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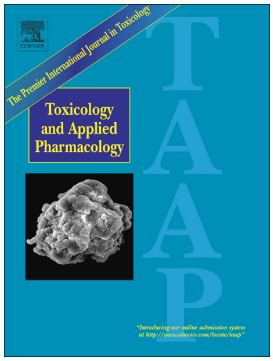

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Maternal exposure to di(2-ethylhexyl)phthalate (DEHP) promotes the transgenerational inheritance of adult-onset reproductive dysfunctions through the female germline in mice

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Running title: DEHP induction of transgenerational inheritance of reproductive disease in mice.

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ABSTRACT

Endocrine disruptors (EDs) are compounds known to promote transgenerational inheritance of adult-onset disease in subsequent generations after maternal exposure during fetal gonadal development. This study was designed to establish whether gestational and lactational exposure to the plasticizer di(2-ethylhexyl)phthalate (DEHP) at environmental doses promotes transgenerational effects on reproductive health in female offspring, as adults, over three generations in the mouse.

Gestating F0 mouse dams were exposed to 0, 0.05, 5 mg/kg/day DEHP in the diet from gestational day 0.5 until the end of lactation. The incidence of adult-onset disease in reproductive function was recorded in F1, F2 and F3 female offspring.

In adult F1 females, DEHP exposure induced reproductive adverse effects with: i) altered ovarian follicular dynamics with reduced primordial follicular reserve and a larger growing pre-antral follicle population, suggesting accelerated follicular recruitment; ii) reduced oocyte quality and embryonic developmental competence; iii) dysregulation of the expression profile of a panel of selected ovarian and pre-implantation embryonic genes. F2 and F3 female offspring displayed the same altered reproductive morphological phenotype and gene expression profiles as F1, thus showing transgenerational transmission of reproductive adverse effects along the female lineage.

These findings indicate that in mice exposure to DEHP at doses relevant to human exposure during gonadal sex determination significantly perturbs the reproductive indices of female adult offspring and subsequent generations. Evidence of transgenerational transmission has important implications for the reproductive health and fertility of animals and humans, significantly increasing the potential biohazards of this toxicant.

Keywords: Di(2-ethylhexyl)phthalate (DEHP), maternal exposure, ovary, reproductive toxicity, transgenerational, mouse

INTRODUCTION

Phthalates (phthalic acid esters) are plasticizers that are added to polymers, especially PVC, to impart softness and flexibility. They are widely used in the manufacture of a broad range of consumer goods such as medical devices, clothing, packaging, food containers, personal-care products and children's toys (Kavlock *et al.*, 2002). The most widely used phthalate is di(2-ethylhexyl) phthalate (DEHP), a hormone-interfering compound. With production of approximately one to four million tons per year, DEHP is one of the most widespread environmental contaminants worldwide (Akingbemi *et al.*, 2001; McKee *et al.*, 2004). Phthalates do not form strong linkages with the polymer, so they can diffuse throughout the matrix and leach into the environment (Petersen and Breindahl, 2000; Bosnir *et al.*, 2003). As a result, the general population is widely and continuously exposed to them through ingestion, inhalation or dermal absorption. Therefore, since phthalates exert endocrine disrupting activity, they arouse significant public health concerns (Silva *et al.*, 2004; Wittassek and Angerer, 2008).

The reproductive system is particularly susceptible to the endocrine disrupting activity of phthalates. Adverse effects include reduction in female fertility (Agarwal *et al.*, 1989), litter size/viability (Agarwal *et al.*, 1985a; Tyl *et al.*, 1988), sperm density and motility, and increased reproductive tract malformations (Agarwal *et al.*, 1985b). Phthalates can cross the placental barrier and also pass into breast milk. This poses a significant risk of damage for the developing fetus and newborn due to inappropriate effects on hormonal levels which, in turn, can result in permanent structural and functional changes (Dostal *et al.*, 1987; Latini *et al.*, 2003). Worryingly, many of the reproductive abnormalities derived from developmental exposure only become apparent after puberty (long-latency effect), which strongly hinders the development of a cause-and-effect relationship.

Recent studies in various species have demonstrated that pre- and peri-natal exposure to DEHP induces reproductive tract abnormalities in male offspring, such as hypospadias, undescended testis,

underdeveloped epididymis and seminiferous tubules, and reduced daily sperm production and sperm fertilizing ability (Barlow and Foster, 2003; Swan *et al.*, 2005; Pocar *et al.*, 2012a). In females the effects of maternal exposure to DEHP have been less well studied than in males but include negative effects on oocyte growth, meiotic maturation, and ovarian function (Lovekamp and Davis, 2001; Lovekamp-Swan and Davis, 2003). Our previous studies (Pocar *et al.*, 2012a) indicate that in maternally exposed female mice, DEHP induced an imbalance of pituitary-gonadal cross-talk in adulthood, leading to disruption of estrogen biosynthesis pathways in the gonads. This endocrine interference was correlated with impaired oocyte quality and early embryo development.

Of greatest concern is the fact that adverse effects in the ovary have the potential to affect subsequent generation(s) through the female germ cells (Gore *et al.*, 2014). Here a substantial distinction has to be made between intergenerational transmission involving direct exposure to the environmental factor, and transgenerational effects involving germ-line transmission without direct exposure of the affected generation (Skinner, 2008). For an ED to have truly transgenerational effects, exposure must occur during development, and the effects need to be detectable in the third generation (F3). This is because when a pregnant F0 female is exposed to an ED, besides F1, F2 too is directly exposed through the developing germ cells in F1. This makes the F3 generation the first generation not directly exposed to the ED (Crews and McLachlan, 2006; Skinner, 2008; Walker and Gore, 2011).

There are various reports of the ability of different environmental EDs such as bisphenol A (BPA – (Salian *et al.*, 2009)), dioxin (Bruner-Tran and Osteen, 2011), vinclozolin (Skinner and Anway, 2007; Stouder and Paoloni-Giacobino, 2010), polychlorinated biphenyls (PCBs – (Pocar *et al.*, 2012b)), and phthalates (Doyle *et al.*, 2013; Manikkam *et al.*, 2013; Chen *et al.*, 2015) to promote transgenerational inheritance.

An important mechanism for transgenerational transmission of disease as a function of early-life ED exposure is considered to involve epigenomic reprogramming due to the high plasticity of the epigenetic code during development (McLachlan *et al.*, 2001). Therefore, when EDs introduce

epigenetic changes during early development, they permanently alter the epigenome in the germ line, and these changes can be transmitted to subsequent generations. In contrast, when an ED introduces epigenetic changes during adult life, the changes within an individual occur in somatic cells and are not permanent or transmitted to subsequent generations (Christensen and Marsit, 2011; Skinner, 2014).

As mentioned before, phthalates have been reported to induce transgenerational reproductive dysfunction. However, in these studies phthalates were used at pharmacological doses within a plastic compound mixture (Manikkam *et al.*, 2013), or male reproductive dysfunction was recorded (Doyle *et al.*, 2013; Chen *et al.*, 2015). Information about transgenerational transmission of disease through the female lineage is still lacking.

Considering our previous results showing that gestational exposure to environmental doses of DEHP caused adverse effects in female F1 (Pocar *et al.*, 2012a), in the present study we investigated whether these effects were inherited by further generations up to F3 along the female lineage. Treatment covering the whole gestational and lactational period was scheduled, so as to cover the full time window of female reproductive system development in the mouse, including epigenetic DNA reprogramming events in female germ cells (Surani *et al.*, 2008). DEHP dosages and the administration with food were chosen for their relevance to human exposure.

MATERIALS AND METHODS

Animals

Five-week old virgin female CD-1 mice were purchased from Charles River (Calco, Italy) and allowed to acclimatize for two weeks. They were maintained in the animal facilities of the Dept. of Veterinary Medicine, University of Milan, under controlled conditions (23 ± 1°C, 12h light/dark cycle). Standard pellet food (4RF21, Charles River) and tap water were available *ad libitum*. Groups of two or three females were mated with one male overnight and the day of the vaginal plug was considered day 0.5 of gestation (day post-coitum - dpc +0.5). The pregnant females were randomly assigned to the treatment groups and housed individually in type II cages with stainless steel covers and hard wood shavings as bedding. Care and experimental procedures with mice were in accordance with Italian national regulations and were approved by the University of Milan's ethics committee.

Treatments and doses

DEHP (Sigma-Aldrich, Hamburg, Germany) was diluted in commercial sunflower oil and used by a specialized company (Altromin, Lage, Germany) to prepare treated chow. Pregnant mice were given diets formulated to contain known amounts of DEHP or vehicle from dpc 0 throughout lactation until weaning (post-natal day 21 – PND 21). Two or three pregnant mice were randomly assigned to the groups, and the experiment was replicated at least three times (total 7-10 dams/treatment). The exposure period for dams was chosen so as to cover the whole time window of reproductive system development in the mouse.

Specifically, dams received a diet formulated to ensure a mean daily intake of: control group – vehicle only; DEHP – 0.05 mg/kg/day and 5 mg/kg/day. The amount of DEHP added to the chow to obtain the desired doses was calculated on the basis of the mean daily food intake of CD1 mice, previously described (Pocar *et al.*, 2012a). Each batch of diet was tested before use in an accredited

laboratory (SGS Laboratory GmbH, Hamburg, Germany). The doses were selected in order to overlap the DEHP concentrations reported as environmental exposure in humans (Kavlock *et al.*, 2002).

Experimental design

On PND 21 F1 pups in both experimental and control groups were sexed and body weight was recorded. Female pups from each litter were housed in separate groups up to sexual maturity (PND 42), Standard pellet food (Charles River 4RF21) and tap water were available *ad libitum*.

To study the potential transmission of DEHP effects over multiple generations, at least seven F1 females from different litters were randomly selected and naturally mated with CD-1 non-exposed males of proven fertility, in order to obtain F2 offspring. At sexual maturity, at least seven F2 females from different litters were randomly chosen and mated as described for F1. The experiment ended when F3 offspring reached adult age (PND 42) (Figure 1).

To serve as controls, at least seven F1 females from different litters in the vehicle-treated group were similarly arranged into breeding pairs as described for experimental groups to produce control progeny animals in F2 generation. The same breeding scheme was applied to produce control F3 generation from F2 control dams. To minimize possible environment effects, experimental and control groups were processed identically throughout the whole experiment and litters of the same generation were housed together in the same room.

On PND 42, at least three virgin mice per litter from the experimental and control groups were randomly selected in each generation for autopsy. Livers, pituitaries and reproductive organs were removed, weighed and either snap-frozen in liquid nitrogen, or formalin-fixed for later analysis. The remaining F1, F2 and F3 virgin females were fertilized *in vitro* as described below.

In vitro fertilization and embryo culture

F1, F2 and F3 virgin females were superovulated by intraperitoneal (i.p.) injection of 3.5 IU Folligon (PMSG, Intervet International, Netherlands), followed 48h later by an i.p. injection of 5 IU Chorulon (hCG, Intervet).

Spermatozoa were obtained from the cauda epididymis of untreated male mice. Both cauda were dissected out from the body and transferred into 500 µL of Whittingham medium previously equilibrated (37°C at 5% CO2 in air). Sperm were passively released into the culture medium by puncturing the cauda 3-4 times with a 27G needle and capacitated for 60 min in Whittingham medium (37°C at 5% CO2 in air). Fourteen hours post-hCG, cumulus oocyte complexes were recovered from oviducts in M2 medium (Sigma-Aldrich). After rinsing in Whittingham medium, cumulus-oocyte complexes were inseminated with $2*10^6$ capacitated spermatozoa. Putatively fertilized eggs (6h post-insemination) were then transferred to 250 µL drops of M16 medium (Sigma-Aldrich), covered with paraffin oil and incubated at 37°C at 5% CO2 in air for another 96h. Cleavage and blastocyst rates were recorded respectively 24h and 96h post-insemination.

Histological analysis

Ovaries from F1, F2 and F3 female offspring were fixed in 10% neutral buffered formalin then embedded in paraffin. Specimens were serially sectioned (8 µm), mounted on glass slides and stained with hematoxylin and eosin (HE) according to standard procedures. Follicle counts were taken as described by Tomic et al. (Tomic *et al.*, 2002). Briefly, a stratified sample consisting of ten sections was used to establish the number of different follicle classes per ovary. The sections selected from each ovary were randomized and follicles were counted on the whole section. We only counted follicles with a visible nucleolus in the oocyte so as to avoid counting them twice. We then multiplied the number of follicles in the marked sections by 10 (because every 10th section

was analyzed) and then by 8 (to account for section thickness) to obtain the total number in each ovary.

Follicles were classified according to Flaws et al. (Flaws *et al.*, 2001), as i) primordial when they contained an intact oocyte surrounded by a one-layer ring of fusiform granulosa cells, ii) pre-antral if they consisted of an oocyte and one or more layer of cuboidal granulosa cells, iii) antral if they contained more than one layer of granulosa cells and an antral space, and iv) end-stage atretic if they contained zona pellucida remnants. All sections were evaluated "blind", by an operator with with no knowledge of the treatment group of the animals.

RNA Isolation and reverse-transcription PCR

Total RNA was isolated from one ovary of all mice undergoing autopsy, using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Total RNA was checked for integrity and DNA contamination using an ultraviolet (UV) spectrophotometer and 1.3% agarose gel electrophoresis. One μg of total RNA extracted from each sample was used to synthesize the cDNA using a SuperScript kit (Invitrogen). The RT reaction was carried out at 42ºC for 1h, and terminated by heating at 94ºC for 2 min.

Polyadenylated [poly(A)+] RNA from pre-implantation blastocysts was extracted using Dynabeads mRNA DIRECT kit (Deutsche Dynal, Hamburg, Germany). Briefly, pools of six blastocysts were lysed using 50 μL of lysis buffer. After lysis, 10 μL prewashed dynabeads-oligo (deoxythymidine) were pipetted into the tube, and poly(A)+ RNAs binding to oligo(deoxythymidine) was allowed for 5 min at room temperature. The beads were then separated with a Dynal MPC-E magnetic separator and washed twice with 50 μL washing buffer A [10 mmol Tris-HCl (pH 8.0), 0.15 mmol LiCl, 1 mmol EDTA, and 0.1% (wt./vol) sodium dodecyl sulfate] and three times with 50 μL washing buffer B [10 mmol Tris-HCl (pH 8.0), 0.15 mmol LiCl, and 1 mmol EDTA]. Poly(A)+ RNAs were then eluted from the beads by incubation in 11 μL

diethylpyrocarbonate-treated sterile water at 65°C for 2 min. Aliquots were immediately used for RT using the PCR Core Kit (Perkin Elmer, Wellesley, MA), with 2.5 μmol random hexamers to obtain the widest array of cDNAs. The RT reaction was carried out in a final volume of 20 μL at 25°C for 10 min and 42°C for 1h, followed by a denaturation step at 99°C for 5 min and immediate cooling on ice.

Table 1 lists the primers and PCR conditions for the genes. In ovaries specific transcripts were chosen for their correlation with: gonadotropin response and steroidogenesis (*Fsh-R, Lh-R, Star, Cyp17a1, Cyp19a1, Pgr*), and oocyte quality and follicle health (*Gdf9, Bmp15, Pten*). In blastocysts specific mRNAs (*Oct4, Nanog, Gata3, Cdx-2, Eomes, Lif and Lifr*) were selected for their role in embryonic development and trophoblast differentiation and implantation.

For each set of primers, the optimal cycle number at which the transcript was exponentially amplified was established by running a linear cycle series and the number of PCR cycles was kept within this range. Approximately 1 μL cDNA per sample was used for amplification. The cDNA fragments were generated by initial denaturation at 94°C for 3 min. The PCR products were separated by electrophoresis on 1.3 % agarose gel and detected under UV light. To normalize signals from different RNA samples, *Gapdh* transcripts were co-amplified as an internal standard.

Quantitative expression was analyzed with Quantity One software using the Volume Analysis Report of Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Software 5.03, San Diego CA). Differential gene expression was tested by the D'Agostino and Pearson normality test to confirm Gaussian distribution, then examined by one-way ANOVA, with statistical significance at $P \leq 0.05$. For each primer set each amplification run was performed on treatment groups and

corresponding generation-control and replicated at least three times. In order to normalize individual responses within replicates treatment effects were expressed as DEHP/control ratio.

The mean numbers of follicles per ovary were calculated on ovaries from at least five different mice. Differences between the means were examined by one-way ANOVA, with statistical significance at $P \leq 0.05$. When ANOVA gave a significant P value, the Newmann-Keuls' test was used in the *post hoc* analysis.

Data for *in vitro* embryo culture were analyzed by binary logistic regression. Controls were taken as the reference group. Experiments were replicated at least three times, and each replicate was fitted as a factor. The log likelihood ratio statistic was used to detect between-treatment differences and significance was set at $P<0.05$.

RESULTS

Effect of maternal DEHP exposure on ovarian follicular dynamics

To examine the effects of maternal exposure to DEHP on folliculogenesis in adult mouse ovaries of F1 offspring and subsequent generations, different classes of follicles were scored in ovarian tissue sections. Results from F1, F2 and F3 ovaries are shown in figure 2.

In the F1 generation, ovaries showed significantly altered histology ($p \leq 0.001$) in all exposure groups compared to the vehicle-treated control lineage. Specific findings were: 1) significantly fewer primordial follicles (figure 2A), with respectively about 65% and 30% reductions in the 0.05 mg/kg/day and 5 mg/kg/d groups; 2) significantly more growing pre-antral follicles (figure 2B), with about 40% increases in both dose groups; 3) significantly fewer developing antral follicles (figure 2C), with respectively about 35% and 50% reductions in the 0.05 mg/kg/day and 5 mg/kg/d groups; 4) a higher incidence of follicle late atresia (figure 2D) in the 0.05 mg/kg/day DEHP group.

In F2 and F3 generations, similarly to F1, there was a significantly smaller number of primordial follicles together with a larger number of pre-antral follicles in both dose groups ($p \leq 0.01$) (figures 2A and 2B). Since the F3 generation animals had never been exposed to the DEHP, these alterations in follicular dynamics were transgenerationally inherited by F3 female descendants. Conversely, in F2 and F3 females there was no change in the number of antral or atretic follicles, except for a reduction in antral follicles in F2 of the 0.05 mg/kg/day group (figures 2C and 2D). Therefore, these effects, limited to F1 and F2, can be attributed to direct exposure to DEHP. The total number of follicles was unaffected by treatment in all generations.

In vitro oocyte developmental competence in adult female offspring

In vitro embryo production from oocytes from F1, F2 and F3 adult offspring is illustrated in Table 2. Developmental competence of female gametes was tested on a total of 1463 *in vivo* matured oocytes. The oocytes from the F1 mice of the 0.05 mg/kg/day group produced embryos with a significantly lower capacity to complete the first mitotic division and to subsequently reach the blastocyst stage than controls and the 5 mg/kg/day group ($P<0.05$). These results agree with our previous findings (Pocar *et al.*, 2012a) and confirm a non-monotonic DEHP dose-response curve on oocyte developmental competence of *in utero* and lactationally exposed female gametes.

The negative consequences on oocyte developmental competence induced by maternal exposure to 0.05 mg/kg/day DEHP were still detectable in the subsequent two generations, pointing to transgenerational transmission of the effect. As in F1, the numbers of embryos recovered from the F2 and F3 0.05 mg/kg/day groups were significantly lower than controls and the 5 mg/kg/day group ($P<0.05$), with respectively about 30% and 60% reductions in cleavage and blastocyst rates.

Expression of genes related to oocyte and follicular development in the ovaries

To investigate the mechanisms by which DEHP affects ovarian physiology, we analyzed the effects of exposure on the expression profiles of a panel of genes involved in both steroidogenesis and folliculogenesis. Results from F1, F2 and F3 ovaries are shown in Table 3.

In F1, maternal exposure to DEHP at 0,05 and 0,5 mg/kg/day significantly down-regulated the genes related to gonadotropin response in adult offspring ovaries, in agreement with our previous findings (Pocar *et al.*, 2012a). In both DEHP treated groups the expression levels of *Fsh-R*, *Lh-R*, *Cyp19a1* and *Pgr* in ovaries were significantly lower than in controls. Only the mRNA levels for *StAR* in ovaries of the two DEHP groups and *Cyp17a1* in the lower dose group were not affected. In subsequent generations no alterations were seen in the expression of genes involved in gonadotropin response and steroidogenesis, pointing to a direct effect of maternal exposure to DEHP on pituitary-gonadal cross-talk. As regards the transcripts related to folliculogenesis, *Gdf9* was consistently up-regulated in F1 ovaries in all treatment groups ($p \leq 0.001$), while *Bmp15* and *Pten* were unaffected. This pattern of expression remained unaltered over the two subsequent generations. This indicates that gene expression related to folliculogenesis is affected transgenerationally in mouse adult ovaries and may explain the altered follicular dynamics and oocyte developmental competence observed throughout three generations.

Gene expression profile of pre-implantation blastocysts

To assess the quality of pre-implantation embryos from oocytes in DEHP-treated groups and to gain further information on the molecular bases of transgenerational transmission of adverse effects, we analyzed the expression profile of a panel of embryonic key genes in morphologically normal blastocysts after *in vitro* fertilization. Table 4 reports data related to the embryonic generations, thus from F2 (donor generation F1) to F4 (donor generation F3).

Key genes involved in embryonic development were significantly altered in F2 and F3 embryos of both the DEHP treated groups compared to controls. This indicates a transgenerational effect of the toxicant exposure independently from the dose to which the pregnant F0 females had been

exposed. In F2 and F3 embryos DEHP induced significant upregulation of transcript levels for *Oct-4* and *Gata3*, while down-regulating *Nanog*. No changes in mRNA levels for these genes were detected in F4 compared to controls.

Expression of the genes related to trophoblast differentiation and implantation was significantly affected by DEHP and these effects were transgenerationally transmitted, with some differences between genes and DEHP doses. *Lif-R* expression was upregulated by 0.05 and 5 mg DEHP/kg/day in both F2 and F3 generations, and in the 0.5 mg/kg/day embryos even up to F4. Transcripts for *Cdx2, Eomes* and *Lif* were also upregulated in embryos of the DEHP groups, but surprisingly only from the F3 generation onwards. Specifically, *Cdx2*and *Eomes* mRNA levels were higher than controls in embryos from both DEHP groups, but for *Lif* only in embryos of the lower dose group.

DISCUSSION

To our knowledge, this is the first comprehensive study reporting that maternal exposure to environmentally relevant doses of DEHP have transgenerational adverse effects on ovarian physiology and female gamete developmental competence in mice. Specifically, from F1 through F3 along the female lineage we observed the transmission of an altered reproductive phenotype showing: i) changes in ovarian morphology and functionality; ii) reduced oocyte and preimplantation embryo developmental competence; iii) changes in transcript levels for genes related to oocyte and follicular development in the ovary and iv) changes in gene expression levels for key genes in the blastocysts.

A number of environmental influences (including EDs) during the period of developmental plasticity promote adverse phenotypes that may be transmitted to subsequent generations and beyond through the male and female germ lines (Danchin *et al.*, 2011; Pocar *et al.*, 2012b; Skinner *et al.*, 2013). Peri-natal exposure to pharmacological doses of phthalates induces transgenerational adult-onset reproductive disorders in both male and female rats (Doyle *et al.*, 2013; Manikkam *et al.*, 2013; Chen *et al.*, 2015). However, these studies of phthalates followed the generations along the male lineage, while little is known about the effects of ED exposure transmitted to subsequent generations by the female germ line.

A variety of theories have been recently explored to explain the germline transgenerational transmission of an adverse phenotype. The role of epigenetic inheritance has attracted increasing attention in recent years (Jimenez-Chillaron *et al.*, 2009) despite evidence of extensive reprogramming during germ cell formation and zygotic development (Danchin *et al.*, 2011; Drake and Seckl, 2011; Aiken and Ozanne, 2014). The first wave of epigenetic reprogramming is driven by the female germ line, just after fertilization when the early embryo is transcriptionally and translationally quiescent and the machinery that controls this epigenetic remodeling and first round of DNA replication is orchestrated solely by the oocyte (Gawecka *et al.*, 2013).

The present study did not specifically investigate the epigenetic status of female offspring and their oocytes. However, evidence is emerging that long-term and irreversible alterations in ovarian physiology induced by developmental exposure to phthalates may be related to early changes in epigenetic control of gene expression (Zama and Uzumcu, 2009; Zama and Uzumcu, 2010; Zhang *et al.*, 2013). The high frequency of the morphological phenotype in the ovaries and the highly consistent patterns of gene expression profiles amongst individuals and litters over four generations observed in our study indicate that genetic DNA sequence mutations are not the most likely cause (Barber *et al.*, 2002; Dong *et al.*, 2004). This enables us to hypothesize that the phenotype observed in adulthood may result from epigenetic changes in the germ cells and growing oocytes. Maternal exposure to DEHP could thus have prevented the proper erasure, re-establishment, or maintenance of epigenetic marks in the oocytes, altering the embryonic epigenome, and subsequently enhancing postnatal disease susceptibility.

This hypothesis is supported by recent work indicating that direct exposure of developing ovaries to DEHP, besides inducing sensitive alterations in follicular dynamics, significantly affects the methylation machinery in follicles and growing oocytes *in vivo* and *in vitro* (Xie *et al.*, 2012; Zhang *et al.*, 2014). Further studies are now needed to further clarify the mechanisms of these transgenerational effects.

In considering ovarian function, in the present study follicular distribution was characterized by a low proportion of primordial follicles and a reciprocal increase in the percentage of growing pre-antral follicles in DEHP-exposed animals and subsequent generations. These data are in agreement with recent reports that exposure to phthalates either in adult age (Hannon *et al.*, 2014) or early post-natally (Zhang *et al.*, 2013) induces primordial follicle depletion and increases the population of growing follicles in adult mice and their offspring. Similarly, *in utero* exposure to MEHP (the major DEHP metabolite) also raises the percentage of growing follicles in mice (Moyer and Hixon, 2012). The present finding of the increased number of growing pre-antral follicles is in good

agreement with the concomitant upregulation of *Gdf9* transcript levels in affected ovaries. Recent studies have in fact shown that giving GDF-9 to neonatal rats increases the number of primary and pre-antral follicles and reduces the complement of primordial follicles, collectively suggesting that GDF-9 promotes initial recruitment (Vitt *et al.*, 2000).

According to Beranger et al. (Beranger *et al.*, 2012) the phenotype observed in our study strongly suggests that maternal exposure to DEHP accelerates the rate of follicle recruitment, which would rapidly exhaust the primordial follicle reserve, leading to early reproductive failure. We did not actually see any reduction in fertility in *in utero* treated mice and subsequent generations, but we do have to bear in mind that the animals were mated at an early age (8 weeks), and, depending on the strain, reproductive activity in mice does not decline up to 8-12 months of age. It is therefore likely that there are still enough follicles in the ovary at the age we investigated to support pubertal development and fertility, and ovarian failure due to follicle depletion would be visible only later. Furthermore, a slight reduction in fecundity could easily have been masked by the large size of the litters, characteristic of the mouse strain employed. This too calls for further investigation.

In the present study altered follicular dynamics in the ovary was related to impaired oocyte developmental competence, as demonstrated by the reduced ability of *in vivo* matured oocytes to complete the first mitotic division and reach the blastocyst stage after *in vitro* fertilization. The mechanisms of how the phthalates influence pre-implantation development are not yet fully understood. We previously postulated that adverse effects in oocyte developmental competence, after *in utero* and peri-natal DEHP exposure, might be related to deregulated steroid homeostasis and unbalanced pituitary-gonadal axis crosstalk (Pocar *et al.*, 2012a). However, this is apparently limited to F1 gonads, pointing to a direct effect of phthalate exposure.

Therefore, to cast more light on transgenerational transmission of the effects on gametes we analyzed the transcriptional levels of selected genes in blastocysts from oocytes of treated animals and their subsequent generations. Preimplantation embryos in the DEHP groups presented

dysregulation of the expression profile for key genes involved in early embryonic differentiation and development. Our findings indicate an altered profile of pluripotency factors which may lead to changes in the differentiation patterns of extra-embryonic lineages. This is supported on the one hand by the concomitant overexpression of *Oct4* with the down-regulation of *Nanog* transcript levels, and on the other by the up-regulation of the trophoectoderm differentiation markers *Gata3*, *Cdx2* and *Eomes*. It is recognized that *Nanog* down-regulation orchestrates the differentiation of totipotent embryonic cells into extra-embryonic lineages (Hyslop *et al.*, 2005; Hough *et al.*, 2006), and that there is an interconnecting autoregulation loop to maintain totipotency so that, if *Oct4* levels rise above a steady level, *Nanog* expression is repressed (Pan and Thomson, 2007).

As regards the trophoectoderm markers, both CDX2 and EOMES are transcription factors essential for the specification and maintenance of the trophoectoderm-derived trophoblast lineage (Niwa *et al.*, 2005). In addition, GATA3 is expressed only in the trophoblast lineage and is both necessary and sufficient to promote trophoblast maturation, regulating the process of morula to blastocyst transformation (Ralston *et al.*, 2010).

Our findings as a whole may provide some insights into both the reduced oocyte developmental competence and the transgenerational transmission of adverse effects. Indeed, coordinated epigenetic regulation occurred for the correct timing of expression in genes related to lineage determination including *Oct4*, *Nanog* and *Cdx2* (Hattori *et al.*, 2004; Zhang *et al.*, 2008).

In conclusion, the present study indicates that DEHP administered to pregnant female mice induces alterations in the reproductive health of female offspring. Of particular concern is the fact that effects were observed up to the third generation, pointing to a transgenerational effect of DEHP in female offspring. In all the generations investigated, DEHP altered primordial follicle recruitment, and this can potentially interfere with normal ovarian function and cause premature ovarian failure (POF). In humans, population-based studies estimate that 1% of women experience POF (Coulam *et al.*, 1986; Cramer and Xu, 1996; Luborsky *et al.*, 2003). It appears too that women

occupationally exposed to phthalates during their reproductive life are at greater risk of POF than women of reproductive age in other occupations (Gallicchio *et al.*, 2009). Grindler and co-workers documented a direct association between phthalate exposure and earlier age at menopause (Grindler *et al.*, 2015). The DEHP doses employed were within the range of real environmental exposure in humans. Therefore, our observations of the dangerous effects of DEHP on folliculogenesis as regards reproductive performance throughout generations give reason for concern. In fact, although mouse data must obviously be evaluated very carefully before being extrapolated to the human fetus, folliculogenesis processes and blastocyst development are similar and phthalates can cross the placenta in both species. It is therefore reasonable to hypothesize that human fetal DEHP exposure during critical points in development might also act on follicular development and ovarian function. To gain more information on the potential for POF and infertility in the general population, further work is necessary to completely elucidate the mechanism of DEHP-induced acceleration of primordial follicle recruitment and the toxic effects on gametes after *in utero* exposure.

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FIGURE LEGENDS

Figure 1 Diagram of the experimental approach with focus on multigenerational and transgenerational phenomena and direct exposure of the F0 mother, F1 embryo, and F2 germ-line. The F3 generation is the first without direct exposure. (dpc, day post-coitum; pnd, post-natal day)

Figure 2 Distribution of follicular stages in ovaries of the F1, F2 and F3 offspring. A) primordial; B) pre-antral; C) antral; D) end-stage atretic. Each column represents the mean ± SE of at least three

separate experiments. Asterisks denote significant differences from corresponding generation-control (** $p \leq 0.001$; * $p \leq 0.05$).

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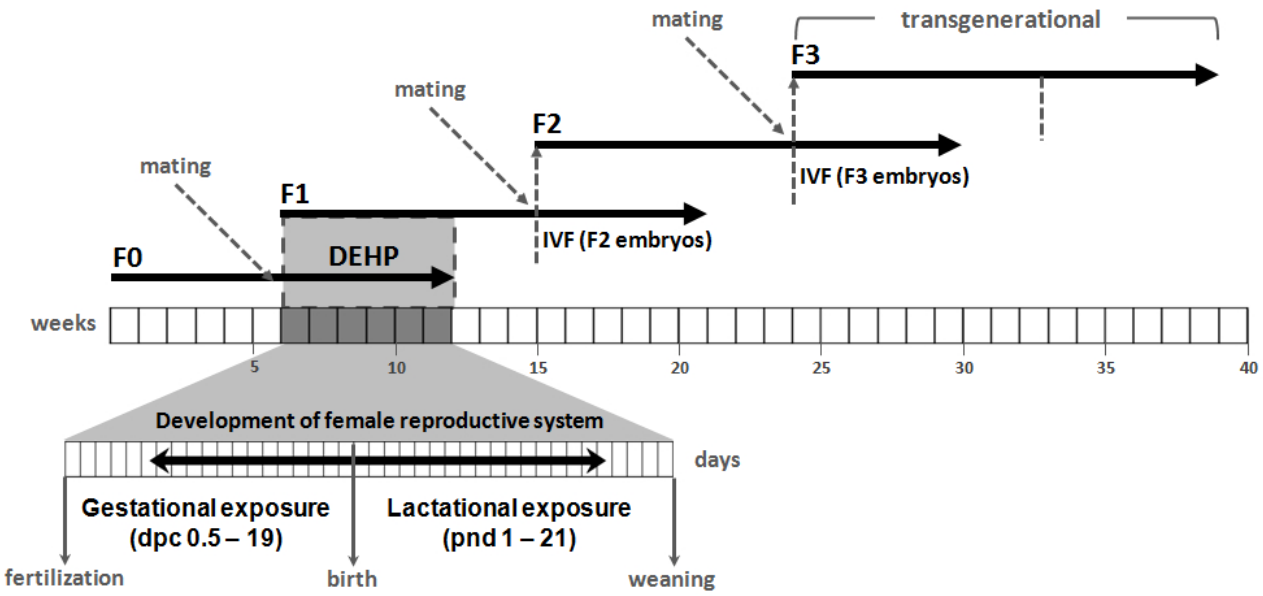

Fig. 1

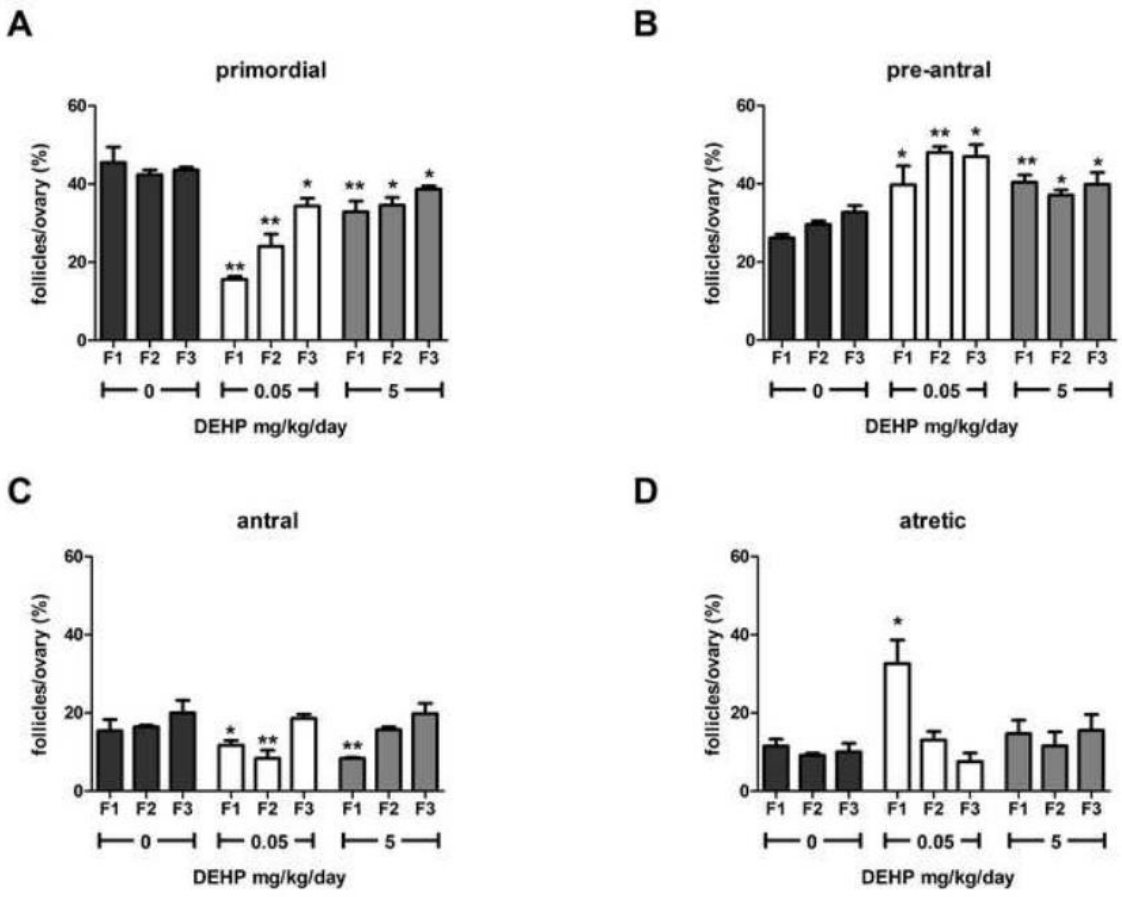

Fig. 2

Gene	Accession No.	Primers	Annealing T°	Product size
gapdh	NM008084	F: TCACCATCTTCCAGGAGCG R: CTGCTTCACCACCTTCTTGA	57	572
lhr	NM013582	F: TCTACCTGCTGCTCATTGCCTC R: AAGGCAGCTGAGATGGCAAAG	57	553
fshr	NM013523	F: ATGTGTAACCTCGCCTTTGCTG R: AACATACAGCTGCGACAAAGGG	57	393
star	BC082283	F: GAAGGAAAGCCAGCAGGAGAAC R: CTGCGATAGGACCTGGTTGATG	58	496
cyp17a1	AY594330	F: ACGGTGGGAGACATCTTTGGG R: CCTTCGGGATGGCAAACTCTC	57	283
cyp19a1	NM007810	F: CCTCTGGATACTCTGCGACGAG R: CGAATGGTGGAAGTTTGTGTGG	56	508
pgr	NM008829	F: GATGAGCCTGATGGTGTTTGGC R: GGGCAACTGGGCAGCAATAAC	57	490
gdf-9	NM008110	F: GGCACTTCCCAGCAACTTCC R: GGTGACTTCTGCTGGGTTTGG	58	326
bmp15	NM009757	F: GCAAGGAGATGAAGCAATGGC R: GAAGCGGAGGCGAAGAACAC	57	458
pten	NM008960	F: TGGAAAGGGACGGACTGGTG R: CCGCCACTGAACATTGGAATAG	55	249
nanog	NM028016	F: TGCCAGGAAGCAGAAGATG R: TTATGGAGCGGAGCAGCATTC	57	471
oct4	NM013633	F: ATCACTCACATCGCCAATCAGC R: CAGAGCAGTGACGGGAACAGAG	58	279
cdx2	NM007673	F: GAAACCTGTGCGAGTGGATGC R: TGCTGCTGCTGCTTCTTCTTG	58	265
eomes	NM001164789	F: GTGGAAGTGACAGAGGACGGTG R: GAGGCAAAGTGTTGACAAAGGG	57	250
lif	NM008501	F: TGGCAACGGGACAGAGAAGAC R: ACGGTACTTGTTGCACAGACGG	58	202
lifR	NM013584	F: CCAAGGACGGAACCAGTAGCAC R: GACGAAGGGTATTGCCGATCTG	57	325
gata3	NM008091	F: CTGGAGGAGGAACGCTAATGG R: TGTGGCTGGAGTGGCTGAAG	57	263

Table 1. Primers and PCR conditions for gene expression analysis

	mg DEHP/kg/day		
	0	**0.05**	**5**
F1			
No. of oocytes	352	368	328
Oocytes/animal	35.20 ± 3.1	36.80 ± 2.3	32.80 ± 5.1
Cleavage rate (%)	74.2 ± 6.6 [a]	47.1 ± 7.3 [b]	74.2 ± 5.7 [a]
Blastocyst rate (%)	64.4 ± 7.8 [a]	26.9 ± 9.8 [b]	57.1 ± 5.8 [a]
Blastocyst/cleaved rate (%)	77.7 ± 7.3 [a]	38.4 ± 9.9 [b]	77.5 ± 5.6 [a]
F2			
No. of oocytes	412	324	304
Oocytes/animal	30.71 ± 3.3	27.00 ± 3.4	30.40 ± 4.6
Cleavage rate (%)	74.3 ± 4.8 [a]	53.2 ± 1.7 [b]	73.2 ± 9.5 [a]
Blastocyst rate (%)	68.5 ± 6.4 [a]	32.9 ± 4.4 [b]	74.0 ± 6.3 [a]
Blastocyst/cleaved rate (%)	87.4 ± 1.1 [a]	59.2 ± 8.6 [b]	93.9 ± 3.8 [a]
F3			
No. of oocytes	378	344	332
Oocytes/animal	26.00 ± 1.9	28.00 ± 3.9	28.00 ± 2.3
Cleavage rate (%)	82.8 ± 3.2 [a]	55.6 ± 6.2 [b]	84.4 ± 3.5 [a]
Blastocyst rate (%)	75.9 ± 3.8 [a]	22.7 ± 6.7 [b]	65.6 ± 8.3 [a]
Blastocyst/cleaved rate (%)	87.8 ± 1.5 [a]	38.5 ± 11.5 [b]	77.4 ± 7.6 [a]

Values are mean ± SEM. *a*, *b*: different superscripts in the same column indicate statistical differences for $P \leq 0.05$.

Table 2. Effects of pre- and perinatal DEHP exposure on developmental capacity of oocytes from F1, F2 and F3 offspring.

	mg DEHP/kg/day					
	0.05			5		
Gonadotropin response and steroidogenesis						
	F1	**F2**	**F3**	**F1**	**F2**	**F3**
Fsh-R	0.61 ± 0.1 ↓	1.1 ± 0.6	1.0 ± 0.1	0.67 ± 0.1 ↓	0.90 ± 0.9	1.20 ± 0.2
Lh-R	0.66 ± 0.8 ↓	1.08 ± 0.3	1.11 ± 0.2	0.64 ± 0.1 ↓	1.00 ± 0.4	1.13 ± 0.2
StAR	0.93 ± 0.6	1.02 ± 0.8	1.02 ± 0.4	1.00 ± 0.8	1.20 ± 0.1	1.12 ± 0.6
Cyp17a1	0.93 ± 0.7	1.01 ± 0.2	1.03 ± 0.3	0.64 ± 0.1 ↓	1.00 ± 0.1	1.06 ± 0.7
Cyp19a1	0.69 ± 0.6 ↓	1.06 ± 0.4	0.89 ± 0.9	0.72 ± 0.1 ↓	0.96 ± 0.4	1.01 ± 0.9
Pgr	0.75 ±0.5 ↓	0.93 ± 0.7	0.94 ± 0.8	0.81 ± 0.4 ↓	0.97 ± 0.1	0.98 ± 0.5
Oocyte quality and folliculogenesis						
Bmp15	1.12 ± 0.5	0.99 ± 0.4	1.16 ± 0.1	0.99 ± 0.6	0.97 ± 0.3	1.22 ± 0.2
Gdf9	1.43 ± 0.1 ↑	1.67 ± 0.2 ↑↑	1.76 ± 0.9 ↑	2.11 ± 0.2 ↑↑	1.69 ± 0.2 ↑	1.99 ± 0.2 ↑↑
pTEN	0.93 ± 0.7	1.05 ± 0.1	1.02 ± 0.1	0.93 ± 0.5	1.09 ± 0.9	0.96 ± 0.2

Table 3. Effects of pre- and perinatal DEHP exposure on expression profile of selected transcripts in ovaries from F1, F2 and F3 offspring. For each primer set each amplification run was performed on treatment groups and corresponding generation-control. Normalized data are expressed as DEHP/control ratio ± SEM (↑↑: $P \leq 0.001$; ↑: $P \leq 0.05$).

	mg DEHP/kg/day					
	0.05			5		
Embryonic development and trophoblast differentiation and implantation						
Embryonic generation	**F2**	**F3**	**F4**	**F2**	**F3**	**F4**
Oct-4	2.20 ± 0.8 ↑↑	2.26 ± 0.2 ↑↑	1.33 ± 0.9	1.94 ± 0.1 ↑↑	1.92 ± 0.2 ↑↑	1.14 ± 0.2
Nanog	0.78 ± 0.2 ↓↓	0.54 ± 0.1 ↓↓	1.05 ± 0.2	0.45 ± 0.1 ↓↓	0.83 ± 0.4 ↓	1.06 ± 0.2
Gata-3	2.27 ± 0.3 ↑↑	2.34 ± 0.2 ↑↑	1.09 ± 0.3	1.91 ± 0.2 ↑↑	1.53 ± 0.2 ↑	0.98 ± 0.2
Trophoblast differentiation and implantation						
Cdx-2	1.26 ± 0.2	1.98 ± 0.2 ↑↑	4.04 ± 0.1 ↑↑	1.39 ± 0.3	1.84 ± 0.2 ↑↑	7.71 ± 0.3 ↑↑
Eomes	1.20 ± 0.2	2.89 ± 0.5 ↑↑	2.47 ± 0.7 ↑↑	1.31 ± 0.2	2.51 ± 0.7 ↑↑	2.54 ± 0.6 ↑↑
Lif	1.29 ± 0.2	2.53 ± 0.9 ↑↑	1.59 ± 0.1 ↑↑	0.98 ± 0.2	0.95 ± 0.1	1.28 ± 0.2
Lif-R	1.51 ± 0.2 ↑	1.73 ± 0.2 ↑↑	0.73 ± 0.1	2.24 ± 0.2 ↑↑	2.23 ± 0.2 ↑↑	2.21 ± 0.2 ↑↑

Table 4. Effects of pre- and perinatal DEHP exposure on expression profile of selected transcripts in blastocysts derived from F1, F2 and F3 oocytes. For each primer set each amplification run was performed on treatment groups and corresponding generation-control. Normalized data are expressed as DEHP/control ratio ± SEM (↑↑: $P \leq 0.001$; ↑: $P \leq 0.05$).

HIGHLIGHTS

- Maternal exposure to DEHP transgenerationally affects female reproductive health
- DEHP reduced ovarian follicular reserve up to the third generation
- DEHP reduced oocyte and blastocyst developmental competence up to F3
- DEHP altered expression levels for key genes in ovary and blastocysts up to F4
- DEHP's adverse effects were observed at doses relevant for human exposure.