Incorporating Kinetic and Dynamic Factors in an *In Vitro* Model of Male Reproductive Toxicity

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

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Program Authorized to Offer Degree:

Environmental and Occupational Health Sciences

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Abstract

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Current models of reproductive toxicity can be expensive, time consuming and require a large number of animals. While new *in vitro* methods have the potential to aid in reducing these burdens, the use of alternative models of male reproductive development has been limited by difficulties in modeling the complex cellular interactions and multiple endpoints which are involved in processes such as germ cell differentiation in the testes. Our lab has developed a new 3-dimensional model of male reproductive development. This cellular co-culture system (3D-TCS) contains several rat testes cell types (Sertoli, germ and Leydig cells) grown in a three dimensional conformation facilitated by an extracellular matrix (ECM) overlay. The addition of ECM in this co-culture model results in a more physiologically stable system and cells form a testicular-like architectural structure representative of *in vivo* characteristics of seminiferous tubules. We sought to characterize the responses in the 3D-TCS to an array of diverse chemicals across multiple

endpoints in order to assess which aspects of *in vivo* testicular toxicity are reflected in the model. Transcriptomic responses after phthalate ester exposure in the 3D-TCS were compared to responses in *in vivo* testes. These analyses showed that transcriptomic responses in the 3D-TCS reflect key aspects of phthalate toxicity *in vivo*. We also analyzed the metabolism of phthalate esters and the kinetic behavior (partitioning) of the both phthalate parent compounds and the main metabolite in the 3D-TCS. In addition, potency of cytotoxic, inflammatory and endocrine disruption endpoints were also evaluated across a diverse set of 5 compounds in order to characterize the range of toxicity signals captured by the model. These studies have demonstrated that the 3D-TCS can be used in a medium throughput format to investigate responses which cover a wide range of testes toxic responses in a manner that in informative of the mechanism of toxicity and the dynamic processes which occur across time and dose.

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List of Abbreviations

3D-TCS	Three dimensional testes co-culture system
BBP	Benzyl butyl phthalate
BMC_{10}	Benchmark Concentration (10%)
CYPP450	Cytochrome P450
DBP	Dibutyl phthalate
DEHP	Diethylhexyl phthalate
DEP	Diethyl phthalate
DMP	Dimethyl phthalate
DNTPE	Developmentally non-toxic phthalate ester
DOTP	Diocytl terephthalate
DPP	Dipentyl phthalate
DTPE	Developmentally toxic phthalate ester
ECM	Extracellular matrix
EPA	Environmental Protection Agency
LDH	Lactate dehydrogenase
MOA	Mode of action
NRC	National Research Council
PoT	Pathway of toxicity
REACH	Registration, Evaluation, Authorization and Restriction of Chemical substances
UGT	Uridine 5'-diphospho-glucuronosyltransferase

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Acknowledgements

I would like to thank my advisor, Dr. Elaine M. Faustman for her knowledge, guidance and support as well as the rest of my committee, Terry Kavanagh, Ed Kelly, Bill Griffith and Carol Ware for their helpful advice during the preparation of my dissertation. I would also like to thank past and present IRARC scientists and administrators who provided invaluable assistance including Xiaozhong Yu, Eric Vigoren, Kethanh Doung, Alison Laing, Marissa Smith, Tomomi Workman, Sara Pacheco, Sanne Hermsen, Carly Strecker and Jim Wallace. I am also extremely grateful to my fellow graduate students in the Faustman lab, especially Susanna Wegner and Julie Park, who worked many hours in preparing cell cultures and running the various assays in order to generate the data used in this work. Sungwoo Hong, our lab manager provided invaluable guidance in the various laboratory techniques. Finally, this work was made possible through a number of funding sources including UW NIEHS Center for Ecogenetics and Environmental Health, US-FDA (1U01FD004242), UW EPA Center for Predictive Toxicology, and the NIH Center on Human Development and Disability.

Chapter 1: Introduction

There currently exist an estimated 80,000 chemicals in production which have yet to be fully evaluated for important toxicological endpoints, such as reproductive and developmental toxicity (Goldman and Koduru 2000; Judson et al. 2009) In order to address the challenging task of evaluating this large number of chemicals for health impacts, the National Research Council (NRC) outlined a vision for improving on current methods of regulatory toxicity testing. The NRC envisioned the use of high throughput *in vitro* models in conjunction with approaches such as transriptomics, metabolomics and proteomics to identify key biological pathways perturbed by environmental toxicants and drug compounds. Rather than relying on high doses and generalized toxicity endpoints, the NRC suggested that testing should be focused on identifying more specific pathways of toxicity at lower (environmentally relevant) doses (Faustman 2000; Gibb 2008). Evaluating pathway based responses represents a powerful method for linking toxicity data across various biological contexts (e.g. *in vitro* to *in vivo* or animal to human), as the molecular aspects of developmental signaling pathways are highly conserved across multiple species (Faustman 2000).

Recently, others have joined the NRC in calling for a major push toward utilizing *in vitro* organotypic models and high throughput testing approaches and applying these data in regulatory and legal fields (Fowle III 2015). These new approaches would have the potential to reduce the number of animals needed for *in vivo* toxicity testing, consistent

with the "3R's" proposed by Russell and Burch (reduction, replacement and refinement of animal testing) (Burch 1959). In order to address the NRC's challenge, federal agencies such as the EPA have implemented programs like ToxCast in order to screen hundreds of chemicals in a high-throughput manner using various *in vitro* assays (Judson et al. 2010). Although *in vitro* tests can be powerful tools for identifying mechanisms of toxicity, in order to appropriately and fully apply knowledge obtained from *in vitro* assays, toxicity signals obtained should be relevant and comparable to modes of toxicity observed in *in vivo* organisms (Bale et al. 2014). In addition, factors such as metabolism, kinetics and bioavailability of compounds in test systems must be taken into account (Wetmore et al. 2012). Integrating information regarding these factors will allow investigators to design experiments which more accurately reflect human exposures and evaluate data in an *in vivo* context, thus moving the field of regulatory toxicity testing closer to the goals set forth by the NRC.

Model of male reproductive toxicity

The current status of methods used in reproductive toxicity testing highlights several of the challenges inherent in implementing the strategies outlined above. Current models of reproductive toxicity can be expensive, time consuming and require a large number of animals. For example, Rovida, et al. estimated a cost of ~€6.9 billion and ~49 million animals would be required to implement all reproductive toxicity testing proposed under Europe's REACH program (Registration, Evaluation, Authorization and Restriction of

Chemical substances) (Scialli 2008; Rovida and Hartung 2009). In particular, male reproductive toxicity testing can be problematic. Identification of testicular toxicity in later animal toxicology studies but not in initial range finding studies can generate large costs in pharmaceutical development (Parks Saldutti et al. 2013). While new *in vitro* methods have the potential to aid in reducing these burdens, the use of alternative models of male reproductive development has been limited by difficulties in modeling the complex cellular interactions and multiple endpoints which are involved in processes such as germ cell differentiation in the testes (Sofikitis et al. 2005; Adler et al. 2011). In the spirit of the research goals outlined by the NRC, we have developed an *in vitro* model of testes development. This cellular co-culture system (3D-TCS) contains several rat testes cell types (Sertoli, germ and Leydig cells) grown in a three dimensional conformation facilitated by an extracellular matrix (ECM) overlay. Earlier experiments in our lab showed that addition of ECM in this co-culture model creates a more physiologically stable system and that cells form a testicular-like structure representative of *in vivo* seminiferous tubules (Yu et al. 2005). We have recently adapted the culture system for use in a 96-well plate format, facilitating a semi-high throughput testing regime that has allowed us to screen for cytotoxicity effects in over 70 compounds to date. We have also observed changes in basal testosterone levels in the 3D-TCS after exposure to well-known male reproductive toxicants (phthalate esters). In addition, we have shown that well known cellular markers of meiosis are expressed in the 3D-TCS (Wegner, et al., manuscript in preparation). These results suggest that the 3D-TCS is a dynamic system which captures important male testes

developmental processes. These processes could provide the basis for sensitive indicators of male reproductive toxicity.

Table 1 (adapted from Parks-Saldutti, et al.) lists some examples of testes culture systems that have been used by various researchers in the past (Parks Saldutti et al. 2013). These include both single cell cultures and co-cultures. Effects of compounds on various endpoints specific to certain cell types have been measured including steroidogenesis and germ cell detachments from Sertoli cell monolayers. One advantage of the 3D-TCS is the presence of all three key testes cell types (Sertoli, Leydig can germ cells) as well as macrophages, so meaning that many of the endpoints listed in Table 1 could be evaluated in an efficient system due to the high yield of experimental samples that the 3D-TCS provides.

Table 1. Examples of testes culture systems

Culture system	Example endpoints evaluated
Single cell	
Leydig cell	Chemical impacts on testosterone or progesterone production
Interstitial cells	Testosterone secretion
Sertoli cells	Lactate and pyruvate secretion, FSH-induced cAMP production, aromatase activity, morphology and cell markers
Germ cells	Cytotoxicity
Peritubular cells	Production of paracrine factors involved in testes development
Co-cultures	
Sertoli-germ cells	Detachment of germ cells from Sertoli cell monolayer, FSH-induced cAMP production, cell viability
Leydig-Sertoli cells	Testosterone production, paracrine signaling (Sertoli cells produce factors that stimulate Leydig cell steroidogenesis)
Fetal testis	Inhibition of steroidogenic gene expression, (authors report culture procedure very time-consuming)
3D-TCS (Sertoli-Germ-Leydig cells and macrophages)	Cytotoxicity, testosterone secretion, pathway based analysis of gene expression, inflammatory responses, cells can be cultured in a medium-throughput manner

Pathway based determination of toxicity

Identifying the molecular basis for perturbations of critical pathways of toxicity (e.g. DNA damage, endocrine disruption, oxidative stress, etc.) in *in vitro* cell systems will allow scientist to better predict outcomes in the various organ systems that are potential targets of toxicity (Faustman 2000; Bale et al. 2014). However, determining which endpoints and what doses are relevant to real life human exposure scenarios presents many challenges. For example, many compounds require metabolic activation in order to induce a toxic response in human or animal cells. In addition, variation in metabolic capability across distinct cell types has the potential to confound the interpretation of *in vitro* assays. Another challenge is implementing a pathway based approach to toxicity evaluation lies in determining the threshold dose at a critical toxicity pathway is "triggered". Under conditions of exposure to a particular compound, cells are able compensate for the disruption of homeostasis, however at certain threshold of exposure, cells will be unable to adjust and toxicity pathway can be said to have been "triggered"(Bale et al. 2014). The threshold dose at which toxicity pathways are activated will no doubt be a function of the concentration of the compound at a certain site of action, such as a membrane receptor or a DNA molecule. However, similar to different levels of uptake the nominal concentration added to the cell media, *in vitro* systems will no doubt vary over time due to multiple processes such as metabolism, binding with cellular proteins and partitioning within the system (i.e. uptake by cells over time) (Blaauboer 2010).

Dissertation Outline

The overall goal of my dissertation is to characterize the dynamic responses of our testicular co-culture system to a diverse set of compounds and compare these responses to those observed *in vivo*. Using a number of different endpoints (transcriptomic profiles, cytokine production, testosterone production and cytotoxicity), my research will identify a number of different toxic modes of action in the testes that are capture in our *in vitro* model.

Chapter 2 will be a comparison of transcriptomic profiles between the 3D-TCS and *in vivo* testes after exposure to phthalate esters. Using developmentally toxic and non-toxic phthalates (DTPE and DNTPE), we will show that the transcriptomic responses in the 3D-TCS can discriminate between DTPE and DNTPE in manner similar to the *in vivo* responses.

In **Chapter 3**, I will characterize the capacity for the 3D-TCS to metabolize phthalate diesters to their monoester metabolites. Phthalate monoester metabolites are considered toxicologically active and the capacity for the 3D-TCS to catalyze this reaction has important implications for interpreting response to these compounds in the 3D-TCS. In addition, many genes which metabolize toxicants such as phthalate serve to catalyze endogenous reactions which play important roles in testes development. Therefore, the metabolic capability of the cells will be an important consideration when assessing the model's ability to replicate *in vivo* testes biology.

In **Chapter 4**, my thesis explores the ability of the 3D-TCS to replicate a highly relevant endpoint in testes toxicity, inflammatory processes. Immunoendocrine signaling involving cytokine and inflammatory signaling molecules are important contributors to normal testes development. In addition, responses in some of these molecules have been shown to modulate the effects of toxicants in the testes. In this chapter, the presence of resident macrophage markers, indicates that our co-culture includes this cell type and that it can modulate some of these key processes in the *in vivo* testes.

In **Chapter 5**, I report on results in which we screened a diverse set of >70 compounds in the 3D-TCS for impacts on cytotoxicity and cell viability. Benchmark dose response modeling was used to rank the potency of these impacts across the compounds. A subset of 5 compounds was then selected for further analysis using assays that are more informative of specific mechanisms of toxicity, i.e. measuring changes in levels of testosterone production and levels of cytokines across 4 doses.

Chapter 6 is a summary of key findings and future directions of research

Specific Aims and Hypotheses

Chapter 2

Specific Aim: Using a pathway based analysis to compare phthalate induced transcriptomic changes in the 3D-TCS with a similar exposure as is seen in developing rats *in vivo*.

Hypothesis: Reproductively toxic and non-toxic phthalate esters will differentially impact steroidogenic and other key developmental pathways in the 3D-TCS as well as in *in vivo* testes and these responses will be predictive of the mechanism of reproductive toxicity.

Chapter 3

Specific Aim: Characterize metabolism and kinetics of phthalate esters in the 3D-TCS.

Hypothesis: Phthalates will be metabolized in the 3D-TCS at a rate similar to what is observed in rat testes *in vivo*. Physiochemical properties will predict phthalate partitioning in the 3D-TCS.

Chapter 4

Specific Aim: Determine if key immune cells (macrophages) and inflammatory signals (gene and protein expression) are present and impacted by toxicants in the 3D-TCS.

Hypothesis: Macrophages will be present in the 3D-TCS and inflammatory markers (cytokines) will be detectable and influenced by exposure to phthalate diesters.

Chapter 5

Specific Aim: Determine how well cytotoxicity and cell viability assays can predict reproductive toxicity potential in a broad range of compounds in the 3D-TCS.

Hypothesis: Cytotoxicity and cell viability will be able to predict reproductive toxicity potential for compounds acting through modes of action such as oxidative stress and cell death pathways. Further testing will be needed to identify potential reproductive toxicants that act through alternative modes of toxicity.

Chapter 6

Conclusions and future directions

Chapter 2: Comparison of toxicogenomic responses to phthalate ester exposure in an organotypic testis co-culture model and responses observed in vivo

This chapter has been submitted to Reproductive Toxicology. *The authors of the manuscript are:*

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Abstract

We have developed a three-dimensional testicular co-culture system (3D-TCS) which mimics *in vivo* testes. In this study, transcriptomic responses to phthalate esters (PE's) were compared in the 3D-TCS with responses in rat testes *in vivo*. Microarray data from the 3D-TCS and from *in vivo* testes were used to compare changes in gene expression patterns after exposure to developmentally toxic (DTPE) or developmentally non-toxic (DNTPE) phthalate esters. DTPE treatments clustered separately from DNTPE treatments based on principle components analysis both *in vitro* and *in vivo*. Pathway analysis using GO-Elite software showed that terms relating to steroid metabolism or reproductive development were enriched both *in vitro* and *in vivo* after DTPE exposure. Processes such as cell cycle, cell proliferation and apoptosis were enriched for DTPE treatments *in vitro*, but not *in vivo.* Based on these analyses we concluded that transcriptomic responses in the 3D-TCS reflect key aspects of *in vivo* phthalate toxicity.

1. Introduction

Current models of male reproductive toxicity are expensive, time consuming and require a large number of animals. Rovida and Hartung (2009) estimated a cost of ~€6.9 billion and ~49 million animals would be required to implement all reproductive toxicity testing proposed under Europe's REACH program (Registration, Evaluation, Authorisation and Restriction of Chemical substances). New *in vitro* methods may aid in reducing these burdens (Scialli 2008; Rovida and Hartung 2009). However, the use of alternative models of reproductive development has been limited by difficulties in modeling the complex cellular interactions and multiple endpoints which are involved in these processes (Adler et al. 2011). Due to these difficulties, there is currently no reliable and reproducible *in vitro* model for testicular toxicity screening (Parks Saldutti et al. 2013). With the aim of addressing some of these issues, we have developed an *in vitro* model of testes development (Yu et al. 2005). This cellular co-culture system (3D-TCS) contains several testes cell types (Sertoli, germ and Leydig cells) grown in a three dimensional conformation facilitated by an extracellular matrix (ECM) overlay. Earlier experiments have demonstrated that addition of ECM in this co-culture model results in a more physiologically stable system and that cells form a testicular-like architectural structure representative of *in vivo* characteristics of seminiferous tubules (Yu et al. 2005).

Phthalate esters (PE's) represent a class of well-known male reproductive toxicants. Male reproductive effects of exposure to certain phthalate esters (PE's) include endpoints such as underdeveloped reproductive organs, hypospadias, cryptorchidism and

decreased anogenital distance (Mylchreest et al. 1998; Gray et al. 2000; Bajkin et al. 2014). Previously, we demonstrated that our 3D-TCS model was able to distinguish between developmentally toxic PEs (DTPE) and developmentally non-toxic PEs (DNTPE) based on observed differences in microarray-based gene expression profiles, with significant changes occurring in the steroid biosynthetic pathway. In contrast, a general cytotoxicity assay (neutral red uptake assay) was not able to distinguish between the toxic and non-toxic phthalates at the same dose. These results suggested that the 3D-TCS system provides a sensitive tool for identifying male reproductive toxicants through alterations in important biological pathways with functional relevance to male reproductive development (Yu et al. 2009).

Previously, Liu et al. exposed pregnant rats to the same group of DTPE and DNTPE's. This was followed by microarray-based gene expression profiling in the testes of male fetuses, which identified a number of cellular pathways disrupted by DTPE exposure. These pathways included lipid and cholesterol homeostasis, insulin signaling, oxidative stress and steroidogenesis. In addition to cellular pathway changes, DTPE exposure was associated with the phenotypic outcome of significantly decreased anogenital distance (Liu et al. 2005). The purpose of the current study was to compare transcriptomic responses induced by PEs in the 3D-TCS model with those observed from the Liu, et al. *in vivo* exposure using analysis of microarray gene expression profiles and pathway based analysis.

Our comparative analyses will allow us to determine the extent to which DTPE induced gene expression changes in the 3D-TCS reflect those occurring under an *in vivo* exposure scenario and aid in the evaluation of a proposed adverse outcome pathway (AOP) for phthalate reproductive toxicity (NRC 2008; Furr et al. 2014). As shown in fig. 1, there were significant differences in the experimental design between the *in vivo* and *in vitro* datasets used in this study, for example the *in vivo* data was a repeated multiple dose exposure while the *in vitro* study was a 24 hours acute exposure. Bearing these differences in mind, we sought to determine whether the transcriptomic response in the 3D-TCS revealed key aspects of the mode of action (MOA) for phthalate reproductive toxicity. In order to accomplish this, we examined overall changes at the pathway level with a particular focus on the steroid biosynthesis pathway because disruption of steroidogenic gene expression has been phenotypically anchored to a number of male reproductive outcomes and is an important MOA for phthalate toxicity (Gray and Gangolli 1986; Mylchreest et al. 1998; Gray et al. 2000). These comparisons have allowed us to link effects on gene expression observed *in vitro* with those observed *in vivo* and provide critical information in establishing an *in vitro* alternative model for evaluating male reproductive toxicity.

2. Materials and Methods

2.1 Three dimensional co-culture model for testes (3D-TCS), treatments and gene expression profiling

Fig. 1 shows the experimental designs for both the *in vitro* and *in vivo* data used in this study. The method for the three dimensional co-culture model for testes (3D-TCS) and PE treatments was described previously (Yu et al. 2009). Briefly, testes were dissected from 5-day-old male pups obtained from mated Sprague–Dawley rats (Charles River Laboratories, Wilmington, USA). A single cell suspension containing primarily Sertoli, germ and Leydig cells was then plated followed by an overlay of extracellular matrix medium (Matrigel^{TM}). Benzyl butyl phthalate (BBP, Sigma # 44-2503, 99% purity), dibutyl phthalate (DBP, Sigma # D2270, 99% purity), diethylhexyl phthalate (DEHP, Sigma # 4-8557, 99% purity), diethyl phthalate (DEP, Sigma #524972, 99.5% purity), dimethyl phthalate (DMP, Sigma # 525081, 99% purity), dioctyl tere-phthalate (DOTP, Sigma # 525189, 96% purity) or dipentyl phthalate (DPP, Sigma # 80154, 99% purity) were obtained from Sigma-Aldrich (MO, USA*).* Phthalate solutions (or vehicle control, DMSO, Sigma-Aldrich, MO,USA) with a final concentration of 100 µM were then added directly to the culture medium 48 hours after the addition of ECM overlay. This dose was selected based on earlier experimental data showing changes in gene expression with a minimum effect on cell viability (90% of the cells were still viable). Total RNA was extracted from cells and used as starting material for probing of Affymetrix Rat Genome 230 2.0 microarrays (3 replicate arrays for

each PE treatment and control).

2.2 Gene Expression Profiling Following In Utero Exposure to Phthalate Esters

In vivo exposure to phthalate esters was reported previously by Liu et al. (Liu et al. 2005). Briefly, time-mated Sprague-Dawley outbred CD rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC) on Gestation Day 0 (GD 0). Dams were treated by gavage daily from GD 12 to GD 19 with corn oil vehicle or BBP, DBP, DEHP, DEP, DMP, DOTP or DPP (Aldrich Chemical Company, Milwaukee, WI) in corn oil at 500 mg/kg per day. This dose was chosen based on earlier experiments showing changes in gene expression with no maternal toxicity or fetal death. DTPE exposure at this dose produced a significant decrease in anogenital distance for male fetal pups. Dams were euthanized on GD 19 by carbon dioxide asphyxiation. Right and left testes were removed from male fetuses. RNA was then isolated followed by synthesis of complementary DNA from 2 μg of total RNA. Equal amounts of purified cDNA per sample were used as the template for subsequent hybridization to GeneChip arrays (RAE230A and RAE230B, which contain the same geneset as Affymetrix Rat Genome 230 2.0 arrays on two separate chips). Three replicate arrays were used for each PE treatment except for DOTP (n=2). The resultant cell intensity (CEL) files were kindly donated by Dr. Kevin Gaido.

2.3 Statistical Analysis

2.3.1 QC/Normalization

Cell intensity files (CEL) for the *in vitro* 3D model and *in vivo* data were normalized using ArrayAnalysis software (http://www.arrayanalysis.org, accessed 11/04/14) for quality control and normalization (Eijssen et al. 2013). Data normalization was performed using GC-RMA (GeneChip-Robust Multiarray Averaging). For each array, intensity values for 31,099 unique probes were normalized to one $\log_2$-transformed expression value for 12,025 EntrezGene ID's. For *in vivo* arrays, A and B chips were analyzed and normalized separately and then combined for the final total of 12,025 genes. For genes which appeared on both A and B chips (1,081 genes), the normalized value from the A chip was used.

2.3.2 Principle Component Analysis

Principle Component Analysis (PCA) was performed on the normalized gene expression values for the *in vitro* and *in vivo* microarrays to visualize similarities and dissimilarities within the data using R statistical software (version 3.1.0). On the PCA plot, shorter distance between two points indicates greater similarity of gene expression profiles between two arrays (Pearson 1901; Ringner 2008).

2.3.3 Pathway analysis for all gene expression values for each experimental condition

ToxProfiler software (http://ntc.voeding.tno.nl/toxprofiler_test/, accessed

12/11/14) was used to conduct a robust, unbiased analysis of significantly impacted biological pathways using the average $\log_2$ (fold-change/mean of controls) for all genes for on each array used in the *in vitro* and *in vivo* experiments. ToxProfiler is a web-based pathway analysis application that identifies enriched biological pathways among sets of genes without the need for statistical cutoffs. T-scores, a measure of statistical enrichment and magnitude of average fold change for genes in each biological pathway, were calculated for each enriched Gene Ontology term (Boorsma et al. 2005). T-scores were input into MeV software for visualization in a heatmap (Saeed et al. 2003). Hierarchal clustering using Euclidean distance and average linkage was performed for all t-scores in order to cluster PE treatment groups based on the similarity of alterations in biological pathways.

2.3.4 Pathway analysis for genes commonly changed by toxic phthalate treatment both in vitro *and* in vivo

In order to identify biological pathways that were specifically impacted by the DTPE exposures, we performed t-tests using R statistical software (version 3.1.0) to find differentially expressed genes (compared to controls) for each phthalate treatment, both *in vitro* and *in vivo* ($p \leq 0.005$). Four hundred-forty (440) genes which were found to be commonly changed across all toxic phthalate treatments in the 3D-TCS; while 35 genes were commonly impacted in the rat fetal testes (4 genes were found to be common between these two lists). These sets of "toxicity signature" genes were used for two

separate pathway enrichment analyses using GO-Elite software (version 1.2.5), which also calculates the average fold-change for genes within each process (Zambon et al. 2012). The average fold-changes for genes within each identified pathway were calculated for both *in vitro* and *in vivo* models. MeV software was used to visualize average expression for all enriched GO terms (Saeed et al. 2003), followed by hierarchal clustering using Euclidean distance and average linkage in order to cluster PE treatment groups based on the similarity of response to PE treatments.

Following GO-Elite analysis, GO terms with particular relevance for male reproductive development (e.g. “male gonad development”, “steroid biosynthesis”, “Sertoli cell differentiation”, etc.) were identified and expression values were obtained for genes related to these terms that were appeared on either of the *in vitro* or *in vivo* “toxicity genelists”. Twenty-one (21) genes were found to be involved in one or more of these terms. Visualization and hierarchical clustering were performed for this set of genes as described above.

2.4 Pathway visualization

PathVisio software (version 3.1.3) was used to construct a visualization of the steroid biosynthetic pathway starting with fatty acid degradation or cholesterol import into the Leydig cell (van Iersel et al. 2008). This pathway was constructed by combining elements from the WikiPathways steroid and cholesterol biosynthesis maps and sources from the literature (Hanukoglu 1992; Barlow et al. 2003; Willighagen 2013). This constructed

pathway was then used to compare gene expression changes for the reproductively toxic phthalate treatments both *in vitro* and *in vivo*.

3. Results

3.1 Principle Component Analysis

Principle Component Analysis was performed for all genes for both *in vitro* and *in vivo* datasets (Ringner 2008). For both datasets, there was a clear distinction between the two classes of phthalates. Fig. 2 shows that the non-toxic PE treatments and controls formed a distinct cluster from the toxic PE treated samples. This was observed for both the *in vitro* and *in vivo* expression data.

3.2 Phthalate induced gene expression changes in testicular co-culture model and in fetal rat testes

One-way ANOVA's were used to determine the number of genes significantly changed compared to controls for each phthalate treatment. As shown in fig. 3, the number of genes significantly changed was greater after exposure to the reproductively toxic phthalates when compared to the non-reproductively toxic phthalates in both the testicular co-culture as well as the *in vivo* rat testes. However, the overall number of genes changes was higher in the *in vitro* model compared to the *in vivo* treatments. In general, there was greater percentage of genes with increased expression compared to controls *in vitro*, while a greater percentage of genes were decreased *in vivo*. Four genes were

identified as significantly changed across all toxic PE treatments in both *in vitro* and *in vivo* models: *Star* (steroidogenic acute regulatory protein), *Gsta1* (glutathione S-transferase alpha 1), *Fdx1* (ferredoxin 1) and *Pgap2* (post-GPI attachment to proteins 2).

3.2 Pathway Analysis

ToxProfiler was used to investigate enriched pathways for all genes on each array. Average expression values across all experimental replicates were used for pathway analysis. Significantly enriched Gene Ontology defined biological processes were given a t-score based on the significance of enrichment and the average $\log_2$(fold-change/control) in gene expression for each gene. These t-scores were then used to cluster treatment groups based on similarity of the changes in these biological processes. As shown in fig. 4, toxic and non-toxic PE treatments clustered separately from DNTPE treatments in both the testes co-culture as well as the *in vivo* treatments. While some processes were affected by the non-toxic phthalates, there was a clearly stronger and more consistent response after exposure to the toxic PE's. Pathways which were altered by DTPE treatments represented several biological themes, including: response to stimuli, steroid or lipid metabolism, cell cycle, redox homeostasis and development or cell differentiation as indicated in the figure. Some of these processes were similarly regulated by DTPE treatment both *in vitro* and *in vivo*. These included the downregulation of processes related to lipid, cholesterol or steroid metabolism (GO terms: "cholesterol biosynthetic process", "isoprenoid biosynthetic process"). By contrast, there were a number of

pathways related to processes such as oxidative stress (“response to oxidative stress”, “glutathione metabolic process”, etc.) and cell cycle (“cell division”, “mitosis”, etc.), which were upregulated *in vitro* and downregulated *in vivo*.

A more targeted pathway analysis using genes that were commonly impacted by all of the DTPE treatments in the 3D-TCS (*in vitro*) and in the fetal rat testes (*in vivo*) was conducted. Multiple biological processes were significantly enriched *in vitro* (n=120) and *in vivo* (n=43). Overall biological themes represented among these pathways included apoptosis, cell cycle, development, hormone metabolism, response to stimulus and cell homeostasis. Analysis of enriched GO terms showed that several cellular pathways with significant mechanistic implications for steroid metabolism or male reproductive development were significantly enriched both *in vitro* ("androgen metabolic process”, “steroid catabolic process”, “reproductive structure development”, see fig. 5a) and *in vivo* (“androgen biosynthetic process”, “male gonad development”, see fig. 5b) after exposure to developmentally toxic phthalates (see Supplementary Tables 1 and 2 for full list of enriched biological processes and z-scores). By contrast, GO terms related to cellular processes such as cell cycle, cell proliferation and apoptosis were enriched for DTPE treatments *in vitro*, but not *in vivo.* Developmentally non-toxic phthalates had comparably little impact on the expression of genes used for pathway analysis. Six processes were commonly enriched *in vitro* and *in vivo* (“cellular response to amine stimulus”, “response to cAMP”, “response to drug”, “response to ionizing radiation”, “response to organic cyclic compound” and “xenobiotic metabolic process”) that were involved with either cellular

response to a stimulus (either exogenous or endogenous) or metabolism of xenobiotics. Figs 5 (a) and (b) show heatmaps for average change in gene expression for biological processes enriched *in vitro* and *in vivo*, respectively.

By comparing the enrichment of gene ontology terms between the *in vitro* and *in vivo* transcriptomic profiles, a list of 21 genes was compiled and which are highly relevant to the pathways of steroid biosynthesis, male reproductive development or both. Fig. 6 shows the direction and magnitude for gene changes both *in vitro* and *in vivo* for this group of genes, as well as which Gene Ontology process the genes belong to. Multiple genes belonged to >1 biological process. The pattern of gene expression changes for these individual genes was consistent with the overall patterns we had observed previously, i.e. the DTPE treatments induced gene expression changes that were clearly distinct from the DNTPE treatments. A few genes, such as *Scarb1* (involved in the uptake of cholesterol for the purposes of steroid biosynthesis) and *Inha*, were downregulated by DTPE exposure both *in vitro* and *in vivo*. However, the majority of gene expression changes showed a distinction between the two models, either in terms of direction of regulation (e.g. upregulated in one, downregulated in the other) or genes that were changed in only one of the model systems (e.g. changed *in vitro* only).

3.3 Steroid biosynthesis pathway

Alterations in steroid biosynthetic gene expression and associated decreases in testosterone are considered the key mode of phthalate testes toxicity (David 2006; Euling

et al. 2011). In addition, the current study identified that processes related to steroid metabolism were impacted by DTPE exposure in the 3D-TCS as well as in the rat fetal testes (*in vitro*: "androgen metabolic process", *in vivo*: "androgen biosynthetic process"). Therefore, we sought to investigate effects on genes involved in the synthesis of testosterone. As shown in figs. 7 (a) and (b), multiple genes were altered after exposure to the reproductively toxic PE's both *in vitro* and *in vivo*, respectively. In our testicular co-culture model, most of the effects appeared to be occurring upstream of cholesterol import into the mitochondria. However, *Star*, the key gene responsible for cholesterol import into the mitochondria (a rate limiting step in testosterone synthesis), was highly upregulated. By comparison all the genes changes in the *in vivo* rat testes were downregulated, including *Star*.

4. Discussion

Gene expression profiling is a powerful tool in toxicology. Changes in gene expression can be more sensitive signals than other toxicity endpoints such as cytotoxicity and can provide key insight into mechanisms of toxicity (Corvi 2002). The comparison of the transcriptomic changes in our testes co-culture (3D-TCS) with effects observed in in vivo fetal rat testes provides important information which aids in the interpretation of data obtained from the 3D-TCS model. Despite differences in experimental design (acute exposure *in vitro* versus subchronic exposure *in vivo*) and the observed differences in expression of single genes and pathways when comparing the *in vitro* and *in vivo* testes

models, we were able to confirm that our *in vitro* co-culture model was able to distinguish between developmentally toxic (DTPE) and non-toxic phthalate esters (DNTPE) based on overall gene expression changes and using a pathway based analysis. This analysis of perturbed cellular pathways showed that changes in the 3D-TCS were consistent with phthalate MOA's *in vivo,* i.e. disruption pathways involved in steroid biosynthesis and overall testes development (Parks et al. 2000; Martino-Andrade and Chahoud 2010).

4.1 Patterns of gene expression changes discriminate between developmentally toxic and non-toxic phthalates in vitro *and* in vivo

An unsupervised principle components analysis (PCA) of all gene expression data on each array showed that the DTPE treatments formed a distinct cluster from the DNTPE treatments (and controls) both for the *in vitro* co-culture model and the *in vivo* testes. In addition, t-tests identifying genes significantly changed compared to controls showed that DTPE treatments were associated with a higher number of gene changes compared to DNTPE treatments both *in vitro* and *in vivo*. This demonstrates that for this class of structurally similar compounds (phthalates), transcriptomic profiles from the 3D-TCS are able to discriminate between those that are potent reproductive toxicants from those that are weak or non-reproductive toxicants.

4.2 Pathway based analysis shows that mechanistic aspects of DTPE toxicity in the 3D-TCS are consistent with phthalate toxicity in vivo

In order to gain insight into the mechanistic aspects of phthalate effects on transcriptomic responses, we performed an unsupervised pathway analysis using expression data for all genes represented on each array. Consistent with the PCA analysis, cluster analysis based on similarities in t-scores for the enriched Gene Ontology terms showed that the *in vitro* and *in vivo* DTPE treatments clustered separately from the DNTPE treatments. In general, DTPE impacts on pathways or processes could be said to be either: upregulated *in vitro*/downregulated *in vivo*, downregulated *in vitro*/upregulated *in vivo* or downregulated both *in vitro* and *in vivo*. Among processes that were downregulated both *in vitro* and *in vivo*, were the GO terms "cholesterol biosynthesis" and "isoprenoid biosynthesis". Impacts on such pathways, which relate to the metabolism of cholesterol and steroid precursors, represent one of the mechanisms of phthalate reproductive toxicity. Altered expression of genes responsible for steroid, lipid and cholesterol metabolism have been shown to be associated with decreased serum testosterone, cryptorchidism, hypospadias and decreased anogenital distance (AGD) in male rats *in vivo* (Foster et al. 2001; Welsh et al. 2008). Indeed, the male rats from which gene expression data were obtained for the current study showed a decreased AGD (Liu et al. 2005). Thus, using a pathway based approach and applying no cutoffs for significant gene changes, we showed that important mechanisms of *in vivo* phthalate toxicity were captured in the 3D-TCS model.

In order to further investigate *in vivo* toxicity responses that were reflected in the 3D-TCS transcriptomic signals, we identified genes which were commonly altered by

reproductively toxic phthalates in our co-culture model (440 genes) and those commonly altered in the *in vivo* rat testes (35 genes). We then used GO-Elite to identify biological processes enriched in each of these genelists and quantified the average change in gene expression for these processes. GO terms enriched by DTPE treatments *in vitro* included a broad range of biological themes, including apoptosis, cell cycle, cell signaling, development, redox homeostasis, steroid metabolism and reproductive development. These results are consistent with previous reports discussing transcriptomic signals for male reproductive toxicity of phthalates. For example, in a recent evaluation of phthalate toxicogenomic data, Euling et al. discuss three main modes of action (MOAs) for phthalate reproductive effects in the male. One involves the decrease in fetal testosterone, which leads to perturbed development of the reproductive tract. The second is a decrease in insl3 expression, which has been demonstrated to be responsible for undescended testes. In addition, a third less characterized MOA is thought to be responsible for effects including apoptosis and multinucleation of gonocytes, altered proliferation of Sertoli cells and altered morphology of seminiferous tubules (Euling et al. 2011). Analysis of pathway enrichment in the 3D-TCS suggests impacts on pathways that are indicative of at least two of these MOAs. Enrichment of processes such as androgen metabolism and steroid catabolic process demonstrate a signal indicative the first (altered testosterone production), while alteration of cell cycle pathways and apoptosis signaling demonstrate potential signals for the third, less well-characterized MOA.

Using the 35 genes which were changed across all toxic treatments *in vivo*, we

identified enrichment for multiple pathways involved in steroid, cholesterol or lipid metabolism as well as reproductive development. By quantifying the average gene expression changes in these pathways *in vitro* and *in vivo*, we were able to confirm that impacts to on these pathways occurred in both models. However, we observed a clear distinction between the two models, with average changes in gene expression showing downregulation *in vivo*, while showing upregualtion *in vitro*.

At the level of biological processes, several similar GO terms with implications for steroid metabolism were identified for both models (*in vitro*: "androgen metabolic process", "steroid catabolic process"; *in vivo*: "androgen biosynthetic process", "cholesterol biosynthetic process"). Similar responses in genes related to cholesterol, lipid and steroid (CLS) metabolism have been reported in other *in vitro* test systems, such as the rat whole embryo culture system, which showed significant impacts on CLS pathways after exposure to the reproductive toxicant MEHP (the main metabolite of DEHP) but comparably little impact after exposure to the non-reproductively toxic MMP (the main metabolite of DMP). In the same study, apoptosis pathways were also upregulated, similar to what we observed in the 3D-TCS (Robinson et al. 2012). Thus, these results demonstrate a consistency in modes of toxicity observed in the 3D-TCS when compared to both *in vitro* and *in vivo* systems, as phthalate exposure in rats *in vivo* has led to disruption of CLS metabolism and apoptosis of testicular cells (Lee et al. 1997; Kasahara et al. 2002; Lehmann et al. 2004). In addition to these CLS and apoptosis pathways, pathways related to general reproductive development were altered by phthalates in the 3D-TCS and in the

in vivo rat testes (*in vitro*: “reproductive structure development”, *in vivo*: “male gonad development”).

Looking more specifically at the steroidogenic pathway, we observed that several genes both up and downstream of cholesterol synthesis were significantly altered by at least one DTPE under both *in vitro* and *in vivo* experimental scenarios. Notably, *Star* and *Scarb1*, which code for key proteins involved in the transport of cholesterol during the process of steroidogenesis, were significantly altered both *in vitro* and *in vivo* (Manna et al. 2009). In addition, several important CYP450’s with important roles in the metabolism of cholesterol and testosterone (*Cyp11a1*, *Cyp17a1* and *Cyp51*) were downregulated by one or more reproductively toxic phthalates both *in vitro* and *in vivo*. Furthermore, 2 of the 4 genes which were commonly altered by all DTPE treatments *in vitro* and *in vivo* have roles in steroidogenesis. *Star* performs the rate-limiting function of importing cholesterol into the mitochondria, while *Fdx-1* is involved in transferring electrons to steroidogenic CYP450’s (Miller 2005).

After performing pathway analysis, we identified a group of individual genes belonging to pathways highly relevant to male reproductive biology which were altered by DTPE treatment. After plotting gene expression for these 21 genes, we observed a response in the *in vitro* system in which genes were either up or downregulated after exposure to DTPE (there were few to no significant changes in the DNTPE exposure groups). In contrast, for the *in vivo* rat testes, almost all of the genes were

downregulated. This suggests a complex and dynamic transcriptomic response to phthalates in the 3D-TCS.

4.3 Mapping changes in the steroid biosynthesis pathway

As we mentioned previously, the downregulation of steroidogenic genes and the decreased production of testosterone in the male testes is a well-studied mode of action for phthalate reproductive toxicity (Lehmann et al. 2004; Foster 2006). In order to investigate the dynamics of gene expression changes in this key pathway, we used Pathvisio to map out the steroid biosynthetic pathway (van Iersel et al. 2008). This pathway includes major reactions from de novo cholesterol biosynthesis (or import into the cell via *Scarb1*) to the formation of various steroids including testosterone and estradiol. By generating this map and showing the associated changes in gene expression, we compared DTPE effects *in vivo* and *in vitro*. Fig. 7 (a+b) clearly shows that there are multiple genes upstream of cholesterol biosynthesis which were downregulated both *in vitro* and *in vivo*. However, *Star*, which performs the rate-limiting function of importing cholesterol into the mitochondria, was highly upregulated by DTPE *in vitro*, while being downregulated *in vivo*. This result shows that, although we can identify dysregulation of certain key pathways after toxicant exposure, it may be necessary to investigate impacts on the level of individual genes in order to fully understand the dynamics of toxicant impacts on cellular processes in the 3D-TCS.

The difference in responses that we observed could be explained by regulatory feedback mechanisms within the steroid biosynthesis pathway. Phthalate disruption of steroid metabolism may lead to decreased availability of steroid hormone precursors and associated adaptive response resulting in an increase in expression of certain steroidogenic genes. These biphasic responses to phthalate exposure have been observed in multiple studies and include reproductive or developmental endpoints such as onset of puberty, steroidogenic gene expression and testosterone production (Ge et al. 2007; Ryu et al. 2008). Differences in response may also be due to developmental stage at which phthalate exposure occurs. For example, some studies in which prenatal animals were exposed to PE's during the critical period of male reproductive development (approximately gestational days 12-19) resulted in damage to testicular tissue and decreased steroidogenic gene expression (Barlow et al. 2003; Hannas et al. 2011). However, other experiments in which treatments occurred outside of this window showed an increase in expression of steroidogenic genes (Ryu et al. 2008). Similarly, cells in the 3D-TCS, cultured from 5 day old rats (outside the GD 12-19 window) showed an increase in *Star* after PE exposure. Differences in response to phthalate exposure have also been noted for different doses administered both *in vitro* and *in vivo*. For example, Ge et al. found that DEHP exposure at a dose of 10mg/kg/day for 28 days resulted in advancement of pubertal onset and increased serum testosterone for Long Evans rats while 750mg/kg/day resulted in delayed onset of puberty and decrease in serum testosterone. In the same study, MEHP (active metabolite of DEHP) treatment of cultured Leydig cells at

100µM increased testosterone production, while 10mM had the opposite effect (Ge et al. 2007).

4.4 In vitro/in vivo *comparisons*

Making comparisons between *in vitro* and *in vivo* transcriptomic data presents multiple challenges. Results can be strongly influenced by experimental design and a number of important factors must be taken into account when interpreting data. For example, timing of exposure and kinetic factors may influence *in vitro* cellular responses to toxicant exposure that may make direct comparisons to *in vivo* exposures difficult. One important difference between the *in vivo* and *in vitro* responses being compared in this study were the relatively large number of gene expression changes observed in the 3D-TCS compared to the *in vivo* exposures. Fortunately, we were able to address these differences in the distribution of gene changes by utilizing a PCA analysis and the approach provided by the ToxProfiler software. Using these approaches we analyzed the entire set of gene expression data for principle components and pathway analysis, as opposed to applying a statistical cutoff for gene lists. This approach allowed us to demonstrate that, regardless of the differences in absolute number of gene changes, both the 3D-TCS and *in vivo* testes transcriptomic profiles are able to discriminate between DTPE's and DNTPE's.

It is possible that some differences in transcriptomic responses were due to kinetic or metabolic processes which occur *in vivo* but are not present in the Sertoli-cell culture

model. Metabolism and kinetics of compounds *in vitro* present a challenge when determining how the data can be can be applied in an *in vivo* context (Coecke et al. 2006). For example, the monoester metabolites of PE's are considered to be mostly responsible for male reproductive toxicity (Gray and Gangolli 1986; Creasy et al. 1988; Ema et al. 2003; Howdeshell et al. 2007). Clewell et al. found a concentration of 100µM of MBP (the active metabolite of DBP) in fetal testes approximately 1 hour after administration of 500mg/kg DBP to pregnant Dams (the same dose that was utilized for the *in vivo* exposure in the current comparison). This was followed by a peak of 150µM after 2 hours (Clewell et al. 2009). By comparison, the *in vitro* exposure to cells in the current study was to 100µM of parent compound. We are currently collecting data on the capability to metabolize phthalate diester compounds to the monoesters as well as kinetics of various phthalates in the 3D-TCS (Harris, et al., submitted manuscript).

4.5 Conclusions

In conclusion, transcriptomic data showed that the 3D-TCS co-culture model was able to differentiate between developmentally toxic and non-toxic phthalate esters based on patterns of gene expression changes and through pathways analysis. Pathway analysis of *in vitro* and *in vivo* transcriptomic data showed that a number of cellular processes related to male reproductive development and hormone signaling were enriched both *in vitro* and *in vivo*. Mapping the steroidogenic pathway allowed us to compare the dynamics of changes within this important pathway between the *in vitro* and

in vivo scenarios. Results indicate that DTPE exposure induced changes in cellular pathways in the 3D-TCS which are related to major modes of action for male reproductive toxicity of phthalates. This study facilitated the evaluation of the 3D-TCS toxicogenomic data within the context of a proposed AOP for phthalate reproductive toxicity (i.e. disruption of steroidogenesis and associated effects on male reproductive development), allowing us to link reproductive and developmental pathways impacted by phthalates in the 3D-TCS with those impacted by phthalates in other *in vitro* cell systems and at higher levels of biological organization (e.g. decreased anogential distance *in vivo*) (Ge et al. 2007; NRC 2008; Robinson et al. 2012; Furr et al. 2014). Despite challenges, the comparison between data obtained from this model with data generated from the *in vivo* study has given us valuable information which improves our ability to interpret responses to phthalate exposure in our co-culture system.

Acknowledgements

This work was supported by in part by the UW NIEHS Center for Ecogenetics and Environmental Health (5 P30 ES007033), UW EPA Center for Predictive Toxicology (RD-83573801), US-FDA (FDA: 1U01FD004242), and the NIH Center on Human Development and Disability (1 U54 HD083091-01) and Niels Stensen Fellowship. We gratefully acknowledge Kevin Gaido for generously providing the *in vivo* transcriptomic data. We would additionally like to thank Kirk Van Ness (Institute for Risk Analysis and Risk Communication, University of Washington) for his helpful comments on this chapter.

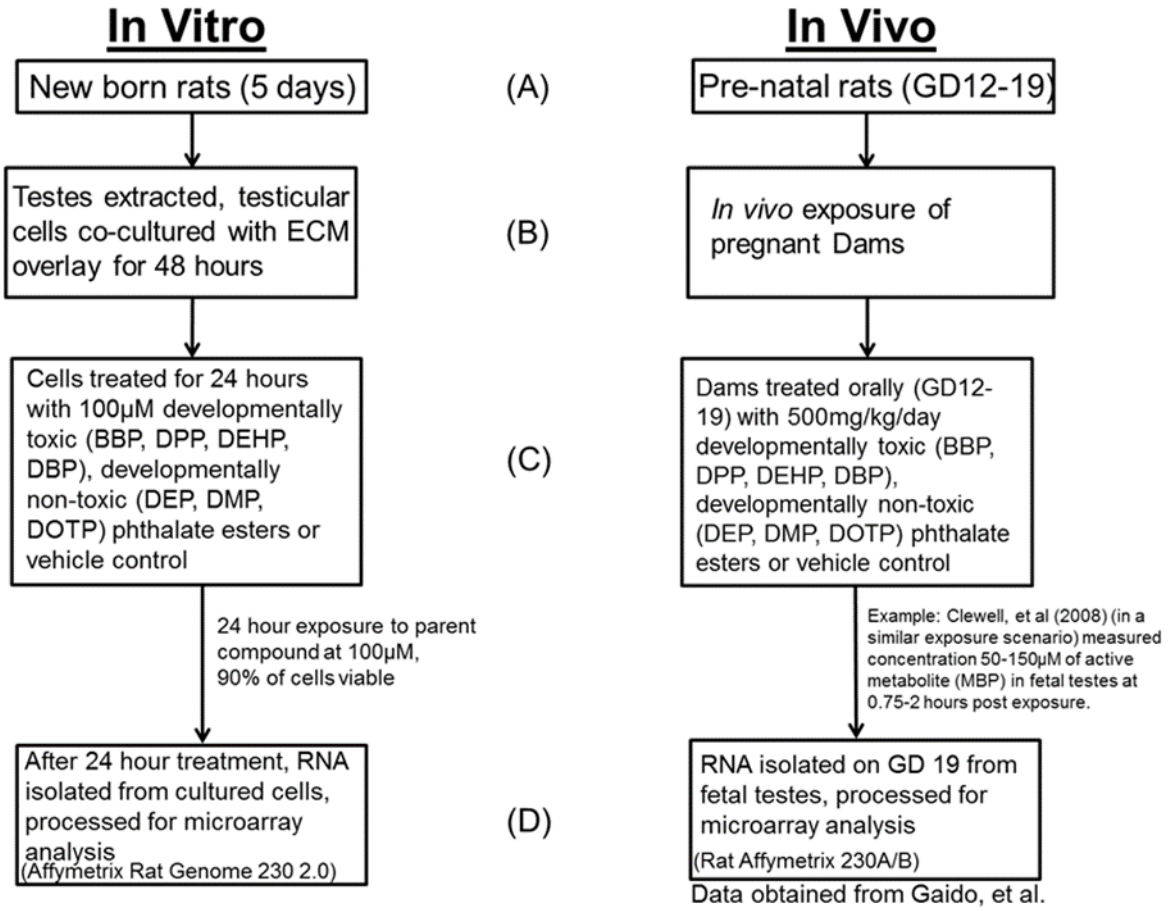

Fig. 1. Experimental design for the *in vitro* three-dimensional testicular cell co-culture system (3D-TCS) and *in vivo* fetal rat testes exposed to phthalate esters

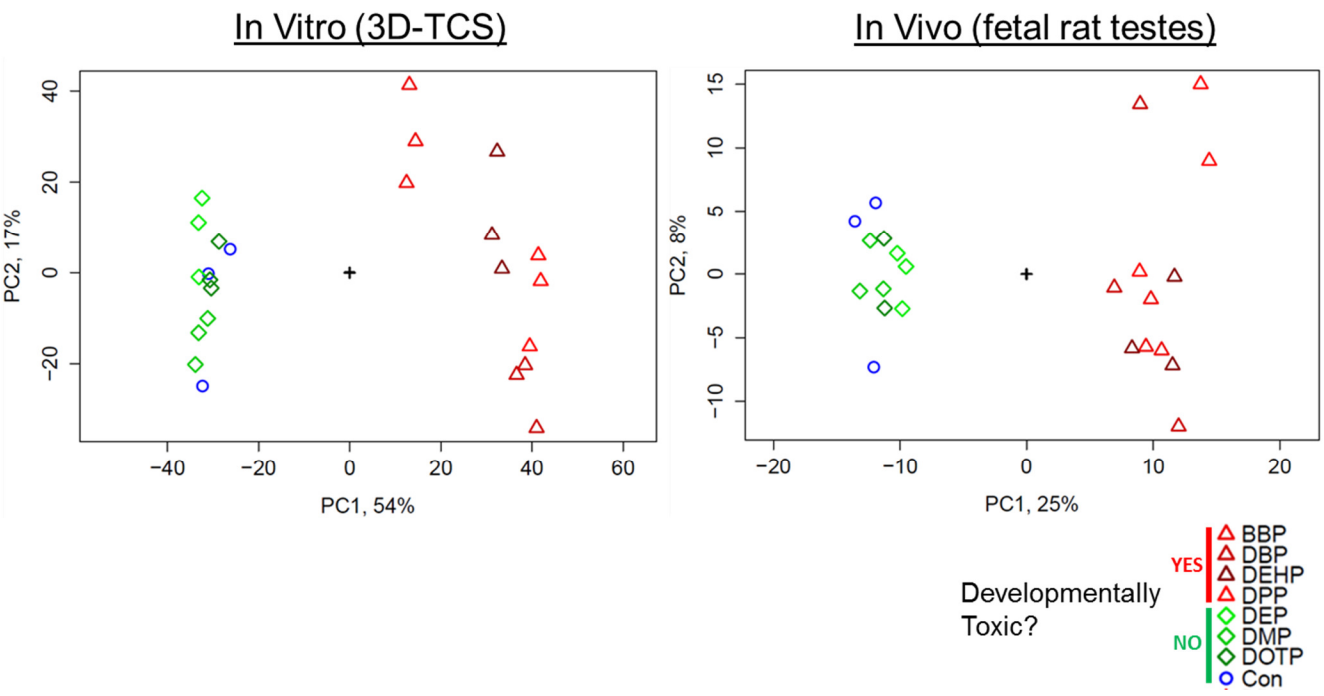

Fig. 2 Principle component analysis of gene expression analysis for microarrays from (a) *in vitro* (3D-TCS) and (b) *in vivo* (fetal rat testes). Genes on each array were analyzed using principle components analysis in order to group samples by the similarity of gene expression profiles. Developmentally toxic phthalate treatments formed a distinct cluster separate from the controls and developmentally non-toxic phthalate treatments for *in vitro* (3D-TCS) and *in vivo* (fetal rat testes) model systems.

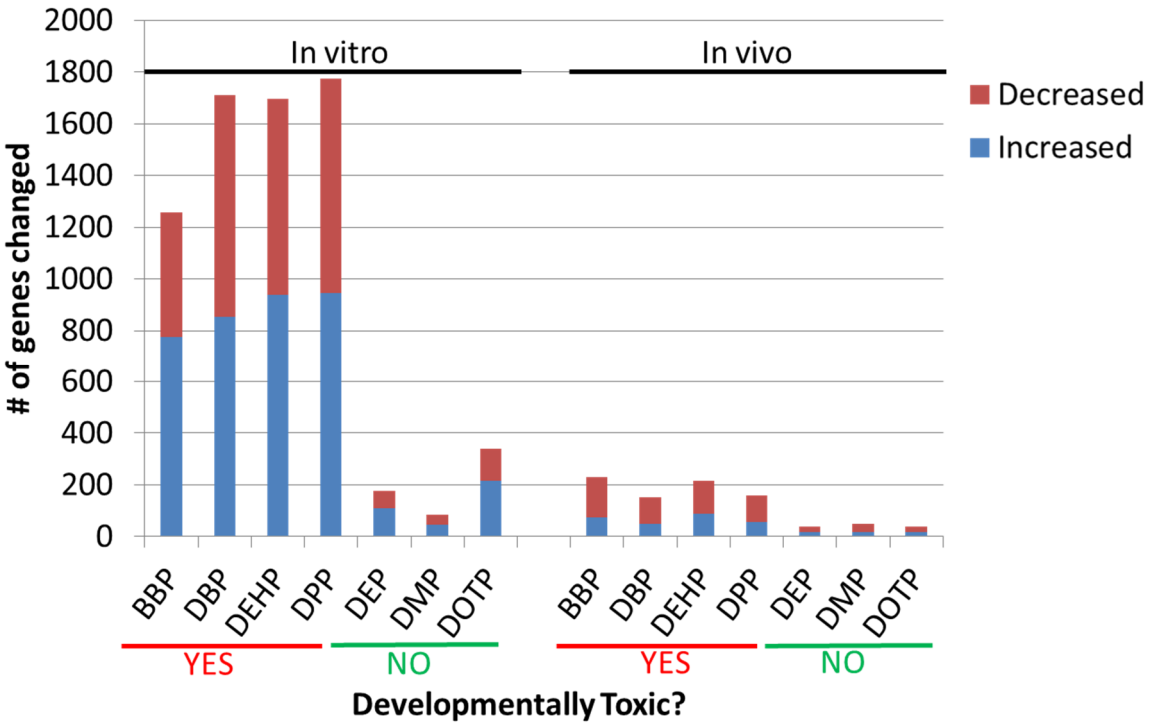

Fig. 3. Gene expression changes following treatment with phthalate esters *in vitro* (3D-TCS) and *in vivo* (fetal rat testes). The number of significantly changed genes was determined using a t-test for significant differences in gene expression between individual phthalate treatment and controls ($p<0.005$). The number of significant gene changes was higher for developmentally toxic phthalates both *in vitro* (3D-TCS) and *in vivo* (fetal rat testes).

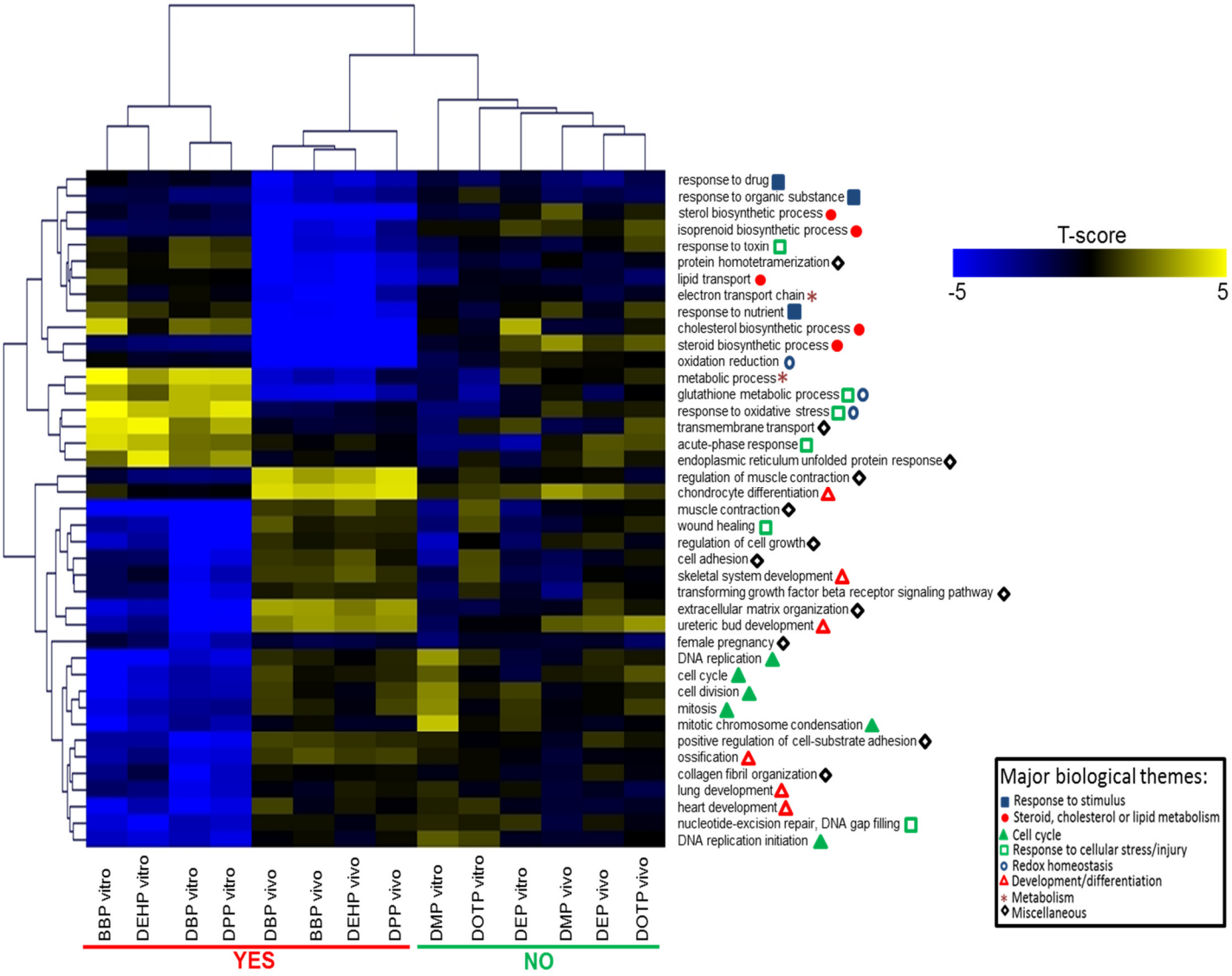

Fig. 4 Identification of enriched Gene Ontology defined biological processes using ToxProfiler software. Enriched processes were identified and t-scores were calculated for transcriptomic profiles from *in vitro* (3D-TCS) and from *in vivo* (fetal rat testes) models after exposure to developmentally toxic and non-toxic phthalate esters.

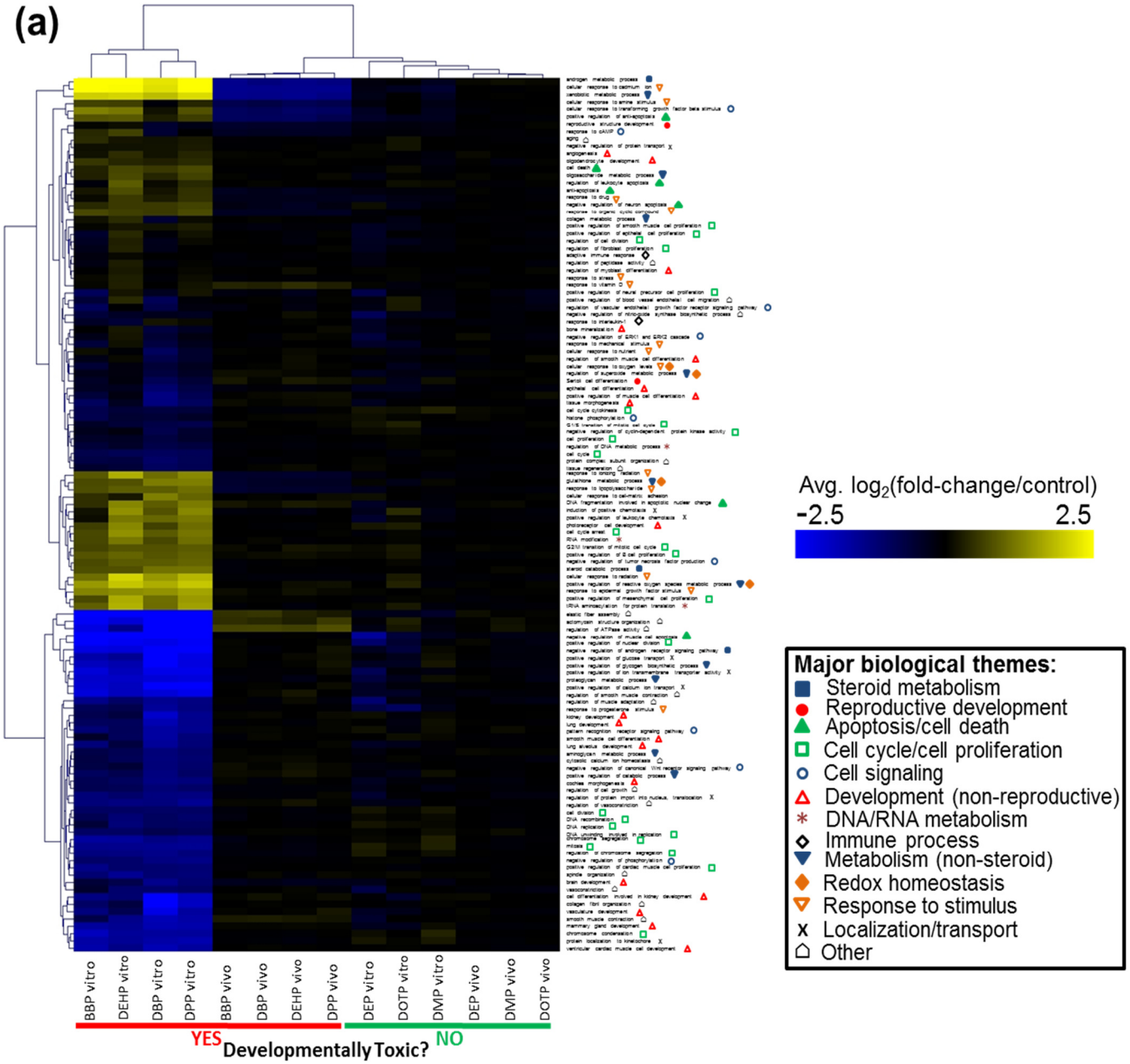

Fig. 5 Identification of enriched Gene Ontology defined biological processes using GO-Elite software. Enriched processes were identified by a z-score>2 and perm. p-value<0.05 for genes changed across toxic phthalate treatments **(a)** *in vitro* (3D-TCS) or **(b)** *in vivo* (fetal rat testes). Average log2 (fold-change/control) for genes changed in each process were calculated for all phthalate treatments.

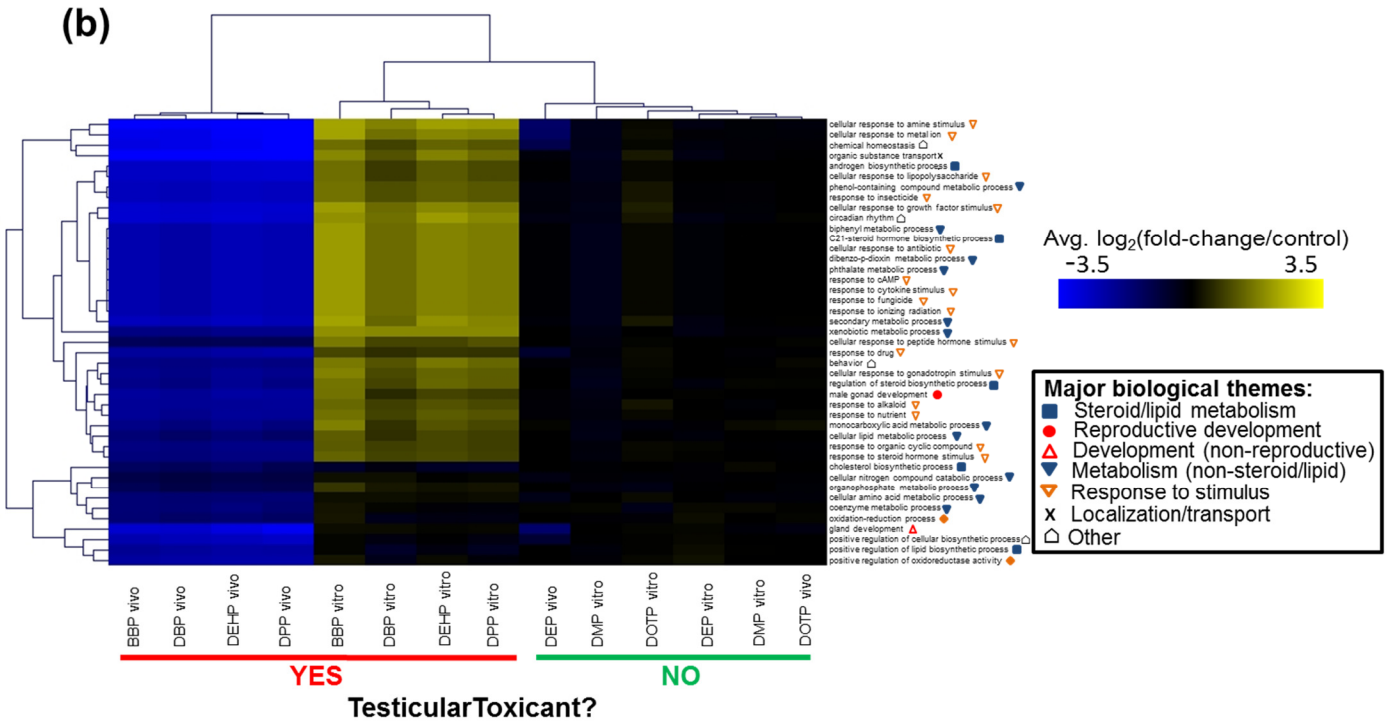

Fig. 5 (cont.) Identification of enriched Gene Ontology defined biological processes using GO-Elite software. Enriched processes were identified by a z-score>2 and perm. p-value<0.05 for genes changed across toxic phthalate treatments **(a)** *in vitro* (3D-TCS) or **(b)** *in vivo* (fetal rat testes). Average log2 (fold-change/control) for genes changed in each process were calculated for all phthalate treatments.

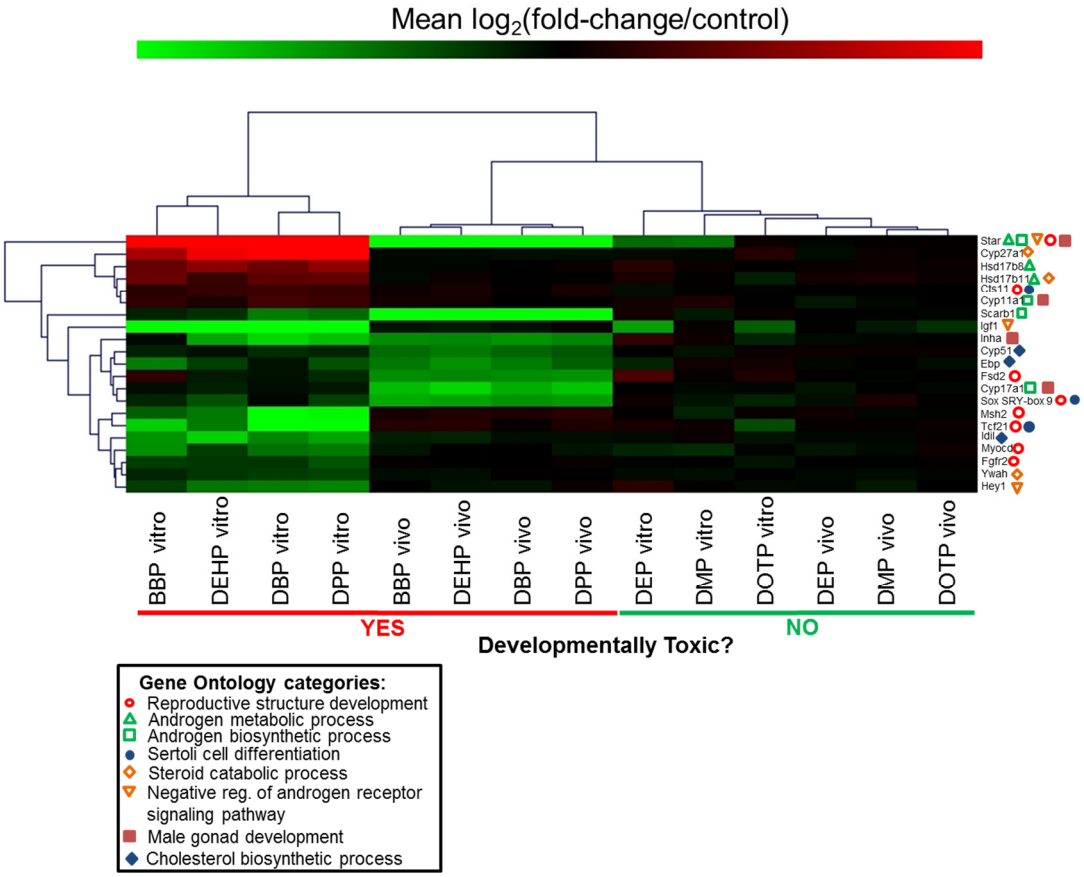

Fig. 6 Individual gene expression changes for genes involved in reproductive development or steroid biosynthesis. Based on the pathway analysis of the *in vitro* (3D-TCS) and *in vivo* (fetal rat testes) data, we identified a group of 21 genes which are involved in either steroid metabolism or reproductive development. Changes in gene expression compared to control samples are shown for both *in vitro* (3D-TCS) and *in vivo* (fetal rat testes) exposures for these 21 genes.

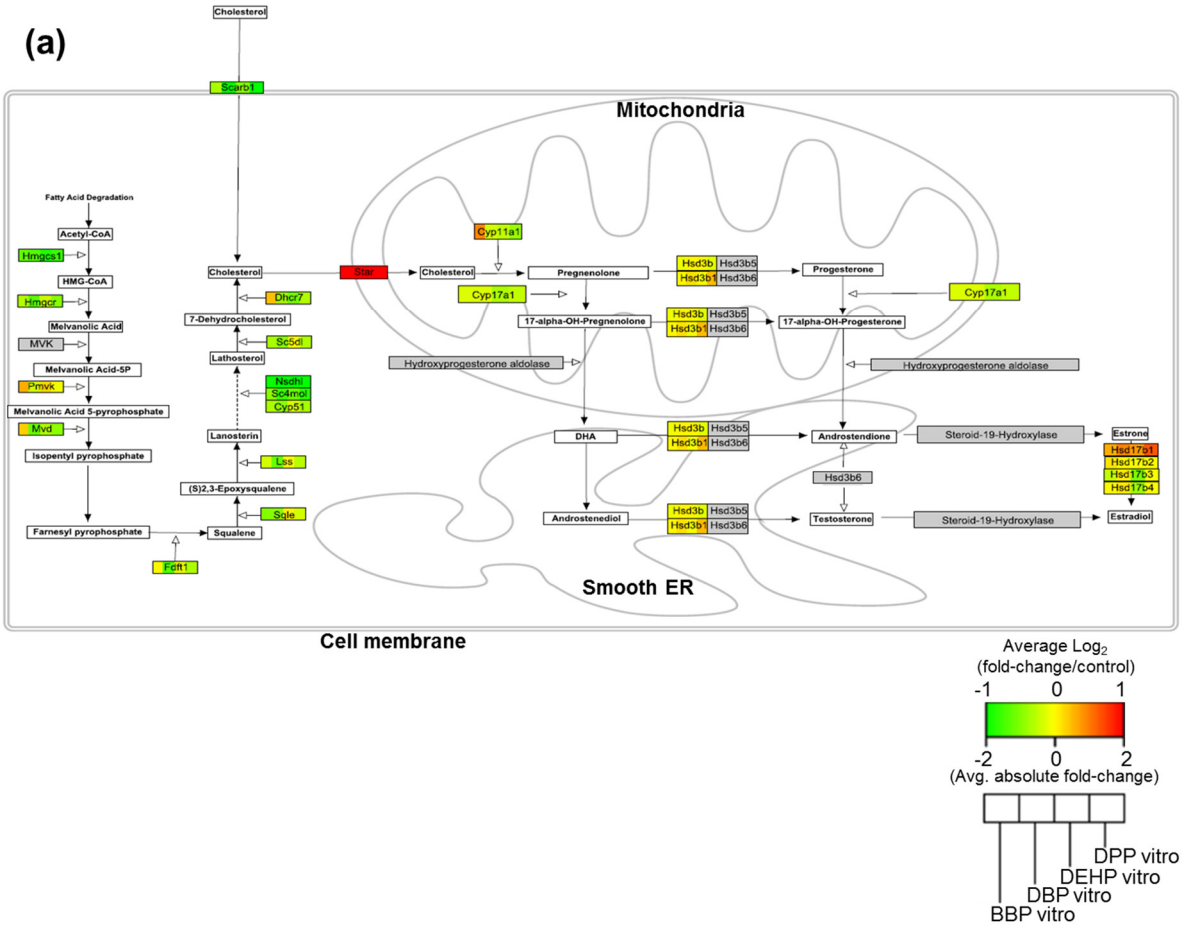

Fig. 7 Visualization of gene expression changes in the steroid biosynthesis pathway after exposure to toxic PE's in the (a) *in vitro* (3D-TCS) and (b) *in vivo* (fetal rat testes). Multiple references were used to construct the rat steroid biosynthesis pathway using Pathvisio software. Gene expression changes (log2fold-change/control) for genes in the pathway are shown, with red=upregulation, green=downregulation, grey=no gene data found and white=metabolite/precursors.

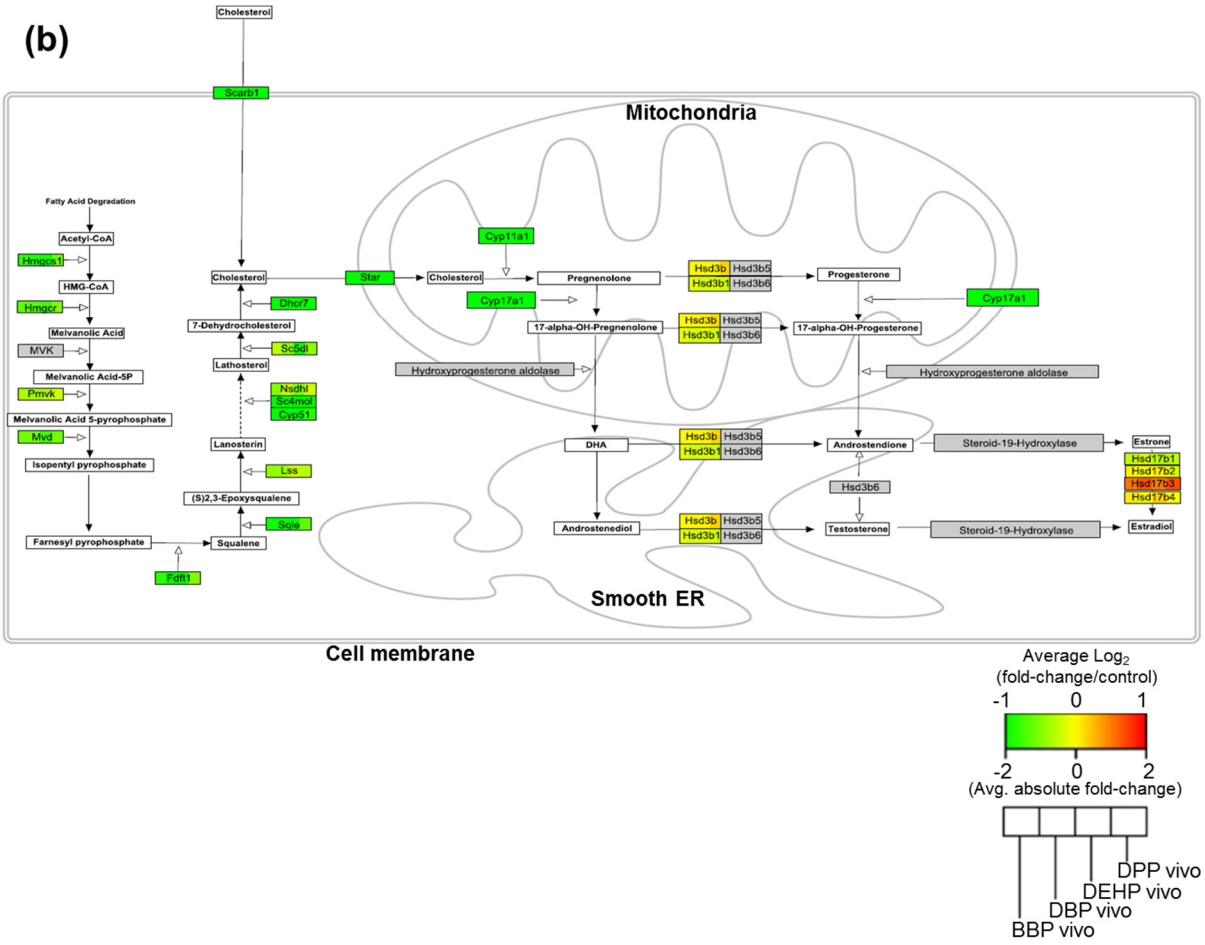

Fig. 7 (cont.) Visualization of gene expression changes in the steroid biosynthesis pathway after exposure to toxic PE's in the (a) *in vitro* (3D-TCS) and (b) *in vivo* (fetal rat testes). Multiple references were used to construct the rat steroid biosynthesis pathway using Pathvisio software. Gene expression changes (log2fold-change/control) for genes in the pathway are shown, with red=upregulation, green=downregulation, grey=no gene data found and white=metabolite/precursors.

Table 1. Gene Ontology Defined Biological Processes Enriched Among Genes Significantly Altered By All Developmentally Toxic Phthalates in 3D-TCS

GOID	GO Name	# Genes in GO	% of Present Genes Changed	% of Genes Present on Array	Z-Score	Parametric p-value
Steroid metabolism/signaling						
8209	androgen metabolic process	24	14.29	87.50	2.44	0.04
6706	steroid catabolic process	20	18.75	80.00	3.05	0.02
60766	negative regulation of androgen receptor signaling pathway	10	42.86	70.00	5.30	0.002
Reproductive development						
48608	reproductive structure development	132	8.08	75.00	2.13	0.03
60008	Sertoli cell differentiation	6	60.00	83.33	6.45	0.001
Apoptosis/cell death						
45768	positive regulation of anti-apoptosis	40	15.15	82.50	3.32	0.01
8219	cell death	417	6.16	81.77	2.15	0.03
2000106	regulation of leukocyte apoptosis	38	21.43	73.68	4.77	0.00
6916	anti-apoptosis	155	10.43	74.19	3.61	0.00
43524	negative regulation of neuron apoptosis	96	8.70	71.88	2.04	0.04
6309	DNA fragmentation involved in apoptotic nuclear change	18	23.08	72.22	3.55	0.01
Cell cycle/cell proliferation						
48661	positive regulation of smooth muscle cell proliferation	59	16.67	81.36	4.55	0.000
50679	positive regulation of epithelial cell proliferation	114	9.64	72.81	2.68	0.01
51302	regulation of cell division	67	17.24	86.57	5.23	0.000
48145	regulation of fibroblast proliferation	62	13.04	74.19	3.19	0.005
2000179	positive regulation of neural precursor cell proliferation	24	15.00	83.33	2.55	0.03
33205	cell cycle cytokinesis	16	20.00	93.75	3.20	0.01
82	G1/S transition of mitotic cell cycle	45	15.63	71.11	3.41	0.01
45736	negative regulation of cyclin-dependent protein kinase activity	16	28.57	87.50	4.75	0.002
8283	cell proliferation	362	6.54	71.82	2.19	0.02
7049	cell cycle	263	9.36	77.19	4.02	0.00
7050	cell cycle arrest	81	10.77	80.25	2.84	0.01
86	G2/M transition of mitotic cell cycle	37	21.43	75.68	4.77	0.001
30890	Positive regulation of B cell proliferation	33	13.04	69.70	2.25	0.05
2053	positive regulation of mesenchymal cell proliferation	35	18.52	77.14	3.90	0.003
51785	positive regulation of nuclear division	31	15.38	83.87	3.01	0.02

51301	cell division	164	13.27	68.90	5.14	0.000
6310	DNA recombination	101	9.21	75.25	2.38	0.02
6260	DNA replication	111	10.26	70.27	2.88	0.01
6268	DNA unwinding involved in replication	7	42.86	100.00	5.30	0.002
7059	chromosome segregation	51	13.79	56.86	2.74	0.02
7067	mitosis	98	12.50	73.47	3.75	0.001
51983	regulation of chromosome segregation	16	25.00	75.00	3.76	0.01
60045	positive regulation of cardiac muscle cell proliferation	16	25.00	75.00	3.76	0.01
30261	chromosome condensation	27	14.29	77.78	2.44	0.04
Cell signaling						
71560	cellular response to transforming growth factor beta stimulus	30	16.00	83.33	3.11	0.01
30947	regulation of vascular endothelial growth factor receptor signaling pathway	16	21.43	87.50	3.37	0.01
70373	negative regulation of ERK1 and ERK2 cascade	20	16.67	90.00	2.78	0.02
16572	histone phosphorylation	16	20.00	93.75	3.20	0.02
32720	negative regulation of tumor necrosis factor production	21	25.00	57.14	3.76	0.01
2221	pattern recognition receptor signaling pathway	28	17.65	60.71	2.91	0.02
90090	negative regulation of canonical Wnt receptor signaling pathway	67	15.69	76.12	4.33	0.002
42326	negative regulation of phosphorylation	84	8.45	84.52	1.96	0.05
Development (non-reproductive)						
1525	angiogenesis	145	8.47	81.38	2.55	0.01
14003	oligodendrocyte development	23	15.00	86.96	2.55	0.03
45661	regulation of myoblast differentiation	20	23.08	65.00	3.55	0.01
30282	bone mineralization	26	18.18	84.62	3.44	0.01
51150	regulation of smooth muscle cell differentiation	14	33.33	85.71	5.24	0.001
30855	epithelial cell differentiation	137	10.91	80.29	3.78	0.002
51149	positive regulation of muscle cell differentiation	30	13.64	73.33	2.34	0.04
48729	tissue morphogenesis	291	8.89	77.32	3.87	0.00
42461	photoreceptor cell development	27	14.29	77.78	2.44	0.04
1822	kidney development	130	8.00	76.92	2.10	0.04
30324	lung development	112	8.79	81.25	2.39	0.02
6939	smooth muscle cell differentiation	37	16.13	83.78	3.50	0.01
48286	lung alveolus development	35	17.24	82.86	3.69	0.00

90103	cochlea morphogenesis	22	25.00	72.73	4.34	0.00
7420	brain development	218	7.78	76.61	2.58	0.01
61005	cell differentiation involved in kidney development	13	33.33	69.23	4.54	0.004
1944	vasculature development	35	13.79	82.86	2.74	0.02
30879	mammary gland development	36	15.38	72.22	3.01	0.01
55015	ventricular cardiac muscle cell development	10	37.50	80.00	4.89	0.002
DNA/RNA metabolism						
51052	regulation of DNA metabolic process	173	9.23	75.14	3.13	0.004
9451	RNA modification	44	16.67	68.18	3.59	0.004
6418	tRNA aminoacylation for protein translation	36	17.24	80.56	3.69	0.001
Immune process						
2250	adaptive immune response	55	12.77	85.45	3.12	0.01
70555	response to interleukin-1	71	10.00	84.51	2.42	0.02
Metabolism (non-steroid)						
6805	xenobiotic metabolic process	36	13.33	83.33	2.65	0.02
14003	oligosaccharide metabolic process	23	15.00	86.96	2.55	0.03
32963	collagen metabolic process	25	18.18	88.00	3.44	0.01
90322	regulation of superoxide metabolic process*	12	30.00	83.33	4.24	0.01
4364	glutathione metabolic process*	28	20.00	71.43	3.70	0.01
2000379	positive regulation of reactive oxygen species metabolic process*	25	15.00	80.00	2.55	0.03
45725	positive regulation of glycogen biosynthetic process	13	25.00	92.31	3.76	0.01
6029	proteoglycan metabolic process	23	14.29	91.30	2.44	0.04
6022	aminoglycan metabolic process	57	13.16	66.67	2.93	0.02
9896	positive regulation of catabolic process	106	8.14	81.13	2.01	0.04
Response to stimulus						
71276	cellular response to cadmium ion	7	50.00	85.71	5.81	0.001
71418	cellular response to amine stimulus	43	23.68	88.37	6.27	0.000
42493	response to drug	471	6.23	81.74	2.37	0.02
14070	response to organic cyclic compound	344	7.25	80.23	2.87	0.002
6950	response to stress	1658	6.22	77.62	4.50	0.000
33280	response to vitamin D	33	20.83	72.73	4.26	0.003
9612	response to mechanical stimulus	166	9.52	75.90	3.25	0.002
31670	cellular response to nutrient	67	10.42	71.64	2.32	0.03
71453	cellular response to oxygen levels*	57	13.33	78.95	3.25	0.01
10212	response to ionizing radiation	126	11.11	71.43	3.52	0.001
32496	response to lipopolysaccharide	260	6.60	81.54	2.02	0.04
71478	cellular response to radiation	55	12.50	72.73	2.79	0.02

70849	response to epidermal growth factor stimulus	19	20.00	78.95	3.20	0.01
32570	response to progesterone stimulus	44	11.11	81.82	2.22	0.04
Other						
7568	aging	230	7.81	83.48	2.79	0.01
52547	regulation of peptidase activity	224	9.04	79.02	3.52	0.000
51771	negative regulation of nitric-oxide synthase biosynthetic process	5	60.00	100.00	6.45	0.000
71822	protein complex subunit organization	499	6.92	78.16	3.10	0.003
42246	tissue regeneration	45	11.43	77.78	2.28	0.04
48251	elastic fiber assembly	6	60.00	83.33	6.45	0.002
31032	actomyosin structure organization	34	12.90	91.18	2.57	0.02
43462	regulation of ATPase activity	21	15.79	90.48	2.66	0.03
51150	regulation of smooth muscle cell differentiation	14	33.33	85.71	5.24	0.001
43502	regulation of muscle adaptation	20	23.53	85.00	4.16	0.003
51480	cytosolic calcium ion homeostasis	148	8.13	83.11	2.41	0.01
1558	regulation of cell growth	244	6.63	80.33	1.96	0.05
19229	regulation of vasoconstriction	47	12.20	87.23	2.73	0.01
7051	spindle organization	46	11.11	78.26	2.22	0.04
42310	vasoconstriction	19	17.65	89.47	2.91	0.02
30199	collagen fibril organization	26	15.00	76.92	2.55	0.03

*Also classified in biological theme: redox homeostasis

Note: Enriched processes were identified with GO-Elite software (using a z-score>2 and perm. p-value<0.05) for genes which were commonly changed across toxic phthalate treatments in the 3D-TCS *in vitro* testes co-culture model.

Table 2. Gene Ontology Defined Biological Processes Enriched Among Genes Significantly Altered By All Developmentally Toxic Phthalates in Fetal Rat Testes

GOID	GO Name	# Genes in GO	% of Present Genes Changed	% of Genes Present on Array	Z-Score	Parametric p-value
Steroid, lipid, cholesterol metabolism/signaling						
6702	androgen biosynthetic process	12	33.33	100.00	20.27	33.33
6700	C21-steroid hormone biosynthetic process	14	23.08	92.86	14.54	23.08
50810	regulation of steroid biosynthetic process	50	9.09	88.00	10.33	9.09
6695	cholesterol biosynthetic process	26	15.00	76.92	11.64	15.00
46889	positive regulation of lipid biosynthetic process	43	7.69	90.70	8.17	7.69
Reproductive development						
8584	male gonad development	81	6.90	71.60	8.90	81.00
Metabolism (non-steroid)						
18958	phenol-containing compound metabolic process	54	8.70	85.19	10.09	54
18879	biphenyl metabolic process	11	37.50	72.73	18.63	11
18894	dibenzo-p-dioxin metabolic process	11	33.33	81.82	17.55	11
18963	phthalate metabolic process	8	42.86	87.50	19.94	8
19748	secondary metabolic process	79	4.92	77.22	6.38	79
6805	xenobiotic metabolic process	36	13.33	83.33	12.64	36
32787	monocarboxylic acid metabolic process	291	1.72	80.07	3.82	291
44255	cellular lipid metabolic process	558	1.39	77.24	4.03	558
44270	cellular nitrogen compound catabolic process	210	1.75	81.43	3.35	210
19637	organophosphate metabolic process	214	1.83	76.64	3.45	214
6520	cellular amino acid metabolic process	289	1.75	79.24	3.87	289
6732	coenzyme metabolic process	176	2.21	77.27	3.92	176
Response to stimulus						
71418	cellular response to amine stimulus	7.89	88.37	8.29	7.89	88.37
71248	cellular response to metal ion	5.36	78.87	6.70	5.36	78.87
71222	cellular response to lipopolysaccharide	5.26	89.41	7.66	5.26	89.41
17085	response to insecticide	22.22	85.71	16.47	22.22	85.71

71363	cellular response to growth factor stimulus	2.75	83.85	4.52	2.75	83.85
71236	cellular response to antibiotic	33.33	69.23	17.55	33.33	69.23
51591	response to cAMP	3.70	85.26	5.42	3.70	85.26
34097	response to cytokine stimulus	1.16	80.94	2.42	1.16	80.94
60992	response to fungicide	23.08	92.86	14.54	23.08	92.86
10212	response to ionizing radiation	3.33	71.43	5.09	3.33	71.43
71375	cellular response to peptide hormone stimulus	3.48	80.99	6.03	3.48	80.99
42493	response to drug	2.08	81.74	6.23	2.08	81.74
71371	cellular response to gonadotropin stimulus	25.00	84.21	17.50	25.00	84.21
43279	response to alkaloid	4.85	84.43	8.19	4.85	84.43
7584	response to nutrient	2.02	77.26	4.79	2.02	77.26
14070	response to organic cyclic compound	2.54	80.23	6.61	2.54	80.23
Localization/transport						
71702	organic substance transport	1.17	79.57	2.44	0.04	1.17
Other						
48878	chemical homeostasis	617	0.82	78.77	2.01	617
7623	circadian rhythm	93	4.11	78.49	5.76	93
7610	behavior	429	1.16	80.19	2.82	429
31328	positive regulation of cellular biosynthetic process	1073	0.72	77.45	2.14	1073

Note: Enriched processes were identified with GO-Elite software (using a z-score>2 and perm. p-value<0.05) for genes which were commonly changed across toxic phthalate treatments in fetal rat testes.

Chapter 3: Phthalate Metabolism and Kinetics in an in Vitro Model of Testes Development

This chapter has been submitted to Toxicology In Vitro*. The authors of the manuscript are:*

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Key Words: Phthalate esters, reproductive toxicity, *in vitro* models, testis, metabolism

Abstract

We have developed an *in vitro* model of testes development (3D-TCS) using rat testicular cells overlaid with extracellular matrix. One barrier preventing utilization of *in vitro* models in toxicity testing is absence of metabolic capability. Another challenge is lack of kinetic data for compounds *in vitro*. We characterized metabolic capabilities and investigated the kinetics of phthalate male reproductive toxicants in the 3D-TCS. Cells were treated with three phthalate diesters for 2, 8 and 24 hours. Parent compounds and metabolites were measured in cell culture media and cell lysate via mass spectrometry. Levels of monoester metabolites were used as an indication of metabolism of phthalates via lipase activity. Metabolites were detected in all treated cell media and cell lysate samples, with levels ranging from <0.5-13% of initial mass of parent compound. Phthalates partitioned between media and lysate in a manner consistent with each compound's degree of lipophilicity. UDGPT activity was detected in DBP and DEP treated samples. 3D-TCS microarray data indicated gene expression for lipases and CYPP450's. Results indicate that the 3D-TCS is a metabolically active co-culture and that physiochemical properties can provide information about the kinetics of compounds in the 3D-TCS, improving our ability to interpret results from the model.

1. Introduction

We have developed an *in vitro* model of testes development (Yu et al. 2005; Wegner et al. 2013). This three dimensional cellular co-culture system (3D-TCS) contains the rat testes cell types including: Sertoli, germ and Leydig cells grown in a three dimensional conformation facilitated by an extracellular matrix (ECM) overlay. Previous experiments have demonstrated that addition of ECM in this co-culture model results in a more stable system and that cells form a testicular-like architectural structure representative of *in vivo* testes (Yu et al. 2005). Since the optimization of the 3D-TCS, we have demonstrated that this co-culture was able to inform specific cell signaling pathways involved in the mechanism of cadmium toxicity in the testes (Yu et al. 2008). We have also shown that this co-culture was able to discriminate between reproductively toxic and non-toxic phthalate diesters based on patterns of transcriptomic changes (Yu et al. 2009). These results highlight a promising potential for the use of the 3D-TCS in characterizing compounds for male reproductive toxicity.

One major barrier in the utilization of *in vitro* models in toxicity assessment is the absence of functional metabolic systems which are in place *in vivo* but not represented *in vitro.* For example, primary and cell line derived hepatocyte cultures have been shown to frequently exhibit decreased cytochrome-P450 activity compared to what is observed *in vivo* (Sidhu et al. 1993; Boess et al. 2003; Brown et al. 2007). This presents a problem in terms of utilizing *in vitro* cell models in toxicity screening of chemical

compounds as many environmental toxicants require metabolic activation by enzyme systems in order to exert toxic effects. Conversely, enzyme systems may also metabolize toxicants to non-toxic metabolites. Absence of these processes in *in vitro* test systems makes interpretation of data obtained from these cell culture models more difficult, especially in a risk assessment context. Thus, the metabolic capability of test systems to activate or deactivate toxic compounds is crucial to interpreting *in vitro* data. Addressing this issue, a 2006 workshop report from the European Centre for the Validation of Alternative Methods (ECVAM) advised that basal biotransformation capabilities of *in vitro* test systems should be characterized before use as a tool for toxicity screening (Coecke et al. 2006). Recent reports continue to call for these studies and cite absence of *in vitro* metabolism in these systems in the prevention of their application, indicating that metabolic activity toward test compounds may be an important factor in interpreting toxicity data from *in vitro* testicular models (Hartung et al. 2011; Parks Saldutti et al. 2013). Relevant to our *in vitro* testes model, there are numerous examples of biotransformation systems known to be active in the adult rat testis including cytochrome P450s, glutathione-S-transferases, aryl hydrocarbon hydroxylase, epoxide hydrolase and lipases (Dixon and Lee 1980; Holst et al. 1994). In order to appropriately interpret results of toxicity assays in the 3D-TCS, it is important to determine to what extent these and other enzyme systems are conserved in the *in vitro* context of our co-culture system.

Another challenge in interpretation of *in vitro* results is the absence of data on the kinetics of compounds in *in vitro* systems. In order for *in vitro* methodologies to be

properly utilized in the risk assessment process, doses used to identify adverse effects *in vitro* should be relevant for *in vivo* exposure scenarios. Discrepancies between the nominal toxicant concentration in cell media and the concentration at the site of toxic action could arise from a number of factors, including those with quantitative impact such as binding to proteins, binding to tissue culture plastic, and evaporation of the test compound (Blaauboer 2010; Groothuis et al. 2013). Evaluations of the kinetic behavior of compounds within the 3D-TCS will be critical to understanding the effects observed in toxicity assays because these evaluations will enable us to determine dosimetry-corrected media concentrations, allowing for more accurate translation of *in vitro* to *in vivo* doses for the purposes of risk assessment.

In the current study we have used exposure to three structurally related phthalate esters to investigate metabolic capabilities and characterize the kinetics of compounds in the 3D-TCS. Some phthalate diesters are known male reproductive toxicants and exposure at low levels is widespread in many human populations (Duty et al. 2003; Bustamante-Montes et al. 2013). Effects of phthalates on male reproductive endpoints in rats have been extensively studied and include hypospadias, cryptorchidism, decreased anogenital distance, and decreased testosterone levels in the testes (Mylchreest et al. 1998; Gray et al. 2000; Clewell et al. 2010). Taken together, these male reproductive endpoints in rats have been described by the term “phthalate syndrome” (Swan 2008). Interestingly, the outcomes described by phthalate syndrome closely resemble the human Testicular Dysgenesis Syndrome (TDS) first described by Skakkebaek

et al. in 2001 (Skakkebaek et al. 2001). The origins of TDS are postulated to be due in part to early life-stage exposures to endocrine disrupting compounds such as phthalates (Andersson et al. 2007). Due to rising rates of effects described by TDS in some human populations, the possibility that phthalate exposures are contributing to human male reproductive disorders remains a concern (Martino-Andrade and Chahoud 2010; Bustamante-Montes et al. 2013). In order to characterize the inherent capacity for the cells in the 3D-TCS to metabolize this important class of male reproductive toxicants, we exposed cells to two known reproductively toxic phthalates, dibutyl phthalate and diethylhexyl phthalate (DBP and DEHP, 100µM) as well as one non-reproductively toxic phthalate, diethyl phthalate (DEP, 150µM). Concentrations of parent compound and the monoester metabolite (which is thought to be the main metabolite responsible for testes toxicity *in vivo*) were measured in media and lysate via mass spectrometry (HPLC-ESI-MS/MS). The metabolic pathways for the three phthalates used in this study are shown in Figures 1a-c.

2. Materials and Methods

2.1 Preparation of three dimensional co-culture model for testis (3D-TCS)

Protocol for the preparation of three dimensional testes co-cultures has been explained in detail elsewhere (Yu et al. 2005; Wegner et al. 2013). Briefly, testes were dissected from 5-day-old male pups obtained from mated Sprague–Dawley rats (Charles River Laboratories, Wilmington, USA). Sequential enzymatic digestions were used to create a single cell

suspension containing primarily Sertoli, germ and Leydig cells. Cells were suspended in a solution containing serum-free Eagle's Minimal Essential Medium (Invitrogen, Carlsbad, CA) containing 0.1nM nonessential amino acids, 1mM sodium pyruvate, 3mM sodium lactate, 1% ITS culture supplement (BD Biosciences, Bedford, MA). Cells were then plated at a density of $1.6x10^6$ cells/35mm plate followed by the addition of extracellular matrix medium (Matrigel™). Phthalates are ubiquitous and have been shown to have the potential to contaminate plastic lab supplies (Reid 2007). In order to address this issue, dishes with only Matrigel™ and phthalates added were plated in parallel in order to detect any background levels of phthalate metabolites in absence of cells. Phthalate solutions with a final concentration of 100μM (DBP, DEHP) or 150μM (DEP) diluted in dimethyl sulfoxide (DMSO) were then added directly to the culture medium 48 h after initial plating of cells. Doses were selected based on those that caused minimal cytotoxic impact observed in previous experiments. DBP (Sigma #D2270, 99% purity), DEHP (Sigma #4-8557, 99% purity), DEP (Sigma #524972, 99.5% purity) and DMSO (Sigma #D1435) were obtained from Sigma-Aldrich (MO, USA*).*

2.2 Sample collection and processing

Dishes containing cells (plus Matrigel™) or Matrigel™ only were exposed to phthalate for 2, 8 and 24 hours (n=3). At the end of each exposure period, media (~2mL) was collected from each plate and replaced with 100μL of cell lysis buffer. Plates were

then scraped with cell scraper and cell lysate (lysis buffer plus scraped material) was collected. Samples were immediately placed on dry ice and then stored at -80^{o}C until analysis. All samples were thawed, sonicated (Heat Systems Ultrasonics^{TM}, Farmingdale, New York) for 15 seconds total and centrifuged for 5 minutes at 16,000xG to remove cellular debris. Supernatants were then placed back at -80^{o}C before analysis via mass spectrometry. Protein levels for cell lysate samples were determined using commercially available colormetric assay (BioRad Laboratories, #500-0006).

2.3 Detection of parent compounds and monoester metabolites in 3D-TCS samples

The methods used to detect parent compounds and monoester metabolites were adapted from Centers for Disease Control method for detecting phthalates in urine (Calafat 2010). This method uses high performance liquid chromatrography-electrospray-ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) in order to quantitatively detect phthalate parent compounds and monoester metabolites. Analysis was performed with a ThermoFinnigan LC pump, ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum triple quadropole mass spectrometery with an electrospray ionization interface. The column used was a Chromolith Flash RP-18e SPE column. Mobile phase A was 0.1% acetic acid in water while mobile phase B was 0.1% acetic acid in acetonitrile. The LC program used varying flow rates and A:B mobile phase ratios over a 27 minute extraction period. Electrospray ionization in negative ion mode

was used to ionize analytes and dissociation voltage was 10V. Average background levels of phthalate metabolites detected in cell free (phthalate and Matrigel™-plated only) samples were subtracted from total phthalate metabolite levels. Levels of metabolites observed in phthalate treated "matrigel-only" plates <1% of initial media concentration of parent phthalates. These were assumed to be attributable to non-enzymatic hydrolysis occurring in cell media or cell lysate during incubation or sample processing, as this has been shown to occur for phthalates *in vitro* and in the environment (Daniel and Bratt 1974; Clewell et al. 2009; Lertsirisopon et al. 2009). Total and unconjugated levels of monoester metabolites were measured after incubation of samples with or without glucuronidase for 90 minutes at 37°C. The difference in metabolite levels measured with and without glucurionidase was used to generate an estimate of the total amount of glucuronidated metabolite in each sample. For calculations of mass balance and total compound recovery, the average masses of parent compound and metabolites were determined in each sample. Masses were then converted to percentage of initial mass of phthalate, giving a percentages of initial starting mass that was accounted for by: parent compound in cell media, metabolite in cell media, parent compound in cell lysate, metabolite in cell lysate, or percentage loss of recovery of phthalate (likely due at least in part to phthalate binding to tissue culture plastic). In order to characterize the kinetics of the phthalate parent compounds, (i.e. the partitioning between cell media and cell lysate) pairwise t-tests were used to compare the levels of phthalate observed in cell media and cell lysate at 2, 8 and 24 hours.

2.4 Transcriptomic analysis of metabolic gene expression (lipases and CYPP450s)

In order to further characterize the metabolic capabilities of the 3D-TCS, datasets consisting of microarray gene expression from the 3D-TCS and *in vivo* fetal rat testes were utilized. *In vitro* data were obtained from a study in which 3D-TCS cells were exposed to 100μM of seven phthalates (benzyl butyl phthalate, dibutyl phthalate, diethyhexyl phthalate, dipentyl phthalate, diethyl phthalate, dimethyl phthalate or dioctyl terephthalate) or vehicle control (DMSO) (Yu et al. 2009). *In vivo* microarray data were obtained from testes of fetal rats for which dams were exposed to the same seven phthalates or control (via oral gavage) from gestational days 12-19. Detailed methods for each of these studies are described elsewhere (Liu et al. 2005; Yu et al. 2009). Arrays used for in vitro data were Affymetrix Rat 230 2.0 and in vivo data were 230 (A and B chips), which contain the same probes. All gene expression intensities were normalized to $\log_2$(fold-change/mean of controls) using the corresponding control for each dataset.

In order to investigate gene expression for key metabolic genes present in the 3D-TCS, the microarray data were queried for gene expression signals for lipases and CYP450s. We identified expression signals for 16 lipase genes and 10 CYP450s with relevance to male reproductive development and/or steroid and lipid metabolism which were changed in the 3D-TCS by at least one phthalate exposure (F-test, FDR<0.1). Gene expression data for the corresponding lipases and CYP's were identified in the *in vivo* fetal rat testes data for comparison. We then used the Gene Ontology (GO) Consortium

database to identify GO terms (biological processes and molecular functions) related to testes or male reproductive processes In order to characterize relevant functions of these lipases and CYP450's in the 3D-TCS model.

3. Results

The levels of monoester metabolites detected varied by phthalate, with detected levels ranging from ~<0.1-13% of initial mass of parent compound (Fig. 2). Levels of parent compound and monoester metabolite in cell media and cell lysate for all samples and all timepoints are summarized in Supplementary Tables 1a (as absolute concentration) and 1b (as a percentage of the initial mass of parent compound). For the purposes of comparing the levels of parent compounds and metabolites in the cell media and lysate fractions, which had different volumes, we used total mass detected and volume of each fraction (2 and 0.12mL for cell media and lysate, respectively) to yield a concentration which we reported in terms of percent of the initial concentration of phthalate in cell media.

3.1 Metabolism analysis

The highest levels of monoester were detected for DEP exposed cells (mean of 13% of the initial DEP mass detected as MEP in cell media after 24 hours). The lowest levels of monoester metabolite were observed for the DEHP exposure (>0.5% detected in cell media after 24 hours). We used the detected levels of monoester metabolites as an

indication of phase I metabolism of phthalate diesters to monoesters by non-specific lipases. Levels of monoester metabolite we detected in our cell culture were compared with metabolic activity of normal *in vivo* rat and human testes tissues. Measured rates of lipase activity for these tissues from the literature (Terner et al. 1975; Murase et al. 1982; Choi et al. 2012) were used for comparison. The reported rates of lipase activity from *in vivo* were compared with the rates of monoester metabolite formation over time in our culture *in vitro*. As shown in Table 1, the rates of lipase activity estimated based on formation of monoester metabolites in our culture were within the ranges of activity levels reported in the literature for DBP and DEP, while the rate of DEHP was below this range (~one-sixth reported rate of DEHP metabolism in human testes tissue).

Levels of glucuronidation were estimated by measuring the levels of monoester metabolites pre- and post-incubation with glucuronidase, with the estimated levels of glucuronidated metabolites equal to the difference between these two values. These estimated rates of phthalate glucuronidation occurring in the 3D-TCS with were compared with rates observed in rat testes *in vivo.* Using this method, we detected significant levels of glucuronidated MBP and MEP (for DBP and DEP treated samples, respectively) in cell media samples after 24 hours of exposure as well as in the cell lysate of DEP treated samples after 2 hours. We did not detect any glucronidated MEHP at any timepoint tested. In order to compare to levels of conjugated metabolites detected in our co-culture samples we calculated rates of activity in our samples (normalized by protein levels and time) with rates of UGT activity reported for rat testes in the literature. We specifically

examined the rates of glucuronidation of bisphenol A (BPA) and 1-napthol (reported in nanomoles glucuronidated/mg protein/hour) measured by Yokota, et al. (2002) in rat testes microsomes (Yokota et al. 2002) . As summarized in Table 2, the estimated rates in the 3D-TCS for DBP and DEP exposures were two orders of magnitude lower than the rates reported in that study.

3.2 Gene expression analysis for metabolic genes

Fig. 4 shows changes in gene expression for 16 lipase and 10 CYP450 genes in the 3D-TCS after a 24 hour exposure to seven different phthalates. We observed a distinct profile of lipase gene changes (both up and downregulation) for reproductively toxic phthalate exposures which was quite distinct from non-reproductively toxic phthalate exposures. The 16 lipases were associated with a number of major biological themes including lipid metabolism (lipid catabolic process, cholesterol metabolic process, sterol esterase activity), inflammatory processes (arachidonic acid metabolism, cytokine production, regulation of inflammatory response), intracellular signaling (response to cAMP) and male reproductive development (acrosomal membrane). Similarly, we observed phthalate induced gene expression patterns for 10 CYP450 enzymes in our cell culture model. Similar to the results reported for the lipase genes, reproductively toxic phthalate exposures impacted gene expression for this class enzymes differently than non-reproductively toxic phthalates.

3.3 Phthalate kinetics in the 3D-TCS

In order to characterize the kinetics of phthalates in our co-culture model, we sought to determine to what extent physiochemical properties such as $\log K_{ow}$ could be used to predict the kinetics of phthalates in the 3D-TCS. The three phthalates used in this study had a range of $\log K_{ow}$ values: 2.47 (DEP), 4.72 (DBP) and 7.5 (DEHP). We predicted that the compound with the highest $\log K_{ow}$ (i.e. the most lipophilic compound) would partition more to the cell lysate fraction compared to the cell media and that for the least lipophilic phthalate, the reverse would be true. As shown in Fig. 6, the three phthalate diesters differentially partitioned between cell media and cell lysate, following our hypothesis that differences in lipophilicity would lead to different kinetic of compounds in the co-culture system. The concentration of DEHP was significantly higher in the cell lysate versus the cell media fraction at all timepoints investigated. In fact, DEHP accumulated in the cell lysate to a degree that the concentration of DEHP in the smaller volume (80µL) of cell lysate was higher than the original concentration in the larger volume of cell media (2mL), with average DEHP concentrations of 124µg/mL and 0.8µg/mL in cell lysate and cell media, respectively. In contrast, DEP maintained a significantly higher concentration in the cell media compared to cell lysate. There appeared to be no discernible tendency for DBP to concentrate in either fraction, as the concentrations in cell media and lysate were not statistically different at any timepoint.

Fig. 6 shows percentages of the total mass of total phthalate diester found as either parent compound or monoester metabolite in various fractions of the cell culture

system (percentages were adjusted for differences between molecular mass of parent compounds and metabolites). In addition to differing rates of metabolism and partitioning between cell media and cell lysate, we also noticed differences in total mass recovery across the three phthalates. We assumed that loss of recovery of phthalate was due to binding to tissue culture plastic because others have shown that phthalates exhibit binding activity with such materials (Desdoits-Lethimonier et al. 2012).

4. Discussion

4.1 Phthalates are metabolized in the 3D-TCS

Uncertainty about the metabolic capabilities in cell culture models is one of the challenges in the utilization of *in vitro* models in toxicity screening. We have demonstrated that metabolism of phthalate diesters is occurring in our model of male testes development (3D-TCS), as evidenced by detection of monoester metabolites in cell lysate and cell media. Phthalates were metabolized at different rates over time and levels of metabolites ranged from <0.1%-13% of initial concentration of phthalate parent compound. The phase I reaction of phthalate metabolism (see Figures 1. a-c) produces the monoester metabolites and is catalyzed by class of enzymes called lipases. These enzymes also play important roles in lipid metabolism and in male reproductive development (Holst et al. 1994; Chung et al. 2001). The rate of metabolism observed (in units of picomoles/mg of protein/minute) for DBP and DEP was within the range of reported rates for lipase activity in rat testes and for human testes subcellular fractions,

while the rate of metabolism of DEHP was about one-sixth of the lowest of these reported rates (see Table 1). Interestingly, the rates of monoester metabolite formation across the three phthalates appeared to follow a trend similar to that reported by Lake et al., who observed in rat and human intestinal preparations, that phthalates with shorter alkyl side chain length were metabolized at a faster rate compared to those with longer side chains. For example, DBP was metabolized at a slower rate than DEP (Lake et al. 1977). Further experiments will be needed to confirm this observation.

Levels of glucuronidated monoester metabolites were measured indirectly by calculating the difference in metabolite levels observed before and after incubation with glucuronidase. Glucuronidation is a phase II reaction catalyzed by a group of enzymes known as UDP-glucuronosyltransferases (UGTs). This group of enzymes plays a role in the metabolism of both endogenous and xenobiotic compounds by converting lipophilic substrates into water-soluble compounds, facilitating excretion. UGTs are expressed in a number of organs in the rat, including the Sertoli cells in the testes and in testicular Leydig cells of humans (Shah and McLachlan 1976; Brands et al. 2000; Uhlen et al. 2010). In our cultures, the average estimated rates for UGT activity toward MBP and MEP (metabolites of DBP and DEP) after 24 hours were 0.16 and 0.18 nanomoles/mg protein/hour, respectively. These rates are lower than rates of glucronidation reported for various other compounds in the *in vivo* rat testicular homogenates. For example, Yokota et al. (2002) found a rate of 60 and 48 nanomoles/mg protein/min. in rat testes microsomes when bisphenol A and 1-napthol were used as a substrates, respectively (Yokota et al. 2002).

There are several potential explanations for these results including differences in activity toward substrates (e.g. activity toward BPA may be higher than toward MBP for certain UGT's) as well as kinetic factors (MBP and MEP may not have sufficient uptake into cells to induce similar levels of UGT activity). In contrast to MBP and MEP, no detectable levels of MEHP conjugation were observed in our *in vitro* model. These results are consistent with observations in rat DEHP exposures *in vivo*, in which MEHP has mostly been observed in its free form and is not thought to be conjugated in any significant amount (Keys et al. 2000; Kurata et al. 2012). It should be noted that β-glucuronidase activity has been reported in the rat testes, meaning that the levels of glucuronidated metabolites measured in the current study are detected despite any endogenous deconjugation due to β-glucuronidase (Foster et al. 1983). In our study only parent compounds and free or glucuronidated monoester metabolites were analyzed. Some products of phthalate metabolism exist (such as phthalic acid) that would not have been detected using our current experimental design. Further experiments will need to be performed in order to fully characterize the phthalate metabolic pathways as well as lipase and UGT activity profiles for the 3D-TCS.

4.2 Phthalates impact expression of key metabolic genes in the 3D-TCS

In addition to the indication of lipase activity suggested by the formation of phthalate metabolites in the 3D-TCS, we were able to demonstrate that gene expression signals were present for these enzymes as well and we compared these data with fetal rat

testes *in vivo*. Both *in vitro* and *in vivo* data showed that gene expression for lipase genes were significantly impacted by exposure to reproductively toxic phthalates, consistent with what has been shown in previous studies. For example, DEHP has been shown to inhibit the activity of lysosomal acid lipase in rat liver fractions and increase lipoprotein lipase activity in rat epididymal tissue. MEHP, the toxic metabolite of DEHP, has been shown to increase expression of hormone sensitive lipase transcript in mouse Leydig tumor cells (Teruyoshi 1986; Gunnarsson et al. 2008; Martinelli et al. 2010). In our 3D-TCS model, we observed a trend of significant downregulation for lysosomal acid lipase after exposure to developmentally toxic phthalates and a significant increase in lipoprotein lipase expression.

Phthalate impacts on lipases have a number of implications for elucidating testes function *in vivo*. Lipases regulate lipid metabolism and one of the modes of action for phthalates in the disruption of lipid profiles in the testes, which in turn can lead to altered steroid levels. The Gene Ontology database was used to identify additional biological processes and molecular functions associated with these genes, which revealed more functions relevant to male reproductive development. For example, in addition to their well-known roles in lipid metabolism, several of the affected lipases were shown to be involved inflammatory processes such as cytokine production (GO:1816) and arachidonic acid metabolism (GO:19369). These processes have important implications for male reproductive toxicity pathways due to the regulatory roles that some cytokines and arachidonic acid play in the regulation of steroidogenesis (Romanelli et al. 1995; Hedger

and Meinhardt 2003). For example cytosolic Phospholipase A_2 ($cPLA_2$) is responsible for the release of arachidonic acid (AA) from membrane phospholipids (Iovannisci et al. 2007). In addition to steroidogenesis AA plays a role in numerous biological processes relevant to testis development including apoptosis signaling and cell differentiation. When comparing changes occurring in these lipases *in vivo* and *in vitro*, we observed that relatively fewer changes were present in the *in vivo* testes. There are a number of reasons for this, including the differences in times of exposure *in vivo* and *in vitro* as well as kinetic and metabolic differences between the two model systems. The gene expression changes observed in the *in vitro* testes model after 24 hours may represent an early response to phthalate exposure that is not maintained after the repeating dosing performed for the *in vivo* data. For example, similar to what we observed in our model, Lahousse, et al. (2006), observed increased expression of *Pla1a* (phospholipase A1, member A) in *in vivo* rat testes 12 hours after exposure to the MEHP, the active metabolite of DEHP (Lahousse et al. 2006). Our observations indicate that this important class of enzymes is functional within the 3D-TCS and chemical impacts on these enzymes could mediate toxicity in this *in vitro* model.

CYP450s are another important class of enzymes with numerous functions including metabolism of exogenous compounds and endogenous metabolism of steroids, cholesterol or lipids (Nebert and Russell 2002). Investigating gene expression data from the 3D-TCS, we observed signals for 10 CYP450s involved in endogenous metabolic processes with relevance to male reproductive development, such as steroid hormone

metabolism (*Cyp11a1*, *Cyp17a1* and *Cyp19a1*) or development of male gametes (*Cyp26b1*). We showed that exposure to reproductively toxic phthalates differentially affected the expression of these CYPs compared to exposures to non-reproductively toxic phthalates. We saw a similar pattern of differential expression for the reproductively toxic phthalate exposures in the in vivo testes, although the specific CYP's affected and pattern of expression changes were divergent. Taken together, these results indicate that the 3D-TCS is a metabolically active culture that is able to metabolize exogenous compounds (phthalates), while expressing transcriptomic signals for important endogenous metabolic functions. Furthermore, these classes of enzymes which have important roles in the proper development of male reproductive organs (CYPP450s and lipases), are impacted by exposure to reproductive toxicants in the 3D-TCS, suggesting roles as important markers of toxicity. Characterizing responses mediated by these enzymes will aid in the elucidation of toxicity pathways and facilitate linkages to *in vivo* pathways using our testes co-culture model.

4.3 Analysis of phthalate kinetics

Another barrier to the use of *in vitro* toxicity models is uncertainty regarding the kinetics of compounds. Evaporation, binding to tissue culture plastics and binding to serum protein have all been identified as factors that could lead to differences between nominal concentrations of test compound in cell media and concentrations observed at cellular targets. Prediction of kinetic behaviors of test compounds *in vitro* will allow for

better interpretation of data generated in these model systems (Groothuis et al. 2013). In the present study, we showed that $logK_{ow}$ (a measure of lipophilicity) was able to predict phthalate diester partitioning between media and cell lysate. This suggests that physiochemical properties are driving phthalate kinetics in the 3D-TCS. Additional kinetic information was provided by a mass balance calculation of phthalate compounds in the co-culture system. We observed a loss of total mass for DBP and DEHP treatments. For the purposes of this study, we assumed that loss of mass for these phthalates was due to binding to plastic materials used in culturing cells or sample collection, as lipophilic compounds (those with higher $logK_{ow}$) and DEHP in particular have been shown to bind to plastic materials used in *in vitro* experiments (Palmgren et al. 2006; Desdoits-Lethimonier et al. 2012).

4.4 Conclusions

In summary, phthalate monoester metabolites were detected in cell media and cell lysate samples in the 3D-TCS. These results, along with observed transcriptomic responses, provide evidence for the presence of functional lipases and CYP450s (enzymes involved in important metabolic processes in the testes) in our testes co-culture (Lobo et al. 2009). In addition we have obtained important information about the kinetic behavior of phthalates in the 3D-TCS, Our results will greatly contribute to the optimization of accurate dosimetry in order to determine the *in vitro* concentrations which correlate with concentrations in testes tissue *in vivo* under relevant exposure scenarios and more

accurately interpret toxicity data obtained from our model.

Acknowledgements

This work was supported by in part by the UW NIEHS Center for Ecogenetics and Environmental Health (5 P30 ES007033), UW EPA Center for Predictive Toxicology (RD-83573801), US-FDA (FDA: 1U01FD004242), and the NIH Center on Human Development and Disability (1 U54 HD083091-01). We gratefully acknowledge Kevin Gaido for generously providing the *in vivo* transcriptomic data. We would additionally like to thank Kirk Van Ness (Institute for Risk Analysis and Risk Communication, University of Washington) for his helpful comments on this chapter.

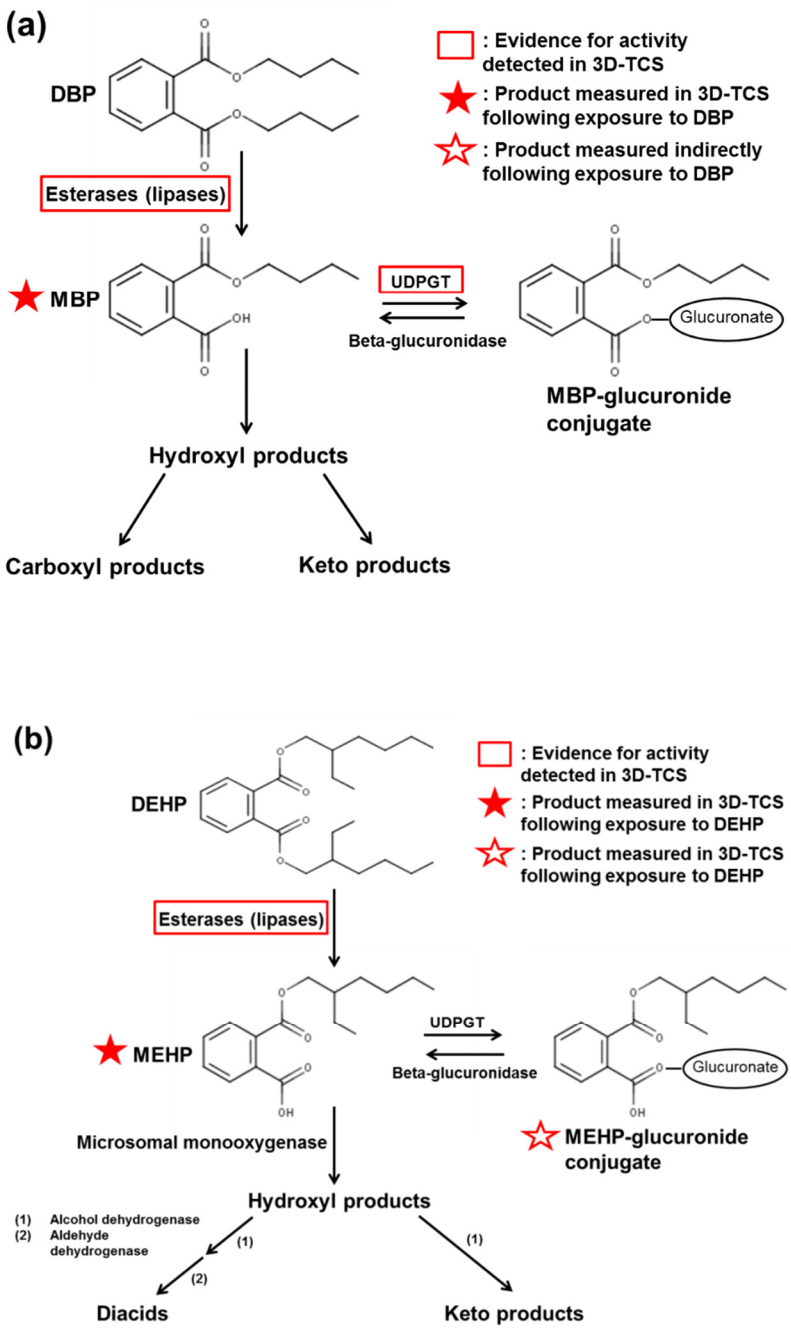


Fig 1. Pathways of phthalate metabolism. Metabolism pathways are shown for the three phthalate diesters used in the current study: (a) dibutyl phthalate (DBP), (b) di(ethylhexyl) phthalate (DEHP) and (c) diethyl phthalate (DEP). Phthalate parent compounds are initially converted to monoester metabolites by a class of enzymes known as lipases. This phase I reaction is followed by either phase II glucuronidation or the formation of alternative metabolic products.

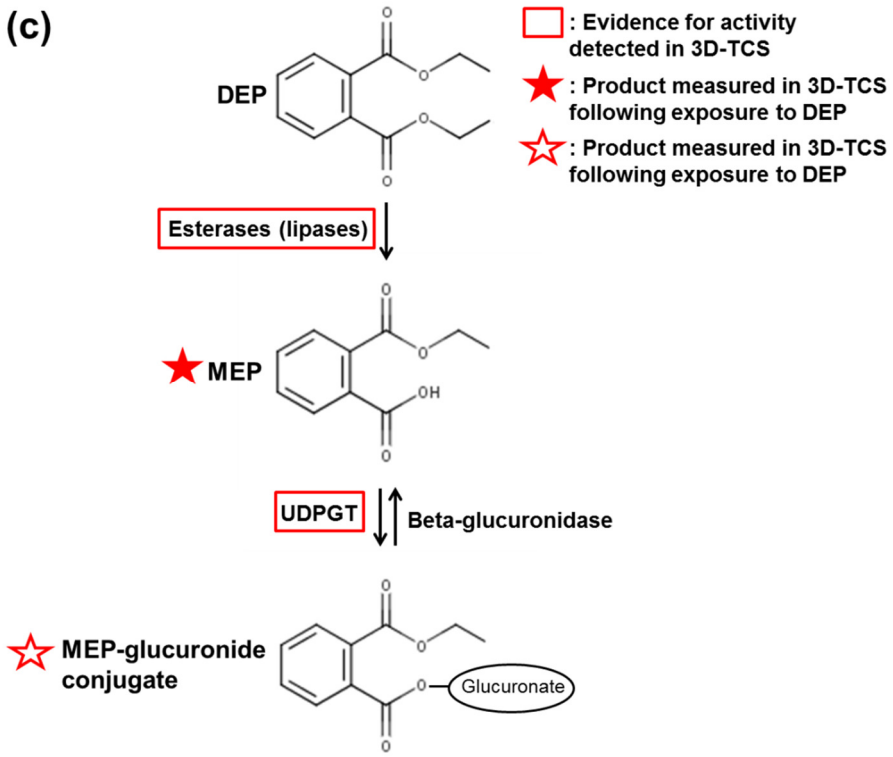


Fig 1 (cont.). Pathways of phthalate metabolism. Metabolism pathways are shown for the three phthalate diesters used in the current study: (a) dibutyl phthalate (DBP), (b) di(ethylhexyl) phthalate (DEHP) and (c) diethyl phthalate (DEP). Phthalate parent compounds are initially converted to monoester metabolites by a class of enzymes known as lipases. This phase I reaction is followed by either phase II glucuronidation or the formation of alternative metabolic products.

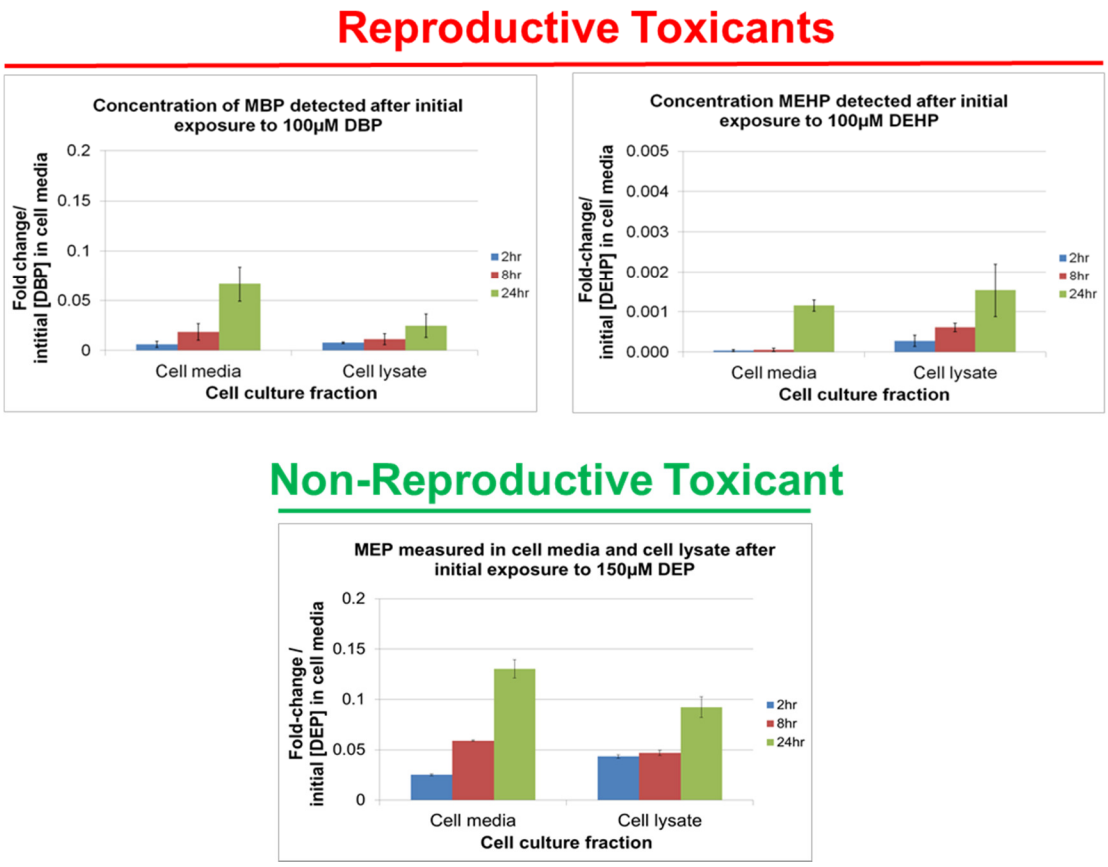


Fig.2.Concentrations of 3 phthalate monoester metabolites (MBP, MEHP and MEP) found in cell media or cell lysate after exposure to phthalate diester parent compounds (DBP, DEHP or DEP) for 24 hours. Testis cells exposed to 100µM concentrations of either dibutyl phthalate (DBP) or bis(2ethylhexyl) phthalate, diethyl phthalate (DEHP) or to 150µM diethyl phthalate (DEP). Levels of monoester metabolites were measured in cell media or cell lysate using HPLC/mass spectrometry at 2, 8and 24 hours post exposure. Concentrations are reported as fold-change from initial concentration of parent compound. Values are mean and standard errors for 3 replicate treatments.

Table 1. Estimated rate of lipase activity in 3D-TCS: Comparison with rates reported in testes tissue in the literature

Reaction	Mean (SD) reaction rate (picomoles/mg protein/minute)
DEHP -> MEHP	0.5 (0.3)
DBP -> MBP	57 (20)
DEP -> MEP	242 (113)

Range of calculated metabolic rate for phthalates in the 3D-TCS:
0.5-242 picomoles/mg protein/min.

Reaction	Reported reaction rate (picomoles/mg protein/minute)
Conversion DEHP->MEHP (Human testes, subcellular fractions)[a]	~3
Lipoprotein lipase activity (Rat testes, whole tissue)[b]	18
Triglyceride lipase activity (rat germ cells)[c]	283

Reported rates for lipases and phthalate metabolism in rat and human testes in literature: **~3-283 picomoles/mg protein/min.**

[a]:Choi, et al. 2012
[b]:Murase, et al. 1982
[c]:Terner, et al. 19751

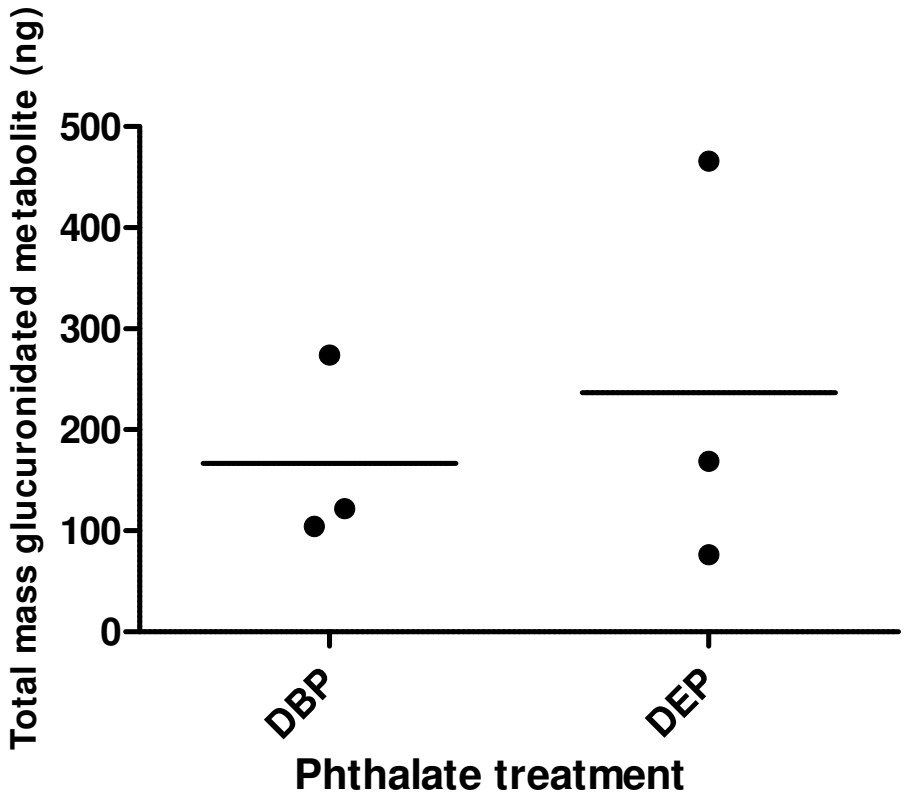
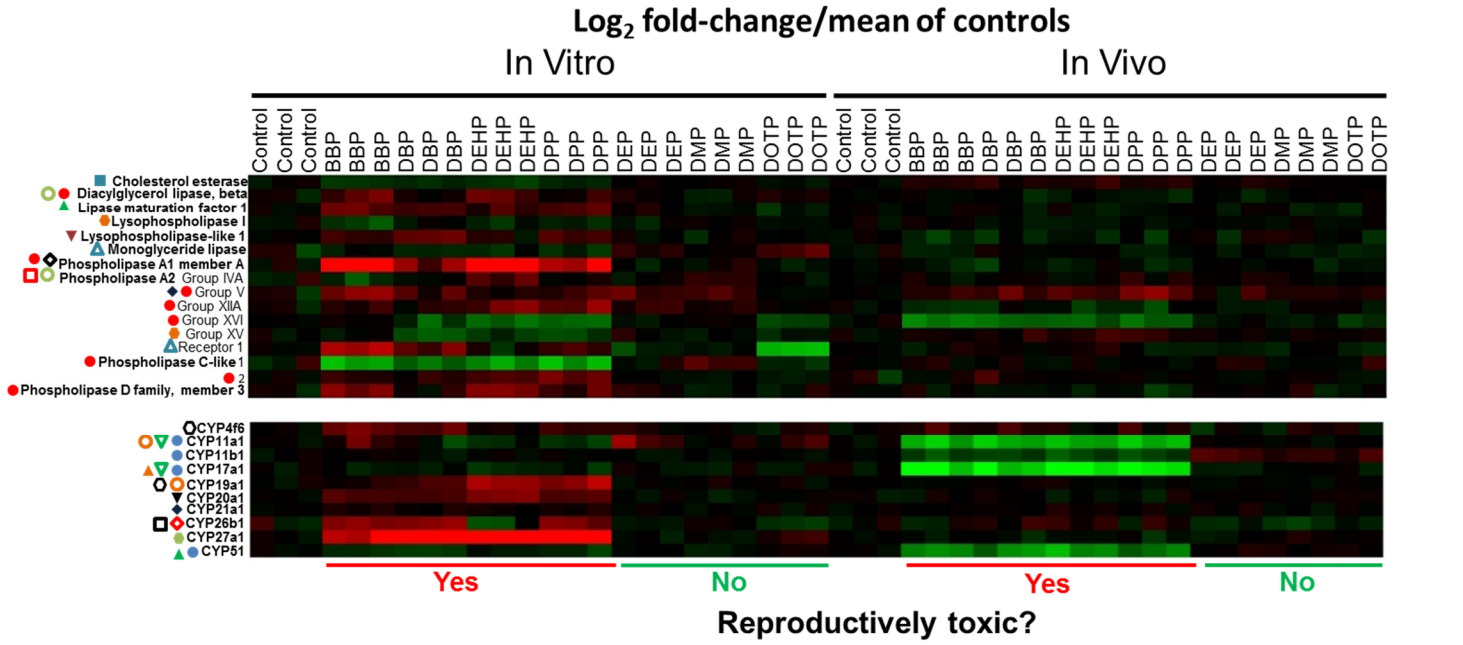


Fig 3. Total mass of glucuronidated metabolite in organotypic culture cell media after treatment with phthalate esters for 24 hours. Testes co-cultures were treated with phthalates (DBP, DEHP-100µM, DEP-150µM) and samples were collected after 2, 8 and 24 hours. Levels of monoester metabolites were measured before and after glucuronidase treatment in order to determine level of glucronidation in each sample. No detectable levels of glucuronidase were found at 2 and 8 hours for DBP and DEP treated or in any DEHP treated samples. n=3

Table 2. Rate of UGT activity *in vitro* (testes co-culture) and *in vivo* (rat testicular microsomes)

Substrate	Rate of glucuronidation (nanomoles/mg protein/hour)
Testes co-culture (current study)	
MBP	0.16
MEP	0.18
Testes tissue (values reported in literature)[1]	
BPA	60
1-napthol	48

[1]Data obtained from: Yokota, et al. (2002), J. Biochem. 132, 265-270



Major Biological Themes:

Lipid/cholesterol/fat metabolism
- Lipid catabolic process
- Regulation of cholesterol metabolic process
- Sterol esterase activity
- Fatty acid metabolism

Inflammatory pathways
- Arachidonic acid metabolic process
- Cytokine production
- Positive regulation of inflammatory response

Signal Transduction
- Response to cAMP

Male Reproductive Development
- Acrosomal membrane
- Leydig cell differentiation
- Male gonad development
- Spermatogenesis
- Male meiosis

Steroid metabolism
- Steroid biosynthetic process
- Testosterone biosynthetic process
- Steroid catabolic process
- C21-steroid hormone biosynthetic process
- Androgen metabolic process

Enzymatic activity
- Aromatase activity
- Hydrolase activity
- Monooxygenase activity

Fig.4. Heatmaps showing changes in gene expression for 16 lipases (top) and 10 cytochrome P450's (bottom) after phthalate exposure, both in the 3D-TCS (in vitro) and in rat fetal testes (in vivo) after exposure to reproductively toxic and non-toxic phthalate esters. These genes belong to multiple Gene Ontology categories covering biological themes relevant to male reproductive processes (e.g. lipid/steroid metabolism, male reproductive development and aromatase activity). In the key to the right, major biological themes are listed in bold with specific Gene Ontology terms listed below next to symbols.

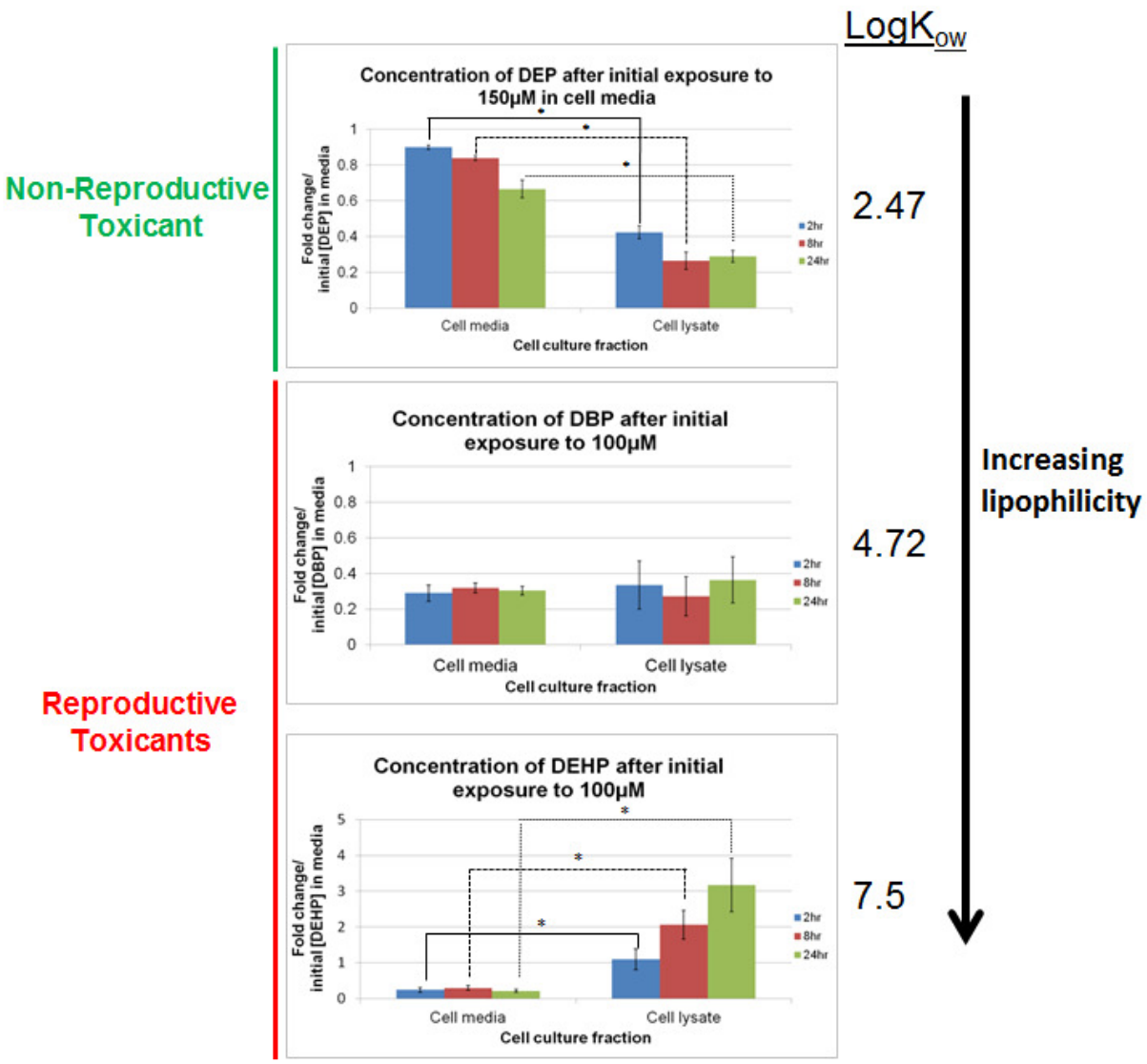


Fig 5. Concentrations of 3 phthalate diesters detected in cell media or cell lysate after exposure to either DBP, DEHP (100µM) or DEP (150µM) for 24 hours. Testis cells were cultured for 48 hours followed by exposure to 100µM concentrations of either dibutyl phthalate (DBP) or bis(2-ethylhexyl) phthalate diethyl phthalate (DEHP) or to 150µM diethyl phthalate (DEP). Values are mean and standard errors for 3 replicate treatments. *indicates concentration in cell lysate sample was significantly different than concentration in cell media at corresponding time point (t-test, $p<0.05$).

Chapter 4: Presence of Macrophages and Inflammatory responses in an In Vitro Testicular Co-Culture Model of Male Reproductive Development Enhances Relevance to In Vivo Conditions

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Key Words: reproductive toxicity, *in vitro* models, testis, macrophages, inflammation, cytokines, phthalate esters

Abstract

Our 3-dimensional testis co-culture system (3D-TCS) represents a promising model of male reproductive toxicity which captures sensitive processes of male reproductive development and contains the main testes cell types (germ, Leydig and Sertoli cells). Macrophages are another cell type important for testicular function and help to modulate immuno-endocrine processes during testes development. It has been established that chemicals such as phthalate esters (PE's) affect macrophage function and testosterone production in the testes *in vivo*. Furthermore, it has been hypothesized that PE induced testicular toxicity may be partially mediated by macrophages. The aim of this study was to determine whether macrophages were present in the 3D-TCS as well as investigate responses in our model that may be related to immune-endocrine functions. Using Western blots, we observed consistent expression of the resident macrophage marker ED2. Levels of three inflammatory cytokines produced by macrophages and testes cells (IL-6, TNF-α and KC/GRO) were increased after exposure to toxic PE's. Furthermore, pathway analysis of genes expression changes after exposure to PE's showed that IL-6 and TNF-α signaling pathways were enriched after treatment with reproductively toxic, but not non-reproductively toxic phthalates. These results indicate that macrophages and inflammatory processes are both captured in the 3D-TCS and that these processes are impacted by exposure to developmental toxicants. These processes represent a major

mode of action for *in vivo* testes toxicity for a wide variety of compounds, thus capturing these key processes was a critical step in characterizing our *in vitro* model.

1. Introduction

The impact of environmental chemicals on male reproductive endpoints are a significant public health concern. In 2001, Skakkebaek, et al. first proposed the concept of the testicular dysgenesis syndrome, which describes several related male reproductive outcomes that have origin in early fetal exposure to environmental chemicals (Skakkebaek et al. 2001). The possibility of chemical exposures leading to TDS are a continuing concern, as several recent publications have reported a decline in semen quality and increases in male reproductive disorders for some human populations and the role that environmental chemicals play in these outcomes is not entirely clear (Hauser and Calafat 2005; Nordkap et al. 2012). These concerns are compounded by the fact that a majority of chemicals in commerce have not gone through adequate reproductive and developmental toxicity testing (Goldman and Koduru 2000). Toxicity screening for these reproductive and developmental endpoints is expensive, time-consuming and requires a large number of animals. Indeed, reproductive and developmental toxicity screening required by Europe's REACH program was estimated to require 49 million animals and cost 6.9 billion Euros or $9.3 billion (Scialli 2008; Rovida and Hartung 2009).

Beyond cost and animal welfare considerations, the prediction of male reproductive toxicity can be particularly difficult. Unlike tissues such as the kidney and liver, there are currently no reliable and convenient biomarkers for testicular injury. For this reason, testicular toxicity can be discovered relatively late in the drug development

process (Dere et al. 2013; Parks Saldutti et al. 2013). Due to these difficulties, new *in vitro* models of testes development are in demand. However, as with any new *in vitro* models of organ systems, these models must be evaluated for their ability to capture relevant *in vivo* biology.

In order to address these challenges and concerns regarding male reproductive toxicity, our lab has established a three dimensional testicular co-culture model (3D-TCS). The goal of this model is to provide an *in vitro* alternative to *in vivo* models of male reproductive toxicity by using a 3D ('organotypic') culture that resembles the testis in structure and function. Staining for important cell markers has confirmed the presence of the major testes cell types, including germ, Sertoli and Leydig cells in the co-culture (Wegner et al. 2013). We have also demonstrated that the addition of a Matrigel overlay at an optimized concentration not only gave the testes cells a three dimensional structure on which to grow, but also improved germ cell survival, enhanced cell survival pathways and reduced cell stress pathways (Yu et al. 2005). Taken together these data indicate that our co-culture system provides an *in vivo*-like niche for the testes cells. Treatment with a variety of toxicants has indicated that the 3D-TCS captures multiple modes of action for testicular toxicity. These include activation of cell death pathways (cadmium), decreased testosterone production (vinclozolin) and impacts on steroidogenic gene expression (phthalates) (Yu et al. 2008; Yu et al. 2009) (Harris et al. submitted manuscript). Inflammatory responses represent another important mode of action for testes toxicity. Although the testes are considered to be “immune-privileged” due to their ability to

tolerate autoantigens that are expressed by germ cells, inflammation can occur due to a number of factors like pathogenic infection or exposure to toxic compounds (Guazzone et al. 2009). There have been a number of recent reports demonstrating impacts on inflammatory responses in the *in vivo* rat testes after exposure to various toxicants. Observed effects include increases in macrophages and macrophage produced cytokines after exposure to phthalates and benzo(a)pyrene, as well as effects on macrophage function after exposure to lead (Zheng et al. 2010; Shamim Ahmed S.K. Barbhuiya 2013; Murphy et al. 2014). Some have proposed that macrophages partially mediate the toxic action of phthalates in the testes *in vivo* (Murphy et al. 2014).

Inflammatory cytokines are produced by multiple testes cells including macrophages, Sertoli and Leydig cells and cytokine signaling plays an important regulatory role in testes development and function (Lee et al. 1999; Richburg et al. 1999; Hedger and Meinhardt 2003). For example, pro-inflammatory cytokines like interleukin-1 and interleukin-6 are known to have direct effects on spermatogenic cell differentiation and testicular steroidogenesis (Boockfor et al. 1994; Hayes et al. 1996; Yan et al. 2000). As mentioned previously, one class of chemicals that are associated with inflammatory responses in the testes are the phthalate esters (PE's). Phthalates are a class of industrial compounds that are used in various consumer products, including food packaging, make up and children's toys (Wilkinson and Lamb 1999; Koo and Lee 2004; Lhuguenot 2009). In the current study, treatment with phthalates was used to investigate inflammatory responses in the testes co-culture model. Two of these phthalates are known to be

potent male reproductive toxicants (DBP, DEHP) and one phthalate is not known to be associated with potent male reproductive effects (DEP). The purpose of this study was to investigate the capability of our testicular co-culture model to capture testes inflammatory responses to toxicant exposure. This was done by determining the presence of an important immunologically active cell type (macrophages), analyzing changes in inflammatory pathways using transcriptomic analysis, and by quantifying changes in protein expression for important inflammatory cytokines after exposure to phthalate esters. In addition, a pathway based analysis of micrarray data was used to investigate gene expression changes for pathways realted to inflammtory processes. Using phthalates with differing degrees of potency for effects on male reproductive end points, we were able to assess whether inflammatory markers can be used in our model to distinguish between toxic and non-toxic compounds of a similar chemical class. Because signaling from cytokines and other immune related molecules is part of normal testes physiology, changes in expression of related genes over time was analyzed and compared to expression changes in *in vivo* testes.

2. Materials and Methods

2.1 Preparation of three dimensional co-culture model for testis (3D-TCS) and chemical treatment

The protocol for the preparation of three dimensional testes co-cultures has been described in more detail by Wegner, et al. (Wegner et al. 2013). Briefly, testes were dissected from 5-day-old male pups obtained from mated Sprague–Dawley rats (Charles River Laboratories, Wilmington, USA). Sequential enzymatic digestions were used to create a single cell suspension containing primarily Sertoli, germ and Leydig cells. Cells were suspended in a solution containing serum-free Eagle's Minimal Essential Medium (Invitrogen, Carlsbad, CA) containing 0.1nM nonessential amino acids, 1mM sodium pyruvate, 3mM sodium lactate, 1% ITS culture supplement (BD Biosciences, Bedford, MA). Cells were then plated at a density of $1.6x10^6$ cells/dish in 35mm plates, followed by the addition of extracellular matrix medium (Matrigel^{TM}) at a density of 200µg/mL. For chemical treatment, media was prepared at final concentration of 100µM of either dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP) or diethyl phthalate (DEP) diluted in dimethyl sulfoxide (DMSO). Media was then added directly to cells 48 h after initial plating. Doses were selected based on those that caused minimal cytotoxic impact observed in previous experiments. DBP (Sigma #D2270, 99% purity), DEHP (Sigma #4-8557, 99% purity), DEP (Sigma #524972, 99.5% purity) and DMSO (Sigma #D1435) were obtained from Sigma-Aldrich (MO, USA).

2.2 Western Blot for Presence of Macrophage Markers

Total protein was collected from testicular co-cultures at various timepoints from initial plating of cells to 7 days in culture using the following protocol. Cells were harvested in lysis buffer (Cell Signaling Technology). Protein was isolated using three freeze-thaw cycles followed by centrifugation (16,300xG for 15 min). Protein concentration was measured using a Protein Assay kit (Bio-Rad Laboratories). Protein samples were then diluted in sample buffer (Novex) and reducing agent, then brought up to volume with lysis buffer, generating samples with equal protein concentrations. Samples were loaded in 4-12% Bis-Tris NuPage precast minigels (Invitrogen) and separated by running at 200V for 45 minutes in running buffer containing 500µL Antioxidant (Invitrogen). Protein was then transferred to polyvinylidene difluoride nylon membranes (Bio-Rad Laboratories) by running at 100V for 2 hours in cold transfer buffer. Efficiency of transfer was confirmed using Coomassie stain of the gel after transfer was completed. Membranes were then rinsed in Tris-buffered saline (TBS) at pH 7.6 and blocked with 5% nonfat dry milk in TBS with 0.1% Tween 20 (TTBS) for one hour. Membranes were rinsed with TTBS and incubated overnight with primary antibodies for ED1 and ED2 (AbD Serotec) and for 2 hours with secondary antibody conjugated to horseradish peroxidase (BD Biosciences). Membranes were then washed 5 times for 5 minutes with TTBS followed by incubation with enhanced chemiluminescence detection reagent (GE Lifescience) and exposure to X-ray films. Blots shown are representative of 3 biological replicates.

2.3 Cytokine assay

Proinflammatory Panel 1 (rat) kit was obtained from Meso Scale Discovery (Rockville, MD). Testes co-culture cells were incubated in 35mm tissue cultures dishes with one of three phthalates or vehicle control (DBP, DEHP or DEP) for 2, 8 or 24 hours. After incubation, cell media was collected and frozen at -80^{o}C for future analysis. Cell media samples were thawed and 50µL of each sample was added to the Proinflammatory Panel 1 plate. Wells in the plate were pre-coated with a capture anti-body for 9 different cytokines/well (IFN-γ, IL-1β, IL-10, IL-13, IL-4, IL-5, IL-6, KC/GRO, TNF-α). Antibodies were conjugated with electrochemiluminescent labels. A voltage was then applied to the plate causing the captured analyte/antibody complexes to emit light at a 620nm. Wavelengths were measured using an SECTOR Imager 2400 and analyzed with MSD Discovery Workbench software. Levels of cytokines in each sample were determined by comparing signals to known standards.

2.4 Transcriptomic impacts from phthalate exposure

We analyzed a previously generated microarray dataset containing gene expression changes in the 3D-TCS after treatment with seven different phthalates. The seven treatments include the potent reproductive toxicants BBP (benzyl butyl phthalate), DBP (dibutyl phthalate), DEHP (bis(2-ethyl hexyl) phthalate) and DPP (dipentyl phthalate), the relatively weak to non-reproductive toxicants DEP (diethyl phthalate), DMP (dimethyl

phthalate) and DOTP (dioctyl terephthalate) or vehicle control (DMSO). Methods for this experiments are reported in more detail by Yu, et al. (Yu et al. 2009). Briefly, cells in 3D-TCS were exposed to 100µM for 24 hours. Microarray chips were obtained from Affymetrix (Rat 230 2.0 arrays). Gene expression data were normalized using GeneChip RMA (GC-RMA) in arraytools.org. Gene expression data for DBP, DEHP and DEP treatments were obtained and uploaded into IPA (Ingenuity Pathway Analysis) software. Significantly changed genes were identified using t-test of phthalate treatment (DBP, DEHP or DEP) vs. vehicle control with false-discovery rate (FDR)<0.05. Enriched canonical pathways (curated by IPA) for DBP and DEHP treatments were identified by a Fisher's exact test ($p<0.05$). The map of the IL-6 signaling pathway available in IPA software was then modified to visualize individual gene expression data across all seven phthalate treatments that were contained in the dataset.

3. Results

3.1 Presence of macrophage marker ED2 in testicular co-culture (3D-TCS)

Using Western blots to probe for expression of the macrophage markers ED1 and ED2, we observed continuous expression of ED2 across all the time points investigated (Fig. 1). Presence of this marker indicates that resident macrophages are present in the testicular co-culture. Expression of ED1 one was not consistently detected, indicating the cells in the testicular co-culture include either the circulating or transitory macrophage populations.

3.2 Impacts on inflammatory markers and transcriptomic signals after exposure to phthalate esters

3.2.1 Effects of phthalate exposure on cytokine levels in cell media

Three of the nine cytokines analyzed (IL-6, TNF-α and KC/GRO) were significantly increased compared to controls after exposure to reproductively toxic phthalate esters (DBP and DEHP). DEP, which is comparatively non-toxic, did not generate a significant increase in any of the nine cytokines analyzed (Fig. 2). Two of the three responsive cytokines were impacted by both of the reproductively toxic phthalates (TNF-α levels increased after DEHP treatment only). Cytokine levels generally increased over 24 hours, with the exception of TNF-α in DBP treated cells, which saw a brief (non-significant) spike at 8 hours followed by a return to lower levels at 24 hours.

3.2.2 Pathway based analysis of gene changes induced by DBP and DEHP treatments

There were 2,212 and 2,241 differentially expressed genes identified for DBP and DEHP treatments, respectively. For the DEP treatment, 0 genes identified with FDR<0.05. Among the enriched pathways (144 and 104 pathways for DBP and DEHP respectively, with 63 of these commonly enriched for both treatments). Enriched pathways included several pathways related to cytokine function, such as IL-6, IL-8 and TNF-α (TNFR1) signaling. Consistent with what was observed in the protein data, the canonical IL-6 signaling pathway was found to be enriched for both DBP and DEHP (Fig. 3). Within this pathway,

levels for *Il-6*, *Cyp19a1*, *Mdr1*, *A2m*, *Vegf* and *Nf-Il6* were all upregulated for toxic phthalate treatment and not the non-toxic phthalate treatments.

4. Discussion

4.1 Presence of macrophages in 3D-TCS

Immune processes mediated by immune capable cells and inflammatory cytokines play an important role in normal testes development *in vivo* as well as in response to certain disease states or toxic injury. In the current study, we sought to characterize the immune cells and inflammatory responses in our testicular co-culture system, thus enhancing our ability to understand *in vitro* responses in our model in an *in vivo* context. Several important results were observed which are relevant to *in vivo* testes immune function. We detected the presence of macrophages in the 3D-TCS through the use of protein and transcriptomic signals. The presence of macrophages in our testicular co-culture indicates both a potential to investigate toxicity responses that are mediated by macrophages in our model and the presence of an important cell type that may aid in maintaining cell signaling processes that are important components of *in vivo* testes physiology.

4.2 Phthalate impacts on cytokine levels in the 3D-TCS

In order to investigate whether our *in vitro* model is able to detect signals for inflammatory responses which could be mediated by macrophages, levels of inflammatory

cytokines were measured in the cell media after exposure to several phthalate diesters. Increased expression of several pro-inflammatory cytokines in our model was observed, suggesting that the model captures aspects of testes inflammatory responses to phthalate exposure. One cytokine that was upregulated in our model after exposure to the toxic phthalates (DBP and DEHP) was IL-6. IL-6 is produced by Sertoli cells, Leydig cells and macrophages. However, it has been reported that in an experimental model of orchitis, only the ED1+ macrophages produced IL-6 (Guazzone et al. 2009). This suggests that either the IL-6 in the 3D-TCS was produced by another testicular cell or that the inflammatory response induced by phthalates is somehow distinct from that of the orchitis model. Il-6 production is stimulated in the testes in response to a number of endogenous signals including testosterone and FSH as well as under conditions of inflammation (Hedger and Meinhardt 2003). Furthermore, exposure to metabolites of DBP (MnBP and MBzP) have been shown to induce the release of IL-6 from cultured human epithelial cells (Jepsen et al. 2004). In the current study, cells in the testes co-culture responded in a similar manner by increased levels of IL-6 over time. Interestingly, Jepsen et al. found that in cultured epithelial cells, phthalates with fewer than eight carbon atoms in their side chains tended to be weaker cytokine inducers compared to those with more carbon atoms. This observation was consistent with results observed in the current study. This response provides relevant mechanistic information regarding male reproductive toxicity signals in our model.

4.3 Microarray analysis reveals inflammatory pathways are impacted by phthalate exposure in the 3D-TCS

Consistent to what was observed cytokine levels, pathway analysis of gene expression data showed enrichment of IL-6 signaling after 24 hours of exposure to toxic phthalate esters. Activation of the IL-6 signaling pathway is usually indicative of a pro-inflammatory response with the potential source of the cytokine coming from most of the testicular somatic cells, interstitial macrophages and ED1+ monocytes (Guazzone et al. 2009). However, IL-6 expression and signaling can occur within the testis that are not associated with pro-inflammatory endpoints as IL-6 has been shown to be an integral part of the regulation of Sertoli cell steroidogenesis, germ cell differentiation and spermatogonial proliferation (Hedger and Meinhardt 2003). IL-6 pleiotropy is, in part, controlled by the type of IL-6 receptor complex assembled by the responding cell as IL-6 bound to the membrane-bound IL-6R/gp130 complex leads towards anti-inflammatory properties while IL-6 binding to a proteolytically-cleaved soluble IL-6 receptor complexed to gp130 leads to an inflammatory state. One component of the IL-6 signaling pathway not represented in our microarray was gp130 (IL-6 signal transducer[IL6ST]) which is involved in binding the IL-6R/IL-6 complex as a homodimer and helps direct IL-6 signaling from either a pro- or anti-inflammatory pathway (Scheller et al. 2011). IL-6 and IL-6R transcripts are usually reserved for a few cell types but in the testis, there are several cell types capable of producing the message as these cells include: germ cells, macrophages, neutrophils, Sertoli and Leydig cells (Rival et al. 2006). Indeed, IL-6 and IL-6R transcripts

were for the most part upregulated in testes co-cultures exposed to reproductively toxic phthalates indicating that this pathway was active.

We also observed a significant elevation of TNF-α at the transcript level and increased cytokine concentrations in the media of treated cultures. TNF-α is cytotoxic and is a cell death mediator, but also inhibits testosterone secretion by Leydig cells. TNF-α is typically secreted by macrophages but has also been shown to be expressed by spermatocytes and spermatids (Hedger and Meinhardt 2003; Theas et al. 2008). It is thought that TNF-α may play role in the regulation of the normal apoptotic processes that occur during normal spermatogenic development. In the current study, it was not possible to deduce whether the observed increase in TNF-α levels was from the resident macrophages in the culture, the germ cells, or both. No significant impacts on cytotoxicity or testosterone levels were observed at any of the time points after phthalate exposure (data not shown), indicating that increases in TNF-α levels are a more sensitive endpoint than cytotoxicity or testosterone secretion in our model. TNF-α levels have important relevance for detecting mechanisms of male reproductive injury in the 3D-TCS, as TNF-α levels in the reproductive tract have been reported to be associated with lower levels of sperm production and TNF-α has been shown to decrease sperm motility *in vitro* (Eisermann et al. 1989; Yao et al. 2009) .

KC/GRO (otherwise known as CXCL1) is a chemokine that is also expressed by multiple cell types in the testes, including macrophages, Sertoli and Leydig cells (Guazzone et al. 2009). KC plays a role in the attraction of neutrophils and its expression is stimulated

by TNF-α. In addition, KC/GRO has been hypothesized to be involved in germ cell proliferation, due to its role as a growth stimulator in certain cells such as human umbilical vein endothelial cells and melanocytes (Aubry et al. 2000). Consistent with the current study, short term DEHP exposure (3 hours) has been shown to upregulate gene expression of KC in human macrophage-like THP-1 cells *in vitro* (Nishioka et al. 2012). Similarly, 3 hours after DBP exposure (500mg/kg), Johnson, et al. observed increased KC gene expression in rat testis *in vivo*. In the 3D-TCS, increased levels of KC protein were observed at early timepoints (significant impacts detected at 8 hours) suggesting that KC mediates early responses to phthalate exposure in the 3D-TCS in manner similar to what is observed *in vivo* (Johnson et al. 2007).

Pathway based analysis of gene expression changes after DBP and DEHP treatment revealed enriched canonical pathways correlated with the upregulation of two of the cytokines we observed (IL-6 and TNF-α). As shown in Fig. 3, IL-6 and TNF-α both are both part of the IL-6 signaling pathway which leads to the upregualtion of VEGF and Cyp19a1. VEGF is a growth factor that serves multiple functions including vasculogenesis, angiogenesis and inhibition of apoptosis. VEGF is known to be involved with cancer progression and has been shown to be induced by xenoestrogens in breast cells (Buteau-Lozano et al. 2008). Upregulation of *Vegf* has important implications for testes development, as *Vegf* is highly expressed in germ cell tumors and angiogenesis is an important aspect of normal testes development (Samson et al. 2004). *Cyp19a1* (otherwise known as aromatase) also serves important functions in the testes, notably serving the

function of converting testosterone to estrogen. The upregulation of this enzyme after exposure to phthalates has direct functional relevance to the physiological role of the testes in male reproductive development, as increased levels of *Cyp19a1* could lead to a decrease in testes testosterone levels. Altered aromatase levels and activity have been shown to be associated with multiple male reproductive effects *in vivo* and is a well-known target of endocrine disruptors. Thus, analysis of this pathway shows a plausible link between the increase in inflammatory cytokine levels and impacts on endocrine function, giving us key insight into the mechanism behind the toxicity signals demonstrated in our testes co-culture model.

4.4 Conclusions

Inflammation is emerging as an important mode of action for phthalate induced testes injury *in vivo*. Taken together, the presence of macrophage markers, the induction of pro-inflammatory cytokines and the enrichment of inflammatory pathways indicate that functional inflammatory pathways are induced by toxic phthalates in the 3D-TCS and that these pathways may be mediated by testicular macrophages. The implication of the early upregulation of IL-6 and TNF-α proteins was further elucidated by analyzing the gene expression networks altered by phthalates exposure. Pathway analysis of gene expression data confirmed that increases in these cytokines are part of signaling cascade that results in testicular tumor formation and (other testes outcome) in testes *in vivo*. Thus, we have demonstrated that using this approach we are able to capture early responses in cytokines

in the 3D-TCS that correlate with testes injury or disease states, allowing us to link early inflammatory events in our *in vitro* testicular model with adverse outcomes *in vivo*. As we have shown here, we can use gene expression changes in inflammatory pathways and in cytokine protein expression to discriminate between toxic and non-toxic phthalate esters. This demonstrates that alteration of cytokine protein expression and impacts on inflammatory pathways are sensitive markers for testicular toxicants in our model, which correlate with specific modes of testes toxicity observed *in vivo*.

Acknowledgements

This work was supported by in part by the UW NIEHS Center for Ecogenetics and Environmental Health (5 P30 ES007033), UW EPA Center for Predictive Toxicology (RD-83573801), US-FDA (FDA: 1U01FD004242), and the NIH Center on Human Development and Disability (1 U54 HD083091-01).

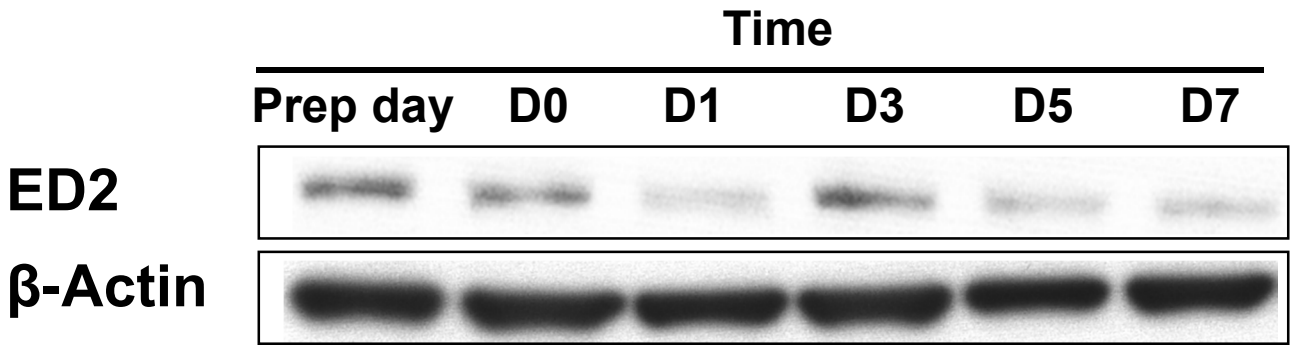

Fig. 1 Expression of macrophage marker ED2 in 3D-TCS (testicular co-culture) over time. Total protein was collected from testicular co-cultures at various timepoints from initial plating of cells ("Prep day") to 7 days in culture. Using Western blots to probe for ED1 and ED2 expression, continuous expression of ED2 (but not ED1) was observed. Blot is representative of 3 replicate experiments.

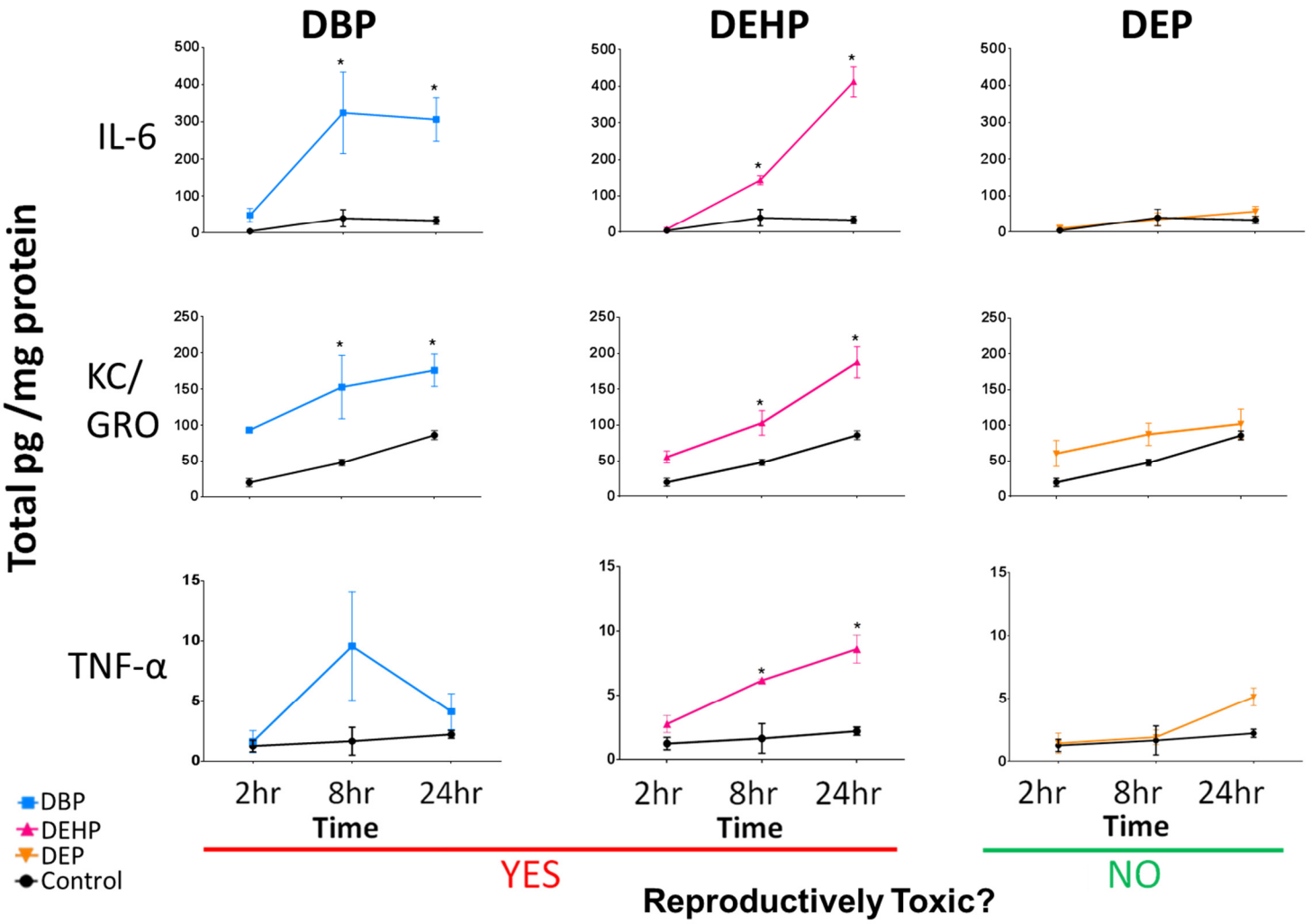

Fig. 2. Cytokines altered after exposure to phthalate esters. Levels of cytokines were analyzed in testes co-culture media via ELISA after exposure to several phthalate esters. Several cytokines produced by both macrophages and testes cells were altered after exposure to toxic (DBP, DEHP), but not non-toxic (DEP) phthalates. *:$p<0.05$ (post hoc t-test)

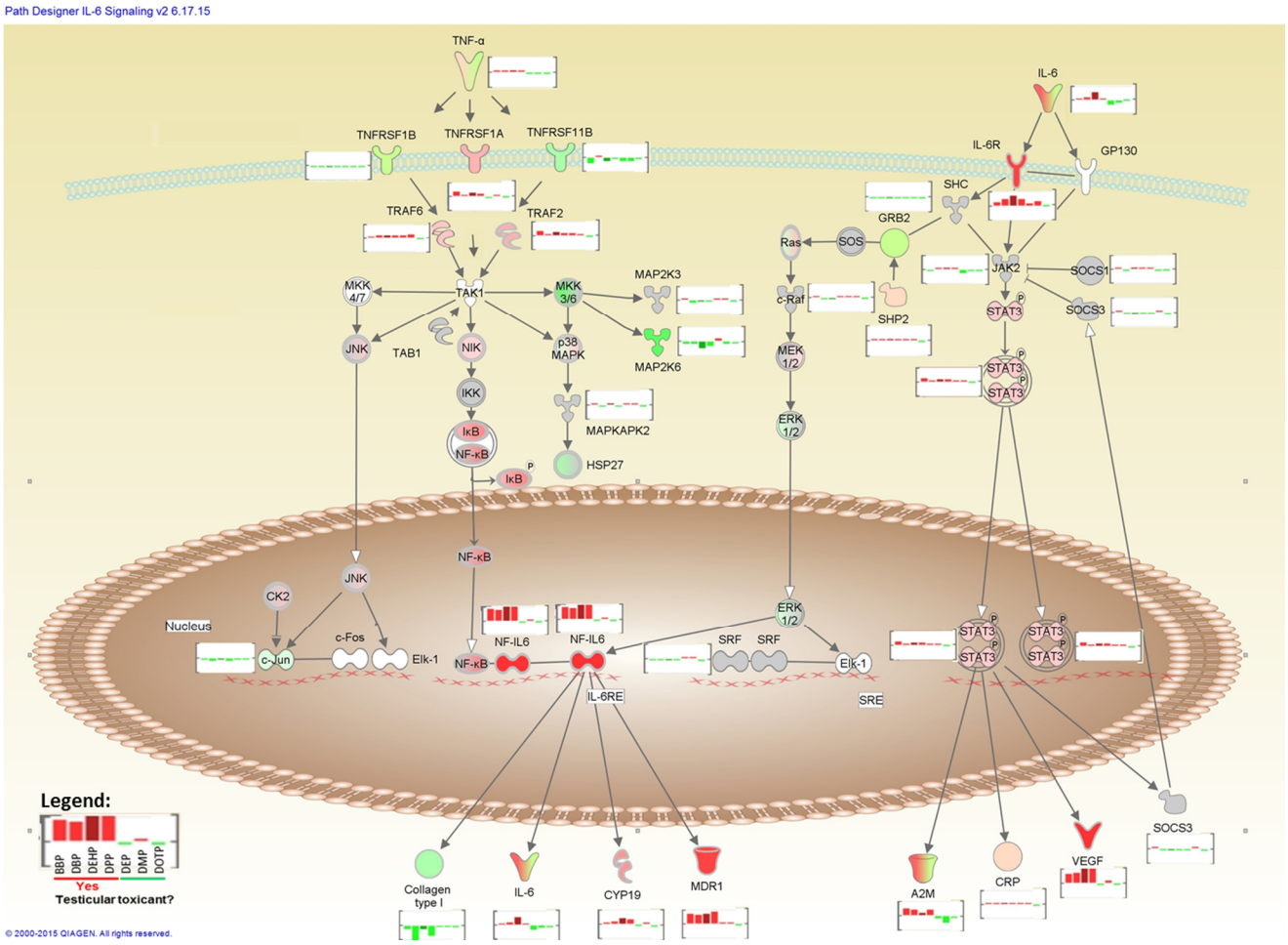

Fig. 3. Phthalate ester effects on the IL-6/TNF-α signaling pathway. Microarray gene expression in testes co-culture was after exposure to 7 different phthalates and ingenuity Pathway Software were used to identify canonical pathways overrepresented among differentially expressed genes. The overrepresented pathway "IL-6 signaling" is shown with associated up or downregulated genes (up or down regulation represented by bar graphs next to each gene).

Chapter 5: An Organotypic Model of Testes Development: Evaluation of Diverse Compounds and Implications for High-Content Toxicity Screening

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Key Words: reproductive toxicity, *in vitro* models, testis

Abstract

We have developed a medium throughput, high content *in vitro* model of male reproductive development using neonatal rat testes. This model includes a co-culture of testes cell types cultured with a three-dimensional extracellular matrix, creating an *in vivo*-like environment (3D-TCS). To determine the breadth of toxicity signals that the 3D-TCS can assess, we screened over 70 compounds for cytotoxicity. Using relevant exposures and experimental toxicity data to set initial screening concentrations, cytotoxic doses were identified for 35 of the 70 compounds. Cytotoxicity was observed for compounds which act through general toxicity mechanisms (such as oxidative stress) as well as through mechanisms more specific to male reproductive endpoints (such as endocrine disruption). For some compounds, such as crizotinib, cytotoxicity was a sensitive indicator of male reproductive toxicity at concentrations near relevant therapeutic levels. For other compounds such as vinclozolin, cytotoxicity was not a good predictor of reproductive toxicity, indicating the need for further analysis. Five compounds (arsenic, crizotinib, nicotine, valproic acid and vinclozolin) were then selected for further testing using additional end points, including effects on testosterone and cytokine production. Significant impacts were observed on one or both of these endpoints below cytotoxic concentrations for all 5 compounds. This study has allowed us to build a preliminary set of "toxicity profiles" across a range of diverse compounds. These profiles will incorporate dose-response data across several key endpoints representing multiple modes of action for testes toxicity, including cytotoxicity, inflammation and endocrine

disruption. Characterizing responses across these modes of action will allow us to further develop our *in vitro* model of testes toxicity and allow us to interpret crucial reproductive toxicity data while reducing the number of animals used.

1. Introduction

Potential impacts of environmental toxicants on male reproductive endpoints are a significant public health concern. In 2001, Skakkebaek and colleagues proposed the Testicular Dysgenesis Syndrome to describe a group of related male reproductive outcomes which were hypothesized to have origins in early fetal exposure to environmental chemicals (Skakkebaek et al. 2001). Recent studies have noted declines in semen quality in some human populations and have led to continued concern regarding the effects of environmental chemicals on male reproductive development (Nordkap et al. 2012). In addition to impacts of environmental compounds, predicting male reproductive effects such as testicular toxicity is a significant challenge in the process of therapeutic drug development. Testicular toxicity is often discovered late in the drug development process, leading to significant cost and male reproductive development is often one of the most sensitive toxicological endpoints. Furthermore, there are currently no reliable biomarkers of testes toxicity, as there are with the liver and kidneys (Parks Saldutti et al. 2013).

There are currently an estimated 80,000 chemicals in production which have yet to be fully evaluated for certain toxicological endpoints, including reproductive and developmental toxicity (Goldman and Koduru 2000; Judson et al. 2009). In 2007, the National Research Council (NRC) outlined a vision for addressing such data gaps in regulatory toxicity testing. The NRC envisioned the use of high throughput *in vitro* models

in conjunction with approaches such as transcriptomics, metabolomics and proteomics to identify key biological pathways perturbed by environmental toxicants and drug compounds. Such approaches could help to reduce the number of animals needed for toxicity testing. Furthermore, rather than relying on high doses and generalized toxicity endpoints, the NRC suggested that testing would be more focused on identifying more specific pathways of toxicity at lower (environmentally relevant) doses (Gibb 2008). In order to address the NRC's challenge, federal agencies such as the EPA have implemented programs like ToxCast in which hundreds of chemicals are screened in a high-throughput manner using various *in vitro* assays (Judson et al. 2009).

However, the implementation of *in vitro* models in toxicity testing faces a number of significant challenges. While new *in vitro* methods have the potential to aid in reducing the burden of animals usage, the use of alternative models of male reproductive development has been limited by difficulties in modeling the complex cellular interactions and multiple endpoints which are involved in processes such as germ cell differentiation in the testes (Sofikitis et al. 2005; Adler et al. 2011). Thus, it is generally agreed that wholesale replacement of animal models of complex biological processes such as reproductive development will not be possible in the near future. However, alternative *in vitro* methods that capture certain key aspects of the reproductive cycle are desirable end products for incorporation into a testing scheme for prediction of toxicity (Parks Saldutti et al. 2013). In the spirit of the research goals outlined by the NRC, we have developed an *in vitro* model of testes development. This cellular co-culture system (3D-TCS) contains

several rat testes cell types (Sertoli, germ, Leydig cells as well as macrophages) grown in a three dimensional conformation facilitated by an extracellular matrix (ECM) overlay. Earlier experiments have demonstrated that addition of ECM in this co-culture model results in a physiologically stable system and that cells form as structure representative of *in vivo* seminiferous tubules (Yu et al. 2005).

As mentioned above, testes development is quite complex and toxic impacts on this process can arise through a number of modes of action. These include direct effects on spermatogenesis such as increased apoptosis in germ cells or abnormal sperm motility, indirect effects such as impacts on the Hypothalamic Pituitary Gonadal axis, decreased expression of steroidogenic enzymes and inflammatory responses (leading to interference with testosterone, LH or FSH production))(Richburg and Boekelheide 1996; de Oliva and Miraglia 2009; Ceribasi et al. 2012; Xiong et al. 2014). In the development of *in vitro* models of male testicular toxicity, it will be important to consider which of toxic modes of action (MOA) are represented in any particular test system. One advantage of the 3D-TCS is that multiple endpoints relating to a number of these MOA's can be monitored after exposure to toxicants. For example, as reported in this study, we have recently adapted the culture system for use in a 96-well plate format, facilitating a medium- throughput testing regime that has allowed us to screen for effects on cytotoxicity for over 70 compounds. We have also observed changes in basal testosterone levels and in transcriptomic profiles in the 3D-TCS after exposure to well-known male reproductive toxicants (phthalate esters). In addition, we have shown that well known cellular markers

of meiosis are expressed in the 3D-TCS (Wegner, et al. submitted manuscript). These results suggest that the 3D-TCS is a dynamic system which captures important male testes developmental processes. Endpoints related to these processes could provide the basis for sensitive indicators of male reproductive toxicity.

In this study, we report results of our initial screen of 70 compounds for effects on cytotoxicity in our testes co-culture model. Based on this initial screen, we then selected a subset of five compounds for further analysis using endpoints that reflect alternate mechanisms of toxicity: endocrine disruption (testosterone levels) and inflammation (cytokine levels). Investigating chemical impacts on testosterone production in our testicular co-culture system is important because testosterone production is one of the main physiological roles of the testes. Testosterone in the testes is an important regulator in both male development at certain key life stages and for regulation of other physiological processes throughout adulthood (Mooradian et al. 1987). Cytokines also play an important regulatory role in the development and normal function of the testis (Hedger and Meinhardt 2003). Pro-inflammatory cytokines such as interleukin-1 and interleukin-6 have direct effects on spermatogenic cell differentiation and testicular steroidogenesis (Boockfor et al. 1994; Huleihel and Lunenfeld 2004). Our previous study investigating the impact of phthalate ester exposure in the testicular co-culture showed that cellular pathways involved in cytokine activity were altered at the transcriptomic level and that protein expression for certain cytokines was upregulated after 24 hours of exposure to toxic phthalates (Harris et al., submitted manuscript). This suggests that

protein or gene expression of cytokines are a sensitive marker of toxicity in our co-culture model. Therefore, in order to investigate the utility of using these more specific markers of toxicity in our co-culture system, the subset of five compounds was evaluated for dose response effects on testosterone secretion and cytokine profiles.

2. Materials and Methods

2.1 Preparation of three dimensional co-culture model for testis (3D-TCS)

Protocol for the preparation of three dimensional testes co-cultures has been explained in detail elsewhere (Wegner et al. 2013). Briefly, testes were dissected from 5-day-old male pups obtained from mated Sprague–Dawley rats (Charles River Laboratories, Wilmington, USA). Sequential enzymatic digestions were used to create a single cell suspension containing primarily Sertoli, germ and Leydig cells. Cells were suspended in a solution containing serum-free Eagle's Minimal Essential Medium (Invitrogen, Carlsbad, CA) with 0.1nM nonessential amino acids, 1mM sodium pyruvate, 3mM sodium lactate, 1% ITS culture supplement (BD Biosciences, Bedford, MA). Cells were then plated at a density of $9.6x10^4$ cells/well in 96-well plates, followed by the addition of extracellular matrix medium (Matrigel™) at a density of 200µg/mL.

2.2 Compound and Dose Selection

The 70 compounds assayed for cytotoxicity were were compiled from numerous sources including two validation studies assessing *in vitro* and *in vivo* reproductive and

developmental tests and prioritization schemes (Brown 2002; Schenk et al. 2010). Additional environmental and drug compounds were identified in collaboration with the US-FDA, including several new molecular entities approved by the FDA in 2011 (Parks Saldutti et al. 2013). This last set of compounds is particularly concerning because their reproductive effects were not identified until after FDA approval. Human exposure to these compounds comes from a variety of sources such as through consumer products like food packaging (dibutyl phthalate, bisphenol A) (Schettler 2006; Vandenberg et al. 2007; Rudel et al. 2011), environmental sources such as groundwater (arsenic) (Rahman et al. 2009) as well as through therapeutic treatment (methotrexate, carbamazepine) (Pellock 1987; Klareskog et al. 2004).

A review of the literature was conducted in order to generate a preliminary classification of the 70 compounds as either reproductive/developmental or non-reproductive/developmental toxicants. This literature review was then used to select 4 concentrations for dosing in the 3D-TCS based on the range of doses associated with toxic effects for each compound. Both in *in vitro* cell models and *in vivo* animal models were utilized for selecting concentrations. For comparisons to *in vivo* concentrations in blood, it was assumed that the entire dose of the chemical was present in systemic blood circulation and that systemic blood concentration was equal to the tissue concentration in the testes. This most likely represented an overestimate of compound concentration in the testes for most cases and was considered an upper bound for dosing of cells.

2.3 Treatment of cells and cytotoxicity assays

48 hours after initial cell plating, cell culture media was replaced with media containing each of the 70 compounds at four different concentrations (or vehicle control). All compounds were diluted in DMSO or cell culture media. Cytotoxicity was measured using CytoTox 96® non-radioactive cytotoxicity assay kits (Promega Corporation, WI, USA). Cytotoxicity was evaluated through measurement of released lactate dehydrogenase (LDH) into cell media after treatment with compounds or vehicle controls for 24 or 72 hours (LDH is a cytosolic enzyme that is released into media by damaged or lysed cells). All cytoxicity assays were run with 4 replicate wells per treatment. Samples were incubated with 50µL LDH substrate mix in the dark. After 30 minutes, reactions were stopped with stop solution and absorbance at 490nm was read with a plate reader SpectraMax 190™ microplate reader, giving an indication of the amount of LDH in each sample. Media blank corrected absorbance values were normalized to percentage of an "LDH max." (i.e. untreated control wells in which all cells were lysed, giving the putative maximum reading of total LDH at a particular time point).

2.4 Benchmark Concentration calculations

Benchmark concentrations (BMC_{10}) were calculated for 10% response in cytotoxicity and cell viability for each compound. Concentration response curves were fit to cytotoxicity data using EPA's Benchmark Dose Modeling approach and a modified version of the Hill model for continuous data (Davis et al. 2011). Curves were fit to toxicity

data using the Hill function, which has been shown to be a biologically plausible model when modelling dose responses in both *in vitro* and *in vivo* systems and is commonly used to model continuous dose-response data (Jodrell et al. 1992; Murrell et al. 1998). The Hill function has the form:

$$R(d)=b+vd^n/k^n+d^n$$

where *R(d)* is the dose response at dose *d*, *b* is the response in the controls, *v* is the maximum response found for the *in vitro* assay and substance being tested, *k* is the dose at which half of the value of *v-b* is estimated to occur, and n is a coefficient which describes the shape of the dose-response. Based on preliminary analysis of the shape of the dose response curves, *n* was given a fixed value of 3.5.

The potency of cytotoxic impacts for each chemical across the 70 compounds was visualized by using the $-\log(BMC_{10})$ for each compound, then using these values to generate a heatmap using MeV software (Saeed et al. 2003). Compounds for which cytotoxicity was <10% of LDH max. response or for which the p-value for model fit was <0.1 were given default a BMC_{10} of 500mM.

2.5 Testosterone assays for subset of 5 compounds

Testosterone in cell media was measured using an ELISA assay kit (Neogen™). Cell media was pooled from four replicate wells and 50µL was diluted 1:1 in Ultrapure Water. Diluted

samples were incubated on plates coated with antibodies against testosterone, followed by addition of enzyme linked conjugate with affinity for the testosterone antibody. In the assay, testosterone in media competes with the enzyme conjugate, leading to color change in the absence of testosterone. Plates were then read in a plate reader and absorbance at 650nM was measured (absorbance is inversely proportional to amount of testosterone in samples). Cell lysate was collected and pooled from four replicate wells and protein concentration across replicates was measured using a protein assay kit (Bio-Rad Laboratories). Testosterone levels were normalized to total protein content.

2.6 Cytokine assays for subset of 5 compounds

Proinflammatory Panel 1 (rat) kit was obtained from Meso Scale Discovery (Rockville, MD). Wells in the plate were pre-coated with a capture anti-body for 9 different cytokines/well. Cytokines included IFN-γ, IL-1β, IL-4, IL-5, KC/GRO, IL-10, IL-13 and TNF-α. Pooled media samples from four replicate wells/dose were incubated in assay plate with wells containing capture antibodies as well as detection antibodies conjugated with electrochemiluminescent labels. A voltage was then applied to the plate causing the captured analyte/antibody complexes to emit light at 650nm. Plates were read using MSD SECTOR Imager 2400 and data were analyzed using MSD Discovery Workbench software. Similar to testosterone assay, pooled protein concentration was measured and cytokine levels were normalized to total protein content.

2.7 Statistical analysis of cytokine and testosterone data

For the five compounds selected for follow-up testing, doses associated with significant effects on cytokine and testosterone production were identified by ANOVA using mixed effects models. Statistical analysis and graphs were generated using R. In the mixed effects models we controlled for the preparation date by treating it as a random effect, while dose was treated as a fixed effect. Graphs show mean and standard deviation of mean each dose and chemical treatment.

3. Results

3.1 Cytotoxicity and cell viability

Cytotoxicity was identified in 35 of the 70 compounds, as determined by statistical difference from controls and a LDH response ≥10% of positive controls. Fig. 1 shows the potency of the cytotoxic response across the 70 compounds. Twenty-three compounds were cytotoxic after 24 hours, while an additional 12 were identified as cytotoxic only after 72 hours of exposure.

3.2 Identification of subset of compounds for evaluation of alternate mechanisms

After the initial screening of ~70 compounds for effects on cytotoxicity and cell viability in our model, we identified a subset of four compounds that had consistent impacts on these endpoints (arsenic, crizotinib, nicotine and valproic acid) and one that did not (vinclozolin). All five of these compounds have been shown to impact male reproductive

endpoints either in *in vivo* or *in vitro* test systems (Sanghamitra et al. 2008; Lehraiki et al. 2011; Nishimura et al. 2000; Jana et al. 2010; Weickhardt et al. 2013). These compounds were selected based on the fact that we had observed effects on cytotoxicity in our model at high doses (except vinclozolin), the diversity of the compounds and known male reproductive toxicity *in vivo*.

3.3 Testosterone assays

Fig. 2 shows the effects the compounds had on total testosterone in the cell media. Significant decreases in testosterone levels in cell media were observed for 4 of the 5 compounds at least one of the doses tested (crizotinib, nicotine, valproic acid and vinclozolin).

3.4 Cytokine assays

Levels of cytokines normalized by volume and total protein content are shown in in fig. 3. Significant increases in levels of one or more cytokines were observed for crizotinib, nicotine, sodium arsenite and valproic acid treatments after 24 hours while vinclozolin induced significant effects only after 72 hours of exposure. For crizotinib, nicotine and valproic acid, effects were detected only at the highest doses. For sodium arsenite treatments, significant increases in IL-6 and KC were observed at doses much lower (1, 10μM) than those at which cytotoxicity was observed (20μM).

4. Discussion

4.1 Cytotoxicity detected across male reproductive toxicants which act through multiple mechanisms

We investigated cytotoxicity responses for 70 compounds in our testes co-culture model. As expected, compounds inducing the highest degree of cytotoxicity in the 3D-TCS are known to associated with male reproductive toxicity *in vivo* (e.g. sodium arsenite, vinblastine, crizotinib and dipentyl phthalate) (Creasy 2001; Liu et al. 2005; Sanghamitra et al. 2008; Parks Saldutti et al. 2013). These compounds are known to act through both relatively general toxicity mechanisms (such as oxidative stress, e.g. nicotine) as well as through mechanisms more specific to male reproductive endpoints (such as endocrine disruption, e.g. dipentyl phthalate) (Liu et al. 2005; Lagunov et al. 2011). When comparing these results to the literature, we observed that for some compounds, such as crizotinib, cytotoxicity was an indicator of male reproductive toxicity at concentrations relatively close to relevant therapeutic blood levels. For example, significant cytotoxicity was observed in the 3D-TCS after exposure to 10µM crizotinib while blood levels recorded in patients treated with this compound can range from 0.2-1.9µM (Timm and Kolesar 2013; Kurata 2015). However, we hypothesized that cytotoxicity alone would not be sufficient in identifying all male reproductive toxicants, as many compounds have alternate mechanisms of toxicity such as endocrine disruption or induction of inflammatory responses. Indeed, for certain compounds such as vinclozolin,

cytotoxicity was not a good predictor of reproductive toxicity, as no increase in cytotoxicity was detected despite its well-known effects on male reproductive end points *in vivo*. These results highlight the need to utilize a range of assays which cover multiple modes of toxicity in the 3D-TCS, due to the fact that testicular toxicants act via a wide range of mechanisms.

4.2 Impacts on testosterone and cytokine levels were detected across 5 compounds selected for evaluation of alternate mechanisms of toxicity

In order explore alternate modes toxicity in the 3D-TCS, 5 of the 70 compounds (crizotinib, nicotine, sodium arsenite, valproic acid and vinclozolin) were selected for further analysis of additional endpoints relevant to testes toxicity. These compounds were selected due to their diversity of uses (therapeutic compounds, environmental toxicants and consumer products) and demonstrated impacts on male reproductive health *in vivo* (Nishimura et al. 2000; Lehraiki et al. 2011). We assessed impacts on testosterone secretion and cytokine production, two end points indicative of distinct modes of action for testicular toxicity (although it should be noted that there is potential for these two endpoints to influence each other, as certain cytokines are known to partially mediate steroid production in the testes) (Hedger and Meinhardt 2003).

Testosterone secretion by the testes is an important aspect of male reproductive development. A number of compounds tested in the current study have been shown to impact testosterone levels *in vivo* and *in vitro*, including nicotine, dibutyl

phthalate and vinclozolin (Villeneuve et al. 2007; Oyeyipo et al. 2010; Zhou et al. 2013). Identifying compounds with the potential to cause impacts on testosterone secretion from the testes is an important function of our co-culture model. We have shown here that four of the compounds selected for in-depth analysis decreased testosterone levels in the cell media at doses lower than those causing cytotoxicity. These effects could arise though a number of mechanisms including effects on steroidogenic gene expression, targeted cytotoxicity of Leydig cells or impacts on steroidogenic enzymes (Sanderson et al. 2002; Kim et al. 2005; Liu et al. 2005). For example, vinclozolin is a well-known endocrine disruptor that has been shown to induce Cyp19a1 activity in multiple *in vitro* systems at comparable doses that were utilized in the current study (~10-100µM). This induction of Cyp19a1 has been shown to coincide with an increase in estradiol with concurrent decrease in testosterone levels, suggesting a potential mechanism for vinclozolin effects on testosterone level in the 3D-TCS (Sanderson et al. 2002; Villeneuve et al. 2007). Similarly, the other 3 compounds which caused decreased in testosterone levels in the 3D-TCS have all been shown to cause related effects in rodents or humans *in vivo.* For example, nicotine and crizotinib have both been shown to cause decrease steroid hormone levels in rats and humans, respectively, while valproic acid has been shown to impact sperm motility and sperm count in rats (Nishimura et al. 2000; Jana et al. 2010; Weickhardt et al. 2013) These results, along with previously published studies in which we demonstrated the effects of phthalate esters on steroidogenic gene expression and

testosterone secretion, demonstrate that our *in vitro* model is able to reflect this highly relevant mechanism of testes toxicity (Yu et al. 2009; Wegner et al. 2014).

Similarly, we found that the 5 selected compounds had significant effects on levels of multiple cytokines in the 3D-TCS. Inflammation is an important pathway for testes injury from toxicants and in certain disease states. Inflammatory responses caused by toxicant exposure include increased cytokine levels and increases in the number of testicular macrophages and have been induced *in vivo* by various compounds including phthalates, benzo[a]pyrene and lead (Zheng et al. 2010; Shamim Ahmed S.K. Barbhuiya 2013) . As mentioned above, we have previously shown that macrophage markers are present in our testicular co-culture and that levels of IL-6, KC and TNF-α increased in response to toxic phthalate exposure (Harris, et al., submitted manuscript). In the current study, we have shown that exposure to 4 of the 5 compounds tested caused increases in IL-6 levels. IL-6 plays a role in the regulation of inflammation and is produced by Sertoli and Leydig cells in the testes, in addition to macrophages. IL-6 has been reported to inhibit DNA synthesis in rat spermatocytes, highlighting this cytokine as an important potential marker of toxicant impacts on spermatogenesis in our *in vitro* model (Hakovirta et al. 1995). In addition, KC/GRO (otherwise known as CXCL1) was increased after exposure to crizotinib, sodium arsenite, valproic acid and vinclozolin after either 24 or 72 hours of exposure. KC is a chemokine expressed by multiple testes cell types (Guazzone et al. 2009). KC has been hypothesized to mediate several important function in the testes such as germ cell proliferation (Aubry et al. 2000). Previously, we demonstrated that

reproductively toxic phthalate esters increased levels of KC in the 3D-TCS (Harris, et al. submitted manuscript). In the current study, we have identified 4 new compounds which induce KC expression in the 3D-TCS, indicating a role for KC in response to toxicant exposure. Finally, three of the five compounds tested caused a significant increase in levels of TNF-α (crizotinib, nicotine and valproic acid). TNF-α signaling is involved in numerous functions related to both inflammation and apoptosis as well as mediation of testosterone secretion by Leydig cells. TNF-α is typically secreted by macrophages but has also been shown to be expressed by spermatocytes and spermatids (Van Antwerp et al. 1996; Hedger and Meinhardt 2003; Theas et al. 2008). Consistent with effects that we noted for reproductively toxic phthalates, increases in cytokine levels detected in the current study were detected at doses lower than those inducing cytotoxicity (for sodium arsenite and vinclozolin) or at timepoints prior to when cytotoxicity was detected (crizotinib, nicotine and valproic acid). Thus, these results further highlight the role of cytokines as an important indicator of toxicity in the 3D-TCS that part of a relevant mode of action for testes injury *in vivo*.

4.3 Conclusions

This study demonstrates that our testicular co-culture models is able to exhibit biological responses representative of those observed in *in vivo* testes under conditions of toxic injury. These responses include effects on cytotoxicity, hormone production and inflammation. These data highlight the need to look across multiple mechanisms of action

when evaluating reproductive toxicity within our testes model, as compounds may act through a diverse set of toxicity pathways.

These early data evaluating various modes of action in our model have allowed us to identify responses in the 3D-TCS which are predictive of male reproductive toxicity *in vivo*. Evaluating the dynamics of the responses to various compounds (i.e. which endpoint is detected at the lowest dose and which is detected at the earliest timepoint?) will allow us to begin building "toxicity profiles" for compounds in the 3D-TCS. As shown in fig 4., we are currently developing a framework for evaluating the various modes of action by which toxicity can arise in our model. Using the data generated in the current study, we can classify compounds based on responses observed across the various assays (i.e. cytotoxicity, testosterone levels and inflammatory responses). These responses represent a number of potential modes of action, including oxidative stress, necrosis, inflammation or endocrine disruption. The toxicity profiles will allow us to evaluate responses in our model in a broad biological context, allowing us to capture the breadth of responses present in our *in vitro* model. Further analysis using transcriptomic and metabolomics analysis will help to supplement the data obtained from these diverse assays and further clarify what specific mechanisms lead to effects on these endpoints.

Acknowledgements

This work was supported by in part by the UW NIEHS Center for Ecogenetics and Environmental Health (5 P30 ES007033), UW EPA Center for Predictive Toxicology (RD-

83573801), US-FDA (FDA: 1U01FD004242), and the NIH Center on Human Development and Disability (1 U54 HD083091-01).

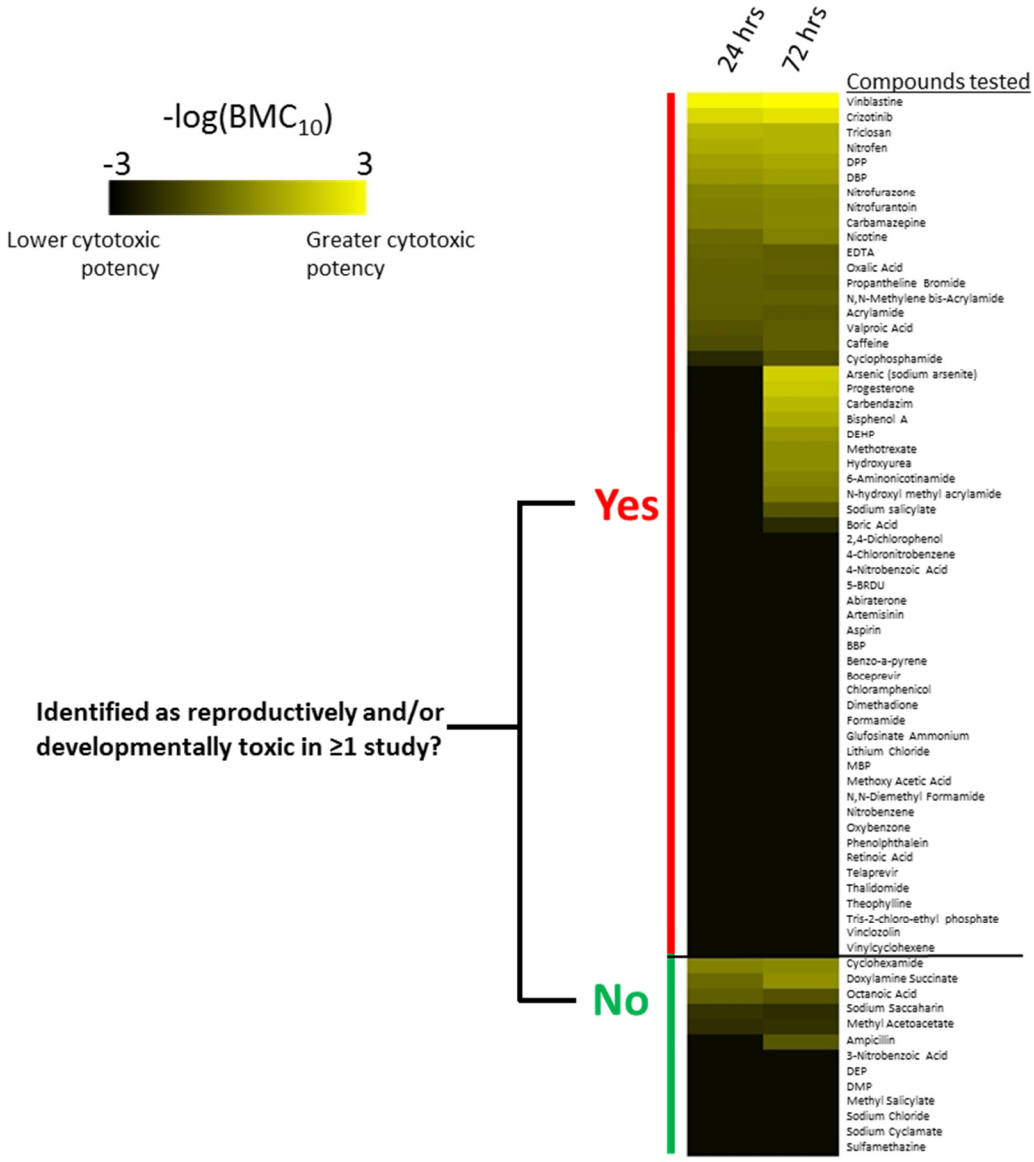

Fig 1. Heatmap showing potency of cytotoxic effects for 70 compounds in an organotypic testes co-culture model. Compounds were preliminarily classified as reproductive/developmental toxicants or non-reproductive/developmental toxicants based on review of the literature. LDH cytotoxicity assay was performed 24 or 72 hours after dosing. BMC_{10} values were calculated using a modified version of EPA's Benchmark Dose modeling approach.

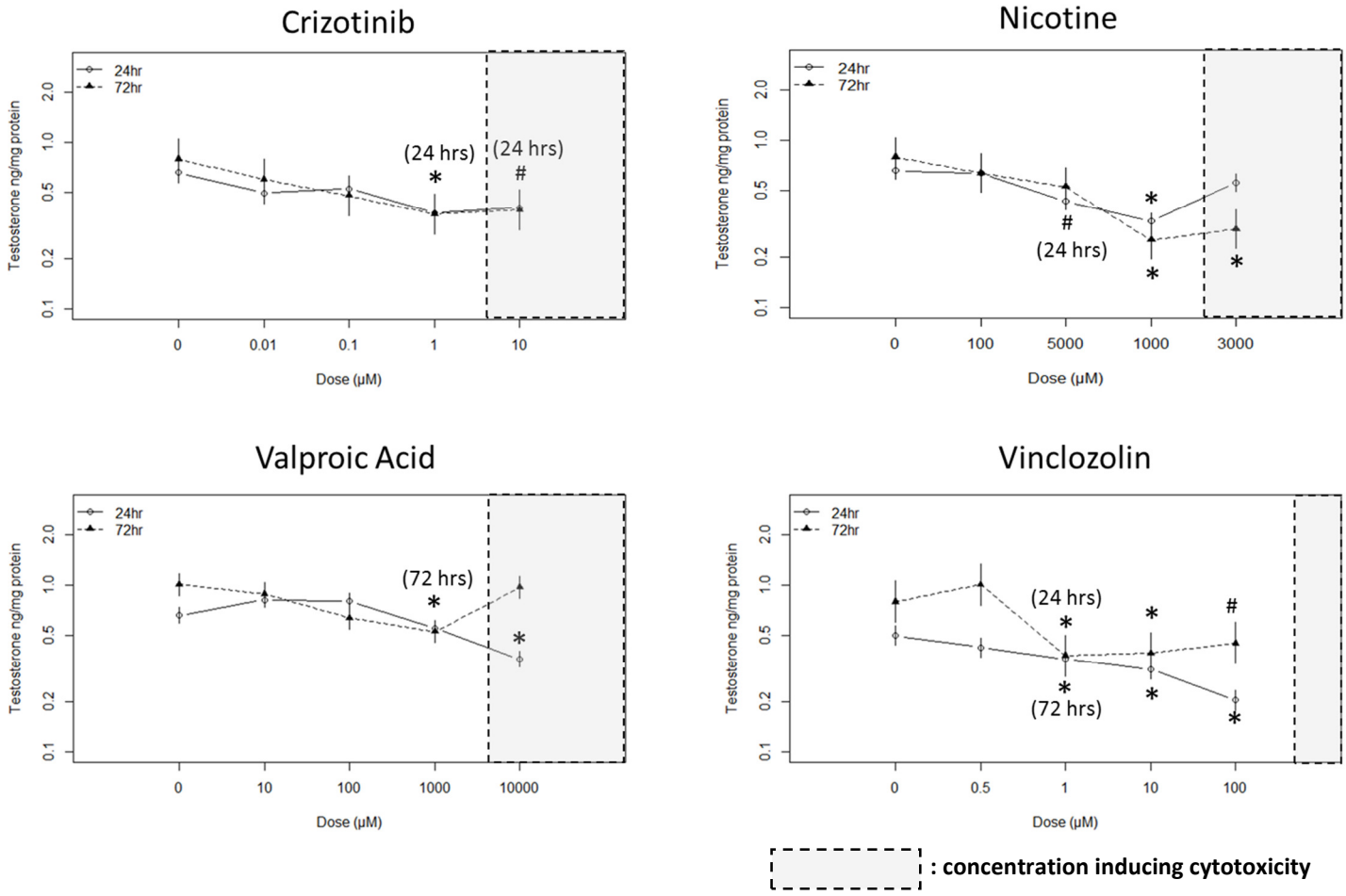

Fig. 2 Chemical impacts on testosterone production in testes co-culture model. Testosterone levels were measured in cell culture media 24 and 72 hours after exposure to either: crizotinib (0.01, 0.1, 1, 10μM), nicotine (0.1, 0.5, 1, 3mM), valproic acid (0.01, 0.1, 1, 10mM) or vinclozolin (0.5, 1, 10, 100μM). *: $p<0.05$ (post hoc) test vs. control, #: $p<0.1$ (post hoc test vs. control)

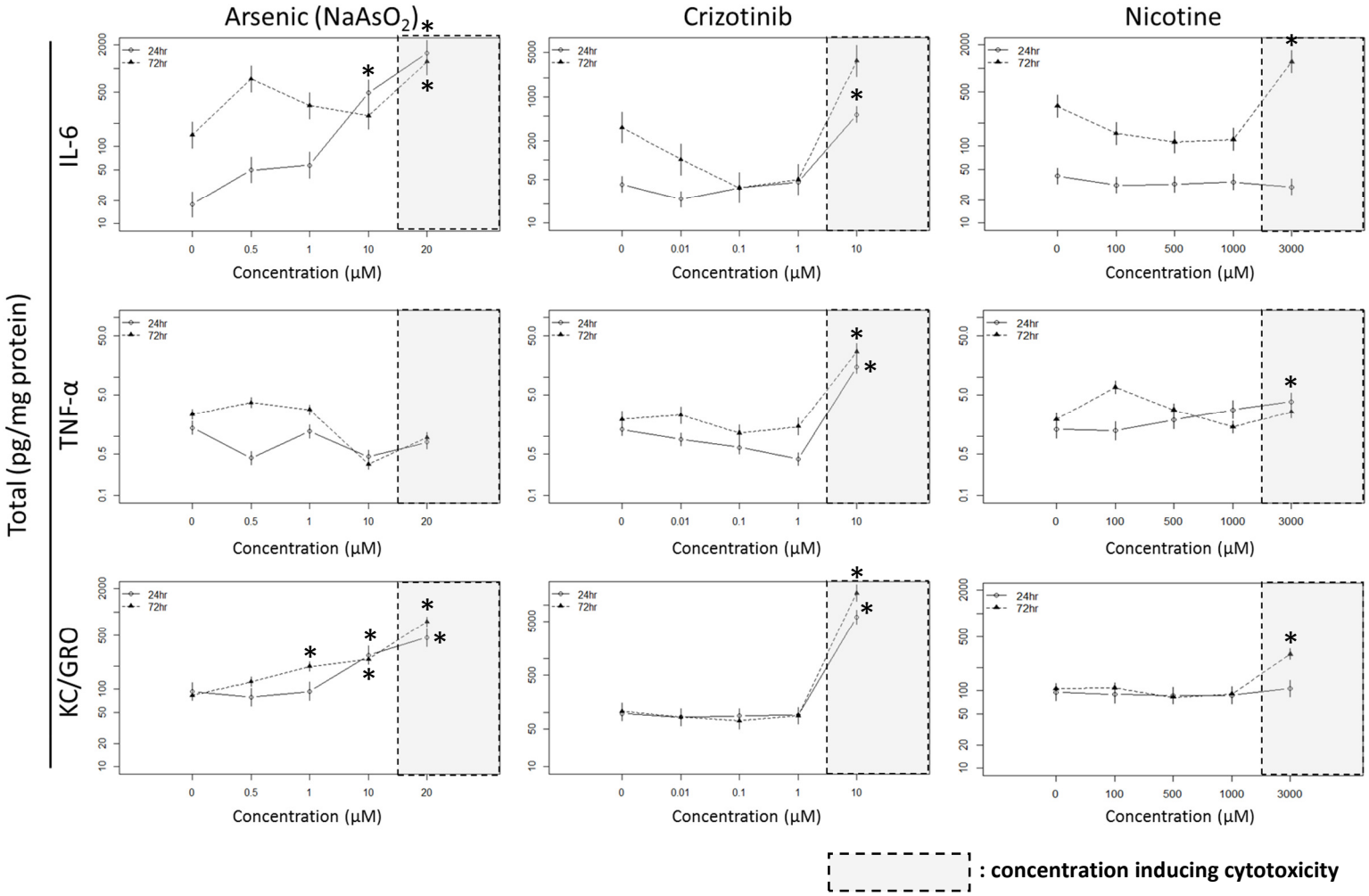

Fig. 3 Effects of five compounds on levels of 3 different cytokines (IL-6, TNF-α and KC/GRO) in testes co-culture model. Cytokine levels were measured in cell culture media 24 and 72 hours after exposure to 1 of 5 compounds: sodium arsenite (0.5, 1, 10, 20μM), crizotinib (0.01, 0.1, 1, 10μM), nicotine (0.1, 0.5, 1, 3mM), valproic acid (0.01, 0.1, 1, 10mM) or vinclozolin (0.5, 1, 10, 100μM). *: $p<0.05$ (post hoc) test

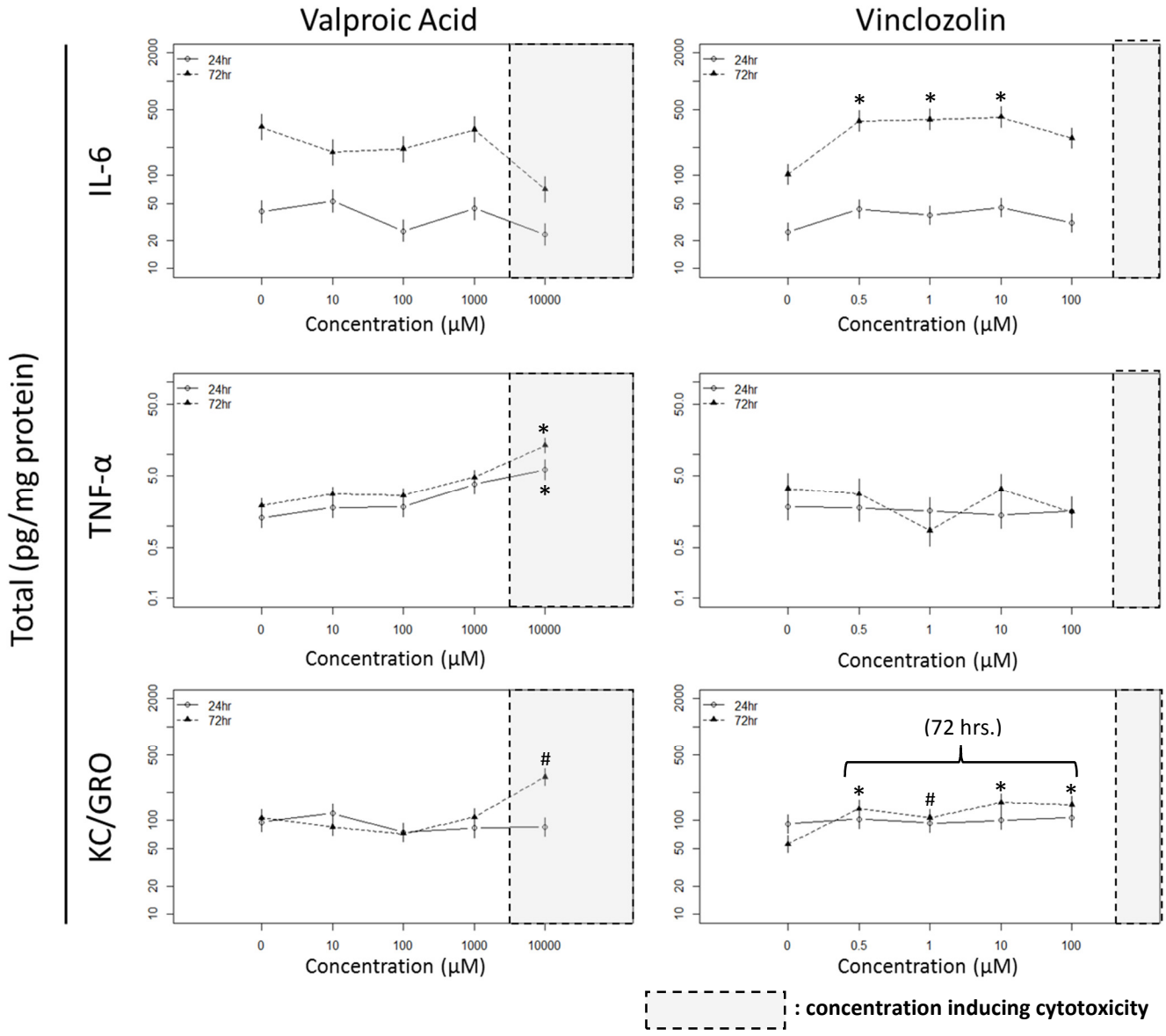

Fig. 3 (cont.) Effects of five compounds on levels of 3 different cytokines (IL-6, TNF-α and KC/GRO) in testes co-culture model. Cytokine levels were measured in cell culture media 24 and 72 hours after exposure to 1 of 5 compounds: sodium arsenite (0.5, 1, 10, 20μM), crizotinib (0.01, 0.1, 1, 10μM), nicotine (0.1, 0.5, 1, 3mM), valproic acid (0.01, 0.1, 1, 10mM) or vinclozolin (0.5, 1, 10, 100μM). *: $p<0.05$ (post hoc) test

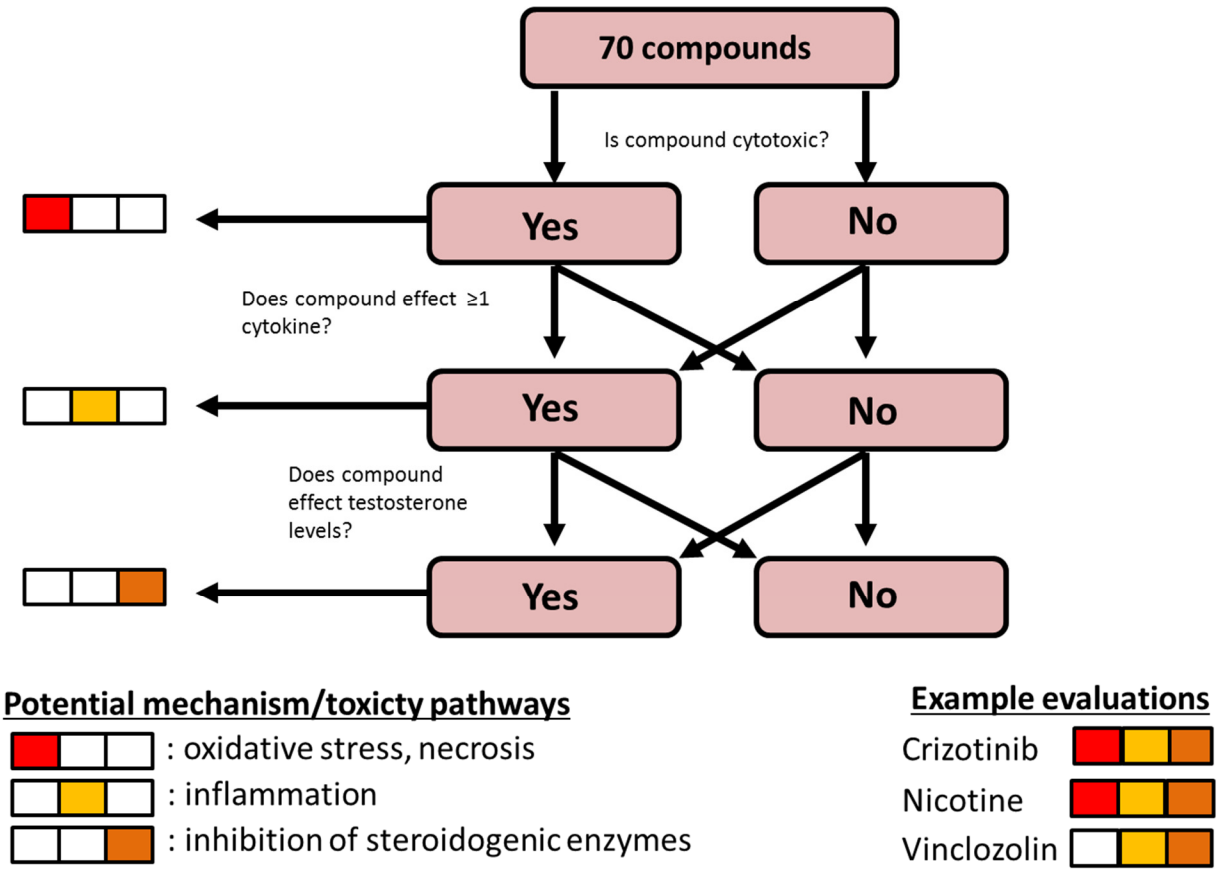

Fig. 4. Preliminary framework for evaluating compounds in the 3D-TCS. In order to capture the full breadth of toxicity signals that the organotypic testes co-culture is capable of detecting, it will be necessary to develop a framework for generating "toxicity profiles" for compound being evaluated. Compounds act through one or more modes of action (e.g. oxidative stress, endocrine disruption, inflammation, etc.) when inducing testicular toxicity. Evaluating which toxicity pathways are being triggered at the earliest timepoints and at the lowest doses will allow us to determine which endpoints are the most sensitive for a particular compound.

Chapter 6: Conclusions and Future Directions

Screening chemicals for reproductive and developmental toxicity is a difficult and expensive process. In order to address the challenges inherent in predicting impacts on these endpoints, researchers are turning more to *in vitro* models and pathway based analysis for toxicity screening. Our *in vitro* co-culture model of testes development represents a promising new tool for investigating chemical impacts on male reproductive health. As discussed in Chapter 1, one of the main challenges in reproductive and developmental toxicity testing is the large number of animals (and associated cost) required, thus there is a high demand for *in vitro* models of key reproductive or developmental processes that reduce the number of animals needed. One of the key goals of developing the 3D-TCS is to reduce the number of animals used for evaluating male reproductive toxicity. We have calculated that using the 3D-TCS, we can increase the number of experimental units by ~33-fold compared to similar *in vivo* methods. Thus the 3D-TCS represents a promising model for evaluating male reproductive toxicity while reducing animal usage.

There are several challenges involved in the developing any new *in vitro* model of organ toxicity. Comparison of toxicant perturbation of biological signaling is essentially only useful when interpreted in a comparable *in vivo* context and so characterizing which toxicity pathways are represented in organotypic models is crucial. In addition, the metabolic capability of many *in vitro* cultures has not been characterized and in some cases has been shown to differ significantly from their respective *in vivo* tissues (Boess et

al. 2003). Kinetics of compounds in culture media can also vary and thus concentrations at specific targets of toxicity (e.g. cell receptors or organelles) can differ significantly from what is typically observed for *in vivo* exposures. Thus, characterizing both kinetics and metabolism of compounds is essential to the interpretation of toxicity signals in cell culture systems (Blaauboer 2010). In addition, it is important to identify which pathways of toxicity (PoT's) are represented in any particular *in vitro* model (Kleensang et al. 2014). For example, testes toxicity can arise though a number of different independent pathways, including endocrine disruption (decreased capacity of the testes to produce testosterone), inflammation and induction of germ cell apoptosis (Parks Saldutti et al. 2013).

In the preceding chapters, we have made several steps in characterizing our organotypic testes co-culture system. We demonstrated that using microarray analysis of gene expression data, the 3D-TCS can discriminate between potent male reproductive toxicant phthalate esters from non-reproductively toxic phthalates. Furthermore, transcriptomic analysis revealed that pathways targeted by these compounds (such as androgen biosynthesis) in the 3D-TCS reflect those targeted by phthalates in the developing testes *in vivo*. We have also shown that our co-culture was able to metabolize phthalate parent compounds to their active monoester metabolites. Finally, we identified impacts on toxicity pathways beyond disruption of steroidogenesis by investigating toxicant impacts on inflammatory responses and cytotoxicity.

Our findings indicate that a number of key testicular PoT's are present are induced by well-known testicular toxicants. For example, in Chapter 2, our microarray analysis

demonstrated that phthalate esters impacted the steroid biosynthetic pathway, a key mode of action for testes toxicity. Within this key pathway, a number of steroidogenic enzymes were downregulated after exposure to reproductively toxic phthalates in a manner consistent with what was observed in the *in vivo* testes. Furthermore, in Chapter 5, we demonstrated that the well-known male reproductive toxicants crizotinib, nicotine, valproic acid and vinclozolin decreased testosterone production in a dose dependent manner. This indicates that the functional end point of testosterone production can be used to identify toxicants in the 3D-TCS. Similarly, in Chapter 4 we demonstrated that another key pathway of testes toxicity was present and detectable in the 3D-TCS. Using phthalate exposure, we showed that important inflammatory markers were upregulated after exposure to phthalates, consistent with gene expression patterns. These two pathways (endocrine disruption and inflammatory signaling) are well characterized in the *in vivo* testes and part of proposed adverse outcome pathways for male reproductive toxicity. Still another PoT for testes toxicity is induction of necrosis and other cell death signaling processes which may manifest as overt cytotoxicity in the 3D-TCS. In order to investigate a diverse set of compounds and their potential to impact these endpoints, we evaluated cytotoxic responses in the 3D-TCS after exposure to a set of 70 chemicals. Using relevant exposures and experimental toxicity data to set initial screening concentrations, we identified cytotoxic doses for 35 of the 70 compounds tested in the 3D-TCS. Due to the nature of the assay used (LDH leakage assay) it is likely that the responses observed were apical responses that represent an overall cell death response through a process

such as necrosis. However, we hypothesized that effects would be observed at lower doses than those causing cytotoxicity and that these could be detected using assays evaluating more specific end points. Indeed, this is what we observed in the 5 compounds that we chose for higher content evaluation (sodium arsenite, crizotinib, nicotine, valproic acid and vinclozolin). For this group of compounds, impacts on cytokine or testosterone levels were observed either at concentrations lower than those inducing cytotoxicity or at timepoints prior to when a significant cytotoxicity signal was observed (24 vs. 72 hours post exposure). Notably in the case of crizotinib (a chemotherapeutic approved for treating metastatic lung cancer), decreased testosterone levels have been observed in humans taking this drug and testicular degeneration has been observed in rats, highlighting the relevance for impacts in the 3D-TCS when comparing to *in vivo* effects for this compound (Weickhardt et al. 2013). These results highlight the need to evaluate a broad suite of endpoints and define their dose response curves in order to determine the most sensitive endpoint for risk assessment purposes.

There are numerous promising areas of future research that would be extremely useful in the development of this model. Characterization of a broader set of metabolic enzymes would enhance our ability to predict and interpret responses to various toxicants in the model as well as provide crucial information on the extent to which the testes co-culture maintains the metabolic activity of *in vivo* testes. In addition, methods such as metabolomic profiling could be employed for evaluating responses in the culture. Such analyses would provide invaluable information to compliment transcriptomic responses

that have been observed. It is well known that gene expression changes do not necessarily correspond to protein or metabolite changes and metabolomic analysis would shed light on the levels of key products of gene expression changes. Further work could also investigate kinetics of key compounds over a greater number of time points than has been analyzed thus far in order to generate a more complete kinetic model of compound kinetics.

In conclusion, we have developed a promising *in vitro* model of testes development. This model has the potential to aid in the reduction of animal use in male reproductive toxicity screening though the analysis of specific pathway perturbations relevant to male reproductive toxicity *in vivo*. We have characterized several important kinetic and dynamic factors that will aid in our interpretation of the toxicity data generated. Future experiments will further clarify the dynamic aspects (i.e. effects across dose and time) of chemical impacts on male reproductive processes evaluated in this model.

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