Paternal exposure to the pesticide DDT induces intergenerational programming of breast cancer predisposition via sperm miRNA

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Abstract

Background: DNA sequence accounts for the majority of disease heritability, including cancer. However, it is becoming clear that environmentally-induced epigenetic inheritance can also occur. Epidemiological studies have shown that maternal exposure to the pesticide DDT in pregnancy is associated with increased breast cancer risk in women. Yet, the effects of paternal exposure to this and other pesticides on the progeny's breast cancer development has not been investigated.

Methods: Male mice (*c57bl/6*) were exposed to DDT or to a control-vehicle (CO) solution and used for sperm collection or mating with unexposed females to produce the DDT or CO daughters. In another experiment, normal mouse embryos (zygote stage) were injected with miRNA-10b and implanted into surrogate mothers to produce miR-10b offspring. DDT daughters or miRNA-10b females were used to study breast cancer development and metabolic parameters. Paternal sperm was used for RNA-seq analysis and miRNA expression levels.

Results: Pre-conception paternal DDT exposure altered the sperm small non-coding RNA load, with an increase in miRNAs and a specific surge in miRNA-10b levels. DDT offspring weighed less at birth and at weaning, but became overweight and showed metabolic dysfunction in adulthood compared to CO. DDT daughters also showed increased mammary tumorigenesis, developing more aggressive tumors that grew faster than in CO. This tumor phenotype was linked to suppression of the AMPK energy sensing pathway and mTOR activation in mammary tissues. Remarkably, embryonic injection of miRNA-10b recapitulated the mammary gland and tumor phenotypes observed in DDT daughters.

Conclusions: To our knowledge, this is the first report of an association between paternal DDT exposure and breast cancer in offspring. Paternal DDT-induced programming of breast cancer

development in daughters is mechanistically linked to sperm miRNA-10b. The impact of DDT and other endocrine disrupting chemicals on sperm and programming of breast and other cancers in offspring needs to be evaluated in humans.

Introduction

DNA sequence is responsible for the majority of disease heritability, including cancer. It is becoming clear, however, that epigenetic inheritance can also occur. At conception, parents not only contribute their genome but also transmit a molecular memory of past environmental experiences to their progeny [1, 2]. This can result in offspring's predisposition to chronic diseases later in life [3]. This environmentally-induced risk of diseases, including breast cancer, has been shown to be transmitted to the offspring via epigenetic mechanisms through both the female and male germ-lines [3-5]. Although most of the evidence for this mode of disease inheritance comes from maternal exposures in pregnancy, we [6-9] and others [10-12] have shown that paternal exposures in the pre-conception window are also important in determining disease outcomes in the offspring.

Several recent published reports demonstrated that the small RNA load in paternal sperm can transmit environmentally-induced phenotypes to the offspring [2, 5, 13, 14]. Some of those studies implicate specific classes of small RNA and both miRNAs and tRFs obtained from sperm can recapitulate the effect of specific paternal exposures in offspring when injected into normal embryos following in vitro fertilization [5, 13-15].

Dichlorodiphenyltrichloroethane (DDT), one of the first synthetic organochlorine pesticides, was widely used in the United States (U.S.) from the 1940s to the 1970s and up to the 1980s in Europe [16, 17]. While the use of DDT has been restricted in the U.S. and Europe for decades, developing countries with endemic malaria and other vector-borne diseases continue to use it [17-19]. This pesticide has a prolonged half-life and is lipophilic, bio-accumulating in the food chain, and is still detected in fatty tissues and circulation of animals and humans [17]. In the U.S., the highest concentrations of DDT are detected in minority populations and recent

immigrants [20, 21]. Like other organochlorines, DDT is an endocrine disruptor [17, 22] and it has been linked to metabolic syndrome [23], obesity [24] and cancer [18, 25]. Because the mammary gland bud first emerges during fetal development, it has been proposed that some breast cancers may originate *in utero* [26] and multiple reports on maternal exposure in pregnancy have offered support to this hypothesis [4, 18, 27, 28]. In line with that, a recent epidemiological analysis using the Child Health and Development Studies cohort (CHDS) showed that daughters of women exposed to high levels of DDT in pregnancy have increased rates of breast cancer [18] and are more likely to be diagnosed with advanced stage and HER-2 positive tumors. Other studies in the same cohort found that women exposed to this pesticide before age 14 have increased breast cancer risk, with the highest risk if the exposure occurred in early infancy [25, 29].

While there is a clear link between maternal exposure to DDT and breast cancer in the next generation, no studies to date have investigated the contribution of pre-conception paternal exposure to this or other pesticides to offspring's breast cancer development. Here, we tested the hypothesis that paternal exposure to DDT can alter the sperm non-coding RNAs and program breast cancer development in daughters, using a mouse model of this disease. We also examined whether paternal DDT-induced programming of breast cancer development in daughters is mechanistically linked to sperm miRNAs.

Materials and Methods

DDT exposures and breeding: Animals were purchased from Taconic Biosciences. The *c57bl/6* strain of mice was used in all experiments. Adult male mice (8 weeks of age) were either treated with a DDT solution (1.7 mg/kg) or vehicle-control solution (vegetable oil) via oral gavage for

two weeks. The DDT solution used mimics the formulation of DDT before its ban in the U.S.: 77.2% p,p'-DDT and 22.8% o,p'-DDT as described before [23]. Animals body weight was monitored weekly. At the end of the exposure period, DDT-exposed and control male mice were mated to unexposed females to generate the female offspring (**Fig. S1**). Female mice were kept on a standard chow diet during the breeding period, for the extent of pregnancy (21 days) and after giving birth. The birth weight of pups and number of pups per litter was determined. To avoid litter-effect, pups were cross-fostered one day after birth. Pups from 2-3 dams were pooled and housed in a litter of 8-10 pups per nursing dam. All pups were weaned on postnatal day (PND) 21. Offspring's body weight was recorded weekly. The female offspring of control or DDT-exposed fathers were used to study body weight, metabolic function, and mammary tumorigenesis and for mammary gland collection as described in the following sections. The number of contributing fathers and female offspring used in each experiment is listed in **Table S1**. All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were performed following the National Institutes of Health guidelines for the proper and humane use of animals in biomedical research.

DDT and Phenobarbital (PB) treatment: PB treatment is known to promote hepatic enzymes activation, accelerating DDT metabolism and excretion in humans [30, 31] and animals [32-34]. A sub-set of control-vehicle or DDT exposed males were concomitantly treated with phenobarbital (20 mg/kg of body weight, i.p.) or saline and used for sperm harvesting.

Mature spermatozoa collection and purification: Control and DDT-exposed males as well as those treated with PB were euthanized and caudal epididymis dissected for sperm collection. The epididymis was collected, punctured, and transferred to tissue culture dish containing M2 media (M2 Medium-with HEPES, without penicillin and streptomycin, liquid, sterile-filtered, suitable

for mouse embryo, SIGMA, product #M7167) where it was incubated for 1 hour at 37°C. Sperm samples were isolated and purified from somatic cells. Briefly, the samples were washed with PBS, and then incubated with SCLB (somatic cell lysis buffer, 0.1% SDS, 0.5% TX-100 in Diethylpyrocarbonate water) for 1 hour. SCLB was rinsed off with 2 washes of PBS and the somatic cell-free purified spermatozoa sample pelleted and used for RNA extraction.

Measurement of DDT metabolites: Liver tissues from control, DDT and DDT-PB treated males (n=3 pooled samples/group), collected within 24 h after last dose, were used to determine the levels of DDT main metabolites. Measurements were performed commercially by Pacific Rim Labs, a fully accredited laboratory providing analysis of DDT and other persistent organic pollutants. Briefly, samples were fortified with each of the six ¹³C-DDT isotopes and then extracted with hexane. The extract was columned on Florisil and analyzed by GC-MS/MS. The instrument was calibrated with a five-point calibration covering the range of 0.2-400 ng/g or mL.

Body weight monitoring: DDT female offspring's body weight was measured at birth (n=8-17 litters/group) and at weaning and weekly after that (n=25/group). Birth weights were analyzed by t-test and longitudinal body weight analyzed using two-way ANOVA (group, time), followed by post-hoc analyses.

Small RNA-Seq Analysis: Total RNA was isolated from paternal sperm (n=4/group) using Qiagen's miRNeasy extraction kit, according to the manufacturer's instructions. Small non-coding RNA transcript libraries were constructed according to Illumina TrueSeq Small RNA Pre-Kit. Indexed, paired-end sequencing libraries were prepared from quality total RNA (RIN ≥ 8). For each library, 10 million reads (raw data) were generated by Illumina Hi-Seq 4000. The raw reads were subject to adapter trimming and low quality filtering using Trimmomatic program. The high quality clean reads were aligned on the mouse genome. Non-coding RNA

tags were mapped to the mouse genome (GRCm38/mm10 reference genome) in order to analyze their expression and distribution on the genome. Small RNA tags were annotated with miRNA, t-RNA and pi-RNA to miRBase, Ref-seq, GenBank and Rfam database using blastn with standard parameters. To analyze the differential expression of miRNA between control and DDT groups, miRNAs were normalized to TPM (Transcripts Per Kilobase Million). miRNAs with a P value less than 0.05 were considered significant, with an appropriate correction for multiple testing [35].

Quantitative real time PCR validation (qRT-PCR): cDNA was synthesized from total RNA samples using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems). PCR products were amplified from cDNA using TaqMan® Fast Advanced Master Mix and sequence-specific primers from TaqMan Assays (Applied Biosystems). Fold change was calculated from Ct values and the expression levels of specific miRNAs or genes were determined by normalizing these values with the fold change values for appropriate endogenous controls (miR-26a or *GAPDH*). Fold differences between the groups were analyzed using t-test or two-way ANOVA (group, treatment), followed by post-hoc analyses.

Carcinogen-induced mammary tumorigenesis: Mammary tumors were induced in control and DDT female offspring (n=25/group) by administration of MPA (medroxyprogesterone acetate, 15 mg, s.c.) at 6 weeks of age, followed by three weekly doses of 1mg of 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma, St. Louis, MO) dissolved in peanut oil by oral gavage. This established model of breast cancer has been used by us and others [6, 36]. Mice were examined for mammary tumors by palpation once per week, starting on week two after the last dose of DMBA and continue for a total of 20 weeks. Tumor growth was measured using a caliper and the width and height of each tumor were recorded. Other end-points for data analysis

were latency to tumor appearance, the number of animals with tumors (tumor incidence), and the number of tumors per animal (tumor multiplicity). During follow-up, those animals in which tumor burden approximated 10% of total body weight were euthanized, as required by our institution. Histopathology of tumors was determined by our pathologist. All tumors included in our analyses were classified as mammary carcinomas. Differences in tumor latency and multiplicity were analyzed by t-test (or corresponding non-parametric test). Kaplan-Meier survival curves were used to compare differences in tumor incidence, followed by the log-rank test. Tumor growth was analyzed using two-way ANOVA (group, time), followed by post-hoc analyses.

Embryonic miRNA microinjections. To induce super-ovulation, female mice (*c57bl/6*) received intra-peritoneal injections of 5 IU of pregnant mare's serum gonadotropin (PMSG). About 48 hours after the PMSG injection, mice were injected with 5 IU of human chorionic gonadotropin (hCG). Super-ovulated females were mated with *c57bl/6* male mice. About 16 hours later, fertilized eggs were harvested. Zygotes were injected with either synthetic miRNA-10b (*mirVana*®, ThermoFisher) diluted in TE solution (10mM Tris-HCl, pH7.4, 0.25 EDTA) or TE solution only (to control for microinjection). Each zygote received ~10 pL of TE or miRNA preparation at 2ng/μL. Microinjections were confirmed by observing the swelling of the male pro-nucleus. Injected zygotes were transferred into recipient female mice to produce offspring as described before [15]. Briefly, recipient females were mated with vasectomized males overnight and identified by copulation plug before the zygote transfer. Each recipient female was implanted with 25-30 zygotes. Dams were housed in groups of two with free access to food and water. Female offspring were used to study tumor growth, metabolic function and for mammary tissue collection. Mature sperm cells contain approximately 20 femtograms of total RNA, of

which about 20% are small non-coding RNAs and 0.4% are miRNAs [37]. Thus, the amount of miRNA injected into zygotes did not exceed physiological levels. Embryonic injections were performed at the Johns Hopkins Transgenic Core Facility. Resulting offspring were subsequently transferred to our institution for follow-up studies.

Syngeneic orthotopic tumor implantation: Mouse EO771 breast cancer cells were cultured in complete medium [Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS and 1% L-glutamine, at 37°C in 5% CO₂ (v/v)] to 80% confluence, trypsinized, counted, checked for viability and re-suspended in PBS for injection into both 4th inguinal mammary glands. A total of 1x10⁶ cells were injected into each of the 4th inguinal mammary gland fat pads of miRNA-10b females (n=6-8/group). Tumor growth was monitored for at least six weeks.

Immunohistochemistry analysis. Cell proliferation (Ki-67) was evaluated by immunohistochemistry in mammary tumors (n=5-7/group). Briefly, tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned (5 µm). Sections were deparaffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed by immersing the tissue sections at 98°C for 40 minutes in 1X Diva Decloaker (Biocare). Tissue sections were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 minutes, and were incubated with the primary antibody (**Table S2**), overnight at 4°C. After several washes, sections were treated to the appropriate HRP labeled polymer for 30 min and DAB chromagen (Dako) for 5 minutes. Slides were counterstained with Hematoxylin (Fisher, Harris Modified Hematoxylin), blued in 1% ammonium hydroxide, dehydrated, and mounted with Acrymount. The sections were photographed using an Olympus IX-71 Inverted Epifluorescence microscope at 40x magnification. Proliferation index (Ki-67 staining) was determined by

immunoRatio, a plugin Image J software (National Institute of Health, Bethesda, MD, USA) to quantify hematoxylin and DAB-stained cells. Results were analyzed by t-test (or corresponding non-parametric test).

Analysis of cell apoptosis: Cell apoptosis analysis was conducted in mammary tumors (n=14-17/group) by morphological detection. Tissues were fixed in neutral buffered 10% formalin and stained with hematoxylin and eosin (H&E). Cells presenting loss of adhesion between adjacent cells, cytoplasmic condensation and formation of apoptotic bodies were considered apoptotic as described before [38]. The sections were photographed using a Olympus IX-71 Epifluorescence microscope at 40x magnification. Twenty areas were photographed randomly and the number of apoptotic bodies counted. The apoptotic index was determined as the percentage of apoptotic bodies per total number of cells. Images were evaluated with Image J software (NIH, USA). Results were analyzed by t-test (or corresponding non-parametric test).

Mammary gland development: Inguinal mammary glands (4th pair, n=5-16/group) collected on post-natal day 50 (PND 50) were stretched onto a slide, placed in a fixative solution and stained with a carmine aluminum solution (Sigma Chemical Co.) as previously described [39]. Whole mounts were examined under the microscope (AmScope) and ductal elongation and number of TEBs (undifferentiated structure considered to be the targets of malignant transformation), as previously described [39]. In addition, whole-mount slides were digitized (Olympus SZX12 Stereomicroscope) and analyzed using the ImageJ software (National Institute of Health, Bethesda, MD, USA): The area and integrated density of a manually drawn perimeter around the mammary epithelial tree of each whole-mount were measured. The average area as well integrated density measurements are reported for each group. Differences were statistically tested using t test (or corresponding non-parametric test).

Analysis of protein expression: Protein levels were assessed by western blot on PND50 mammary tissues (n=6-7 group) and tumors (n=6/group). Total protein was extracted from mammary tissues using RIPA buffer with Halt™ Protease Inhibitor Cocktail (Thermo Fisher). Ten micrograms of protein extracts were resolved on a 4-12% denaturing polyacrylamide gel (SDS-PAGE). Proteins were transferred using the iBlot® 7-Minute Blotting System (Invitrogen, USA) and blocked with 5% non-fat dry milk for 1h at room temperature. Membranes were incubated with the specific primary antibodies (for antibody specifications and dilutions see **Table S2**) at 4°C overnight. After several washes, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1h. Membranes were developed using the Chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., USA), and exposed to blot imaging systems (Amersham™ Imager 600, GE Healthcare Life Sciences). Optical density of the bands was quantified using Quantity-one software (BIO-RAD, USA). To control for equal protein loading, expression of the proteins of interest was normalized to the β -actin or β -tubulin signal.

Metabolic Parameters: Glucose tolerance test (GTT) was performed after overnight fasting in mice. Glucose (0.75 U/kg body weight) was administered intraperitoneally and tail blood samples were collected at 0, 15, 30 and 60 minutes after injection (n=8/group). Circulating insulin levels (n=6-7/group) were measured using an ELISA kit (Ultra Mouse insulin EIA Kit, Crystal Chem). Differences in GTT were analyzed using two-way ANOVA (group, time), followed by post-hoc analyses. Insulin levels were statistically tested using t test (or corresponding non-parametric test).

Results

DDT offspring have altered body weight gain and metabolic function

Male mice were either exposed to a vehicle-control or an environmentally relevant dose of DDT (as described in the Material and Methods section) and mated with unexposed females to generate the offspring (**Fig. S1**). The phenotypes in offspring from control and DDT-exposed male mice were then examined. No differences in gender distribution or litter size were observed between offspring born from DDT and control males (**Table S3**). However, paternal DDT exposure was associated with a significant decrease in offspring's birth and weaning weights ($P<0.05$, $P<0.01$ **Fig. 1a-b**). After weaning, DDT daughters underwent a catch-up growth period and by 6 weeks of age no differences in body weight were observed between the groups. In fact, DDT females became significantly heavier than controls by 28-weeks of age ($P<0.05$, $P<0.01$, **Fig. 1c**). They also showed metabolic dysfunction with higher circulating levels of insulin and impaired glucose tolerance compared to controls in adulthood (**Fig. 2a-b**, $P<0.05$).

DDT daughters have accelerated breast cancer growth

Maternal DDT exposure has been linked to a near 4-fold increase in breast cancer in daughters [18]. Low birthweight, a phenotype observed in offspring DDT-exposed fathers, has also been linked with higher risk for breast cancer in humans [40, 41] and animal models [8, 42]. Thus, we studied mammary cancer development in control and DDT daughters using the well-established [6, 36] DMBA-induced mouse model of breast cancer. Tumor growth was significantly increased in DDT daughters compared to controls ($P<0.001$, **Fig. 3a**), with tumors in DDT offspring weighing significantly more at the end of the monitoring period ($P<0.01$, **Fig. 3b**). The incidence of mammary tumors in the DDT offspring was also higher and tumor latency slightly shorter than in controls but results did not reach statistical significance (**Fig. 3c-d**).

Given the increased mammary tumor growth in the DDT daughters, we assessed the cell proliferation and apoptosis rates in their tumors. We found a slight increase in cell proliferation rates ($P < 0.1$, **Fig. S2**) as well as a significant decrease in apoptosis rates in tumors of DDT daughters compared to controls ($P < 0.05$, **Fig. S2**).

DDT daughters' mammary gland morphological development is mostly normal

Because DDT daughters showed accelerated breast cancer growth, we next analyzed the effects paternal DDT on offspring's mammary morphological development in post-natal day (PND)50 inguinal mammary glands. We assessed number of terminal end buds (TEB), ductal elongation and epithelial area/density. No major morphological changes were detected, except for a slight increase in mammary ductal elongation in DDT daughters compared to controls (**Fig. S3**).

DDT daughters' mammary tissues have rewired nutrient sensing mechanisms

We previously showed that low birth weight is linked to suppression of the nutrient/energy sensing AMPK pathway (**Fig. 4b**) in mammary tissues and tumors [8]. Indeed, we found that both AMPK expression and phosphorylation levels were decreased in DDT daughters' mammary tissues. Consistent with that, we also detected an increase in expression and activity of mTOR and its down-stream target, 4E-BP1 (**Fig. 4b, Fig. S4a-f**). Similar patterns of mTOR and 4E-BP1 expression and activity were also found in tumors of DDT daughters, although results were not statistically significant (**Fig. 4c, Fig. S4g-l**).

Pre-conception paternal DDT exposure alters the sperm non-coding RNA content

To determine the impact of DDT exposure on paternal sperm, we performed RNA-seq analysis to assess the small RNA patterns in mature cauda sperm of control and DDT-exposed males. The distribution of the major small RNA species detected is in line with previous reports [13, 14], with tRNA-derived fragments (tRFs) being the most abundant sperm small RNA sub-type,

followed by miRNAs and piRNAs in both groups (**Fig. 5a**). Interestingly, sperm of DDT-exposed males showed higher abundance of miRNAs (6.7%) compare to controls (1.4%) (**Fig. 5a-b**). A total of 16 miRNAs were differentially expressed (nine down- and seven up-regulated) in sperm of DDT-exposed mice compared to controls (**Fig. 5c**). Of the seven miRNAs upregulated in sperm of DDT-exposed males, miRNA-10b had the highest surge, representing close to 60% of the overall miRNA increase (**Fig. 5d**).

To establish whether upregulation of miRNA-10b in sperm was, in fact, induced by DDT exposure, we treated DDT-exposed mice with phenobarbital (PB), a drug known to promote hepatic enzymes activation and accelerate DDT metabolism and excretion in humans [30, 31] and animals [32-34]. Indeed, we observed an increase in hepatic phase I enzymes ($P < 0.001$, **Fig. 5e**) and a reduction in DDT metabolites in males treated with both DDT and PB (**Table S4**). Compared to mice exposed to DDT only, miRNA-10b was significant decreased in sperm of mice treated with both DDT and PB with levels reversing to those observed in controls ($P < 0.05$, **Fig. 5f**).

miRNA-10b embryonic injection replicates DDT daughters' phenotypes

miRNA-10b along with other members of the miRNA-10 family play critical roles in embryonic development and cell differentiation through the regulation of Homeobox (*Hox*) genes [43]. These genes regulate organogenesis including development of the mammary gland [44-46]. We next examined whether miRNA-10b was responsible for the phenotypes observed in DDT offspring using embryonic (zygote) microinjections. While no major changes in mammary gland morphology were detected (**Fig. S3**), female mice derived from the miR-10b zygote injections (miR-10b females from now on) showed mammary tissue suppression of AMPK and increased expression and activity of mTOR and down-stream effector, 4E-BP1, compared to controls (**Fig.**

6a, Fig. S5), a phenotype also observed in mammary glands of DDT daughters. More importantly, we also found increased orthotopic (EO771 cells) mammary tumor development in miR-10b females, which showed both increased tumor growth and higher tumor weights compared to controls ($P < 0.05$, $P < 0.01$, **Fig. 6b-c**). Survival after tumor implantation was also worse in miRNA-10b females (**Fig. 6d**, $P < 0.1$). Further, embryonic miR-10b injection phenocopied the metabolic dysfunction of DDT daughters, with adult miR-10b females showing hyperinsulinemia ($P < 0.01$) compared to controls (**Fig. 6e**).

Discussion

Paternal environmental exposures have been shown to modulate offspring's health phenotypes via sperm non-coding RNAs [13-15]. Here, we show that pre-conception paternal exposure to the pesticide DDT modulates the sperm non-coding RNA load, particularly miRNAs, and programs female offspring's breast cancer development in a pre-clinical animal model. We also found that miRNA-10b, the miRNA with the highest increase in sperm of DDT-exposed fathers, can recapitulate phenotypes observed in DDT daughters including alterations in the mammary tissue AMPK/mTOR axis and accelerated breast tumor growth. These findings are outlined in the model presented in **Fig. 7**. Our results lend support to the notion that predisposition to diseases such as cancer can be determined by factors other than genetic background and suggest that it could be transmitted through sperm miRNAs. We showed before that paternal obesity, malnutrition or specific nutrient intake can increase breast and other types of cancer in the progeny [4, 6, 8, 9]. This study further implies that environmentally-induced non-genetic or epigenetic predisposition to cancer can occur and should be evaluated in human studies.

Published reports using the CHDS cohort showed that daughters of women exposed to DDT in pregnancy have increased rates of breast cancer [18] and are more likely to be diagnosed with advanced stage tumors. Likewise, we found that paternal DDT exposure in the pre-conception window leads to increased breast cancer growth in daughters in a mouse model. Our new observations and previously published data [6-9] indicate that paternal exposures are as important as maternal ones in programming breast and other cancers in offspring and ought to be examined in epidemiologic studies. The overlap between the effects of maternal and paternal exposure on offspring's breast cancer phenotypes in animal models suggest a common underlying mechanism that also needs further investigation.

While no studies in humans have directly investigated the link between paternal DDT exposure and breast cancer in their descendants, evidence for a role of related paternal exposures exists. For instance, an association between father's exposure to pesticides and a number of childhood cancers in offspring has been reported [47, 48]. Epidemiologic studies also show that paternal exposure to DDT and other pesticides is linked to low birthweight [49] and our study supports those findings. Low birthweight is more prevalent in minority populations [50, 51] which are more likely to be exposed to environmental toxicants such as DDT [20, 21, 52] and to develop more aggressive breast tumors [53].

Our study shows that DDT exposure alters the non-coding small RNA load in sperm. While the proposed mechanisms by which sperm cells acquire their small RNA load in response to environmental insults are still being worked out, studies have shown that extra cellular vesicles produced by epididymal cells play a role [14, 54]. Further, it has been proposed that during the normal fertilization process, sperm small RNAs are delivered to the oocyte and modulate the transcriptome during the first few cell divisions, setting a signaling cascade that can impact

embryonic development [55] and recent reports support this view [2, 14]. In line with that, embryonic injection of miR-10b, the miRNA with the highest surge in sperm of DDT-exposed males, replicates most of the major phenotypes observed in DDT daughters including higher circulating levels of insulin, altered AMPK/mTOR signaling in mammary tissues and increased tumor growth.

Although we have focused on miRNA-10b given its DDT-induced surge in sperm and its role during embryonic development [43], we acknowledge that the full phenotype observed in DDT offspring may reflect the interaction of that miRNA not only with other miRNAs but also other non-coding RNAs delivered by the sperm of DDT-exposed males during fertilization as reported by others[13, 15]. It is important to note, however, that studies examining different paternal environmental exposures—including obesity and malnutrition which also program breast cancer risk in offspring—report a surge in levels in miRNA-10b and other members of this family in sperm [7, 8, 13, 56], indicating that alterations in the miRNA-10 family could represent a common mechanism underlying the effects of different paternal factors on offspring's phenotypes.

MicroRNA-10b along with other members of the miRNA-10 family play critical roles in embryonic development and cell differentiation through the regulation of Homeobox (*Hox*) genes [43]. These genes regulate organogenesis including development of the mammary gland and other skin-derived tissues [44-46]. However, whether alterations in *Hox* genes during embryonic development of the mammary bud play a functional role in the increased breast cancer development in daughters of DDT-exposed fathers will need to be examined in futures studies.

We previously showed that low birth weight (induced by paternal malnutrition) leads to re-wiring of the nutrient sensing AMPK/mTOR pathway both in normal mammary tissues as well as in tumors. Similarly, we found that DDT daughters—who are also born small—have alterations in the mammary AMPK/mTOR axis. Whether these molecular alterations in nutrient sensors are directly programmed by pre-conception paternal DDT exposure or are a consequence of the metabolic dysfunction observed in DDT daughters remains to be determined. However, miRNA-10b embryonic injection recapitulated the nutrient sensing machinery phenotype in mammary tissues, indicating that dysregulation of the AMPK/mTOR axis is likely initiated through sperm miRNAs after fertilization.

Given its documented role in cancer, the local mammary changes in AMPK/mTOR signaling are likely mechanistic linked to accelerated tumor growth in both DDT daughters and miR-10b females, although this remains to be studied experimentally. In normal cells, proliferation is coupled to nutrient availability to ensure proper cell growth and division [57]. The AMPK pathway functions as a cellular energy sensor mechanism and provides a link between nutrient supplies and cell growth and metabolism. This pathway controls mTOR activity and associated energy-consuming biosynthetic activities such as protein synthesis [58]. Not surprisingly, suppression of AMPK and increased mTOR activity (due to loss of LKB1, the major AMPK kinase) is responsible for the inheritable Peutz-Jeghers syndrome, in which patients have an 18-fold increase in cancer risk, including breast cancer [59]. Alterations in AMPK/mTOR axis have been shown to lead metabolic reprogramming in cancer cells [60-62]. Interestingly, we previously reported that paternal nutrition can lead to metabolic reprogramming in mammary tumors [8], suggesting that ancestral exposures can not only alter cancer predisposition but also its biological phenotype.

In summary, our study shows that the environmental toxicant DDT alters the male germline non-coding RNAs and increases cancer susceptibility in the progeny. While many questions remain, our findings could have important implications for human health: According to published studies and the Center for Disease Control and Prevention (CDC), minorities such as African Americans and Mexican-Americans and recent immigrants have the highest circulating concentrations of this pesticide in the U.S. [20, 21, 63]. In addition, DDT is still used in developing countries and Americans who could have been affected by paternal exposure to DDT before its ban [18] are still alive today and reaching an age when the incidence of breast and other cancers increases. While our study focused on DDT exposure, our findings are likely relevant to other commonly used pesticides and flame retardants which, like DDT, have anti-androgenic properties [64-66].

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The small RNA-seq (small RNA) data has been deposited in GEO (Gene Expression Omnibus) database with accession code pending.

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462 **Author contributions:** S.D.A. conceived the study, oversaw the research and wrote the
463 manuscript; R.S. supervised the animal work and tissue collection, performed molecular analysis
464 and wrote the manuscript; H.C and C.C.F performed animal work. A.N. and A.K. processed
465 tissues and/or helped perform molecular analysis; M.I.C. assisted with animal studies; L.J.
466 performed RNA-seq data.

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Figure Legends

Fig-1. Paternal DDT exposure programs offspring's body weight. Body weight (grams) at (a) birth (n=8-17 litter/group), at (b) weaning and in (c) adulthood [longitudinal body weight from 6 to 30 weeks of age (n=25/group)] in control (CO) and DDT offspring. All data are mean $\pm$ SEM. *p<0.05, **p<0.01 by t-test (birthweight and weaning) or repeated measures ANOVA (longitudinal body weight).

Fig-2. Paternal DDT exposure programs metabolic dysfunction in daughters. (a) Serum insulin levels (n=6-7/group) in adult control (CO) and DDT female offspring; (b) Glucose tolerance test (GTT) in adult control and DDT female offspring (n=8/group). All data are mean $\pm$ SEM. *p<0.05 by t-test (serum insulin levels) or repeated measures ANOVA (GTT).

Fig-3. Paternal DDT exposure programs breast cancer development in daughters. Carcinogen-induced mammary tumorigenesis in control (CO) and DDT female offspring: (a) Mammary tumor volume, (b) tumor weight (grams), (c) tumor incidence and (d) tumor latency (n=25/group). Tumor incidence is shown as percentage of animals with tumors. All other data are mean $\pm$ SEM. *p<0.05, **p<0.01 by t-test (tumor weight and tumor latency), repeated measures ANOVA (mammary tumor growth) or Kaplan-Meier analysis followed by log-rank test (tumor incidence).

Fig-4. Paternal DDT exposure programs the AMPK/mTOR signaling pathway in DDT daughters' mammary tissues. (a) Simplified schematic representation of the pathway; (b-c) Representative western blots showing protein expression (phosphorylated and total) levels of members of the AMPK/mTOR axis in (b) normal mammary tissues and in (c) tumors (n=6-7/group) of control (CO) and DDT female offspring. Protein expression levels were normalized by either β -actin or β -tubulin. Densitometry data is shown in Fig. S4.

Fig-5. DDT exposure reprograms the sperm non-coding RNA load. (a) Small RNA subtype distribution and (b) miRNA abundance in sperm of DDT-exposed and control (CO) males (n=4/group) assessed by RNA-seq; (c) Heat-map showing differentially expressed miRNAs in sperm from DDT-exposed and CO males; (d) miR-10b as a fraction of all upregulated miRNAs in sperm of DDT-exposed males; (e) Expression levels of Cyp2B10 assessed by q-PCR in liver tissues of CO and DDT-exposed male mice treated with phenobarbital (PB, 20mg/kg) or vehicle (n=4-5/group); (f) Expression levels of miRNA-10b, assessed by q-PCR, in sperm of control or DDT-exposed male mice treated with PB or vehicle (n=8-20/group). Expression levels of Cyp2B10 and miRNA-10b are relative to GAPDH and miR-26a, respectively. All data are mean $\pm$ SEM. *p<0.05, ***p<0.001, ****p<0.0001 by two-way ANOVA.

Fig-6. Embryonic (zygote stage) injection of miRNA-10b recapitulate the phenotypes observed in DDT daughters. (a) Representative western blots showing protein expression (phosphorylated and total) levels of members of the AMPK/mTOR axis in normal mammary tissues of control (CO) and miRNA-10b females (Protein expression levels were normalized by β -actin,

n=5/group. Densitometry data is shown in Fig. S5); (b-d) Orthotopic tumor growth (EO771 cells, n=6-8/group) in adult CO and miRNA-10b females [(b) tumor volume, (c) tumor weight (grams) and (d) survival]. (e) Serum insulin levels in adult control (CO) and miR-10b females (n=6/group). Survival is shown as percentage of animals still alive at a given time; All other data are mean $\pm$ SEM. *p<0.05, **p<0.01 by t-test (insulin levels, tumor weights, repeated measures ANOVA (mammary tumor volume) or Kaplan-Meier analysis followed by log-rank test (survival).

Fig-7. Proposed mechanisms by which paternal DDT exposure programs offspring's breast cancer development. DDT exposure increase levels of miRNA-10b and other non-coding RNAs in sperm which are delivered to the oocyte during fertilization. This, in turn, leads to epigenetic programming of mammary bud in the developing fetus (perhaps through modulation of *Hox* genes). After birth, the ensuing mammary suppression of AMPK and sustained mTOR activity increase cell growth and survival allowing breast tumors to develop. M.G., mammary gland.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

SUPPLEMENTARY INFORMATION

Supplementary Figures

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Supplementary Figure Legends

Fig-S1. Experimental design: C57BL/6 male mice were exposed to DDT (1.7mg/kg body weight) by oral gavage for two weeks. DDT or control-vehicle (CO)-exposed males were mated with unexposed female mice to generate the offspring. To avoid litter-effect, pups were cross-fostered one day after birth. Pups from 2-3 dams were pooled and housed in a litter of 8-10 pups per nursing dam. Pups were weaned from mothers at 21 days of age.

Fig-S2. Cell proliferation is increased and apoptosis rates decreased in mammary tumors of DDT female offspring. Representative images and quantification (percentage of the positively stained nuclear area) of proliferation staining (ki67; a-c, arrows, n=6/group) and apoptosis (d-f, morphological assessment, H&E sections, arrows, n=14-17/group) in control (CO) and DDT tumors. All images captured at 40x. Values are mean $\pm$ SEM. *P<0.05 by t-test.

Fig-S3. No major changes in mammary gland morphology on PND50 in DDT or miRNA-10b female offspring. (a,b) Representative photomicrography of mammary gland whole mount (6.25x) and digitized image of the whole mount. (c,g) Number of TEBs; (d,h) Epithelial elongation (distance between lymph node and the end of the mammary epithelial tree); (e,i) Mammary epithelial area measurement (MEA); (f,j) Branching density values; (n=5-16/group) in control (CO), DDT or miRNA-10b females. All data are mean $\pm$ SEM. PND, post-natal day.

Fig-S4. Paternal DDT exposure programs the AMPK/mTOR signaling pathway in DDT offspring's mammary tissues. (a-l) Quantification of protein expression levels of members of the AMPK/mTOR axis in (a-f) normal mammary tissues and in (g-l) tumors (n=6-7/group) of control (CO) and DDT offspring. Protein expression levels were normalized by either β -actin or β -tubulin. Optical density of individual bands was quantified using Quantity-one software. Values are mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.01 by t-test.

Fig-S5. Embryonic injection of miR-10b recapitulate the DDT-induced programming of the AMPK/mTOR signaling pathway in mammary tissues. (a-f) Quantification of protein expression levels of members of the AMPK/mTOR axis in normal mammary tissues (n=5/group). Protein expression levels were normalized by β -actin. Optical density of individual bands was quantified using Quantity-one software. Values are mean $\pm$ SEM. ***p<0.01, ****p<0.01 by t-test.

Supplementary Tables

Table S1: Number of offspring and number of contributing fathers per experiment.

Samples	Assay	Group	Father	Female
Mammary gland	Terminal End Buds	CO	2	12
		DDT	3	16
	Western Blot	CO	2	6
		DDT	2	6-7
Mammary tumor	Apoptosis	CO	6	14
		DDT	9	17
	Ki67	CO	5	6
		DDT	5	6
	Tumorigenesis	CO	5	25
		DDT	8	25
	Western Blot	CO	3	6-7
		DDT	4	6
Plasma	ELISA	CO	1	6
		DDT	3	7

Table S2 - List of Antibodies

Antibody	Application	Dilution	Description	Supplier
AMPK α	Western Blot	1:1000	Rabbit polyclonal	Cell Signaling #2532
Anti-Ki67	IHC	1:7	Rabbit monoclonal	BIOCARE #CRM325
mTOR	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#2983
Phospho-AMPK α	Western Blot	1:1000	Mouse monoclonal	Cell Signaling #2535
Phospho-mTOR	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#2971
Phospho-4E-BP1	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#2855
4E-BP1	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#9452
β -Actin	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling #8457
β -Tubulin	Western Blot	1:1000	Mouse monoclonal	Thermo Fisher Scientific #MA5-16308
Anti-mouse IgG, HRP	Western Blot	1:1000	Goat	Cell Signaling # 7076S
Anti-rabbit IgG, HRP	Western Blot	1:1000	Horse	Cell Signaling # 7074S

Table S3. Gender distribution (proportion) and number of pups per litter in control and DDT groups

Group	Female	Male	Number of pups per liter (mean±SEM)
CO	0.53	0.47	8.7±0.37
DDT	0.55	0.45	8.2±0.29

Table S4 – Levels of DDT metabolites in liver of mice treated with vehicle-control, DDT or DDT+ phenobarbital (**PB**)

DDT metabolite	Group (concentration, ng/g)		
	CO	DDT	DDT- PB
o,p' DDE	ND	ND	ND
p,p DDE	ND	0.6	ND
o,p DDD	ND	ND	ND
p,p DDD	ND	0.3	ND

Levels of DDT metabolites in liver (n=3 pooled samples/group) of control (CO), DDT (1.7/mg/kg) and DDT+phenobarbital (1.7mg+20mg/kg; DDT-PB) treated male mice measured by GC-MS/MS 24h after the last treatment. ND, non-detectable (readings below assay detection limits and not distinguishable from the blank control).