Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate *in utero*

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Exposures to di-(2-ethylhexyl) phthalate (DEHP) have been shown to be associated with decreased adult testosterone (T) levels and increased Leydig cell numbers. As yet, little is known about DEHP effects *in utero* on fetal Leydig cells (FLC). The present study investigated effects of DEHP on FLC function. Pregnant Long–Evans female rats received vehicle (corn oil) or DEHP at 10, 100, or 750 mg/kg by oral gavage from gestational day (GD)2–20. At GD21, T production, FLC numbers and distribution, and testicular gene expression were examined. The percentage of FLC clusters containing 6–30 cells increased in all treatment groups, with 29 ± 2% in control vs. 37 ± 3, 35 ± 3, and 56 ± 4% in rats receiving 10, 100, and 750 mg/kg DEHP, respectively. In contrast, FLC numbers were 33% and 39% lower than control after exposures to 100 and 750 mg/kg DEHP, respectively. At these doses, mRNA levels of leukemia inhibitory factor (LIF) increased. LIF was found to induce cell aggregation in FLCs *in vitro*, consistent with the hypothesis that DEHP induced FLC aggregation. Testicular T levels were doubled by the 10 mg/kg dose and halved at 750 mg/kg. The mRNA levels of IGF-1 and c-*Kit* ligand (KITL) were induced by 10 mg/kg DEHP. These results, taken together, indicate that fetal exposures to DEHP have effects on FLC number, distribution, and most importantly, steroidogenic capacity and suggest that abnormal expressions of IGF1, KITL, and LIF genes may contribute to the reproductive toxicity of phthalates.

di-(2-ethylhexyl) phthalate | testosterone | reproduction | endocrine disruptor | steroidogenesis

Phthalates, widely used as plasticizers and solvents, are commonly found in a variety of consumer products including cosmetics, toys, medical tubing, and catheters and in the environment as an industrial waste product. Increasing public concern over lack of regulation on their use in the United States, in contrast to the European Union and 14 other countries (1), has arisen in response to reports that exposures to phthalates may be linked to abnormal reproductive development in the human male (2, 3). Epidemiological studies show statistical correlations between serum concentrations of phthalate monoesters, the primary metabolites of phthalates, and the incidence of anomalies such as cryptorchidism and shortened anogenital distance (AGD) (4, 5). Di-(2-ethylhexyl) phthalate (DEHP), the most abundant phthalate in the environment, has been shown to have adverse effects on androgen synthesis in the rodent (6).

The Agency for Toxic Substances and Disease Registry reported that, although exposure to DEHP is generally low, the exposures of preterm infants can be as high as 10–20 mg per day (7). Controversy exists over whether DEHP, at the levels found in the environment, is harmful to humans, because most studies have been conducted in rodents administered high doses. In previous studies, we showed that the administration of low-dose (10 mg/kg body weight) DEHP for 28 days during pubertal development caused elevations in testosterone (T) (8, 9). This was in contrast to doses >750 mg/kg, which significantly reduced serum and testicular T (10). Similar results were obtained in rats treated by inhalation with a dose of DEHP comparable to 1~10 mg/kg per day orally (11) and in boars exposed intramuscularly to DEHP at 50 mg/kg twice per week during puberty (12).

Fetal Leydig cells (FLCs) are a distinct population of Leydig cells that originate in the fetus. These cells secrete high levels of T that are critical for development of the penis and sex accessory glands and, along with another FLC product, insulin-like growth factor 3 (INSL3), for the scrotal descent of the testis. FLCs reach their peak numbers around birth and then gradually involute after postnatal day 7 such that few, if any, are present in the adult testis (13). The goal of the present study was to examine the effects of DEHP dose on the prenatal production of T by the FLCs. We postulated that FLCs might have distinctive responses to DEHP compared to adult Leydig cells (ALCs), given that these cells respond to different regulatory factors. For example, FLCs develop normally and secrete T in luteinizing hormone (LH) receptor knockout mice, whereas LH is required for the formation and steroidogenic function of ALCs (14). Given the essential role of fetal T production, our objective was to determine whether and how DEHP treatment *in utero* perturbs these cells and thus steroidogenic function in the fetus.

Results

General Reproductive Toxicology. As shown in Table 1, none of the DEHP doses affected the body weights of dams, birth rates (number of dams that delivered litters divided by the number of dams with an established pregnancy as defined by the presence of a vaginal plug), numbers of pups per dam, male-to-female sex ratio, or male pup body weight at gestational day (GD)21 in comparison to the controls. The AGD of the male pups was significantly reduced ($P < 0.001$) at a dose of 750 mg/kg DEHP (Table 1). Because AGD is a function of androgen action, we examined whether phthalate treatment caused a reduction in T synthesis by the fetal testis.

Testicular T Production. Intratesticular T concentrations at GD21 were measured to assess the steroidogenic function of the FLCs.

Author contributions: H.L. and R.-S.G. contributed equally to this work; R.-S.G., Q.-Q.L., and X.-K.L. designed research; H.L., R.-S.G., G.-R.C., G.-X.H., L.D., D.O.H., C.M.S., and M.P.H. performed research; R.-S.G. and X.-K.L. analyzed data; and R.-S.G., D.O.H., X.-K.L., and M.P.H. wrote the paper.

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Table 1. Reproductive parameters before and after exposure to DEHP for 19 days

Parameters	DEHP, mg/kg per day			
	0	10	100	750
Dams				
Number of dams	6	6	6	9
Body weight before, g	224 ± 7	218 ± 3	222 ± 2	218 ± 6
Body weight after, g	335 ± 11	323 ± 14	352 ± 6	320 ± 12
Pup numbers per dam	11 ± 2	12 ± 1	13 ± 1	11 ± 3
Birth rate	6/6	6/6	6/6	7/9
Pup male, %	40 ± 10	44 ± 7	56 ± 7	57 ± 7
Male pups				
Number of pups	30	34	41	51
Body weight, g	6.0 ± 0.1	5.6 ± 0.1	5.8 ± 0.1	5.6 ± 0.1
AGD, mm	4.5 ± 0.1	4.3 ± 0.1	4.8 ± 0.1	4.1 ± 0.1*†
Testicular T, ng/mg	0.89 ± 0.13	1.4 ± 0.19*	0.69 ± 0.24	0.29 ± 0.07*

Dams of Long–Evans rats were gavaged with DEHP from GD2 to GD20. Parameters were measured at GD21. Values are mean ± SEM, $n = 6$ for dam's data. *, $P < 0.001$.
†One-way ANOVA with Dunnett's Multiple Comparison Test vs. control, significantly higher than control values.

Relative to controls, T values were 50% higher in the 10 mg/kg DEHP exposure group but 66% lower in the 750 mg/kg group (Fig. 1*A* and Table 1). T values were not significantly different from controls in the 100 mg/kg group.

Leydig Cell Distribution, Number, and Size. FLCs are not uniformly distributed in the interstitial space of the GD21 testis but rather are found in discrete clusters (15); only 20 ± 1% were present as single cell (Table 2). The percentage of single cell decreased significantly from controls with DEHP exposures, and the average number of FLCs per cluster also was greater after DEHP (Table 2). This is evident in micrographs (Fig. 1 *B* vs. *C*) and particularly so after quantification. Thus, as seen in Table 2, the numbers of FLCs per cluster increased even at the 10 mg/kg dose. At 750 mg/kg, some clusters contained >100 cells, and the frequency of FLC clusters containing 30 or more cells was 7% compared with 1% in vehicle controls. The increased FLC cluster sizes associated with DEHP exposures of 100 and 750 mg/kg were associated with reduced testis weights (Fig. 1*D*), reduced Leydig cell numbers per testis (Fig. 1*E*), and reduced average Leydig cell volume (Fig. 1*F*).

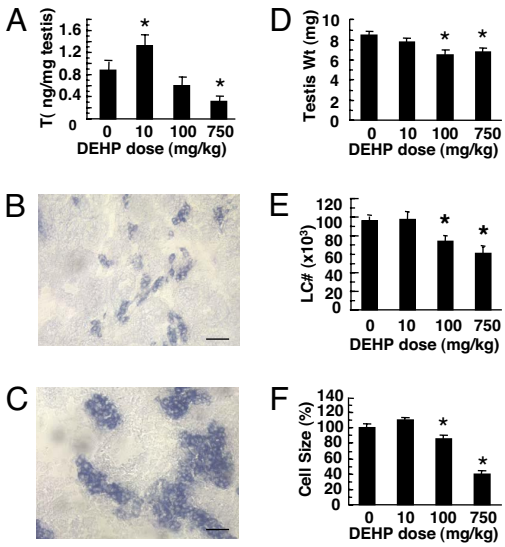

Fig. 1. Effects of *in utero* DEHP exposure on testicular T levels, testis weights, Leydig cell aggregation, number, and distribution frequencies. Pregnant dams were gavaged with 0 (control), 10, 100, or 750 mg/kg DEHP from GD2 to GD20. Measurements were made by using male pups on GD21. Data are represented as mean ± SEM. (*A*) Testicular T levels ($n = 10$–11). (*B* and *C*) Histochemical staining of 3βHSD in FLCs of control (*B*) and after 750 mg/kg DEHP (*C*). (Scale bar, 50 μm.) (*D*) Testis weights ($n = 8$–10). (*E*) FLC numbers ($n = 8$). (*F*) FLC volume as percentage of control ($n = 8$). *, Significant difference compared with control (DEHP 0 mg/kg) at $P < 0.05$.

Effect of LIF on Leydig Cell Aggregation. As shown in Fig. 2, *Lif* expression was increased in response to 750 mg/kg DEHP dose. This dose also was associated with larger-sized FLC clusters (Fig. 1). To determine whether there might be a cause–effect relationship between increased LIF and FLC aggregation, the effects of LIF on cultured FLCs were analyzed. In response to LIF at 1 ng/ml (IC_{50} for LH-stimulated T production) or 10 ng/ml (concentration showing maximal inhibition) of LIF for 12 h *in vitro*, FLC aggregations were increased in frequency (Table 3). LIF antibody significantly antagonized the effects of LIF (Table 3).

Testicular Cell Gene Expression. A panel of genetic markers was selected to assess cell type-specific function in the testis after prenatal exposure to phthalate. As shown in Fig. 2, the markers included genes encoding growth factors (*Igf1*, *Kitl*, *Lif*), their receptors (*Igf1r*, *Kit*, *Lhcgr*, *Pdgfra*), cholesterol transporters (*Scarb1*, *Star*), and steroidogenic enzymes (*Cyp11a1*, *Cyp19*, *Sdr5a1*). In all, transcript levels of 37 testicular mRNAs were examined by real-time PCR. The gene names, symbols, and functions are listed in supporting information (SI) Table S1. Among the growth factors, *Igf1* and *Kitl* were elevated in response to the 10-mg/kg DEHP dose, whereas *Lif* and *Pdgfb* mRNAs were increased in response to the 750-mg/kg dose. *Amh*, *Pdgfa*, and *Fgf2* expression levels were unchanged (data not shown). mRNA levels of growth factor receptors *Lhcgr* and

Table 2. Frequency distribution of cluster sizes of FLCs after *in utero* exposure to DEHP

Frequency, %	DEHP dose, mg/kg			
Cell no. per cluster	0	10	100	750
1	20 ± 1	10 ± 2**	12 ± 2**	4 ± 1***
2–5	50 ± 2	51 ± 2	51 ± 1	34 ± 5
6–30	29 ± 2	37 ± 2*	35 ± 3*	56 ± 4***
>30	1 ± 1	1 ± 0.4	1 ± 0.4	7 ± 2***
Average	2 ± 0.2	3 ± 0.4	4 ± 0.3	9 ± 1***

Mean ± SEM, $n = 6$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; in comparison to vehicle control (0 mg/kg).

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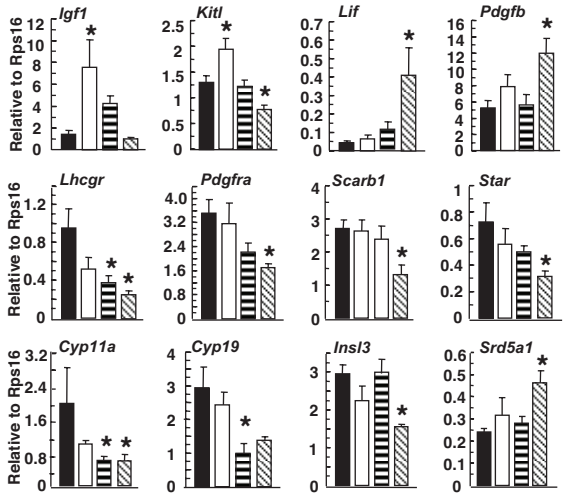

Fig. 2. Real-time PCR analysis of testicular gene expression after *in utero* DEHP exposures. Pregnant dams were gavaged with 0 (control, ■), 10(□), 100 (▤), or 750 (▧) mg/kg DEHP from GD2 to GD20. Data, from male pups on GD21 are presented as mean ± SEM (n = 5–6). *, Significant difference compared with control (DEHP 0 mg/kg) at $P < 0.05$.

Pdgfra and the cholesterol transporters *Scarb1* and *Star* were decreased in the 750-mg/kg DEHP group. No change was observed in the expression levels of *Kit*, *Igf1r*, *Amhr2*, *Fshr*, or *Pbr* (data not shown). In addition, there was no effect of DEHP exposure on the nuclear receptors (*Ar*, *Esr1*, *Nr5a1*, *Ppara*, *Pparg*, and *Pcna*) and transcription factors (*Gata4* and *Rcl*) (data not shown). The steroidogenic enzyme genes, *Cyp11a1* (100 and 750 mg/kg) and *Cyp19* (100 mg/kg) had decreased levels of expression in response to DEHP, whereas the *Sdr5a1* mRNA level (5α-reductase, 750 mg/kg) increased (Fig. 2). These changes are consistent with reduced circulating T levels (Fig. 1*A*). The suppression of steroidogenic function appeared to result from selective rather than generalized modulation of gene expression. For example, mRNA levels of *Hsd3b1*, *Cyp17*, *Hsd17b3*, *Hsd17b12*, *Gja1*, and *Trpm2* were unchanged (data not shown). *Insl3*, the protein product of which is involved in testis descent, was significantly reduced in the 750-mg/kg DEHP group.

T Biosynthetic Enzyme Protein Levels. T biosynthetic enzyme protein levels were also evaluated. As shown in Fig. 3, P450scc was reduced at high DEHP dose, but 3βHSD, P450c17, and 17βHSD were not affected. This also was true of their mRNAs. These results suggest the reduced levels of P450scc might be integrally involved in DEHP-mediated inhibition of T production at the higher DEHP doses.

Discussion

DEHP and T Production. Steroidogenic activity in FLCs peaks 1–2 days before birth on GD19 (16). The T produced at this time is

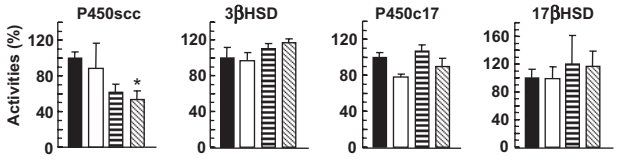

Fig. 3. Steroidogenic enzyme levels in testes after *in utero* DEHP exposures. Pregnant dams were gavaged with 0 (control, ■), 10 (□), 100 (▤), or 750 (▧) mg/kg DEHP from GD2 to GD20. Enzyme activities were measured in the testes of GD21 pups. Mean ± SEM (n = 4). *, Significant difference compared with control (DEHP 0 mg/kg) at $P < 0.05$.

critical for male secondary sexual differentiation (i.e., development of the penis and sex accessory glands) (17). FLCs also produce the hormone INSL3, which binds to the leucine-rich repeat-containing G protein-coupled receptor 8 (LRG8). INSL3 specifically binds LRG8s in the gubernaculum (18) and, together with androgen, induces scrotal descent of the testis (18, 19). Thus, interference with the development of FLCs may be a precipitating cause of cryptorchidism (20). Indeed, the incidence of cryptorchidism is significantly increased during the neonatal period after exposures to high doses of phthalates (20). Exposure to doses of phthalates at 250 mg/kg per day or higher also have been reported to be associated with increased frequencies of underdeveloped epididymides, testicular atrophy, hypospadias, and ectopic or absent testes (21).

The present study shows that low-dose exposure to DEHP (10 mg/kg) *in utero* resulted in increased testicular T levels, whereas a higher dose of DEHP (750 mg/kg) resulted in reduced T levels and AGD. Previous studies similarly showed that low and high doses of DEHP, when administered postnatally, had differential effects on T levels (6, 11, 12). Low-dose exposures that abnormally elevate T levels are likely to be as undesirable as the suppressive effects documented at high doses. The explanation for the differential effects of low- and high-DEHP doses is uncertain. Our and previous results suggest several plausible possibilities. With respect to the observation that testicular T levels in the 10 mg/kg group were higher than the controls, IGF-1 has been reported to stimulate T production by FLCs (22). Among the 36 genes that potentially affect FLC development and steroid production, *Igf1* and *Kitl* had significantly elevated expression levels. IGF-1 is produced by both Leydig and Sertoli cells, and c-*Kit* ligand (KITL) is exclusively produced by Sertoli cells. IGF-1 and KITL are required to stimulate T production by FLCs. Levels of the mRNA for *Igf1* increased 5-fold after exposure to 10 mg/kg DEHP; *Kitl* increased 50%. These increases are consistent with the increased T production seen in response to 10 mg/kg DEHP. Thus, one possibility is that increases in IGF-1 and KITL may explain the significant elevations of T in animals exposed to 10 mg/kg DEHP. It should be pointed out that elevations in T levels in response to low-dose DEHP exposures also were noted in DEHP-treated pubertal rats (9, 11, 12).

Higher doses of DEHP resulted in reduced FLC numbers, cell size, and steroidogenic enzyme activities, consistent with the

Table 3. Aggregation of FLC after leukemia inhibitory factor treatment *in vitro*

	Frequency, %			
	Control	1 ng/ml LIF	10 ng/ml LIF	10 ng/ml LIF + AB
Single cells	96 ± 2	75 ± 1***	61 ± 3***	90 ± 2
Clusters (two or more cells)	5 ± 2	25 ± 1***	39 ± 3***	10 ± 2

Values are mean ± SEM, n = 4–5 preparations. AB as LIF antibody (1:100 dilution). One-way ANOVA with Newman–Keuls Multiple Comparison Test. ***, Significant difference compared with control at $P < 0.001$ in single cells or clusters.

reduced testicular T levels seen after high-dose exposures. In previous studies, similar high-dose effects of DEHP also were demonstrated in studies by using 500 mg/kg DBP, another phthalate ester that is structurally related to DEHP (23). Additionally, as seen in this study, decreased AGD in male fetuses has been observed after exposure of pregnant dams to either DBP or DEHP at doses of 500 mg/kg and above (23).

DEHP and FLC Aggregation. FLCs were aggregated rather than dispersed when doses of DEHP were increased. Previous studies similarly reported that high-dose exposures to DEHP or DBP *in utero* resulted in focal disruptions in the structure of the seminiferous epithelium and in abnormal aggregations of FLCs (24, 25). Stereological analyses have suggested that the increased FLC number per cluster in response to DEHP does not result from increased Leydig cell numbers (our results and ref. 26). In our study, a dose as low as 10 mg/kg per day for 20 days (GD2–GD20) elicited Leydig cell aggregation. The physiological consequences of FLC aggregation *per se* are uncertain, although there is evidence that it is associated with reduced fertility (27) and increased incidence of cryptorchidism (20). It seems unlikely that the expanded clusters of FLCs observed with DEHP represent tumors. Support for this comes from observations that FLC numbers were reduced, not increased, in response to DEHP, and that expression levels of the tumor transformation gene *Rcl* were unchanged (data not shown).

The mechanism by which FLCs become grouped in clusters is not well understood. One possible explanation is that these cells are initially more diffusely distributed but then form aggregates under the influence of local growth factors. One such growth factor that may be active in this respect is LIF, which is primarily produced by peritubular myoid cells (28). We herein showed that LIF caused dose-dependent increases in the cluster size of FLCs *in vitro*. This was consistent with the known ability of LIF to stimulate aggregation in other cell types such as platelets (29) and embryonic stem cells (30).

In conclusion, these results, taken together, indicate that fetal exposures to DEHP have effects on FLC number, distribution, and most importantly, steroidogenic capacity, and suggest that abnormal expressions of IGF1, KITL and LIF genes may contribute to the reproductive toxicity of phthalates.

Materials and Methods

Animals and Treatments. Long–Evans rats were purchased from Charles River. All studies were approved by The Rockefeller University's Animal Care and Use Committee (Protocol #04059). Adult pregnant dams were treated from GD2 to GD20 with 0 (control, $n = 6$), 10 ($n = 6$), 100 ($n = 6$), or 750 ($n = 9$) mg/kg DEHP (Sigma–Aldrich) in 1 ml/kg corn oil, administered daily by oral gavage. The body weights and AGD of male pups at GD21 were measured. The pups were killed by inhalation of CO_2 on GD21.

Immunohistochemical and Histochemical Staining. Frozen testes from rats (0, 10, 100, and 750 mg/kg DEHP) were embedded in the same blocks and cryostat-sectioned (8 μm). FLCs were identified by staining for 3βHSD (31). Cells also were immunohistochemically stained for P450scc by using a rabbit polyclonal antibody against P450scc (RDI Research Diagnostics), by the avidin/biotin method (Vectastain, Elite, ABC Kit, PK-6101, Vector Laboratories). In brief, endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol for 30 min. The sections then were incubated with P450scc antibody (diluted 1:500) and also stained for 3βHSD. The antibody–antigen complexes were visualized with diaminobenzidine (Peroxidase Substrate Kit, SK-4100, Vector Laboratories) and counterstained with Mayer's hematoxylin.

Cell Counts and Computer-Assisted Image Analysis. Testis sections were captured by using a Nikon Eclipse E800 microscope (Nikon) and a SPOT RT digital camera (model 2.3.0.; Diagnostic Instruments) interfaced to a computer. Cell numbers were estimated by using image analysis software (Image-Pro Plus, Media Cybernetics).

Stereological Analysis of FLC Number, Size, and Clusters. To enumerate FLC numbers, testicular tissues were sampled according to the Fractionator technique, as described in ref. 9. The total number of FLCs per testis was calculated by multiplying the number of FLCs counted in a known fraction of the testis by the inverse of the sampling probability, and average FLC numbers per testis per treatment group were determined. Average cell sizes were measured by tracing the profiles of ≈300 FLCs.

Numbers of clusters were counted, and frequency distributions were calculated.

Testicular T Analysis. Testicular steroids were extracted from the testes of control and DEHP-exposed pups ($n = 10$ control; $n = 12$ DEHP), as described. T concentrations were measured by a tritium-based RIA validated for use with rat antiserum, as described (8, 9).

RNA Quantification by Real-Time PCR. Total RNA was extracted from rat testes in TRIzol according to the manufacturer's instructions (Invitrogen). First-strand synthesis and real-time PCR were performed as described in ref. 32. Ribosomal protein S16 (Rps16) mRNA levels were assayed in all samples as internal controls. The primers of the other 36 genes were provided in Tables S1 and S2. These genes were: growth factors including insulin growth factor I (*Igf1*), Kit ligand (*Kitl*), antimulerian hormone (*Amh*), leukemia inhibitory factor (*Lif*), platelet growth factor A subunit (*Pdgfa*), platelet growth factor B subunit (*Pdgfb*), fibroblast growth factor 2 (*fgf2*), and insulin-like growth factor 3 (*Insl3*); cell proliferative genes cyclin G1 (*Ccng1*), cyclin D3 (*Ccnd3*), c-myc related gene Rcl (*Rcl*), and proliferation cell nuclear antigen (*Pcna*); membrane receptor genes including LH receptor (*Lhcgr*), insulin-like growth factor I receptor (*Igf1r*), platelet growth factor receptor α (*Pdgfr*), c-*kit* (*Kit*), anti-Mullerian hormone receptor 2 (*Amhr2*), and follicle stimulating hormone receptor (*Fshr*); cholesterol-transporting genes including cholesterol HDL receptor (*Scarb1*), steroidogenic acute regulatory protein (*Star*), and peripheral benzodiazepine receptor (*Pbr*); nuclear receptors and transcription factors estrogen receptor α (*Esr1*), androgen receptor (*Ar*), steroidogenic factor I (*Nr5a1*), peroxisome proliferative activated receptor α (*Ppara*), and peroxisome proliferative activated receptor γ (*Pparg*); Gata 4 (*Gata4*), steroidogenic enzyme genes including P450scc (*Cyp11a*), 3βHSD1 (*Hsd3b1*), P450c17 (*Cyp17*), 17βHSD3 (*Hsd17b3*), 17βHSD12 (*Hsd17b12*), 5α-reductase 1 (*Sdr5a1*), and P450 aromatase (*Cyp19*); and cell junction proteins including connex 43 (*Gja1*) and Clusterin (*Trmp2*). The relative mRNA levels of targeted genes were normalized to Rps16 (internal control gene) by using the standard curve method.

Leydig Cell Steroidogenic Enzymes. P450scc and 3βHSD intensities were measured in immunohistochemically stained cryostat sections by using image analysis software. The total intensity in cells from treated animals was expressed as a percentage relative to the untreated control. For measurements of P450c17 and 17βHSD3, 1 μM radiolabeled substrates (^{3}H-progesterone) or ^{3}H-androstenedione were added to 100 μg of protein (homogenized testis). After 120 min, products were examined by TLC separation, and radiometric scanning was performed as described.

Percoll Purification and Culture of FLCs. FLCs were isolated by the same procedure that was used successfully for immature Leydig cells (33). In brief, testes from 60 GD21 male rats were removed and decapsulated. Interstitial cells were dissociated with 0.25 mg/ml collagenase (collagenase-D, Roche Molecular Biochemicals) in medium 199 for 10 min at 34°C. The separated cells were resuspended in 55% isotonic Percoll. After centrifugation at 25,000 $\times$ *g* for 45 min at 4°C, the FLC fraction was collected between densities of 1.064 and 1.070 g/ml. The cells were resuspended in DMEM:F12 (1:1) supplemented with 1 mg/ml bovine albumin and plated to dishes. After plating the interstitial cells, FLCs attached to the dishes, resulting in enrichment ≈30–50% purity. FLCs ($0.1–0.5 \times 10^6$) were cultured in the presence of increasing concentrations of LIF for 24 h. Neutralization of LIF was performed by using LIF receptor antibody (1:100 dilution, sc-20087, Santa Cruz).

Statistical Analysis. Values are expressed as mean ± SEM, and data were analyzed by using one-way ANOVA. At $P < 0.05$, Dunnett's test was used to compare values from DEHP-treated animals to control values. For this purpose, GraphPad Prism (version 4, GraphPad Software) was used. Data for Leydig cell cluster number per testis were log-transformed before statistical analysis by ANOVA test because of their skewed distribution.

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