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Phthalate-induced pathology in the foetal testis involves more than decreased testosterone production

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Abstract

Foetal exposure to phthalates is known to adversely impact male reproductive development and function. Developmental anomalies of reproductive tract have been attributed to impaired testosterone synthesis. However, species differences in the ability to produce testosterone have been noted; e.g., following foetal exposure, abnormal clustering of Leydig cells or decreased production of testosterone that is manifested in rats does not occur in mice or humans. Nonetheless, other facets of testicular dysgenesis occur in both rats and mice as well as in some other species tested. We recently published a comprehensive evaluation of the foetal rat testis proteome, following *in utero* exposure to diethylhexyl phthalate (DEHP), which revealed changes in individual proteins that are known to be factors in cellular differentiation and migration or related to the capacity of the foetal Leydig cell to produce testosterone and fit a pathway network in which each is regulated directly or indirectly by oestradiol. Plasma oestradiol indeed was found to be elevated approximately twofold in 19-day-old DEHP-exposed foetal male rats. In this brief review, we discuss our new findings vis-à-vis 'oestrogen hypothesis' as a cause for testicular dysgenesis syndrome.

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Testicular dysgenesis syndrome and phthalates

The term testicular dysgenesis syndrome (TDS) was coined to describe the reported, albeit controversial, rise in alterations in reproductive health in men, such as reduced semen quality, testicular cancer, hypospadias and cryptorchidism (Skakkebaek *et al*. 2001, Sharpe & Skakkebaek 2008). The notion that TDS is predicated on a significant decline in testosterone production by the foetal testis is based on many studies on phthalates using the laboratory rat. Indeed, all of the TDS phenotypes except testicular cancer have been associated with gestational exposure of rats to 500–750 mg phthalate/kg body weight, exposures which result in significant declines in foetal testis testosterone.

It is important to note that gestational exposure of rats to oestradiol (Howdeshell *et al*. 2007) or diethylstilbestrol (DES; Hutson *et al*. 1990) results in alterations similar to those found following phthalate exposure, such as alterations in the testis and accessory sex organs and cryptorchidism. The relationship between oestradiol/DES exposure vs phthalate exposure may be linked to disruption of the androgen–oestrogen balance during foetal development (Rivas *et al*. 2002). However, androgen supplementation cannot prevent induction of cryptorchidism by oestradiol/DES. Thus cryptorchidism has been linked to oestradiol/DES-induced down-regulation of ESR1 and INSL3 (Cederroth *et al*. 2007); androgens do seem to prevent some oestradiol/DES-induced ESR1 alterations in the reproductive tract (Rivas *et al*. 2003).

Aggregation of Leydig cells

Parks *et al*. (2000) first reported foetal rat Leydig cell aggregation resulting from exposure to 750 mg/kg

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diethylhexyl phthalate (DEHP) from gestational day (GD) 14–postnatal day (PND) 3. Mahood *et al*. (2005) exposed dams to 500 mg/kg dibutyl phthalate (DBP) from GD13–20, and determined that foetal Leydig cell aggregation was not associated with an increase in the number of foetal Leydig cells. However, the volume of these cells was reduced significantly. Later, Lin *et al*. (2008) investigated foetal Leydig cell aggregation following phthalate exposure more carefully with lower dose exposures. Testosterone content in GD21 testes was not reduced significantly by exposure to 10 or 100 mg DEHP/kg from GD2–20. Moreover, the activities of key steroidogenic enzymes such as P450 side-chain cleavage, 3β-hydroxysteroid dehydrogenase and P450 C17 hydroxylase/lyase were not decreased. Morphometric analysis revealed that the size of the foetal Leydig cells was reduced after exposure to 100 mg/kg consistent with Mahood *et al*. (2005), but a significant decrease in the number of foetal Leydig cells also was observed. Interestingly, there was a significant increase in the size of foetal Leydig cell clusters beginning at 10 mg/kg. These were the first data suggesting that the aggregation of foetal Leydig cells following phthalate exposure precedes compromised production of testosterone, although *ex vivo* production was not evaluated. Additional support for the notion that compromised testosterone production by foetal Leydig cells and the aggregation of these cells are distinct phenotypes was provided by a study by Van den Driesche *et al*. (2012*a*, 2012*b*). In this study, exposure to 500 mg/kg DBP late in gestation, i.e. GD19–20, resulted in decreased intratesticular testosterone production in the absence of foetal Leydig cell aggregation.

The only other gestational exposure that produced Leydig cell aggregation that we are aware of involved exposure of mice to the Leydig cell toxicant ethane dimethanesulphonate from GD11–17, but the aggregation was not evident until the male offspring were adults (Tarka-Leeds *et al*. 2003). In this study, morphometric analysis revealed an increased interstitial area containing significantly fewer and smaller Leydig cells. In this instance, each Leydig cell was found to produce more testosterone both without and with luteinising hormone (LH) stimulation compared with those in control testes. Thus, adult Leydig cell aggregation was not related to compromised testosterone production. Interestingly, Sertoli-cell-only seminiferous tubules were observed in the adult testes exposed during gestation. How this exposure of foetal Leydig cells resulted in permanent alterations in the adult remains a mystery. Taken together, these studies suggest that compromised testosterone production is not associated with Leydig cell clustering.

Leydig cell hyperplasia presents a slightly different scenario. When Leydig cells were exposed to 10 and 100 mg DEHP/kg from PND21–90, a significant increase in the number of adult Leydig cells was observed (Akingbemi *et al*. 2004). However, these Leydig cells produced less testosterone *in vitro* both without and with LH stimulation compared with control Leydig cells. Despite the steroidogenic compromise in the DEHP-exposed Leydig cells *in vitro*, serum levels of testosterone, oestradiol and LH were elevated. Inhibition of testosterone biosynthesis along with elevated LH is considered to be an hormonal scenario causing Leydig cell hyperplasia with human relevance (Clegg *et al*. 1997, Cook *et al*. 1999). In fact, testicular biopsies from infertile men with impaired spermatogenesis showed Leydig cell clusters which have the appearance of Leydig cell hyperplasia. These men have decreased testosterone:LH ratio (Holm *et al*. 2003) and testosterone:oestradiol ratio (Andersson *et al*. 2004). This indicates a role of oestrogen in testes with poor spermatogenesis, although the mechanism is not understood. Similar lesions also have been noted in domestic animals (Veeramachaneni *et al*. 1986). In contrast to Leydig cell clustering, all putative causes of Leydig cell hyperplasia appear to involve compromised production of testosterone.

A multifaceted approach to better understand phthalate-induced dysgenesis

Proteomics has been in the rumble seat of the omics technologies. This is attributed to the fact that it is costly and time-consuming to provide high-quality, meaningful data. We felt it is important to study dysgenesis in the foetal testis following gestational exposure to phthalates from a comprehensive, non-targeted proteomic perspective (Klinefelter *et al*. 2012). We focused initially only on proteins that were significantly altered by both of the relatively low doses tested (i.e. 10 and 100 mg DEHP/kg). We correlated the proteins altered at both doses with i) the capacity of the Leydig cells in the foetal testis to produce testosterone *ex vivo* when challenged with LH and ii) the signature phenotype of dysgenesis in the rat testis following gestational phthalate exposure, i.e. Leydig cell aggregation or clustering.

We discovered that Leydig cell aggregation clearly precedes compromised steroidogenic capacity in the foetal Leydig cell exposed to DEHP. However, when steroidogenic capacity was compromised, there was a significant increase in the formation of large Leydig cell clusters (>5 Leydig cells/cluster). Three of the proteins significantly altered by DEHP exposure, HSPA8, YWHAE and HSP90B1, were actually capable of predicting the formation of small Leydig cell clusters with 75–100% accuracy. Heat shock protein 71 kDa (HSPA8), 4-3-3 Epsilon (YWHAE) and dihydropyrimidinase-related protein 2 (DPYL2) were able to predict the formation of large Leydig cells clusters with 75–100% accuracy. HSPA8 and YWHAE predicted testosterone production in control as well as 10 mg/kg incubations with 100%

accuracy, but only 50% of the 100 mg/kg incubations were correctly identified.

It occurred to us that by ignoring proteins that were only significantly altered at 100 mg/kg, we might have missed protein(s) that may better account for the decreased steroidogenic capacity at 100 mg/kg. We determined which proteins were altered only in the higher dose group and which of those best correlated with and were predictive of *ex vivo* testosterone production. In total, there were 12 proteins significantly altered only at 100 mg/kg. Five of these proteins were significantly correlated with the capacity of the foetal Leydig cell to produce testosterone; each of these was upregulated, but only one, namely; HSP90AB1, heat shock protein HSP 90-Beta (molecular chaperone) was predictive. When this protein was added into the discriminant analysis along with HSPA8 and YWHAE, 100% of the testosterone incubations were correctly classified in the control, 10 and 100 mg/kg groups. To our surprise, the proteins that were correlated with and/or predicitive of Leydig cell clustering or testosterone production fit a common pathway network. The majority of these proteins have one upstream regulator in common, i.e. oestradiol. As the notion that oestradiol might somehow be involved in phthalate-induced toxicity in the foetal testis was completely novel, we went on to determine that oestradiol was indeed significantly increased in the plasma of male foetuses exposed *in utero* to phthalate; oestradiol was unchanged in plasma from female foetuses (Klinefelter *et al.* 2012). The protein pathway network for the seven original proteins that were correlated with and/or predictive of endpoints plus HSP90AB1 is shown in Fig. 1; notice that HSP90AB1 is also regulated by oestradiol.

During his presentation at this Workshop, Dr Richard Sharpe noted that there have been 1792 citations of the 'oestrogen hypothesis' that he and Dr Niels Skakkebaek put forth as an explanation for falling sperm counts and male reproductive anomalies (Sharpe & Skakkebaek 1993) but no new substantive data. After all, our observations may provide the first glimpse and offer support for the 'oestrogen hypothesis'. We still have to determine the site(s) of increased aromatase activity in the exposed male offspring. Sites of interest include the testis, the liver, the brain or even adipose tissue. Interestingly, aromatase has been shown to be significantly higher in the hypothalamic/preoptic area of the brain in PND1 male rats compared with females. Moreover, when exposed to >5 mg DEHP/kg during gestation, aromatase activity in this region increases significantly (Anderson *et al.* 2006). Exogenous oestradiol has been shown to decrease testosterone production by the foetal rat Leydig cell (Delbes *et al.* 2006) and this appears to be mediated through ESR1.

HSP90 proteins have previously been implicated in prenatal exposure of rats to oestradiol (Wang *et al.* 2004). These proteins are known to associate with the oestrogen receptor and were associated with increased expression in gonocytes following exposure to oestradiol. Our work revealed differential expression of HSP90B1 and HSP90AB1. HSP90B1 was decreased at both 10 and 100 mg/kg and found to be correlated with and predictive of small Leydig cell clusters. HSP90AB1 was increased at 100 mg/kg and found to be correlated with and predictive of the decreased capacity of the foetal Leydig cells to produce testosterone in this group. DYPL2, known to be involved in cell differentiation and migration, is indirectly regulated by oestradiol and found to be correlated with small Leydig cell clusters and predictive of large Leydig cell clusters.

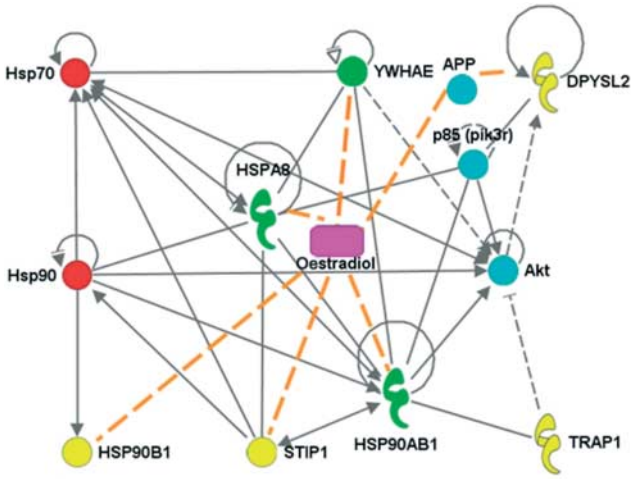

Figure 1 Pathway network for the eight proteins that were correlated with and/or predictive of foetal Leydig cell clustering and the capacity of the foetal Leydig cell to produce testosterone. Upregulated proteins are filled in green, downregulated proteins are filled in yellow. The upstream regulator oestradiol is filled in pink and the proteins regulated by oestradiol are indicated by the brown, dashed lines. Protein intermediates are filled in cyan and protein families are filled in red. Akt, serine/threonine-protein kinase; APP, amyloid beta A4 protein; p85, extracellular matrix protein 1.

Less scrutinised histologic changes in the testis may be the key

In most studies with phthalate exposures, certain histological changes associated with various components of the interstitium (such as vasculature, fibroblasts and wandering monocytes or macrophages called histiocytes) and seminiferous cords of the foetal testis other than the fate of the Leydig cells have not been considered or documented. In a companion study, that first reported Leydig cell aggregation following exposure to 750 mg DEHP/kg (Parks *et al.* 2000), interstitial vascular damage and also haemorrhage was observed (Gray *et al.* 2000). The sequelae of the vascular lesions – extravasation of erythrocytes followed by migration of histiocytes and subsequent inflammatory reaction with aggregation of various components of interstitial tissue over time – have been pictorially documented (Fig. 4 in

Gray *et al.* 2000). These conspicuous vascular lesions in the interstitium along with changes in the basement membranes of the adjacent seminiferous cords also have been documented at much lower exposure levels (10 or 100 mg DEHP/kg; Supplemental Figures 2 and 3 in Klinefelter *et al.* (2012)). Similar focal haemorrhage and aggregation of interstitial cellular components have been observed in PND5 rabbit testis following gestational exposure to 400 mg DBP/kg (Higuchi *et al.* 2003, Veeramachaneni, unpublished electron micrographs). These lesions in the rat and rabbit are shown in Fig. 2. One must wonder whether changes in vascular response are not one of the proximate consequences of elevated oestradiol. Alterations in proteins such as PDIA6, decreased in our study, and known to be oestradiol regulated (Fu & Zhu 2009) and involved in platelet aggregation may facilitate histological observations such as free-floating erythrocytes in the interstitial space, formation of erythrophagosomes and focal granulomata. When the haemorrhage was multifocal and extensive, coagulative necrosis involving both interstitial and seminiferous epithelial elements was observed at higher exposure levels (750 mg DEHP/kg; Gray *et al.* (2000)).

It is likely that similar vascular damage also occurs in other developing tissues and organs following phthalate exposures. The putative role of estrogen in phthalate exposure is probably not limited to the foetal testis. Indeed, xenoestrogens bisphenol A and phthalates have been implicated with aggravated symptoms of asthma via the action of oestrogen on oestrogen receptors that are widely expressed on immunocompetent cells (Bonds & Midoro-Horiuti 2013).

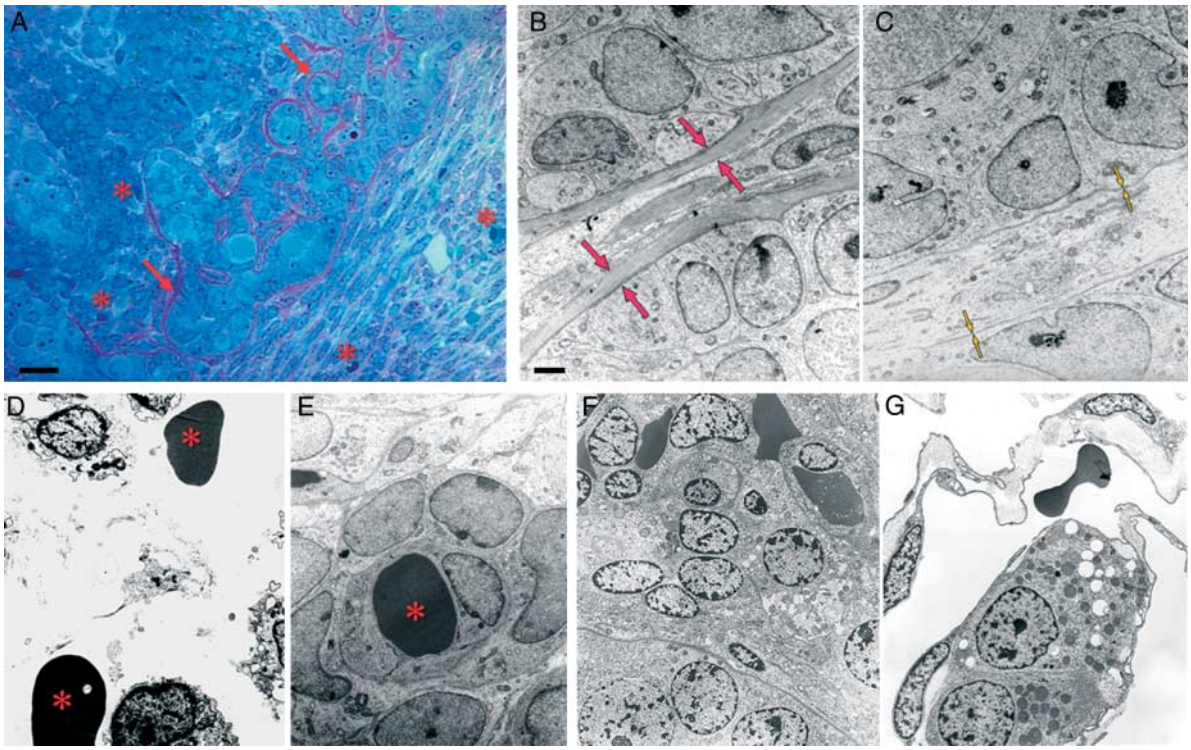

Figure 2 Light and transmission electron micrographs of testicular lesions in the rat and rabbit exposed to phthalates. All electron micrographs are produced at the same magnification (scale bar: 2 µm). (A, B, C, D, and E) Rats exposed to 0 or 100 mg DEHP/kg from GD13–19. (F and G) Rabbits exposed to 400 mg DBP/kg from GD15–29. (Panels B, C and D have been reformatted from supplemental figures published in Klinefelter *et al.* 2012 *Reproduction* **14** 747–761). (A) Dysgenetic seminiferous cords with thickened basement membranes (red arrows) and unusually large numbers of Leydig cells surrounding the dysgenetic cords in 100 mg DEHP-exposed rat. Note erythrocytes in the interstitial tissue and testicular capsule (asterisks), which are shown in electron micrographs in panels D and E. Semi-thin section stained with methylene blue and basic fuchsine staining of semi-thin section, Scale bar = 20 µm. (B and C) Ultrastructure of basal laminae of seminiferous cords in the GD19 foetal rat testes. Compare the relative thickness and number of layers of basal laminae of dysgenetic seminiferous cords of 100 mg DEHP-exposed rat (B) with those in unaffected seminiferous cords in control (C). Note the profiles shown in panel B are from the same block of tissue shown at a light microscopic level in panel A. (D and E) Erythrocytes in the interstitium of 100 mg DEHP-exposed GD-19 rat testes. Note erythrocytes (asterisks) in panel D are not bounded by capillary wall and are dispersed in the interstitium (haemorrhage). The erythrocyte in panel E is engulfed by a histiocyte forming an erythrophagosome, which is surrounded by aggregated fibroblasts or Leydig cell precursors indicating an inflammatory change. (F and G) Erythrocytes in the interstitium of rabbit testes. In this PND5 DBP-exposed rabbit testis (F), note gray irregular structures on the top are remnants of erythrocytes undergoing phagocytosis and accompanying aggregation of fibroblasts or Leydig cell precursors similar to that of DEHP-exposed rat testis. Panel G shows an erythrocyte (biconcave, dumb-bell shaped structure) outside a vessel in 24-week-old rabbit exposed to DBP *in utero*. Note two adult-type Leydig cells. This shows the impact of developmental exposure continues to manifest into adulthood.

Important species differences and human relevance

There is a significant problem with an attempt to relate testosterone data derived from phthalate exposure of the foetal rat testis to potential foetal human testis risk (reviewed by Johnson *et al.* (2012)). Phthalates do not seem to compromise testosterone production in other species studied. In the foetal mouse testis, while 500 mg DBP/kg exposure does result in increased seminiferous cord diameter and the formation of multinucleated germ cells, testosterone production is not altered (Gaido *et al.* 2007). Subsequent *in vitro* work indicated that exposure of the foetal mouse testis to mono ethyl hexyl phthalate alters germ cell development in an androgen- and oestradiol-independent manner (Lehraiki *et al.* 2009). However, this was based only on the number of germ cells lost during culture; formation of multinucleated germ cells was not evaluated. Interestingly, abnormal development of the genital tubercle including hypospadias was evident in foetal mice exposed to as low as 100 mg DEHP/kg; unfortunately, the litter was not used as the statistical unit and androgen levels were not measured (Liu *et al.* 2008).

Recently, one molecular difference between foetal mouse and rat Leydig cells exposed to DBP has been reported (Van den Driesche *et al.* 2012*a*, 2012*b*). The expression of chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), known to repress testosterone biosynthesis before the masculinisation window in the rat, is maintained by DBP in foetal rat Leydig cells but not in foetal mouse Leydig cells; the increased expression of COUP-TFII is further associated with decreased expression of pivotal steroidogenic regulators such as StAR. Interestingly, a significant increase in COUP-TFII was observed in foetal rat Leydig cells exposed to only 20 mg/kg DBP, and this expression doubled when intratesticular testosterone decreased at 100 mg/kg.

The only report of a phthalate-induced decrease in testosterone in mice was documented in the Kummings mouse strain exposed to 500 mg DEHP/kg (Wu *et al.* 2010). How this Chinese mouse strain compares with the more commonly used strains such as the CD1 and the C57Bl/6 remains to be determined. What is also unique is that their data show that testosterone is not only reduced significantly at GD19 and PND3 but also at PND21 and PND56. This persistent effect of the foetal exposure is not seen in rats (Klinefelter, unpublished). When rabbits were exposed *in utero* to 400 mg DBP/kg, a significant decrease in serum testosterone observed at 6 weeks was not observed later on during development (Higuchi *et al.* 2003). There is one report of gestational plus lactational exposure to 750 mg DEHP/kg, resulting in reduced serum testosterone on PND49 in the rat (Lin *et al.* 2009).

In vitro work demonstrated that monobutyl phthalate reduced testosterone production and induced Leydig cell aggregation in foetal rat testis but not in foetal human testis (Hallmark *et al.* 2007). More recently, Mitchell *et al.* (2012) demonstrated that the foetal human testis is also refractory with respect to testosterone production. They used a testis xenograft model, in which a human foetal testis graft is transplanted into castrate nude recipient mice before phthalate exposure (500 mg DBP/kg) for several days. Serum testosterone and seminal vesicle weight in these mice were no different from comparable vehicle control mice. In contrast, when rat foetal testis grafts were implanted before treatment, serum testosterone and seminal vesicle weight were significantly reduced compared with controls.

More recently, Heger *et al.* (2012) published results of a similar study. Their work also used a foetal testis xenograft model but compared the response of mouse, rat and human xenografts with intact nude rat and mouse hosts rather than castrate nude mouse hosts. Hosts were exposed to 100, 250 or 500 mg/kg DBP. Multinucleated germ cells were evident in DBP exposed xenografts of all three species. While exposed rat xenografts produced significantly less testosterone *ex vivo*, exposed mouse xenografts failed to produce less testosterone. Importantly, by using both mouse and rat hosts for both mouse and rat xenografts, the host was ruled out as contributing to these observed differential responses. When human foetal testis xenografts were transplanted into rat hosts, DBP was found to increase the formation of multinucleated germ cells but failed to alter any of the steroidogenic enzymes altered in rat grafts. Interestingly, clustering of foetal Leydig cells was not reported in any of these models.

To summarise, gestational exposure to phthalates causes significant reduction in testosterone production by the foetal Leydig cell in the rat and rabbit, but not in the mouse (most strains) and the human, which appear to be refractory. However, other facets of dysgenesis, specifically alterations in the seminiferous tubule, still occur even when testosterone remains unaffected. Whether Leydig cell aggregation that is typically observed in foetal rat testis following phthalate exposure also occurs in other species is not known.

That ESR1 is expressed in both the foetal rat and mouse testes, specifically the Leydig cell (O'Donnell *et al.* 2001), obviously does not help explain the observed differences in phenotypes between the rat and the mouse. It does however support the difference observed with foetal human xenografts, as human foetal Leydig cells appear to be devoid of ESR1 (Boukari *et al.* 2007). This is also consistent with the recent report by Mitchell *et al.* (2013) which demonstrated that DES failed to reduce testosterone production by human testis xenografts. These authors also did not detect ESR1 in human foetal Leydig cells and concluded that the presence or absence of ESR1 expression was the distinguishing

feature in the differential response of the foetal rat and human testis to DES.

Recently, Savchuk *et al.* (2013) have investigated two strains of mice with vastly different serum testosterone:oestradiol ratios; CBA/Lac which has a high ratio and C57BL/6 which has a low ratio. They found that testosterone is not reduced when immature mouse Leydig cells of either strain were exposed to oestradiol *in vitro*. To our knowledge, oestradiol has not yet been measured in the plasma or testis of the phthalate-exposed male mouse or human; there may be no elevation of oestradiol. At least in the rat, it is possible that phthalate exposure to the foetal rat testis elevates oestradiol, which in turn activates ESR1 and ultimately decreases the expression of proteins such as DPYL2, known to be a factor in cellular differentiation and migration. This could prompt Leydig cell aggregation and lead to decreased testosterone production as the Leydig cells further dedifferentiate. Indeed, the decrease in DPYL2 at 10 mg DEHP/kg was correlated with the formation of small Leydig cell clusters and was predictive of large Leydig cell clusters at 100 mg/kg with compromised testosterone.

Figure 3 is our attempt to model events which appear to be involved in the paths-to-phenotypes when the foetal rat testis is exposed to phthalate. Phthalate enhances aromatase in a variety of tissues, resulting in elevated circulating oestradiol. The increased oestradiol has proximate action on specific proteins such as PDIA6 and alterations in the vasculature ensue. The most obvious sequel to vascular change appears to be haemorrhage, which results in as inflammatory response in organs such as testes and lungs. In the testis, the inflammatory response appears to alter the composition of interstitial fluid, and this plus activation of specific proteins (e.g. DPYL2) facilitates the aggregation of foetal Leydig cells. Following the onset of Leydig cell aggregation, steroidogenic capacity decreases, perhaps resulting from facets of dedifferentiation of the foetal Leydig cell (e.g. persistent COUP-TFII signaling?). This in turn leads to a significant decrease in the testosterone:oestradiol ratio which results in impaired differentiation of the seminiferous tubules and accessory sex organs. The extent to which this is influenced solely by decreased testosterone vs a decrease in the testosterone:oestradiol ratio remains unknown. However, comparing Sertoli cell maturation in oestrogen- and antiandrogen-treated adult men (in gender reassignment cases) with that of typical (spontaneous) cases of cryptorchid adults, Nistal *et al.* (2013) concluded that therapeutic oestrogen and antiandrogen exposure causes true Sertoli cell dedifferentiation in adults and thus implicated environmental oestrogen exposure as the potential cause for impaired differentiation and development of foetal testis.

The linkage between adverse outcome pathways in the phthalate-exposed foetal testis and predictive biomarkers of dysgenetic phenotypes of the foetal testis is obvious and can only improve our ability to predict dysgenesis in an animal model following numerous exposures. This could lead to new directions for research on the relevance of exposure of the human foetal testis, including early detection of TDS in the human foetus. This may be best done by evaluating those proteins that are upregulated as these are more likely to appear in accessible matrices such as amniotic fluid, blood or urine. In our final pathway network of eight proteins that were correlated with and or predictive of endpoints, three were upregulated. We are currently trying to determine if any of these proteins, i.e. HSPA8, YWHAE or HSP90AB1, can be measured in the plasma of either the dams or male foetuses exposed to DEHP.

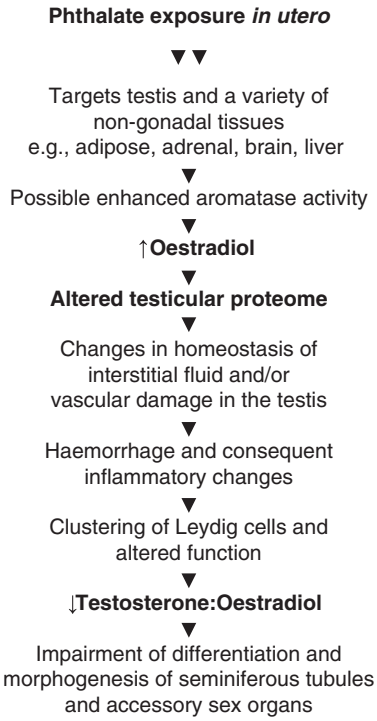

Figure 3 Diagram to model the paths-to-phenotypes in the foetal rat testis exposed to phthalate. An initial elevation in circulating oestradiol results in vascular changes (i.e. haemorrhage) in organs such as the lungs and testes via altered proteome. The ensuing inflammatory response alters the interstitial tissue compartment of the testis. This plus an activation of oestradiol-dependent proteins prompts aggregation of the foetal Leydig cells. Once significant aggregation occurs, the Leydig cells dedifferentiate with respect to their ability to produce testosterone. The resulting decline in the testosterone:oestradiol ratio compromises differentiation of the seminiferous cords and sex accessory glands.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. This manuscript has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. EPA, and

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