Novel molecular targets associated with testicular dysgenesis induced by gestational exposure to diethylhexyl phthalate in the rat: a role for estradiol

Gary R Klinefelter, John W Laskey, Witold M Winnik¹, Juan D Suarez, Naomi L Roberts, Lillian F Strader, Brandy W Riffle² and D N Rao Veeramachaneni³

United States Environmental Protection Agency, Office of Research and Development, Toxicology Assessment Division, National Health and Environmental Effects Research Laboratory, Reproductive Toxicology Branch, MD#72, Reproductive Toxicology Facility, Durham, North Carolina 27713, USA, ¹National Health and Environmental Effects Research Laboratory, Proteomic Research Core, Durham, North Carolina 27711, USA, ²Toxicology Assessment Division, National Health and Environmental Effects Research Laboratory, Endocrine Toxicology Branch, MD#72, Reproductive Toxicology Facility, Durham, North Carolina 27713, USA and ³Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523-1683, USA

Correspondence should be addressed to G R Klinefelter; Email: klinefelter.gary@epa.gov

Abstract

Significant research has been focused on phthalate-induced alterations in male reproductive development. Studies on rodents have prompted the notion that a syndrome exists in the human male which includes phenotypic alterations such as hypospadias, cryptorchidism, poor semen quality, and even testicular cancer. Each phenotype in this 'testicular dysgenesis syndrome' is predicated on reduction in testosterone production by the fetal Leydig cell. We sought to examine the relationship between dysgenesis and steroidogenic capacity in the fetal rat testis more stringently by incorporating lower exposures than those typically used, conducting a comprehensive, non-targeted quantitative evaluation of the fetal testis proteome, and relating alterations in individual proteins to the capacity of the fetal Leydig cell to produce testosterone, and histopathology of the fetal testis. Pregnant dams were dosed orally from gestation day (GD) 13–19 with 0, 10, or 100 mg diethylhexyl phthalate (DEHP)/kg body weight per day. Each endpoint was represented by 16 l. Clustering of Leydig cells occurred before any significant decrease in the capacity of the GD19 Leydig cell to produce testosterone. At 100 mg DEHP/kg, testosterone production was reduced significantly, Leydig cell clusters became quite large, and additional dysgenetic changes were observed in the fetal testis. Of 23 proteins whose expression was altered significantly at both DEHP exposure levels, seven were found to be correlated with and predictive of the quantified endpoints. None of these proteins have been previously implicated with DEHP exposure. Notably, pathway analysis revealed that these seven proteins fit a pathway network in which each is regulated directly or indirectly by estradiol.

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Introduction

A potential decline in human male reproductive health (i.e. semen quality, hypospadias, cryptorchidism, and testicular cancer) and a link to exposure to endocrine active chemicals in the environment have been controversial for almost two decades (Carlsen *et al.* 1992, Fisch 2008, Sharpe & Skakkebaek 2008, Akre & Richiardi 2009). Numerous epidemiology studies have focused on exposure to phthalates and their ability to compromise semen quality in men (Pant *et al.* 2008, Swan 2008). There has also been an effort to relate phthalate exposure to developmental landmarks such as anogenital distance, penile size, and testis descent (Meeker *et al.* 2009). Definitive correlations between

gestational exposures and adverse outcomes at adulthood are lacking.

Studies on laboratory animals have shown that exposure to 500–750 mg dibutyl phthalate (DBP)/kg during the critical period of male reproductive development, i.e. gestation day (GD) 12–19, results in remarkable phenotypic alterations in normal development (Mylchreest *et al.* 1998, 2000). At birth, males presented with reduced anogenital distance. At adulthood, the phenotypes included cryptorchidism, epididymal agenesis, testicular atrophy with germ cell loss, hypospadias, and absent or smaller seminal vesicles and prostate. These phenotypes can be linked to androgen deprivation during the critical period of sexual differentiation. Rats exposed to 750 mg diethylhexyl phthalate (DEHP)/kg

between GD12 and 19 resulted in testes containing large aggregates or clusters of Leydig cells in the interstitial spaces, multinucleated germ cells in the seminiferous cords, and significant reductions in testosterone levels in the GD19 testis (Parks *et al.* 2000).

By virtue of these phthalate-induced alterations in the rat, a link between comparable human male reproductive disorders and androgen deprivation during fetal development has been suggested. Thus, manifestation of reproductive anomalies such as hypospadias, Sertoli cell-only seminiferous tubules, low sperm counts, and testicular cancer has been termed as the testicular dysgenesis syndrome (TDS; Sharpe & Skakkebaek 2008). However, it should be noted that Sertoli cell-only tubules and reduced sperm numbers can result from fetal or adult exposures to chemicals that do not decrease testosterone production by the Leydig cells.

In contrast to rat studies, when fetal male mice were exposed to 500 mg DBP/kg during gestation, some signs of testicular dysgenesis were observed such as increased seminiferous cord diameter and increased numbers of multinucleated germ cells, but there was no decrease in testicular testosterone (Gaido *et al.* 2007). Doses up to 1500 mg/kg failed to decrease fetal testicular testosterone. Later, it was suggested that phthalate-induced alterations during germ cell development were independent of steroidogenic status (Lehraiiki *et al.* 2009). These authors used fetal mouse testis organ cultures to establish that mono-(2-ethylhexyl) phthalate (MEHP) induced germ cell apoptosis. The number of multinucleated germ cells increased and the number of gonocytes decreased in the absence of steroidogenic change. Interestingly, in both of these studies (one *in vivo*, one *in vitro*), there was no evidence of phthalate-induced Leydig cell aggregation.

The responses of fetal rat and human testis explants exposed to monobutyl phthalate (MBP) *in vitro* also differed, with modest reductions in testosterone production and modest increases in Leydig cell aggregation observed in rat explants but not in human explants (Hallmark *et al.* 2007). Fetal human testis explants exposed to MEHP also failed to exhibit any changes in testosterone production although germ cell loss was observed; Leydig cell aggregation was not evaluated (Lambrot *et al.* 2009). Recently, when fetal human testis or fetal rat testis explants were xenografted into castrate male nude mice and exposed to DBP, serum testosterone was compromised in mice with rat testis grafts but not in mice with human testis grafts (Mitchell *et al.* 2012). Collectively the data support the notion that fetal mouse and human testes respond differently to phthalates than the fetal rat testis. Although Leydig cell aggregation seems to be consistent with decreased testosterone in fetal rat testis, it is not clear if dysgenesis of the fetal Leydig cell population precedes or follows steroidogenic dysfunction.

In an attempt to explain phthalate-induced steroidogenic compromise observed in the rat, numerous studies have sought transcriptional guidance by quantifying the expression of molecules known to be regulated in steroidogenesis such as StAR protein (StAR), P450 side-chain cleavage enzyme (P450scc), and P450 C-17 hydroxylase/lyase (P450c17). In general, the expression of P450scc and P450c17 is not significantly altered until relatively high doses of phthalates are administered (500 mg DBP/kg; Shultz *et al.* (2001) and Lehmann *et al.* (2004)) whereas StAR's gene expression decreased at 50 mg DBP/kg (Lehmann *et al.* 2004) and 100 mg DEHP/kg (Borch *et al.* 2006). The importance of the post-transcriptional products (i.e. the proteins) in the paths to phthalate is obvious. Yet only a few studies have sought to identify alterations in protein expression in the testis in a comprehensive, non-targeted fashion (Plummer *et al.* 2007, Zhang *et al.* 2007), and these studies used relatively high doses.

Except for one study in which pregnant rats were exposed to doses 10, 100, and 750 mg DEHP/kg (Lin *et al.* 2008), there have been few *in vivo* studies of phthalates where low doses (i.e. <100 mg/kg) were used. Lin *et al.* (2008) observed Leydig cell clusters at 10 and 100 mg and the Leydig cells were decreased in both size and number. As pre-term infants can be exposed to 10–20 mg/day (Agency for Toxic Substances and Disease Registry 2006), we wanted to more carefully evaluate the dose response for the 10–100 mg/kg range. Thus, we sought to evaluate the complete testis proteome in the fetal rat testis following exposure to these doses during GD13–GD19 compared with the control testis. The objectives of this study were to i) determine whether dysgenesis in the fetal testis precedes a significant change in the ability of the fetal Leydig cell to produce testosterone, ii) identify proteins that are up-regulated or downregulated at both 10 and 100 mg/kg exposures, iii) correlate proteins that are upregulated or downregulated by DEHP to alterations in both the steroidogenic capacity and aggregation of the GD19 Leydig cells, and iv) select proteins that are predictive of alterations in both steroidogenic capacity and aggregation of the GD19 Leydig cells. Once proteins that are correlated with or predictive of specific endpoints were determined, they were submitted for pathway analysis. The identification of predictive biomarkers and adverse outcome pathways provides a basis for the development of more sensitive and quantitative assays with the potential to predict an adverse outcome in humans.

Results

Alterations in testis histology

Dysgenesis was obvious in the GD19 testis following exposure to both 10 and 100 mg DEHP/kg, hereafter

referred to simply as 10 and 100 mg. Even with 10 mg exposure, Leydig cell clustering in the interstitium of the testis was evident (Fig. 1B). Following 100 mg, the clustering of the cells was more remarkable as large clusters containing many Leydig cells were frequently observed (Fig. 1C). Gonocyte-like cells could be found in the interstitium rather than solely localized within seminiferous cords (Fig. 1C and Supplementary Figure 1, see section on supplementary data given at the end of this article) and erythrophagosomes resulting from extravasated erythrocytes were also evident in the interstitium (Fig. 1C and Supplementary Figure 2, see section on supplementary data given at the end of this article). Finally, it was not uncommon to find malformed seminiferous cords, particularly adjacent to the tunica albuginea, with obvious thickening of the basement membranes (Fig. 1D). At an ultrastructural level, it was found that basal laminar components (lamina lucida and lamina densa) of the basement membranes of seminiferous cords were conspicuously thickened (Supplementary Figure 3, see section on supplementary data given at the end of this article).

Frequency of Leydig cell clusters

Immunostaining of the GD19 testis for the Leydig cell-specific enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSD) allowed for the quantification of Leydig cells in clusters. There was a significant increase in clusters containing 2–5 Leydig cells in the interstitium of the GD19 testis following exposure to 10 mg (Fig. 2A); the mean number of clusters per testis cross section containing 2–5 Leydig cells was 14 compared with nine for controls. The 100 mg exposure caused

a significant increase in the size of the Leydig cell clusters; the mean number of clusters per testis cross section containing >5 Leydig cells was 11 compared with three in controls. The number of these large clusters was also significantly increased in the testes following the 10 mg exposure, with an average of seven compared with three for control testes. Relatively few clusters were observed in cross sections of testes from controls (Fig. 2B), and those that were evident were predominantly 2–5 cell clusters. By contrast, the increase in clusters after exposure to 10 mg was evident (Fig. 3C), and with 100 mg exposure most of the clusters were quite large. These large clusters typically contained 20–50 Leydig cells.

Decreased testosterone production

A single GD19 testis from a control male produced an average of 15 ng of testosterone over the course of a 3 h incubation with LH stimulation (Fig. 3). While there was no decrease in the ability of the testes from male offspring exposed to 10 mg to produce testosterone, it was significantly reduced in males exposed to 100 mg, with only 9 ng testosterone produced during the incubation.

Changes in the testis proteome

The expression of 23 different proteins in the GD19 testis proteome was significantly altered at both 10 and 100 mg exposures (Fig. 4 and Table 1). Those proteins which proved to be significantly correlated with treatment or the altered endpoints (viz., testosterone production and Leydig cell clustering) are shown in red in Fig. 4 and are indicated in bold in Table 1. Of the

Figure 1 (A, B, C and D) Photomicrographs depicting histological changes in the GD19 testis subsequent to DEHP exposure from GD13–GD19. (A) Image of the testis from a control fetus. (B) Leydig cells in testis in the 10 mg exposure group begin to form clusters (cyan arrows). Most of these clusters contain 2–5 contiguous Leydig cells. (C) Leydig cell clusters in testes in the 100 mg group are considerably larger. On occasion gonocytes (yellow arrows) can be observed in the interstitial space along with extravasated erythrocytes and what appear to be erythrophagosomes (red arrows). (D) Dysgenetic seminiferous cords in testes in the 100 mg exposure group (asterisks). Note thickening of the basement membranes surrounding these cords (red arrows). Magnification bars = 20 μ m.

Figure 2 (A) Data showing frequency of Leydig cell clusters expressed as the mean number observed in a testis cross section (mean $\pm$ s.e.m., $n=8$ males/treatment group; ^{a,b,c}denote significance at $P<0.05$). Clusters were classified as those containing 2–5 contiguous Leydig cells (small clusters) and those containing more than five Leydig cells (large clusters). Note the significant increase in small cluster following exposure to 10 mg and the significant increase in large clusters following exposure to 100 mg. (B) A GD19 control testis immunostained for 3β-HSD; Leydig cells are clearly indicated (green arrow). (C) Testes in the 10 mg group showed a dramatic increase in small Leydig cell clusters (orange arrows). (D) The Leydig cell clusters in testes in the 100 mg group were noticeably large (red arrows). Magnification bar shown in (A) = 20 μm.

23 different proteins identified, all but four were downregulated. Interestingly, three of the four that were upregulated were correlated with and predictive of certain endpoints (Table 2). These three proteins were heat shock 71 kDa protein (HSPA8), spermatid synthase (SRM), and 14-3-3 epsilon (YWHAE). Of the 19 that were downregulated, four proteins – stress-induced phosphoprotein 1 (STIP1), heat shock protein 90 kDa (HSP90B1), heat shock protein 75 kDa (TRAP1), and dihydropyrimidinase-related protein 2 (DPYL2) – were correlated with and predictive of certain endpoints.

We confirmed the upregulation of HSPA8 and YWHAE by both western blotting (Fig. 5) and immunohistochemistry (Fig. 6). Immunostaining for HSPA8 was increased in blots of the GD19 testis extract at both 10 and 100 mg exposures. Similarly, immunostaining for YWHAE was increased at both dose levels of DEHP. While we did not identify a significant change in the expression of P450scc (or CYP11A1) at both 10 and 100 mg exposures, there appeared to be a slight decrease in immunostaining for CYP11A1 in extracts of testes exposed to 100 mg. Immunohistochemical staining of the GD19 testis for both HSPA8 and YWHAE also confirms an increase in the expression of these two proteins following exposure to 10 mg (Fig. 6). While inconclusive, the greatest increase in immunostaining appears to reside within the fetal Leydig cells.

Correlations and predictivity

There were five proteins that were significantly ($P<0.05$) correlated with treatment, i.e. exposure to DEHP (Table 2). These were HSPA8, SRM, TRAP1, YWHAE, and STIP1. Three of these – HSPA8, TRAP1, and YWHAE

– were significantly correlated with the decreased capacity of the DEHP-exposed GD19 testis to produce testosterone when stimulated with LH. Two additional proteins – DPYL2 and HSP90B1 – were found to be significantly correlated with the formation of small clusters containing 2–5 Leydig cells. The three proteins that were significantly correlated with the formation of the large clusters containing greater than five Leydig cells were HSPA8, SRM, and YWHAE. It is not surprising that two of these proteins – HSPA8 and YWHAE – were also correlated, albeit inversely, with testosterone production as the correlation between testosterone production and large clusters of Leydig cells were highly significant ($P<0.02$).

Discriminant analysis revealed which proteins were predictive for treatment or a specific measured endpoint (Table 2). HSPA8 and YWHAE were found to be predictive of treatment and each measured endpoint.

Figure 3 Testosterone production by the GD19 testis over 3 h while challenged with maximal (100 ng/ml) LH stimulation. The observed decrease in testosterone production by testes exposed to 100 mg DEHP/kg was highly significant (* $P<0.0001$); mean $\pm$ s.e.m., $n=16$.

Figure 4 Two-dimensional gel profile of the GD19 testis proteome. Proteins which were significantly altered by both the 10 and 100 mg exposures are indicated. Proteins whose expression was upregulated are circled in cyan, all other proteins were downregulated. Proteins that were either correlated with and/or predictive of treatment, testosterone production, or Leydig cell clustering are indicated by the red numbers.

When TRAP1 was used along with HSPA8 and YWHAЕ, 100, 75, and 100 percent of the animals in control, 10 and 100 mg treatment groups were correctly classified (Table 3). When HSP90B1 was used along with HSPA8 and YWHAЕ, 100, 75, and 100 percent of the testes in control, 10 and 100 mg treatment groups were correctly classified based on the frequency of small Leydig cell clusters. Interestingly, DPYL2, which was highly significant when correlated with small Leydig cell clusters ($P < 0.0005$), was actually predictive of the formation of large Leydig cell clusters. When used together with HSPA8 and YWHAЕ, 100, 100, and 75 percent of the testes in the control, 10 and 100 mg treatments were classified correctly based on the frequency of large Leydig cell clusters. There was less certainty predicting decreased steroidogenic capacity of the fetal Leydig cell to produce testosterone; HSPA8 and YWHAЕ provided the best result. These two proteins correctly predicted 100 percent of the incubations of testes from controls and 10 mg exposure but only half of the incubations of testes from the 100 mg exposure.

Pathway analysis

Ingenuity Pathway Analysis software was used to identify pathway networks. Analysis of all 23 proteins whose expression was altered significantly by both levels of DEHP exposure resulted in two pathway networks. One included 17 of the 23 identified proteins (Supplementary Figure 4, see section on supplementary data given at the end of this article). Interestingly, many of these proteins were shown to interact with one another. The second network contained the remaining six proteins (Supplementary Figure 5, see section on supplementary data given at the end of this article). In this network, none of the proteins interacted with one another. A third

network (Supplementary Figure 6, see section on supplementary data given at the end of this article) contained the seven proteins that were correlated with and predictive of DEHP treatment, small and large clusters of fetal Leydig cells, and the capacity of the fetal Leydig cell to produce testosterone. Figure 7 is an attempt to depict the relationships of these proteins as shown in the network. This isolated pathway network reveals that two of the seven proteins (i.e. YWHAЕ and STIP1) are known to interact directly with HSPA8. To our surprise, estradiol was identified as being related to the expression of all seven proteins. All but two of the seven proteins are known to have a direct relationship with estradiol; DYPL2 and TRAP1 appear regulated subsequent to estradiol-mediated regulation of amyloid precursor protein (APP) and proto-oncogene c-myc (MYC) respectively.

Estradiol in the GD19 fetus

Based on the fact that estradiol was at the focal point of the pathway network for proteins correlated with and predictive of various endpoints, we conducted a follow-up study in an attempt to identify any alteration in estradiol following DEHP exposure. Blood plasma from GD19 males exposed to 100 mg DEHP was found to have significantly increased levels of estradiol compared with control males; 118 vs 68 pg/ml (Fig. 8). There was no difference in estradiol levels between 100 mg females and control females (data not shown).

Discussion

We identified novel proteins involved with the onset of dysgenesis of the fetal rat testis and altered capacity of the fetal Leydig cell to produce testosterone following

Table 1 Proteins found to be significantly down- or up-regulated following both 10 and 100 mg/kg DEHP exposures.

Spot	Symbol	Accession numbers	Protein name	Protein MW (kDa)	Percent coverage	Dose			Molecular function
						0	10	100	
2	<u>STIP1</u>	Q3ZCU9	Stress-induced-phosphoprotein 1	63	66	100	58 ^a	52	Protein-protein binding
8	SYG	Q5I0G4	Glycyl t-RNA synthetase	72	15	100	56	66	Protein biosynthesis
22	LMAN1	Q62902	Lectin mannose binding 1	55	20	100	61	71	Protein transport
23	PDIA3	P11598	Protein disulfide-isomerase A3	57	60	100	67	71	Cellular redox homeostasis
47	ENO1	P04764	Alpha enolase	47	29	100	60	60	Glycolysis/plasminogen activation
55	ENO1	P04764	Alpha enolase	47	37	100	78	73	Glycolysis/plasminogen activation
84	<u>HSPA8</u>	P63018	Heat shock protein 71 kDa	71	38	100	143	162	Chaperone/transcriptional repressor
108	ACTG	P63259	Actin, cytoplasmic 2	42	33	100	40	37	Responder to calcium/ATP binding
111	<u>HSP90B1</u>	Q66HD0	Heat shock protein 90 kDa, subunit 1	90	7	100	53	59	Chaperone in the endoplasmic reticulum
112	<u>HSP90B1</u>	Q66HD0	Heat shock protein 90 kDa, subunit 1	90	7	100	51	50	Chaperone in the endoplasmic reticulum
127	<u>TCP1</u>	P28480	T complex protein 1 subunit alpha	60	40	100	62	60	Chaperone involved in protein folding
130	<u>TRAP1</u>	Q5XHZ0	Heat shock protein 75 kDa, Mito.	74	26	100	68	58	Chaperone involved in protein folding
143	<u>SRM</u>	Q99MI5	Spermatine synthase	34	18	100	132	140	Spermatine biosynthesis
174	ATP6V1-B2	P62815	V type proton ATPase subunit B	57	12	100	49	47	Hydrogen ion transport/ATPase activity
191	RPLPO	P19945	60S acidic ribosomal protein P0	34	34	100	67	63	Translational elongation
196	PDIA6	Q63081	Protein disulfide isomerase A6	46	33	100	78	70	Chaperone/platelet aggregation
202	CCT5	Q68FQ0	T complex protein 1 subunit epsilon	59	23	100	71	76	Chaperone involved in protein folding
242	GSTM4	P08009	Glutathione S-transferase	26	6	100	72	73	Conjugation of reduced glutathione
319	TUFM	P49411	Elongation factor Tu, Mito.	45	67	100	78	74	Translation of proteins
333	TRAP1	Q5XHZ0	Heat protein 75 kDa, Mito	74	27	100	62	67	Chaperone/ATPase activity
338	<u>YWHAE</u>	P62260	14-3-3 epsilon	32	32	100	142	177	Signal transduction via protein binding
349	CCT2	Q5XIM9	T complex protein 1 subunit beta	57	57	100	80	75	Chaperone involved in protein folding
365	<u>DPYL2</u>	P47942	Dihydropyrimidinase-related protein 2	62	28	100	70	76	Differentiation/cell migration
366	RPL13A	Q5RKI0	WD repeat containing protein 1	66	26	100	134	152	Disassembly of actin
380	VIM	P31000	Vimentin	54	24	100	59	74	Intermediate filament of cytoskeleton
381	RPSA	P38983	40 S ribosomal protein SA	33	33	100	73	67	Cell surface receptor for laminin/morphogenesis

Statistical results represent the analysis of 16 l per treatment group ($n=16$); one testis from four GD19 males pooled and extracted/litter.

^aThe background-corrected, integrated optical density of each spot was averaged within group and expressed as percent of control. The symbols of proteins that were found to be significantly ($P<0.05$) correlated with and/or predictive of treatment or measured endpoints (bold and underlined).

exposure to lower more relevant DEHP exposures. We first correlated proteins in the fetal testis proteome that were significantly altered at both 10 and 100 mg exposures of the animal, as well as to the endpoints we quantified, i.e. Leydig cell clusters and altered testosterone production. Next, discriminant analysis identified which of the correlated proteins were predictive; these proteins were subjected to pathway analysis. The underlying goal of this approach was to identify novel predictive biomarkers in adverse outcome pathways associated with DEHP exposure. Two proteins, HSPA8 and YWHAE were predictive of both an increased frequency in Leydig cell clusters and reduced capacity

to produce testosterone. Estradiol was linked to the observed alterations in these proteins. These two new proteins and estradiol may be candidates for new assays developed to screen for early onset testicular dysgenesis.

Previous work suggested that clustering of fetal rat Leydig cells occurs following *in utero* exposure to 10 mg DEHP/kg from GD2–GD20 (Lin *et al.* 2008). Testosterone content in the fetal testis was not altered until a 750 mg/kg exposure was reached. Herein we also observed significant Leydig cell clustering following *in utero* exposure to 10 mg from GD13–GD19 assessed by both light microscopy and immunohistochemical staining for 3 β -HSD. Both small and large clusters of

Table 2 Proteins determined to be correlated and/or predictive of treatment and quantified endpoints.

Correlated						Predictive		
Treatment	HSPA8	SRM	TRAP1	YWHAE	STIP1	HSPA8	TRAP1	YWHAE
Testosterone	HSPA8	TRAP1	YWHAE			HSPA8	YWHAE	
Small LC clusters	DPYL2	HSP90B1				HSPA8	HSP90B1	YWHAE
Large LC clusters ^a	HSPA8	SRM	YWHAE			HSPA8	DPYL2	YWHAE

^aCorrelation between large clusters and T ($P < 0.02$).

Leydig cells were more frequent at 10 mg than in control testes. At 100 mg there was a substantial increase in large clusters as the frequency of small clusters decreased to that of controls. Presumably, the return in small clusters to a control frequency reflects the transition and/or further aggregation of many small clusters to large clusters as exposure level increased.

Light microscopy revealed other facets of dysgenesis in the fetal testis with exposure to 100 mg DEHP. Gonocytes were observed in the interstitium along with what appeared to be erythrophagosomes resulting from extravasated erythrocytes. In addition, misshapen seminiferous cords/tubules around the periphery of the fetal testis displayed a marked thickening of the basement membranes. Multinucleated germ cells were first observed following *in utero* exposure to 750 mg DEHP/kg (Gray *et al.* 2000, Parks *et al.* 2000) and later in both rats and mice exposed *in utero* to 250–500 mg DBP/kg (Gaido *et al.* 2007). Leydig cells have been observed in newly formed tubules, along with Sertoli cells in interstitial spaces following 500 mg DBP/kg (Mahood *et al.* 2006). Gray *et al.* (2000) documented extravasated erythrocytes and erythrophagosomes in the interstitium at 750 mg DEHP/kg, but the presence of germ cells in the interstitium with 100 mg/kg phthalate exposure *in utero* has not been reported, nor has the appearance of erythrophagosomes or aberrant basement membranes of dysgenetic seminiferous tubules at this relatively low exposure level.

While testosterone production by the fetal testis *in vivo* is independent of LH (Huhtaniemi & Pelliniemi 1992), the fetal Leydig cell does possess LH receptors as early as GD16 (Zhang *et al.* 1994). As we tested lower exposures than those usually studied, we challenged the GD19 Leydig cell with LH during a 3 h incubation to determine whether the Leydig cell's 'capacity' to produce testosterone was compromised. Our results demonstrate that while the decrease in the capacity of the GD19 testis to produce testosterone following exposure to 10 mg was not significant, the decrease following exposure to 100 mg was significant. A significant decrease in testosterone production by the fetal testis *ex vivo* at an exposure of 100 mg has not been reported. That the increased formation of Leydig cell clusters at 10 mg was significant and the decrease in testosterone production *ex vivo* was not significant, demonstrates that dysgenesis within the fetal testis precedes compromised testosterone production. A recent study has shown that multiple phthalates begin

to compromise testosterone production by the unstimulated fetal testis *ex vivo* at 300 mg/kg (Hannas *et al.* 2011). Thus, the dysgenesis which results from the 10 mg exposure does indeed precede the dysfunction of the fetal Leydig cell *ex vivo* as well as *in vivo*.

We speculate that once aggregated, the fetal Leydig cells become dysfunctional, which results in their decreased ability to produce testosterone. Data from recent studies with the fetal human testis tend to support this notion. When fetal human testis explants were cultured *in vitro* with MBP, neither a decrease in testosterone production nor any aggregation of Leydig cells was observed (Hallmark *et al.* 2007). By contrast Leydig cell aggregation and reductions in testosterone production were observed in similarly cultured fetal rat testis explants. The ability to produce testosterone by human or rat fetal testis explants was studied in castrate nude mice that were exposed to DBP for 2 weeks subsequent to the grafting (Mitchell *et al.* 2012). Serum testosterone levels were unchanged in mice with human testis grafts but were significantly decreased in mice containing rat testis grafts. The histology of the human fetal testis graft following DBP exposure has not been reported; we surmise that there will be no significant aggregation of Leydig cells in the exposed human testis explants.

Figure 5 Western blot demonstrating the increased expression of both HSPA8 and YWHAE following DEHP exposure. P450scc (CYP11A1) was evaluated to confirm the absence of altered expression at both 10 and 100 mg exposures. Samples from 3 l were evaluated, one shown for representation.

Figure 6 Immunohistochemical staining for HSPA8 and YWHAE in the GD19 testis. (A) In control testes HSPA8 is localized (brown reaction product) in germ cells in the seminiferous tubules and throughout the entire Leydig cell in the interstitium (cyan arrow). (B) In testes in the 10 mg exposure group immunostaining of the Leydig cells is visibly increased (cyan arrow). (C) Like HSPA8, YWHAE is localized in control testes in both germ cells and interstitial Leydig cells. (D) And like HSPA8, the increase in immunostaining in testes in the 10 mg exposure group is more pronounced in the Leydig cell. Magnification bar shown in (A) = 20 μ m. $n=8$ males examined/treatment group.

In an attempt to better understand the molecular pathways involved in DEHP-induced Leydig cell clustering and altered testosterone production, we relied on our quantitative proteomic evaluation of the GD19 testis. Of the 23 different proteins that were altered significantly at both exposure levels, not one was previously considered to be pivotal to steroidogenesis. Before proceeding to discriminant and pathway analyses on these newly identified proteins, we probed western blots of extracts from control and DEHP-exposed testes with P450scc (CYP11A1) to confirm the absence of altered expression at both 10 and 100 mg exposures. This protein was selected, as several studies have demonstrated decreased gene expression of P450scc and other steroidogenic molecules following *in utero* exposure to phthalates at doses as low as 100 mg (Shultz *et al.* 2001, Lehmann *et al.* 2004, Hannas *et al.* 2011). We observed no decrease across the DEHP exposures; a slight decrease at 100 mg was suggested. This is consistent with previous results reporting no decrease in expression of P450scc protein (Lehmann *et al.* 2004) or its activity (Lin *et al.* 2008) until at least a 500 mg exposure was reached. Obviously alterations in gene expression occur at lower exposures than those resulting in proteomic changes.

Five proteins were correlated with exposure to DEHP, i.e. HSPA8, SRM, TRAP1, YWHAE, and STIP1. STIP1 is a co-chaperone homologous to heat shock proteins 70 and 90. Its expression was previously shown to be altered in MEHP-exposed mouse testis (Mizrak *et al.* 2006). In addition to HSPA8 and YWHAE, heat shock protein 75 kDa (TRAP1) was predictive of treatment. Trap1 is another heat shock protein shown to protect against ischemic injury (Voloboueva *et al.* 2008). The decrease in this protein may be associated with the observed extravasated erythrocytes resulting from damage to

vessel walls (hemorrhage) and consequent formation of erythrophagosomes observed at 100 mg.

Five proteins were correlated with the increased frequency of Leydig cell clusters following DEHP exposure, i.e. DPYL2, HSP90B1, HSPA8, SRM, and YWHAE. DPYL2 is involved with differentiation and migration of cells (Rozhkova *et al.* 2011) consistent with a role in clustering of Leydig cells. Spermatine, the product of SRM, is important in fetal development, stages of spermatogenesis and steroidogenesis (Lefevre *et al.* 2011). HSP90B1 was recently associated with alterations in sperm chromatin integrity following exposure to chemotherapeutic agents (Maselli *et al.* 2012) and found to be predictive of small clusters. Interestingly, while DPYL2 was not correlated with the

Table 3 Classification of treatment, Leydig cell clusters, and testosterone production by discriminant analysis.

Predictors	% Correctly classified
HSPA8, TRAP1, YWHAE	
Treatment	
Control	100
10 mg/kg	75
100 mg/kg	100
HSPA8, HSP90B1, YWHAE	
Small Leydig cell clusters	
Control	100
10 mg/kg	75
100 mg/kg	100
HSPA8, DPYL2, YWHAE	
Large Leydig cell clusters	
Control	100
10 mg/kg	100
100 mg/kg	75
HSPA8 and YWHAE	
Testosterone production	
Control	100
10 mg/kg	100
100 mg/kg	50

Figure 7 Diagram depicting the pathways common to those proteins that were correlated with and/or predictive of endpoints (i.e. treatment, testosterone production, Leydig cell clustering). Proteins whose expression was increased with DEHP exposure, i.e. heat shock protein 71 (HSPA8), spermatine synthase (SRM), and 14-3-3 epsilon (YWHA8) are shown in green boxes whereas proteins whose expression was decreased with exposure, i.e. stress induced protein 1 (STIP1), heat shock protein 90 subunit 1 (HSP90B1), dihydropyrimidinase-related protein 2 (DPYL2), and heat shock protein 75 kDa (TRAP1) are shown in yellow boxes. Proteins that are predictive of particular endpoints appear in red. YWHA8 and STIP1 are shown to interact directly with HSPA8 (solid lines). Estradiol was identified as central to the regulation of all seven proteins. The expression of DPYL2 appears to be indirectly regulated by estradiol via amyloid precursor protein (APP) and the expression of TRAP1 is indirectly regulated by estradiol via MYC.

formation of large Leydig cell clusters, it was predictive of these clusters. Heat shock protein 71 kDa (HSPA8) and 14-3-3 epsilon (YWHA8) were predictive of both small and large Leydig cell clusters. HSPA8 is now known as one of the pivotal signaling proteins during gonadal development (Wilhelm *et al.* 2006). YWHA8 is involved in signal transduction consequent to phosphoserine/threonine binding and pivotal to proper neuronal migration and brain development (Toyo-oka *et al.* 2003).

Three proteins were correlated with decreased capacity of the LH-stimulated fetal Leydig cell to produce testosterone following DEHP exposure, i.e. TRAP1, HSPA8, and YWHA8. TRAP1 was correlated with testosterone production by the LH-stimulated GD19 fetal testis, but only HSPA8 and YWHA8 proved predictive for testosterone production. And, while this combination predicted 100 percent of the incubations from control and 10 mg testes correctly, only 50 percent of the 100 mg testes were classified correctly. We suspect that a protein(s) needed to predict the significant decrease in the capacity of the GD19 testis to produce testosterone at 100 mg is (are) only significantly altered in 100 mg dose extracts. This further supports the notion that DEHP-induced testicular dysgenesis precedes alterations in testosterone production; as clusters become larger, additional protein(s) become linked to compromised testosterone synthesis.

The pathway network created for the seven proteins that were correlated with and predictive of DEHP exposure and the measured endpoints revealed two

direct interactions. HSPA8 has been shown to bind to both YWHA8 (Ballif *et al.* 2006) and STIP1 (Odunuga *et al.* 2003). Surprisingly, the focal point in the pathway network clearly was estradiol. The expression of HSPA8 itself is known to be increased by estradiol, as it was following DEHP exposure (Mercier *et al.* 2009). YWHA8 which was upregulated by DEHP exposure is also increased by estradiol (Thompson *et al.* 2002). Based on immunohistochemical staining, both HSPA8 and YWHA8 appeared to have increased expression in the fetal Leydig cells in both 10 and 100 mg testes. The third predictive upregulated protein of the seven was SRM, also known to be regulated by estradiol (Kapyaho *et al.* 1980); SRM is also regulated by MYC (Guo *et al.* 2000) which is part of the pathway network.

HSP90B1, another heat shock protein altered in this study, is also regulated by estradiol (Papaconstantinou *et al.* 2002). While normally estrogen might be expected to increase HSP90B1, DEHP exposure resulted in a significant decrease in HSP90B1. Both HSP70 and 90 are known to complex with estrogen receptor (ER) to form a stable, high affinity estradiol-binding state (Whitesell & Lindquist 2005). With DEHP exposure, HSP90B1 may be altering estradiol expression by virtue of decreased levels in the ER-HSP complex. The remaining proteins in the pathway network, i.e. STIP1, DPYL2, and TRAP1 were also decreased by DEHP exposure. STIP1 has been shown to be directly regulated by estradiol (Watanabe *et al.* 2003), while DPYL2 is dependent on estradiol-mediated App expression (Manthey *et al.* 2001). TRAP1 expression is regulated by MYC (McConnell *et al.* 2003), which in turn is known to be regulated by estradiol (Wang *et al.* 2011). ER α (now referred to as ESR1) appears to mediate this regulation (Duplessis *et al.* 2011). While we only localized the expression of HSPA8 and YWHA8, the two proteins that were predictive of exposure and each quantified endpoint, we suspect the expression of some

Figure 8 Plasma estradiol concentrations in GD19 males. The observed increase in the estradiol level in males exposed to 100 mg DEHP from GD13 to GD19 was significant (* $P < 0.05$) compared with control males; mean $\pm$ S.E.M., $n = 7$ males/group.

of the other five proteins that may be changing in the fetal Leydig cell as well.

While estradiol's involvement in this pathway network was unexpected, it is well known that increased exposure to estradiol adversely affects male reproductive development (Delbes *et al.* 2006, Howdeshell *et al.* 2007) including cryptorchidism, germ cell atypia, thickening of basement membranes of seminiferous tubules, and dysplastic adenomyotic lesions in the epididymis (Veeramachaneni *et al.* 1988, Veeramachaneni 2006). Moreover, exogenous administration of androgen to pregnant females exposed to diethylstilbestrol (DES) fails to prevent DES-induced cryptorchidism (Hutson *et al.* 1990). ESR1 is a major contributor to estrogen-mediated dysgenesis of the fetal testis and cryptorchidism (Cederroth *et al.* 2007). Exogenous estradiol has been reported to decrease testosterone production by GD16 and GD21 Leydig cells (Delbes *et al.* 2006). This is also apparently mediated via ESR1, as fetal testes from ER α -knockout mice produce significantly more testosterone than wild type mice under both LH-stimulated and unstimulated conditions. ESR1 is expressed in the testis of mice and rats early in fetal development and has been localized in the Leydig cell (O'Donnell *et al.* 2001). Collectively, this suggests that DEHP exposure may increase levels of estradiol and/or increase the responsiveness of ESR1 to estradiol in the fetal rat testis. Given that DEHP has virtually no binding affinity for the ER (Blair *et al.* 2000), it is unlikely that DEHP binds ESR1 to activate signaling.

We sought to verify in a follow-up study, if there would be an increase in circulating estradiol in male fetuses exposed to 0 or 100 mg from GD13–GD19. We were surprised to find that plasma estradiol levels were increased approximately twofold in males exposed to DEHP compared with control males. Future studies are needed to determine the source of the increased estradiol, i.e. increased aromatase activity in the fetal Sertoli cell, the fetal Leydig cell, or both cell types. Nonetheless, the increase in plasma estradiol following DEHP exposure is consistent with the above discussion and the notion that estradiol is pivotal to the observed dysgenesis of the fetal testis as well as early events resulting in decreased ability of the fetal Leydig cell to produce testosterone. In a previous study in which 10 mg DEHP/kg was administered to rats from weaning until adulthood, Leydig cell hyperplasia was demonstrated (Akingbemi *et al.* 2004). In this study, Leydig cells purified from DEHP-treated animals were found to produce significantly less testosterone in response to LH. Interestingly, these Leydig cells produced increased levels of estradiol. Thus, DEHP may enhance aromatase activity in the fetal rat Leydig cell and/or Sertoli cell resulting in increased estradiol, ESR1-mediated dysgenesis, and ultimately compromised ability of Leydig cell to produce testosterone. However, aromatase activity need not necessarily be enhanced within the

Leydig cell for compromised testosterone production by estradiol. Indeed, both *in vitro* and *in vivo* exposures to estradiol have resulted in significantly decreased testosterone production (Delbes *et al.* 2006). Regardless, the compromise is thought to be mediated via ESR1. While fetal rat Leydig cells possess ESR1, the human fetal testis is devoid of ESR1 (Boukari *et al.* 2007). This may explain, in part, why DEHP exposure fails to alter testosterone production in human fetal testis (Mitchell *et al.* 2012).

In summary, lower DEHP exposures resulted in dysgenesis of the fetal rat testis, specifically clustering of the fetal Leydig cell, which preceded significant compromise in testosterone production. A comprehensive proteomic evaluation of the fetal testis revealed 23 individual proteins that were altered significantly by both DEHP exposures. With correlation and discriminant analyses, we determined that seven of these proteins were predictive of i) DEHP exposure, ii) increased frequency of both small and large Leydig cell clusters in the fetal testis, and iii) the capacity of the GD19 Leydig cell to produce testosterone when stimulated with LH. Pathway analysis and fetal plasma hormone data support the notion that the onset of dysgenesis in the fetal rat testis exposed to phthalates may be mediated by estradiol. The new predictive proteins may be used to develop new assays to screen for the onset of testicular dysgenesis. It remains to be determined if such assays would be applicable to detection of dysgenetic phenotypes in the human fetal testis.

Materials and Methods

Animals and experimental design

The study consisted of two replicate experiments. A total of 60 timed-pregnant Sprague–Dawley rats (Charles Rivers Laboratories, Inc., Durham, NC, USA) were allowed to acclimate for 1 week before the onset of dosing. The U.S. EPA NHEERL IACUC approved all procedures. Pregnant dams were housed individually under room conditions of 12 h light:12 h darkness, $22 \pm 1^\circ\text{C}$, $50\% \pm 10\%$ relative humidity.

For each experiment 30 dams were randomly assigned based on body weight ranking on GD11 to one of three treatment groups (control, 10 mg DEHP/kg, and 100 mg DEHP/kg) to provide a minimum of 8 l per treatment group. DEHP (Sigma–Aldrich) was diluted in corn oil and administered daily in the morning by oral gavage ($2 \mu\text{l/g}$ body weight) from GD13 until necropsy on GD19. Each of the two replicate experiments was performed in two randomized blocks over 2 consecutive days. Each block consisted of 4 l per treatment group and for each litter, four males were randomly selected for testosterone, proteomics, and histology evaluations. For the entire study, each endpoint in each treatment group was represented by 16 l (64 males). Thus, the entire study consisted of 48 l (192 males).

On the day of necropsy, the right testis of each selected male was fixed in either glutaraldehyde (two males/litter) for light and transmission electron microscopy or formaldehyde (two males/litter) for immunohistochemistry. The left testis of each male

was incubated in medium to determine testosterone production (described below). After the incubation, the four testes per litter were pooled and frozen for subsequent extraction and proteomics. Thus, it was possible to correlate testosterone production and the proteins in the testis proteome on a litter basis. Moreover, data from each of the two testes chosen for β -HSD immunohistochemistry could be correlated with the testosterone produced by the contralateral testis in these males.

Testosterone production by the GD19 testis

The left testis from each of four males/litter was placed in a well of a 24-well culture plate in 1 ml pre-warmed medium, Medium-199 (Gibco, #94-038DK), buffered with sodium bicarbonate, and supplemented with BSA (0.2%), insulin-transferrin-selenium mix, sodium pyruvate, and non-essential amino acids. Immediately after the four testes/litter were plated, 100 ng/ml ovine LH (NIDDK-oLH-26) was added to maximally stimulate testosterone production; the goal being to detect any alteration in the capacity of the fetal Leydig cell to produce testosterone. Incubations were carried out in 5% CO₂ at 34 °C for 3 h. Medium from each well was transferred to a microcentrifuge tube and stored at -70 °C for testosterone assay. Testosterone RIA was performed using a radiolabeled antibody kit (Coat-A-Count, Siemens Healthcare Diagnostic, Tarrytown, NY, USA) according to the manufacturer's instructions. Intra-assay and inter-assay coefficients of variation for the assay were both <10%. Each of the four testes/litter following the incubations was transferred to a microfuge tube and stored at -70 °C until extracts for the proteomic evaluation were prepared.

Quantitative evaluation of the GD 19 testis proteome

GD19 testes (4/l) were held at -70 °C until extracts were prepared for 2D SDS-PAGE as previously described (Kaydos *et al.* 2004). Upon thawing, the testes were homogenized in a 1 ml glass-glass homogenizer in 1 ml 10 mM Tris buffer containing 1 mM EDTA, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.25% OBG, pH 7.2, to which 0.2 mM phenylmethylsulphonyl fluoride was freshly added. Each homogenate was desalted and concentrated in Ultrafree-4 centrifugation filter units (Millipore, Bedford, MA, USA) with 1 mM Tris buffer, pH 7.2, and assayed for protein using Micro-BCA protein assay kit (#23235, Pierce, Rockville, IL, USA) before quantitative 2D SDS-PAGE analysis.

Sample volumes containing 30 µg of protein were lyophilized, and protein was solubilized for 30 min at room temperature in 45 µl of sample buffer consisting of 5.7 g urea, 4 ml of 10% NP-40, 0.5 ml ampholytes (3–10 only; Serva, Heidelberg, Germany), and 0.1 g of dithiothreitol per 10 ml. Isoelectric focusing (750 V, 3.5 h) was carried out in capillary tube gels consisting of 6.24 g urea, 1.5 ml acrylamide solution (30% acrylamide, 1.2% bisacrylamide), 2.25 ml of 10% NP-40, and 0.65 ml ampholytes (3–10 only) per 10 ml. Molecular weight separation was carried out in mini 14% acrylamide gels (200 V, 1 h). Gels were fixed in 40% ethanol (v/v) and 10% acetic acid (v/v) in ultrapure water, fluorescence stained using Krypton Protein Stain (Pierce Biotechnology) and, finally, immersed in destaining solution (5% acetic acid

in ultrapure water) for 5 min. A Fluorescent Image Analyzer (FLA-5100; Fujifilm, GE Health Care, Piscataway, NJ, USA) was used to scan gels and capture high-resolution images with a 532 nm laser light source. Progenesis Same Spots Software (2.0) was used for background correction, spot matching, and spot area quantification. Protein spots from every gel were aligned to a reference gel (Supplementary Figure 7) which was selected from among the treatment groups as a representative profile. Once accurately aligned, quantitative analysis was performed on spot optical densities.

Spots corresponding to proteins whose expression was significantly altered (upregulated or downregulated) at both 10 and 100 mg exposures were punched from 2D gels using an automated Ettan spot picker (GE Healthcare). Gel punches were destained twice in i) 50 µl of 200 mM ammonium bicarbonate, 40% (v/v) acetonitrile, and ii) 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile, and dehydrated with acetonitrile under vacuum. Gel punches were rehydrated in 10 µl proteomics grade trypsin (Sigma, Cat # T 6567, 20 µg/ml) dissolved in trypsin reaction buffer (40 mM ammonium bicarbonate, 2% (v/v) acetonitrile). After 2 h, a 20 µl aliquot of the trypsin reaction buffer was added, and the digests were incubated overnight in an Eppendorf thermomixer at 37 °C, 80 g. Finally, 5 µl aliquot of 3% aqueous trifluoroacetic acid was added and the peptide mixture was desalted using C18 Ziptips (Millipore, Billerica, MA, USA). The samples were eluted in 0.7–1.0 µl of α -cyano-4-hydroxycinnamic acid MALDI matrix dissolved in 0.1% trifluoroacetic acid in a 4:1 acetonitrile:water mixture, which was sufficient to create three MALDI spots per sample. The first two spots were used directly for MALDI-MS and data-dependent MS/MS analysis, and the remaining spot was used for mass-selected MALDI-MS/MS.

Protein identification by mass spectrometry

Proteins were identified using a 4800 MALDI TOF/TOF (Applied Biosystems (AB), Foster City, CA, USA) mass spectrometer. The on-line proteomics software 'Aldente' (<http://expasy.org/tools/aldente>) was used to identify proteins from background-subtracted MALDI-MS data. This step was followed by MALDI-MS/MS sequencing, and the final protein identification was done using Protein Pilot 3.0 software (AB), searching against the rat species sub-database of the SwissProt protein database (<http://expasy.org/sprot/>). Mass-selected MALDI-MS/MS spectra acquisition was performed to increase protein sequence coverage. The MS peaks selected by Aldente were used to acquire more MS/MS data from the remaining MALDI spots. The reported proteins were identified based on at least two high-quality peptide sequence identifications characterized by the Protein Pilot probability peptide MS/MS score of 95 or better. Finally, proteins that were predictive of treatment and correlated with testosterone production or Leydig cell clustering were subjected to pathway analysis (Ingenuity Pathway Analysis).

Western blotting

Two proteins that were identified as predictive and both upregulated by DEHP exposure were also probed by western blotting. HSPA8 was predictive of large Leydig cell clusters and

decreased testosterone production. YWHAE was predictive of DEHP treatment. P450scc (Cyp11a1) was probed to confirm lack of altered expression at both 10 and 100 mg/kg. The three polyclonal primary antibodies for these proteins were as follows: goat anti-HSPA8 (sc-1059, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-YWHAE (sc-1020, Santa Cruz Biotechnology), and goat anti-Cyp11a1 (P450scc; sc-1043, Santa Cruz Biotechnology). The secondary antibodies were conjugated with Alexa Fluor 680. These were donkey anti-goat Ig (A21084, Invitrogen) and goat anti-rabbit Ig (21076, Invitrogen).

Samples (10 µg) representing each treatment group were loaded onto precast Novex 14% Tris-glycine mini resolving gels (EC6485BOX, Life Technologies). Following electrophoresis proteins were transferred onto nitrocellulose membrane (iBlot transfer stacks; IB3010-02, Invitrogen) using the iBlot system (Invitrogen). After transfer the membrane was removed from the anode stack and incubated for 1 h at room temperature on an orbital shaker in Odyssey blocking buffer (#927-40000, Licor, Lincoln, NE, USA): membranes were incubated with primary antibody diluted 1:500 or 1:1000 in blocking buffer overnight in the cold. Membranes were washed four times in Dulbeccos PBS (DPBS) with 0.1% Tween-20 before incubating with secondary antibody diluted 1:25 000 for 1 h at room temperature. Finally, the membranes were washed four times in DPBS with Tween followed by three washes in DPBS alone. The membranes were scanned using an Odyssey infrared imaging system (Licor).

Testis histology and immunohistochemistry

Testis histology and immunohistochemistry was carried out as described previously (Tarka-Leeds *et al.* 2003). The GD19 testes were immersion fixed in 5% glutaraldehyde (#01909; Polysciences, Warrington, PA, USA) in 0.05 M collidine buffer with 0.1 M sucrose overnight at 48 °C. The tissue was rinsed twice with 0.2 M collidine buffer for 5 min. Tissue was postfixed in 1% aqueous osmium tetroxide (#223A; Polysciences) in 0.05 M collidine buffer on ice for 1 to 2 h followed by a 5-min rinse in 0.2 M collidine. Dehydration consisted of two 5-min washes in 70, 80, and 95% ethanol on ice, and finally three 20 min washes in 100% ethanol at room temperature. After two 10-min rinses in propylene oxide, a 1 h incubation in propylene oxide:Epon (1:1) and an overnight incubation (48 °C) in 100% Epon, tissues were embedded in 100% fresh Epon at 60 °C for 48 h. Semi-thin (1 µm) sections were cut with an RMS Ultramicrotome (MT-7; RMC, Inc., Tucson, AZ, USA) and stained with an aqueous solution of toluidine blue (#02205; Electron Microscopy Sciences, Fort Washington, PA, USA).

Tissue blocks showing conspicuous dysgenetic lesions were processed for further ultrastructural characterization. Thin sections (60–80 nm) were cut and stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEOL-1200EX, JEOL USA, Inc., Peabody, MA, USA).

For the immunohistochemistry, testes were fixed in formaldehyde, transferred to 70% ethanol, rinsed two times in 95% ethanol for 15 min, three times in 100% ethanol for

20 min, and two times in 100% xylene for 30 min. Tissue was incubated in a 1:1 xylene:paraffin (Paraplast Plus; Oxford Labwares, St Louis, MO, USA) bath at 60 °C, two times for 45 min, and then three times for 30 min in a 100% paraffin bath at 60 °C. Paraffin blocks were allowed to cool overnight and then stored at 4 °C until sections (4–6 µm) were obtained. After sectioning, the slides were held at room temperature overnight and then stored at 4 °C.

To identify Leydig cells, 3β-HSD was immunolocalized. Sections were deparaffinized via a graded ascending series of ethanol and xylene and microwaved at low power for 60 s in 0.01 M sodium citrate buffer containing 0.05% Tween 20, pH 6 for antigen retrieval. Sections were washed two times in DPBS and then blocked in DPBS containing 1% protease-free BSA (#A-3294, Sigma) for 30 min, incubated for 15 min at room temperature in 3% H₂O₂ in dH₂O, and again washed two times in DPBS. Slides were incubated overnight at 4 °C in rabbit anti-3β-HSD antibody (H-143, Santa Cruz Biotechnology), diluted 1:200 in PBS containing 1.0% BSA, and then washed two times in PBS. Following a 1-h incubation at room temperature with biotinylated anti-rabbit IgG (BA 1400: R.T.U. biotinylated universal anti-rabbit/mouse; Vector Laboratories, Burlingame, CA, USA) diluted 1:100 in PBS, sections were washed twice in PBS, incubated for 1 h with avidin–biotin peroxidase complex (PK7100: Vectastain R.T.U. Elite ABC reagent; Vector Laboratories), and washed twice with PBS. Bound antibody was localized with Vector DAB peroxidase substrate kit (#SK-4100; Vector Laboratories). Sections were washed in dH₂O and counterstained with hematoxylin. Slides were rinsed in running tap water and dehydrated through a graded descending ethanol series, and coverslips were mounted with Vectamount (H-5000; Vector Laboratories).

The two proteins that were identified as predictive and probed by western blotting were also immunolocalized in the GD19 testis. HSPA8 was identified as predictive of large Leydig cell clusters and decreased testosterone production. YWHAE was identified as predictive of DEHP treatment. The primary antibodies for these proteins were the same as those used for western blotting and were used at 1:200. The secondary for YWHAE was the same as that used to localize 3β-HSD, the secondary for HSPA8 was a biotinylated anti-goat (BA-5000; Vector Laboratories). Each was used at 1:100. Sections were evaluated using a Leica DM6000B microscope and images were captured with a Leica DFC295 color camera.

Estradiol in the GD19 fetus

Because of the surprising outcome of the pathway analysis indicating a role for estradiol in the observed protein spectrum, we undertook a follow-up study. For this study, 15 timed-pregnant Sprague–Dawley rats (Charles Rivers Laboratories, Inc.) were allowed to acclimate for 1 week and randomly assigned based on body weight ranking on GD11 to either control or 100 mg DEHP/kg groups (*n*=7 each). DEHP was administered from GD13–GD19 as before and after the last dose on GD19, litters were evaluated. Dams were decapitated and pups were delivered via Cesarean section. Pups were then placed in numbered dishes kept on a slide warmer maintained at 37 °C. Fetal trunk blood was collected into heparinized

capillary tubes. Sex of each fetus was ascertained and male and female fetal blood was pooled by litter into siliconized 1.5 ml centrifugation tubes. Plasma was separated by centrifugation and frozen immediately at -70°C . Plasma concentrations of estradiol were measured using the Ultra-Sensitive Estradiol RIA kit following the manufacturer's instructions (DSL4800, Beckman Coulter, Webster, TX, USA). The limit of detection was 1.2 pg/ml.

Statistical analysis

For testosterone and proteomic evaluations 16 l were evaluated per treatment group ($n=16$). Both testosterone and protein data were analyzed using two-way ANOVA (PROC GLM; SAS 9.1, 2002–2003) for treatment effects. As absolute values for individual proteins between control testes in the two experiments differed, individual protein data were converted to percent of control for the ANOVA analysis. A correlation analysis was performed to determine whether significant ($P<0.05$) correlations existed within an animal for treatment, testosterone production, small Leydig cell clusters (2–5 LCs/cluster), large Leydig cell clusters (>5 LCs/cluster), and the individual proteins that were significantly altered at both 10 and 100 mg/kg DEHP exposure. For this, the background-corrected spot density for each protein was used. To determine whether individual proteins could be used to predict treatment, decreased testosterone production, and/or Leydig cell clustering, a linearized, discriminant analysis program was used (PROC DISCRIM; SAS 9.1, 2002–2003). For discriminant analysis treatment, classifications were 0, 10, and 100 mg/kg. For testosterone production, classifications were based on the mean (expressed as % of control) per treatment group, i.e. 100% control (0 mg/kg), 89% of control (10 mg/kg), and 43% of control (100 mg/kg). For Leydig cell, clustering classifications were based on the means of small and large clusters within each treatment group. For small clusters, means were 9 (0 mg/kg), 14 (10 mg/kg), and 5.75 (100 mg/kg). For large cluster, means were 3 (0 mg/kg), 7.25 (10 mg/kg), and 11 (100 mg/kg). Plasma estradiol concentration data were evaluated for a treatment effect using a two-tailed Student's *t*-test with GraphPad InStat 3 (GraphPad Software, La Jolla, CA, USA). Data were tested for normality using the Kolmogorov–Smirnov test before statistical analysis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0266>.

Declaration of interest

Disclaimer: This manuscript has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views or policy of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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