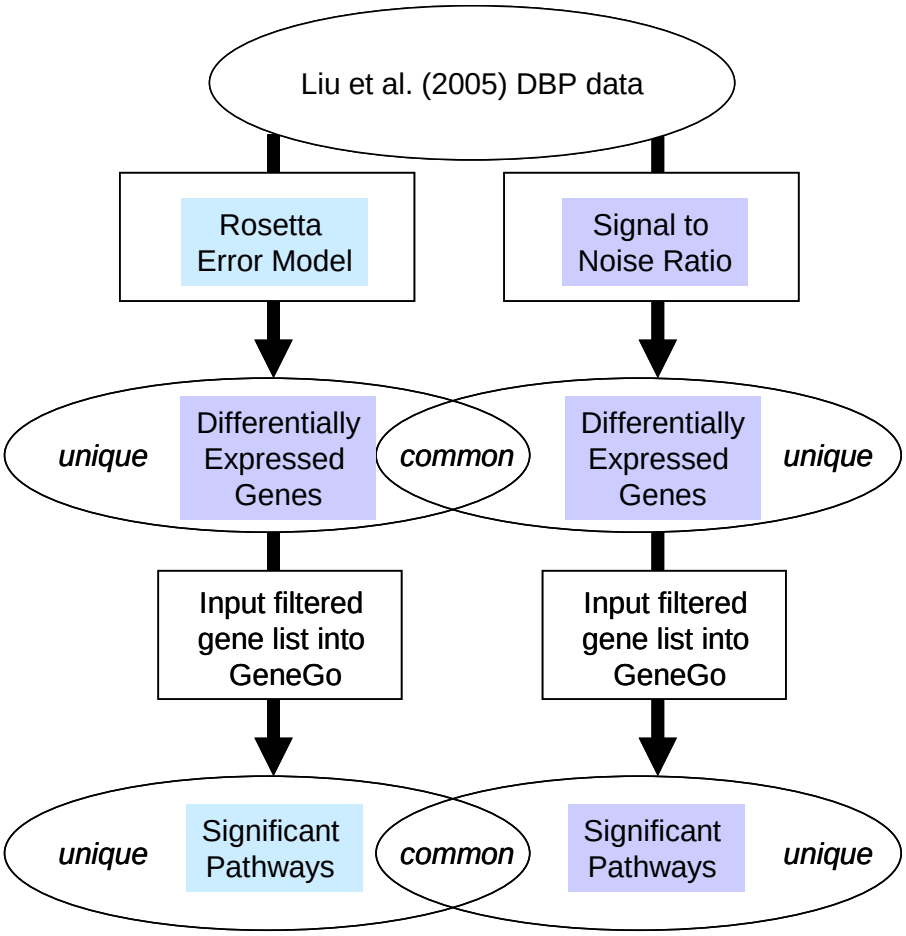

Figure 5-3. Schematic of the two analysis methods (REM and SNR) for identifying differentially expressed genes and subsequent pathway analysis using GeneGo. Two separate analyses, REM and SNR statistical filters, were performed to identify common and unique genes from the Liu et al. (2005) data. The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

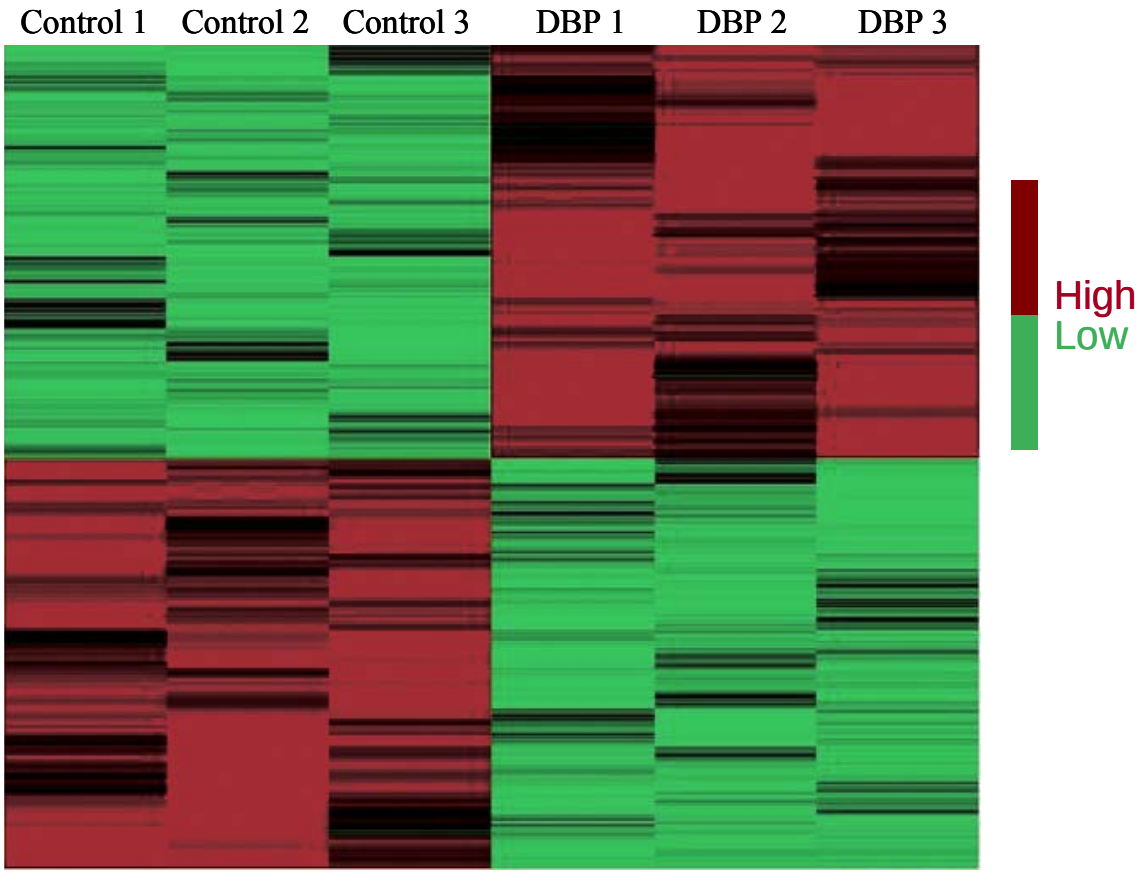

Figure 5-4. Heat map of 1,577 DEGs from SNR analysis method. The three lanes on the left are vehicle treated and the three lanes on the right are DBP treated. Data used for analysis from Liu et al. (2005). Control 1-3 lanes correspond to three replicate control samples. DBP 1-3 lanes correspond to three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

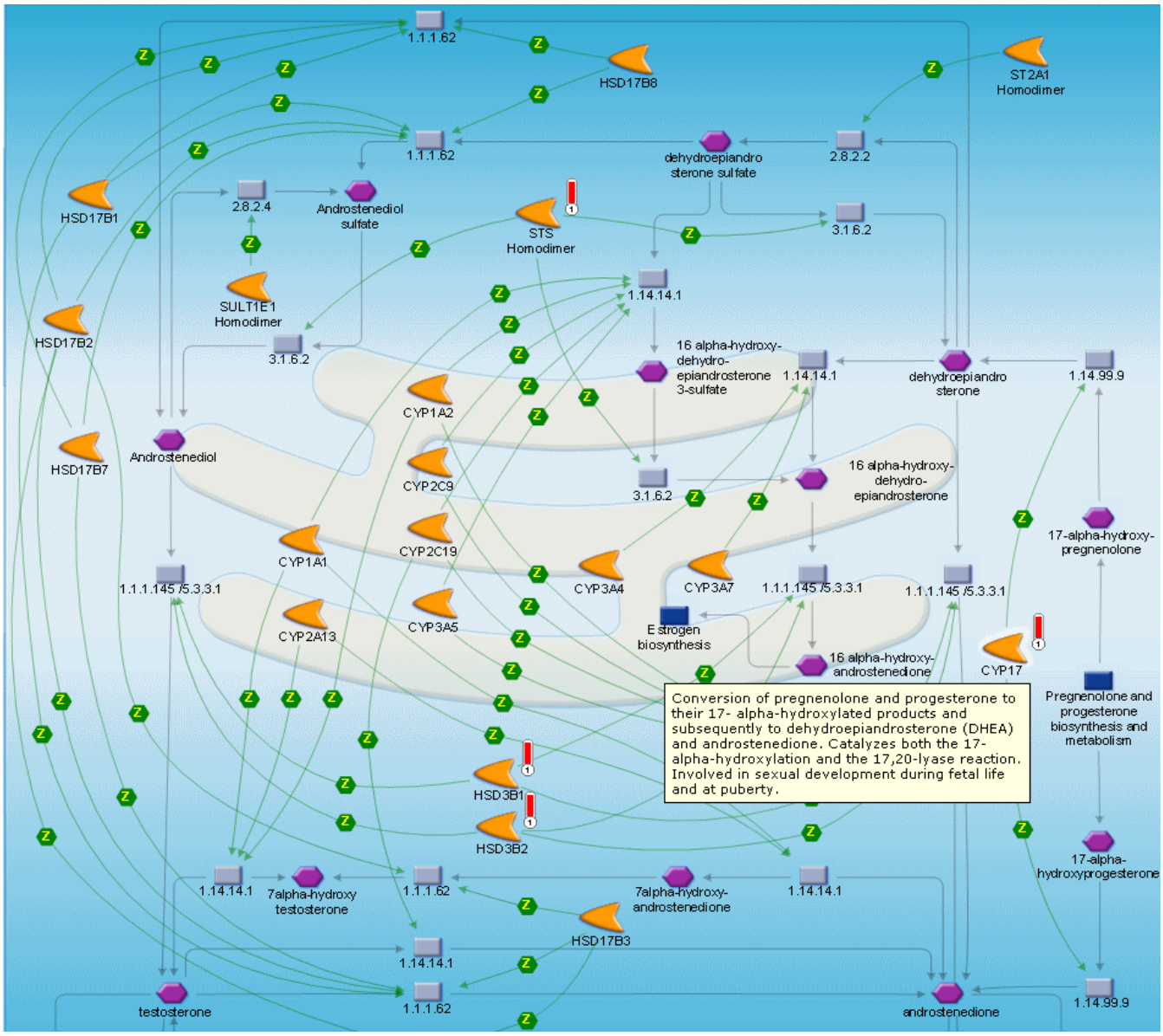

Figure 5-5. Mapping the Liu et al. (2005) data set onto the canonical androstenedione and testosterone (T) biosynthesis and metabolism pathway in MetaCore (GeneGo). Key enzymes activated by DBP are identified by red thermometers.

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.