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Research Article

Paternal developmental toxicant exposure is associated with epigenetic modulation of sperm and placental *Pgr* and *Igf2* in a mouse model†

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Abstract

Preterm birth (PTB), parturition prior to 37 weeks' gestation, is the leading cause of neonatal mortality. The causes of spontaneous PTB are poorly understood; however, recent studies suggest that this condition may arise as a consequence of the *parental* fetal environment. Specifically, we previously demonstrated that developmental exposure of male mice (F1 animals) to the environmental endocrine disruptor 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was associated with reduced sperm quantity/quality in adulthood and control female partners frequently delivered preterm. Reproductive defects persisted in the F2 and F3 descendants, and spontaneous PTB was common. Reproductive changes in the F3 males, the first generation without direct TCDD exposure, suggest the occurrence of epigenetic alterations in the sperm, which have the potential to impact placental development. Herein, we conducted an epigenetic microarray analysis of control and F1 male-derived placentae, which identified 2171 differentially methylated regions, including the progesterone receptor (*Pgr*) and insulin-like growth factor (*Igf2*). To assess if *Pgr* and *Igf2* DNA methylation changes were present in sperm and persist in future generations, we assessed methylation and expression of these genes in F1/F3 sperm and F3-derived placentae. Although alterations in methylation and gene expression were observed, in most tissues, only *Pgr* reached statistical significance. Despite the modest gene expression changes in *Igf2*, offspring of F1 and F3 males consistently exhibited IUGR. Taken together, our data indicate that paternal developmental TCDD exposure is associated with transgenerational placental dysfunction, suggesting epigenetic modifications within the sperm have occurred. An evaluation of additional genes and alternative epigenetic mechanisms is warranted.

Summary Sentence

Developmental dioxin exposure of the paternal parent was associated with changes in the sperm and placental epigenome in association with impaired placental function; selected genes were also found to be differentially methylated in sperm and placentae of progeny without direct toxicant exposure.

Key words: environmental toxicants, pregnancy, progesterone, IUGR, placenta, sperm, DNMT.

Introduction

Preterm birth (PTB), defined as spontaneous delivery prior to 37 weeks' gestation, is a global medical crisis that impacts as many as 15 million babies each year [1, 2]. Multiple maternal factors, including age, smoking status, and race, are known to contribute to the risk of PTB; however, women with no known risk factors can also deliver early [1]. Epidemiological studies suggest that paternal factors, such as obesity and race, can additionally influence pregnancy outcomes in their partners [3–6]; however, the biologic mechanisms associated with a paternal-derived PTB risk remain poorly understood.

Using a murine model, we previously demonstrated that spontaneous PTB was common in the unexposed partners of adult males (F1 males) with a history of in utero exposure to the persistent environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, also known as dioxin) [7–9]. F1 males also exhibited reduced sperm numbers and altered sperm morphology, which was associated with testicular inflammation and subfertility [7]. Importantly, present within fetal F1 animals are germ cells which have the potential to become the F2 generation. Thus, F2 animals are recognized as also being directly exposed following maternal (the F0 generation) toxicant exposure during pregnancy [10]. Not surprisingly, we found that adult F2 males also exhibited testicular inflammation, subfertility, and conferred a risk of PTB to control mating partners [7]. However, in the absence of additional exposure, similar reproductive defects were also observed in F3 males, with 35% of mating partners delivering preterm while none of the control pregnancies delivered early [7]. Adverse reproductive changes in F3 males, animals without a direct TCDD exposure, suggest toxicant mediated epigenetic modifications within the male germ cell have occurred [11, 12].

The male germ cell, the sperm, conveys the father's entire contribution to pregnancy. Following sperm fertilization of the oocyte, the preimplantation blastocyst develops. The blastocyst contains an inner cell mass, which develops into the fetus, and the trophectoderm, which will form the placenta. The placental phenotype plays a critical role in maternal response to implantation and maternal–fetal communication throughout pregnancy [13, 14]. Importantly, seminal studies conducted in 1985 by Barton and colleagues demonstrated that fetal development is markedly stunted in artificially created blastocysts containing only paternally derived genetic material; however, these "embryos" exhibit significant placental development [15]. These foundational studies were the first to demonstrate the substantial contribution of the paternal genome to placental formation. Although to our knowledge, no previous study has attempted to link specific epigenetic marks associated with paternal TCDD exposure to his partner's pregnancy outcome, numerous studies have demonstrated that appropriate expression of paternally imprinted genes is critical for normal placental development and function [16–18].

Herein, we conducted a global methylation analysis of late pregnancy placentae (E18.5) arising from control or F1 male mice. This analysis revealed 2171 differentially methylated CpG regions, many corresponding to the promoters of genes known to be disrupted in association with PTB in mice and/or women. Ingenuity Pathway Analysis (IPA) revealed estrogen receptor-alpha *(Esr1)* as one of the top upstream regulators impacted by developmental TCDD exposure. Thus, in the current study, we examined expression and methylation of progesterone receptor *(Pgr)* and insulin-like growth factor-2 (*Igf2*), two genes known to be modulated by estrogen.

Estrogen, acting via *Esr1*, has long been known to regulate expression of the *Pgr*, both in humans and rodents [19–21]. Progesterone action is critical to establishment and maintenance of pregnancy and we have previously demonstrated reduced *Pgr* mRNA expression in placentae arising from F1 pregnancies [8]. More recently, studies implicate a role for *Esr1* in modulating imprinting of *Igf2* [22]. *Igf2*, a paternally expressed, imprinted gene, is known to regulate fetal growth [23] and studies suggest that loss of normal imprinting may contribute to male infertility [22, 24] and placental dysfunction [25, 26]. Not surprisingly, inappropriate expression of DNA methyltransferases (*DNMT*), which act to maintain imprinted genes (*DNMT1*) or promote de novo methylation (*DNMT3a; DNMT3b*), has also been linked to placental dysfunction and altered embryo development [27–29]. Therefore, to determine whether the observed TCDD-associated methylation changes were due to altered expression of DNA methyltransferases (*Dnmts*), we additionally examined placental expression of *Dnmt1, Dnmt3a, and Dnmt3b* mRNA. Finally, methylation and gene expression data were correlated with functional outcomes (PTB and IUGR).

Materials and methods

Animals

Young adult (8–10 weeks) male and female C57BL/6 mice were purchased from Envigo (Indianapolis, IN). Animals were housed in Vanderbilt University's Barrier Animal Care Facility (free of common mouse pathogens) according to National Institutes of Health and institutional guidelines for laboratory animals. Fresh food and water was provided ad libitum. Animal rooms were maintained at a temperature of 22–24°C and a relative humidity of 40–50% on a 12-h light: dark schedule. Experiments described herein were approved by Vanderbilt University's Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act.

Chemicals

TCDD (99% in nonane #ED-908) was obtained from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

In utero TCDD exposure

Virgin C57BL/6 females, aged 10–12 weeks, were mated with intact males of similar age. Upon observation of a vaginal plug, females were separated and denoted as day 0.5 of pregnancy (E0.5). Pregnant

mice (F0) were exposed to TCDD (10 μg/kg) in corn oil or vehicle alone by gavage at 1100 h local time on E15.5 (when organogenesis is complete). This in utero plus lactational exposure paradigm results in direct exposure of the feti (F1 mice) and direct exposure of the fetal germ cells, which have the potential to become the F2 generation. The selected dose of TCDD reflects the more rapid clearance of this toxicant in mice compared to humans and is well below the LD_{50} for adult mice of this strain (230 μg/kg) [30]. TCDD given at this time and dose is not overtly teratogenic and gestation length was not affected in the F0 animals; pups (F1 mice) were born on E20 ± 0.5 days.

In order to additionally obtain F3 males for use in the current study, 10- to 12-week-old F2 male offspring of F1 males and control females were mated to age-matched control females. F3 males were utilized at young adulthood as described below.

All males were mated to at least three females. For fertile males, one pregnant female was euthanized on E18.5 while two females were allowed to progress to spontaneous delivery. In mice euthanized on E18.5, for all experiments other than the epigenetic microarray, we compared placentae of unexposed pregnant female mice mated to unexposed males (controls) with placentae of unexposed female mated to F1 males (exposed while in utero) and unexposed female mice mated to F3 males (never exposed, the grandfather was exposed in utero). For each group, we examined multiple placentae per litter using a minimum of 5 litters/group. For all E18.5 studies, placentae from the right uterine horn were fixed, while placentae in the left uterine horn were frozen. A schematic of the study design is shown in Supplementary Figure S1.

Monitoring of pregnancy/birth timing

A single control female was placed with a single male (control, F1 or F3) and examined each morning for the presence of a vaginal plug. Following the identification of a plug (considered E0.5), the male was removed. Females were weighed prior to mating and again on E16.5, when they were examined for signs of pregnancy (weight gain, nipple prominence). Beginning at this time, in order to precisely determine timing of parturition, pregnant females were monitored daily by remote surveillance until delivery. Parturition in C57BL/6 mice normally occurs 19.5 days after identification of a vaginal plug [31]; therefore, term parturition is considered E20. For our studies, pups are considered preterm if born ≤ E18.5 [7, 8].

Euthanasia and collection of tissues

Pregnant females were weighed immediately prior to euthanasia, which was conducted at 1400–1500 h local time on E18.5 by cervical dislocation under deep anesthesia. Feti were counted and weighed together as a litter and euthanized as per the AAALAC guidelines. Placentae were also removed. Half of the placentae were carefully dissected from the decidua, weighed, flash frozen, and stored at –80°C until analysis. Remaining placentae, with decidua attached, were formalin fixed. All formalin-fixed samples were processed and subjected to paraffin embedding by the Vanderbilt Translational Pathology Shared Resource (TPSR) using standard methodology.

For collection of sperm, males were euthanized by cervical dislocation under anesthesia following a minimum of 72 h after mating. Sperm was collected from the epididymal cauda using standard methods as previously described [9] and DNA isolated as described below.

DNA methylation array analysis

Placental DNA was isolated by standard methods using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) [8] from control (N = 3) and F1 male (N = 5) tissues following careful removal of the decidua. Methylated DNA was analyzed by MOgene, LLC (St. Louis, MO) using the differential methylation hybridization technique and the Agilent Mouse CpG Island Microarray (Platform G4811A) according to the manufacturer's recommended protocol. Briefly, methylated regions of a DNA sample were isolated with a monoclonal antibody to 5-methylcytosine which was then labeled with Cyanine 5 and competitively hybridized against similarly Cyanine 3-labeled DNA on a single microarray. Relative DNA methylation levels for each probe were determined by examining the Cyanine 5/Cyanine 3 ratios.

The array used encompasses 15,913 CpG islands using 88,737 probes (in or within 95bp of CpG Islands). Data obtained from the microarray were initially analyzed using GeneSpring GX 13.1 software (Agilent Technologies). Scanned arrays were uploaded into GeneSpring for background adjustment, summarization, log transformation, and baseline transformation. Next, a heatmap of Combined Z-scores was created with TIBCO Spotfire 5.0 software. Data were subjected to IPA (QIAGEN Inc., https://www.qiagen.com/us/shop/analytics-software/biological-data-tools/ingenuity-pathway-analysis/#orderinginformation). Finally, the data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [32, 33] and are accessible through GEO Series accession number #GSE111948 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111948).

Methylation specific quantitative assay of sperm and placentae

DNA was extracted from placental samples as described above. Sperm DNA was isolated using the QIAamp DNA Mini Kit (#51304; Qiagen) as described by Wu et al [34]. Sperm and placental DNA samples were subjected to CpG island DNA methylation profiling of individual genes using the Epitect® Methyl II PCR Assay (Qiagen). Specifically, DNA was digested with methylation-sensitive and/or methylation-dependent restriction enzymes (MDRE). Following digestion, the remaining DNA from each individual reaction was quantified using RT-PCR with primers specific for promoter regions of either *Pgr* (EPMM111296-1A) or *Igf2* (EPMM110567-1A). Primers for both genes were obtained from Qiagen (*Pgr*: cat# 141749315; *Igf2*: cat# 142172789). Data were analyzed using the delta-delta Ct method, comparing quantification of each digest to a mock digest [35]. Calculations were performed per the manufacturer's protocol.

Quantitative RT-PCR analysis of *Pgr, Igf2, Dnmts,* and *H19*

Total RNA was isolated from frozen placental tissues with Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit (Qiagen). Complementary DNA from 1 μg of total RNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) and random decamer primers. Reactions were performed in triplicate in a Bio-Rad CFX96 Real-time thermocycler system. *18s* rRNA was used as an endogenous control for all samples. Results were evaluated using the delta-delta Ct method as above [8, 9].

Primers (forward and reverse) were obtained from Integrated DNA Technologies (Coralville, IA), and sequences are listed in Supplementary Table S1. Since two isoforms of *Pgr* are transcribed from a single gene, primers were selected to amplify either *Pgr-b* or

Pgr-a/b. For all genes, the thermal cycling program applied on the CFX96 Real-time System was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 5 s, followed by a melting curve analysis to confirm product purity.

Immunohistochemistry

Immunohistochemical localization of PGR and IGF2 protein was conducted in our laboratory using commercially available antibodies and the Vectastain Elite ABC kit (Vector Laboratories Inc, Burlingame, CA) according to the manufacturer's protocol. Mouse anti-mouse PGR was obtained from BD Pharmingen (Cat # 558387) and used at a dilution of 1:150 (original concentration of 1mg/mL), Rabbit anti-human IGF2 was also obtained from Abcam (Cat #ab170304) and used at 1:100 (original concentration 0.5 mg/mL). Commercially available, species-specific biotinylated secondary antibodies were obtained from Biogenex (anti-mouse secondary, cat#HK335-5M; anti-rabbit secondary cat# HK-3360416) and were used as per the manufacturer's recommendation. Omission of the primary antibody was used as a negative control. All slides were lightly counterstained with Mayer's hematoxylin. Slides were viewed using an Olympus BX51 microscope system and images captured using an Olympus DP71 digital camera.

Morphometry

Histopathological assessments of PGR and IGF2 were performed by assessing the staining intensity using Fiji software (ImageJ) [36] as previously described by Nguyen et al [37]. Reciprocal intensity was calculated by the formula: $r = 255 - y$, where y is the mean intensity of each image and 255 is the maximum intensity value of an RGB image analyzed in ImageJ (Fiji). For each assessment, slides were scanned at low power and the area with the most intense staining photographed at a magnification of ×100. Regional expression of PGR and IGF2 were assessed using the freeform selection tool of Fiji using all available placentae (N = 3–5) from each of 5 dams/group.

Statistical analysis

Analyses were performed with GraphPad Prism©5 software and presented as mean ± SEM. The statistical difference between samples was determined using one-way analysis of variance followed by Tukey post hoc test. $P < 0.05$ were considered significant.

Results

Placenta DNA methylation microarray analysis reveals 15% of genes are differentially methylated in F1 placentae

Our previous studies revealed that a history of developmental TCDD exposure of the murine father is associated with an increased risk of PTB in his female mating partner in a subsequent pregnancy [8, 9]. Since the placenta is known to be influenced by the paternal genome [15, 18, 38], we examined whether reduced gestation length was also associated with epigenetic alterations in this tissue. We conducted a methylation-specific DNA microarray analysis of control (N = 3) and F1 male (N = 5) derived placentae, each from a different litter. Decidua from each placentae was carefully removed and placental DNA was isolated. Unsupervised clustering of the data was performed to determine similarities among replicate samples across both treatment groups. This analysis revealed that the samples do not group together based on paternal exposure (Figure 1A), suggesting only small changes have occurred across the genome. In fact out of 15,913 CpG islands examined, 13,742 (85%) did not exhibit alterations in methylation. Consistent with this observation, the array did not indicate significant alterations in methylation to the promoters of *Dnmt1*, *Dnmt3a,* or *Dnmt3b*. Nevertheless, as shown in Figure 1B, of the 15,913 CpG islands analyzed, the array revealed that 842 were hypomethylated and 1329 were hypermethylated.

Ingenuity Pathway Analysis predicted a large number of regulatory pathways are impacted in our model. The complete list of predicted pathways impacted is shown in Supplementary File S1. Pathways, upstream regulators, and molecules exhibiting the greatest differences between groups, as identified by IPA, are shown in Table 1. Of particular interest to our studies, IPA revealed *Esr1* as one of the major upstream regulators to be impacted by TCDD exposure (Table 1). This gene is known to modulate expression and action of both *Pgr* and *Igf2* genes found to be differentially methylated by the array (Figure 1B). Given the essential role of progesterone in maintaining pregnancy and the known contributions of *Igf2* to fetal development, these genes were selected for further evaluation.

Validation of methylation status of *Pgr* and *Igf2* in sperm and placentae

In order to confirm the altered methylation status of *Pgr* and *Igf2*, placental samples from F1 males were assessed by MDRE analysis. Furthermore, since the paternal contribution to pregnancy and the placenta is conveyed by the sperm, we also examined *Pgr* and *Igf2* methylation status in caudal sperm obtained from F1 males. Next, in order to determine if these epigenetic changes were stable and inheritable, we also examined the methylation status of these genes in DNA obtained from F3 male-derived samples. As shown in Figure 2A and B, consistent with the findings of the array, MDRE revealed a significant increase in sperm *Pgr* methylation and a significant decrease in *Igf2* methylation in the F1 group vs control ($P < 0.05$). Although similar trends were noted in sperm from the F3 males compared to control, these changes in methylation were not significant. Within the placental tissues (Figure 2C and D), we also noted hypermethylation of *Pgr* and hypomethylation of *Igf2* in F1 and F3 tissues compared to control; however, these changes were only significant for *Pgr* ($P < 0.05$ for both F1 and F3 samples compared to controls).

Increased placental expression of *Dnmt1, Dnmt3a,* and *Dnmt3b* mRNA

Although the DNA methylation array indicated that placental DNA methylatranferases were not epigenetically modified as a consequence of paternal TCDD exposure, a number of studies have demonstrated that *Dnmt* gene expression is suppressed by estradiol and progesterone [29, 39–41]. Since the microarray indicated that TCDD exposure was associated with hypermethylation of both *Esr1* and *Pgr,* it is possible that reduced steroid action in the placenta may lead to a failure to downregulate expression of these enzymes. Indeed, as shown in Figure 3, we found that *Dnmt1* mRNA was significantly ($P < 0.05$) increased in placentae obtained from mating partners of F1 and F3 males compared to control tissues (Figure 3A). However, expression of the de novo methylation enzymes *(Dnmt3a* and *3b*) was not significantly impacted in our experimental tissues (Figure 3B and C).

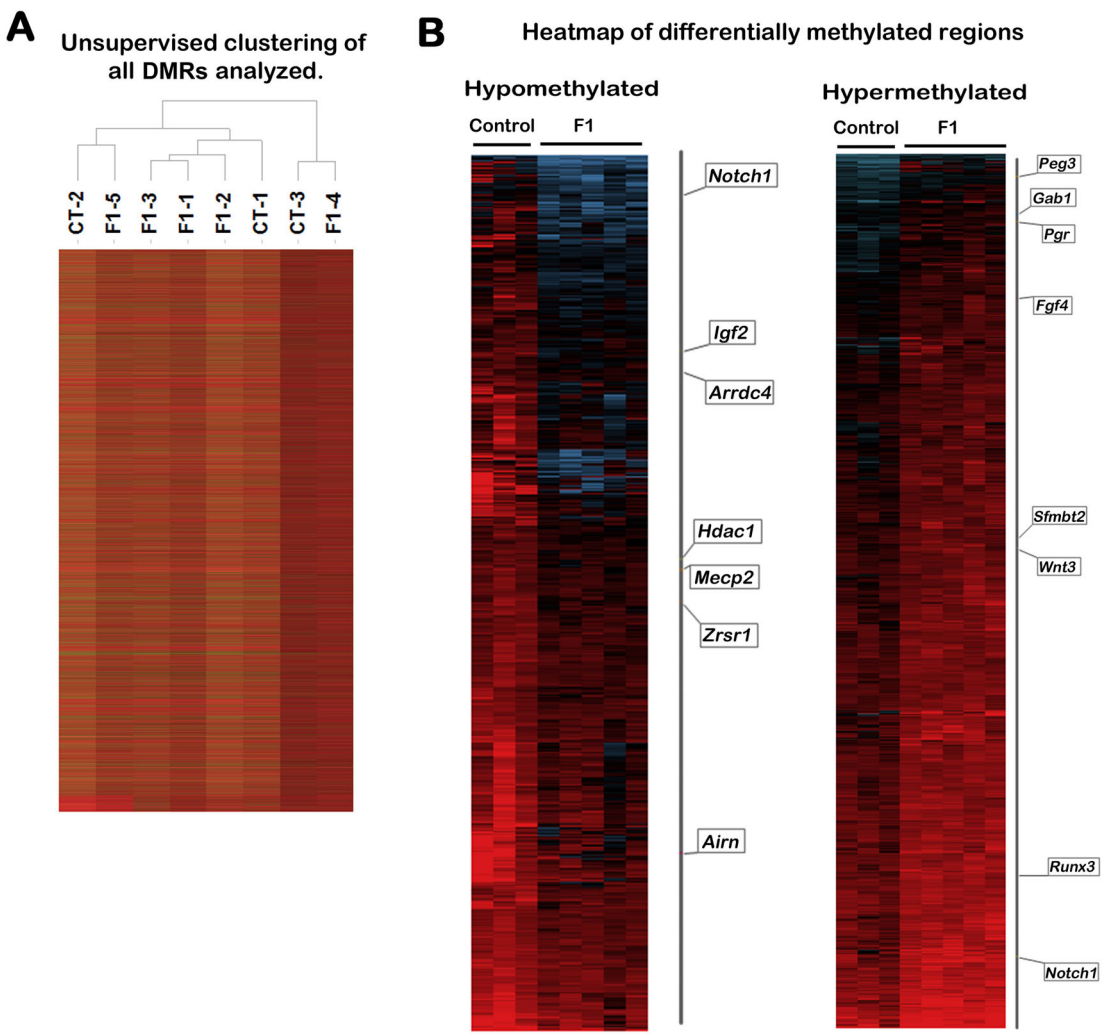

Figure 1. DNA microarray of placental samples. Methylation status of 15,913 CpG islands was analyzed and differentially methylated regions (DMR) between control (N = 3) and F1 male-derived (N = 5) placentae was determined. Unsupervised clustering of all CpG islands revealed that data sets did not cluster by treatment group (Figure 1A). Colors correspond to relative abundance of the transcripts detected, with red indicating high expression and yellow denoting low expression. Regions exhibiting significant ($P < 0.05$) differences in methylation are presented as a heat map (Figure 1B). The array identified 1,329 hypermethylated CpG islands (red, right) and 842 hypomethylated CpG islands (blue, left). The array found that the methylation status of 13,597 CpG islands was not impacted. Selected genes, known to be important in pregnancy maintenance and/or fetal development, are noted.

F1 male-derived placentae exhibit reduced expression of *Pgr, Igf2,* and *H19* mRNA

To further examine the impact of epigenetic modification of our selected genes, we next conducted quantitative RT-PCR to determine mRNA expression of *Pgr-b*, *Pgr-a/b*, and *Igf2*. Consistent with the results of the methylation assessment, compared to controls, expression of these genes was reduced in placental tissues arising from either F1 or F3 males. These changes were significant for *Pgr-b* in both F1 and F3-derived samples ($^{*}P < 0.05$), while the change in *Pgr-a/b* mRNA expression reached significance only in F1-derived tissues ($^{**}P < 0.01$) (Figure 4). Notably, it is known that hypomethylation of *Igf2* is associated with a decrease in mRNA expression [42]. Compared to control samples, *Igf2* mRNA expression was significantly reduced in F1 samples ($^{*}P < 0.05$), but was unchanged in placentae arising from F3 males. Lack of a significant reduction in *Igf2* in F3-derived samples led us to additionally explore the expression of *H19* mRNA. The *H19* gene is located adjacent to *Igf2* and this noncoding RNA is also implicated in regulating fetal growth (rev in [43]). As shown in Figure 4C, H19 mRNA was significantly reduced in both F1 and F3 male-derived placentae ($^{*}P < 0.05$).

Loss of placental PGR is associated with an increased risk of preterm birth

We next examined PGR protein expression in formalin-fixed placentae obtained from control, F1, and F3 males. As expected, the expression of PGR within the maternally derived decidual zone was not significantly different between groups; however, immunohistochemical analysis revealed a significant reduction in placental PGR localization within the junctional and labyrinth zones in partners of F1/F3 males compared to control tissues ($^{*}P < 0.05$; $^{**}P < 0.01$; Figure 5). Premature loss of progesterone action in pregnancy is associated with PTB in women and mice [44–46] and, similar to our previous report [7], control partners of the F1 and F3 males utilized

Table 1. Summary of Results Following Ingenuity Pathway Analysis of Microarray.

Canonical Pathways Predicted to Be Most Impacted by TCDD

Name	p-value	Overlap
Molecular Mechanisms of Cancer	1.14E-13	21.8 % 86/394
Axonal Guidance Signaling	5.66E-10	18.8 % 85/452
Human Embryonic Stem Cell Pluripotency	3.57E-08	25.2 % 36/143
Regulation of the Epithelial-Mesenchymal Transition	1.27E-07	22.2 % 42/189
Huntington's Disease Signaling	6.98E-07	19.6 % 49/250

Top 5 Upstream Regulators Predicted to be Impacted by TCDD

Upstream Regulator	p-value of overlap
SOX2	3.43E-11
ESR1	9.87E-10
Histone h3	5.06E-09
TP53	9.55E-09
KRAS	2.16E-08

Top 10 Up/Down Regulated Molecules

Expr Log Ratio up-regulated		Expr Log Ratio down-regulated	
Molecules	Expr. Value	Molecules	Expr. Value
Oip5	↑ 8.930	**PRPF38A**	↓ -7.992
C12orf29	↑ 8.780	**SLC7A14**	↓ -6.764
Fmnl1	↑ 8.373	**RBBP6**	↓ -6.075
HS3ST2	↑ 8.321	**IL4R**	↓ -5.820
ARPC5	↑ 8.273	**API5**	↓ -5.389
AXIN2	↑ 8.255	**AP1G1**	↓ -4.929
SVOP	↑ 7.763	**Arl4c**	↓ -4.852
TSPAN4	↑ 7.566	**GPX4**	↓ -4.709
ARID1A	↑ 6.988	**CDCA2**	↓ -4.638
ATXN7	↑ 6.566	**SCAF8**	↓ -4.624

in the current study also exhibit an enhanced risk of spontaneous PTB (Supplementary Table S2).

Direct (F1) or ancestral (F3) TCDD exposure is associated with loss of IGF2 and intrauterine growth restriction

Igf2, a paternally expressed imprinted gene, is known to play a role in promotion of fetal growth (rev by [47]). Consequently, hypomethylation, which results in loss of gene expression [48, 49], has been linked to IUGR [42, 50, 51], potentially as a consequence of dysregulated storage and transport of placental glycogen [52]. Therefore, we examined expression of IGF2 in placentae of placentae arising from control, F1 and F3 males. Using immunohistochemical localization, we found that although IGF2 protein is abundant in late pregnancy (E18.5) control placentae (Figure 6A), expression of this protein is markedly lower in both F1 and F3-derived placentae ($^*P < 0.01$; Figure 6B and C). Not surprisingly, offspring of F1 and F3 males consistently exhibited IUGR and placentae of these pregnancies were significantly smaller compared to control pregnancies (Figure 7).

Discussion

Although pregnancy outcome is considered tightly related to women's health, the father contributes substantially to pregnancy outcomes and fetal health [8, 53–58]. Recent research has solidified our understanding of the significant contribution of the paternal parent to placental development in women and mice [8, 15–18, 58, 59]. Moreover, placental inflammation is an important determinant of the timing of parturition [38, 60]; not surprisingly, premature placental inflammation has been associated with PTB [8, 61, 62]. Using a murine model, we previously found that premature placental inflammation and PTB frequently occur in association with a paternal history of developmental exposure to TCDD [8, 9], a common environmental contaminant associated with combustion and known endocrine disruptor. Significantly, the increased risk of TCDD-associated PTB was not confined to partners of directly exposed mice (F1 animals and their offspring, F2 mice), but was also observed in the mating partners of F3 animals, the first generation without direct toxicant exposure [7].

The transgenerational occurrence of PTB in mice following developmental TCDD exposure suggests that stable and inheritable epigenetic modifications have occurred within the male germline. Although of interest to examine the complete sperm epigenome, which would likely provide insight into the infertility frequently observed in

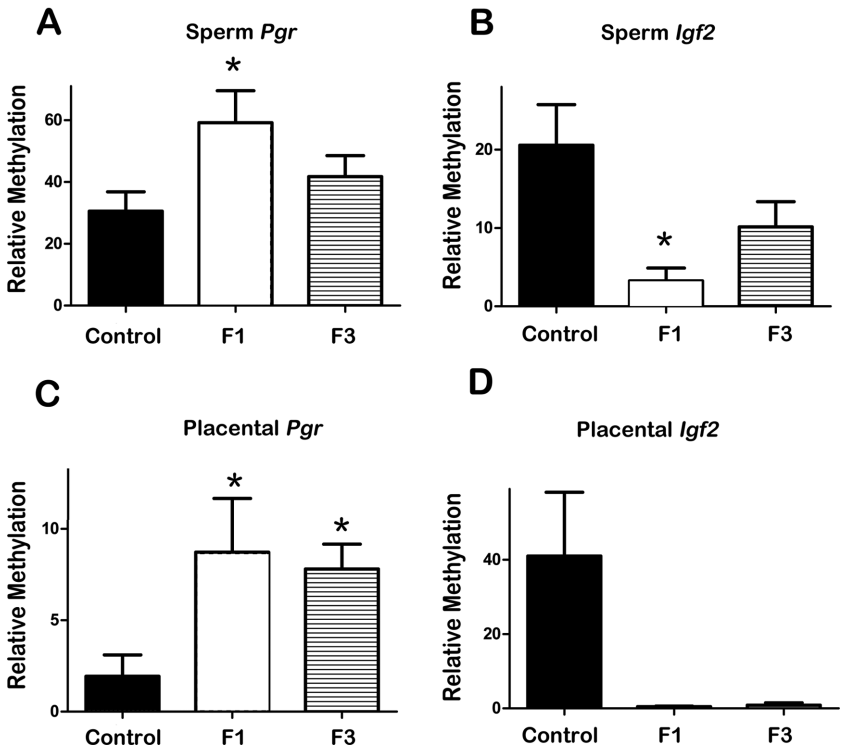

Figure 2. Assessment of methylation status by methylation-dependent restriction enzyme (MDRE): methylation assessment revealed a significant increase in methylation of *Pgr* and a significant decrease in *Igf2* methylation of sperm DNA from F1 males compared to control mice (*$P < 0.05$ for both groups), but only nonsignificant changes in the F3-derived cells (Figure 2A and B). Within placental samples, MDRE of *Pgr* demonstrated hypermethylation of the promoter region in both F1 (*$P < 0.05$) and F3 (*$P < 0.05$) male-derived tissues compared to control samples. Compared to control samples, the assay for *Igf2* showed relative hypomethylation in both F1 and F3 male-derived placentae, but the change in methylation was not significant. Sperm samples were analyzed from ≥5 males/group, while a single placenta from a minimum of 4 dams/group was utilized.

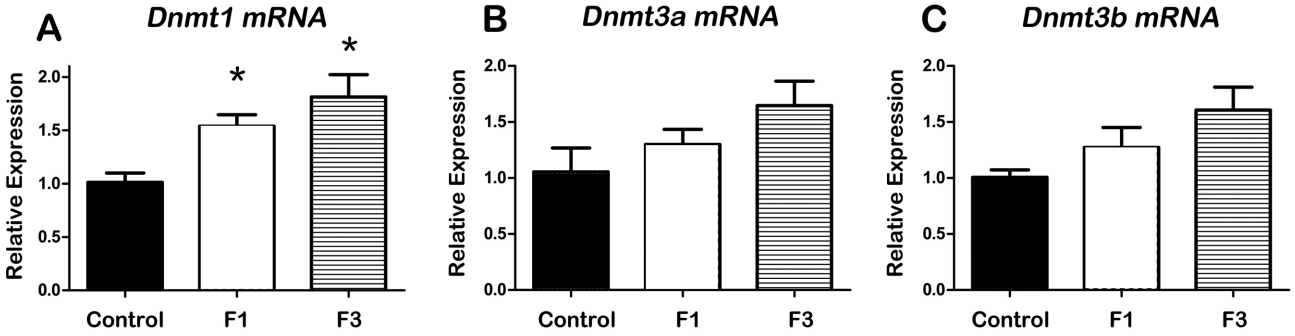

Figure 3. RT-PCR of *Dnmt1, Dnmt3a,* and *Dnmt3b.* RNA from whole placental tissue was extracted, and quantitative RT-PCR was performed to evaluate *Dnmt1, Dnmt3a,* and *Dnmt3b* expression and normalized to *18S.* Compared to control animals, *Dnmt1* mRNA was significantly increased in F1 and F3 male-derived placentae (*$P < 0.05$). Placental expression of *Dnmt3a* and *Dnmt3b* exhibited insignificant increases in both experimental groups compared to control. N ≥ 4/group.

these mice [7, 8], herein, our goal was to identify possible candidate genes which may contribute to the paternal-derived risk of PTB and placental dysfunction. Since the sperm epigenome is known to be capable of influencing the placental epigenome [63, 64], in the current study, we conducted an epigenetic microarray analysis of late pregnancy placentae arising from control female mice mated to control males or males with a direct TCDD exposure (F1 mice). Notably, the majority of CpG regions analyzed by the DNA methylation microarray revealed only a modest impact of paternal in utero TCDD exposure. There are at least two possible explanations for the limited effect of prior TCDD exposure in our model. First, pregnant dams (F0 mice) receive only a single dose of TCDD in on E15.5 of pregnancy, resulting in exposure of F1 feti during the end of pregnancy and early lactation. This timing corresponds only partially with the timing of male germ cell epigenetic mark erasure and remethylation [65]. Secondly, as we previously reported, infertility is common among F1 mice; therefore, it is likely that fertile mice would exhibit fewer alterations compared to mice exhibiting infertility.

Despite these limitations, the microarray analysis revealed ~2200 differentially methylated regions (DMRs), many of which

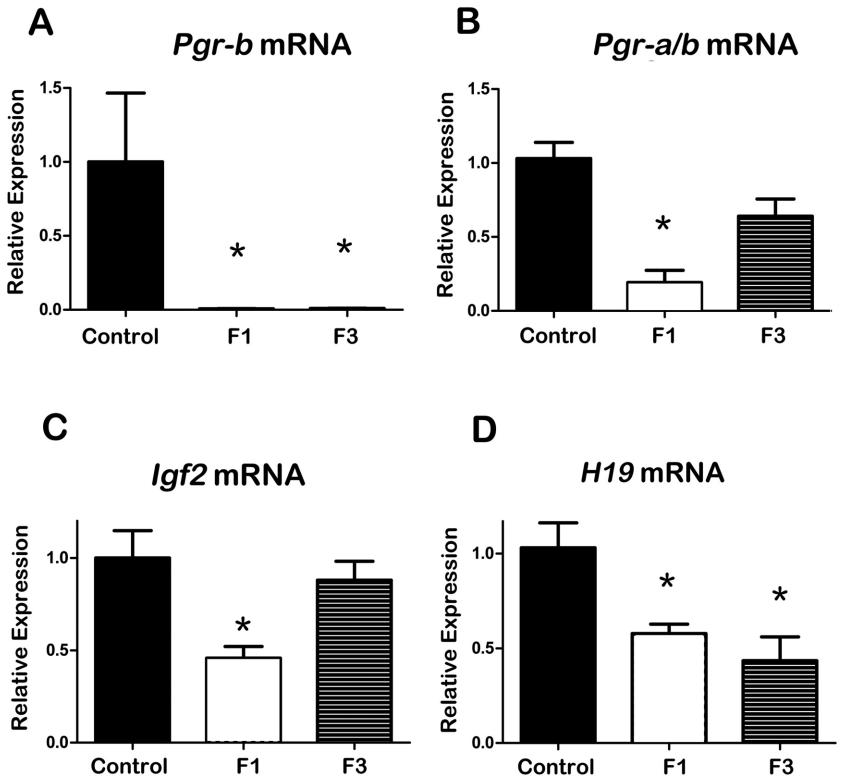

Figure 4. RT-PCR of *Pgr, Igf2,* and *H19* mRNA in placental tissues arising from control, F1 and F3 males. RNA from whole tissue was extracted and quantitative RT-PCR was performed to evaluate *Pgr, Igf2,* and *H19* expression and normalized to *18S*. Compared to control animals, *Pgr-b* mRNA was significantly lower in F1 and F3 male-derived placentae (A, $^{*}P < 0.05$), while the reduction in *Pgr-a/b* mRNA only reached significance in the F1 male-derived samples (B, $^{**}P < 0.01$). Although expression of *Igf2* mRNA was diminished in F1 and F3-derived placentae compared to control, the change was significant only in F1-derived tissues (C). Compared to control samples, *H19* mRNA expression was significantly reduced in both experimental groups (D, $^{*}P < 0.05$). N ≥ 4/group.

correspond to genes previously linked to PTB [66–69]. Ingenuity Pathway Analysis further identified *Esr1* as one of the top upstream regulators impacted in the placental tissues following paternal developmental TCDD exposure (Table 1). Altered expression of *Esr1* would be expected to disrupt expression and regulation of numerous genes, including *Pgr* and *Igf2*, genes which were also identified by the array as being differentially methylated. Appropriate expression of *Pgr* is critical for pregnancy maintenance while placental *Igf2* plays an important role in regulating fetal growth. Our previous studies demonstrate that gestation length in control females mated to F1 males is compromised, while pups exhibit IUGR [8]. Therefore, *Pgr* and *Igf2* were selected for validation and further examination herein. As shown in Figure 2, validation studies confirmed that both *Pgr* and *Igf2* exhibit alter methylation in F1-derived placentae. Similar epigenetic changes were identified in these genes within the sperm, the male germ cell (Figure 2), suggesting the potential for transmission of these changes to future generations. Indeed, examination of sperm and placental samples from F3 males also revealed hypermethylation of *Pgr* and hypomethylation of *Igf2*, although only placental *Pgr* methylation changes were statistically significant (Figure 2).

Next, placental samples were further assessed for *Pgr* and *Igf2* mRNA expression and immunolocalization of corresponding proteins. We demonstrated that mRNA and protein expression were reduced in F1 and F3 male-derived placentae compared to controls (Figures 4–6), with most changes being less marked in tissues from F3 animals compared to F1 males. The limited transgenerational effect of TCDD exposure in our model is not surprising, given our primary focus on pregnancy outcomes. Specifically, as we previously reported, infertility is common in F1, F2, and F3 males [7, 8] and all three generations have higher mortality compared to controls [58]. Since the most severely impacted animals are infertile or die before weaning, these mice cannot be used for placental epigenetic analysis or transgenerational studies. Thus, it is quite remarkable that any epigenetic changes and adverse outcomes are still observed in the F3 animals since they are the descendants of mice exhibiting the best reproductive outcomes.

Nevertheless, the intriguing observation of the persistence of PTB and IUGR in F3 male-derived pregnancies led us to focus the current study on *Pgr* and *Igf2*. Progesterone, known as the "progestational hormone" is essential for both pregnancy establishment and maintenance of pregnancy to term. Loss of progesterone action at the maternal–fetal interface is a prerequisite for parturition, regardless of whether birth occurs at term or prior to term [68]. Our studies demonstrated that in utero TCDD exposure of male mice leads to hypermethylation of *Pgr* in F1 and F3 male-derived placentae and an enhanced risk of PTB in female partners (Figure 2, Supplementary Table S2). Consistent with the observation of IUGR, our study also identified altered regulation and expression of *Igf2*, a paternally expressed imprinted gene that is known to promote fetal growth [71]. However, lack of significant changes in methylation and expression within the F3 derived tissues led us to additionally examine *H19* mRNA expression. *H19*, a maternally expressed imprinted gene, is

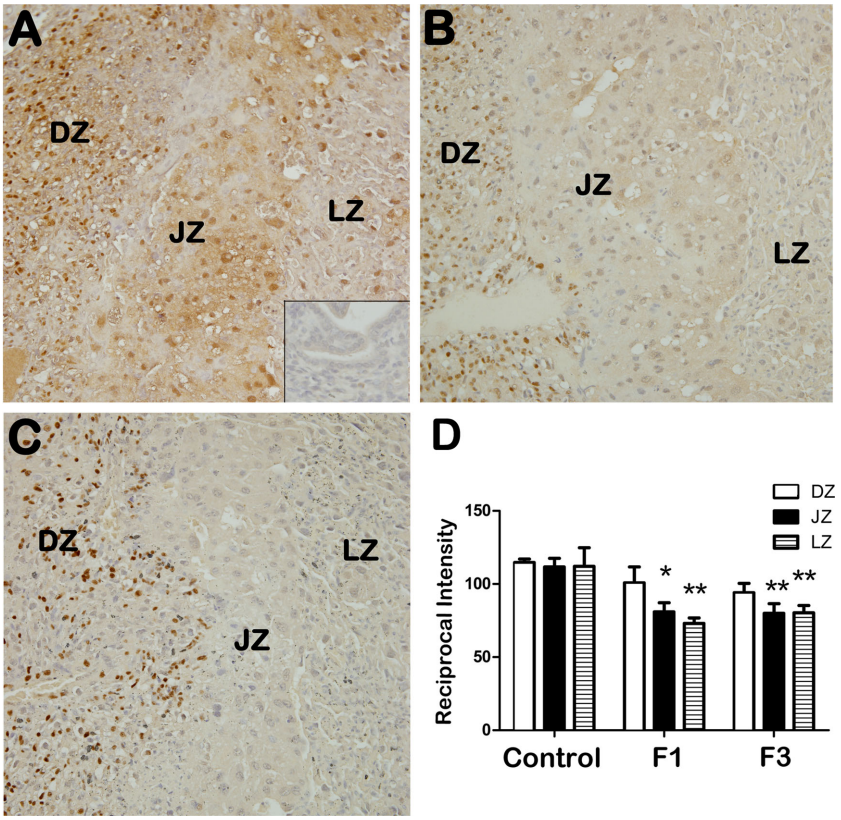

Figure 5. Immunohistochemical localization of PGR in E18.5 placentae. Placental and decidual tissues were obtained on E18.5 from females mated to control (A), F1 male (B), and F3 male (C) mice. Tissues were subjected to immunohistochemical localization of PGR (brown staining). ×200. DZ, decidual zone; JZ, junctional zone LZ; labyrinth zone. Inset in A: Negative control. Computer-assisted semi-quantitative assessment of staining is shown in (D). $^{*}P < 0.05$; $^{**}P < 0.01$. All fixed placentae from a minimum of 5 litters/group were analyzed.

found on the same chromosome and in close proximity to *Igf2*, and these genes are thought to have reciprocal roles in regulating fetal growth [70]. Paternal silencing of *H19* and maternal repression of *Igf2* both depend on a common cis-acting element. Numerous studies have demonstrated that hypomethylation of the *H19/Igf2* promoter within the placenta results in enhanced expression of *H19*, which leads to repression of *Igf2* expression and smaller offspring [48, 49, 71]. Thus, concomitant with the hypomethylation of *Igf2* described herein (Figure 2), we anticipated an increase in *H19* mRNA expression. Surprisingly, we identified a statistically significant *decrease* in expression of this gene in F1 and F3 male-derived placentae (Figure 4). Although unexpected, our data are consistent with studies described by Zuckerwise et al [72], who recently reported decreased expression of *H19* mRNA in placentae of human pregnancies complicated by IUGR, potentially as a consequence of inadequate trophoblast invasion. In another study, treatment of pre-implantation embryos with TCDD in vitro was associated with repression of both *H19* and *Igf2* mRNA, which correlated with a significant reduction in fetal weight following transfer of embryos to untreated Dams [73]. Clearly, the regulation of fetal growth is complex and involves the interaction of numerous factors at the maternal–fetal interface.

In addition to IUGR, our study additionally identified significantly smaller placentae in association with direct (F1) or ancestral (F3) TCDD exposure (Figure 7), which may also contribute to the restricted fetal growth. Kent et al. [74] recently reported that *Igf*$^{-/-}$ mice exhibit reduced placental and fetal weight along with a reduced ability to accumulate placental glycogen. Interestingly, we previously reported reduced placental glycogen in placentae arising from F1 males, but not F1 females [58], potentially exacerbating the loss of paternal imprinting of *Igf2* [75]. Taken together with the findings of others, our data support the hypothesis that paternal exposure to TCDD leads to alterations within the placental epigenome which was associated with placental dysfunction, impaired fetal development, and decreased gestation length.

Finally, we also examined the impact of direct and indirect TCDD exposure on placental expression of the DNA methyltransferases (*DNMTs*), a highly conserved family of enzymes that establish and maintain methylation marks. The most well characterized of these enzymes are *DNMT1, DNMT3a,* and *DNMT3b* (review by [76]). *DNMT1* is the maintenance enzyme, while *DNMT3a* and *3b* are responsible for de novo methylation. In our study, although the DNA microarray did not indicate a change in the methylation of *Dnmt1*, we found a significant increase in mRNA expression of this gene in both F1 and F3-derived placentae compared to control tissue (Figure 3). The microarray indicated that paternal TCDD exposure did not impact the methylation status of placental *Dnmt3a* or *Dnmt3b*; we observed only a nonsignificant trend for increased expression of these genes in experimental tissues compared to controls (Figure 3). Several studies have demonstrated that, in the human endometrium, all three of these *DNMT*s are downregulated during the progesterone-dominant secretory phase [39–41, 77] despite a lack of effect on methylation status [39]. Herein, we report an increase in expression of *Dnmt* mRNA in F1 and F3-derived placentae concomitant with loss of progesterone responsiveness.

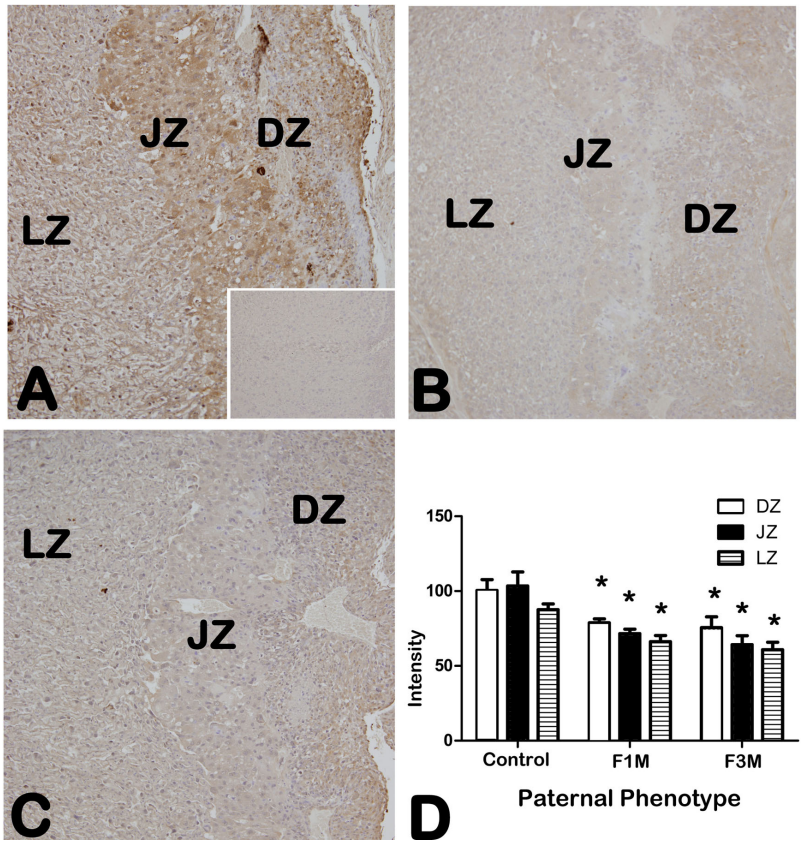

Figure 6. Immunohistochemical localization of IGF2 in E18.5 placentae. Placental and decidual tissues were obtained on E18.5 from females mated to control (A), F1 male (B), and F3 male (C) mice. Tissues were subjected to immunohistochemical localization of IGF2 (brown staining). ×100. DZ, decidual zone; JZ, junctional zone LZ; labyrinth zone. Inset in A: Negative control. Computer-assisted semi-quantitative assessment of staining is shown in (D). $^{*}P < 0.01$. All fixed placentae from a minimum of 5 litters/group were analyzed.

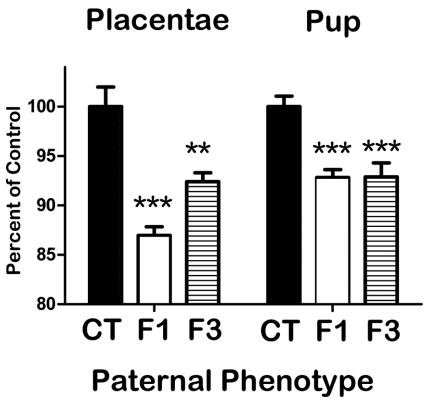

Figure 7. Placental and pup weight on E18.5. Placentae, with decidua removed, were collected on E18.5 from all groups and weighed. Pups were also collected and weighed. Average weight of control placenta was 0.1091 g ± 0.002181 and is shown graphically as 100%. The average weight of placentae arising from F1 males (0.09487 g ± 0.000957) and F3 males (0.1008 g ± 0.0009964) are shown as a percent of control weights. Average weight of control pups was 1.176 g ± 0.01269 and is shown graphically as 100%. The average weight of F2 and F4 pups (offspring of F1 and F3 males, respectively) were 1.092 g ± 0.00937 and 1.093 g ± 0.01682 and are shown as a percent of control. $^{***}P < 0.001$; $^{**}P < 0.01$. At least seven litters were assessed for each group.

In summary, our previous data have demonstrated a transgenerational impact of paternal TCDD exposure on pregnancy outcomes [7, 8], suggesting the likely occurrence of epigenetic modification within the male germline. Our data presented herein clearly demonstrate that developmental toxicant exposure of male mice (F1) can alter the placental epigenome of a subsequent adult pregnancy, thereby contributing to placental dysfunction and adverse pregnancy outcomes in his control partner. Although molecular endpoints only exhibited modest changes in F3 mice, alterations in functional outcomes (PTB and IUGR) remained significant, suggesting additional, TCDD-sensitive genes likely act in concert with *Pgr* and *Igf2* to influence transgenerational pregnancy outcomes. Examination of additional DMRs, identified by the array, should continue to shed light on the role of the paternal parent in mediating the placental phenotype and its role in pregnancy maintenance and fetal development.

In addition to a direct impact of TCDD on DNA methylation, it is possible that developmental exposure of this toxicant may influence histone acetylation and/or expression of microRNAs. Future studies should clarify the mechanisms by which TCDD alters the sperm epigenome; potentially influencing the placental epigenome and pregnancy outcomes. Finally, since the human male also influences the placental phenotype, our findings suggest paternal toxicant exposures may be contributing to the frequent occurrence of PTB in women with no known risk factors.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Overview of study design. Pregnant C57bl/6 mice were exposed to 10 μg/kg TCDD on E15.5 by gavage. Male offspring (F1 males) were mated at adulthood to control females. After mating, males were euthanized and caudal sperm collected. Some females were euthanized on E18.5 for collection of placental tissues; additional animals were allowed to spontaneously deliver, producing F2 mice. At adulthood, F2 males were mated to control females; all pregnancies were allowed to continue to spontaneous delivery. F3 males were subjected to the same experimental endpoints as F1 males.

Supplemental File S1. Canonical pathways identified by Ingenuity Pathway Analysis as being significantly impacted by TCDD exposure. Only entities with a significant difference between groups (with a –log(*P*-value) greater than 1.3) are shown.

Supplemental Table S1. PCR primers.

Supplemental Table S2. Fertility and pregnancy outcomes.

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