A MATERNAL METHYL DONOR-ENRICHED DIET, CAPABLE OF ALTERING FETAL DNA METHYLATION, DOES NOT PREVENT FETAL PROSTATE DEVELOPMENT FROM INHIBITION BY TCDD

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Introduction

The fetal mouse prostate arises from a lower urinary tract compartment known as the urogenital sinus (UGS). The UGS is an expansion of the pelvic urethra and consists of epithelium surrounded by a complex stroma including mesenchyme and other cell types. Androgen receptors in UGS mesenchyme instruct UGS epithelium to initiate prostate development and the epithelium responds by forming prostatic buds beginning around embryonic day (E) 16.5. Mouse prostatic buds arise in a precise pattern to give rise to a bilaterally symmetrical gland organized into ventral, dorsal, lateral, and anterior prostate lobes[1]. The prostatic buds elongate into surrounding stroma, undergo extensive branching morphogenesis, and differentiate to form the mature prostate glandular network.

The mouse developing prostate is particularly sensitive to *in utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD completely prevents ventral prostatic buds from forming and reduces bud formation on the dorsolateral UGS surface. These TCDD-mediated perturbations in fetal prostate morphogenesis associate with persistent changes in susceptibility to prostate disease later in life, even after all TCDD has been eliminated from the body. Included among the persistent effects of perinatal TCDD exposure to the prostate of senescent mice are: age-inappropriate androgen dependence of the dorsolateral prostate and increased incidence of potentially precancerous, hyperplastic lesions in the dorsolateral prostate. In addition, *in utero* and lactational exposure to TCDD increases incidence of prostate cancer in TRAMP (transgenic adenocarcinoma of the mouse prostate) mice[1,2]. An overarching goal of our research is to determine the mechanism by which *in utero* and lactational TCDD exposure disrupts mouse prostate morphogenesis and increases susceptibility to prostate cancer in the adult.

DNA methylation was recently shown to be an active mediator of androgen dependent prostatic bud formation. Numerous genes encoding DNA methylation modifying enzymes are expressed before and during prostatic bud formation and their mRNA expression patterns rapidly change during prostatic bud formation[3]. The influence of chemical DNA methylation inhibitors on prostate development vary depending on developmental stage and can either increase prostatic bud number or impair prostatic ductal outgrowth[4,5]. Therefore, perturbations in DNA methylation are sufficient to disrupt prostate development.

TCDD increases DNA methyltransferase enzyme activity and alters DNA methylation of an imprinted gene in pre-implantation mouse blastocysts[6]. Fetal TCDD exposure also hypermethylates a DNA region within the putative *Pitx1* gene promoter in mouse UGS epithelium. This TCDD-induced DNA methylation mark near *Pitx1*, a tumor suppressor gene, is retained in the dorsolateral prostate of weanling, young adult, and 500 day old senescent mice following *in utero* and lactational TCDD exposure. Inasmuch as DNA methylation primes the prostate primordium to respond to cues that mediate prostate development, TCDD could potentially alter prostate development and susceptibility to adult prostate disease by perturbing DNA methylation during prostate development.

Recent evidence indicates that maternal diets enriched with chemical methyl donors like folic acid can influence fetal DNA methylation and development, and protect against teratogenic actions of environmental contaminants including TCDD. Folic acid serves as a methyl donor for *s*-adenosylmethionine, a DNA methyltransferase cofactor used to generate 5-methylcytosine of methylated DNA. A methyl donor-enriched maternal diet protects against some actions of bisphenol A[7], an endocrine disruptor that modulates prostate development and prostate DNA methylation. Importantly, a folic acid enriched diet consumed by mouse dams decreases incidence of

TCDD-induced cleft palate in fetuses[8]. The purpose of this study was to test the hypothesis that a methyl-donor enriched maternal diet will protect against TCDD-induced inhibition of ventral prostatic bud formation.

Materials and methods

Animals and experimental design. Sexually mature C57BL/6J nulliparous female mice were housed in polysulfone cages containing corn cob bedding and maintained on a 12hr light and dark cycle at 25±5ºC and 20-50% relative humidity. At least two weeks prior to their first mating, female mice were fed a base (control) diet (Harlan Diet 2019, Harlan Teklad, Madison, WI) containing 4mg/kg folic acid or fed the same base diet supplemented with 24mg/kg folic acid (Harlan Diet #120256). Food and water were available *ad libitum*. The two week pre-mating loading period of the folic acid supplemented diet was previously determined to be sufficient to induce changes in DNA methylation of offspring[9]. Timed pregnant dams from both diets were either left untreated or at embryonic day (E) E13.5 given corn oil (5ml/kg, po, maternal dose) alone or containing TCDD (5µg/kg, po, maternal dose). At E18.5, male UGSs were collected, fixed overnight with 4% paraformaldehyde, and dehydrated into methanol for long term storage at -20°C. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Morphometric analysis. Litter size, mass and crown to rump length were measured in E18.5 fetuses from control or folic acid diet dams. Crown to rump length was determined using a digital caliper and was measured from the crown of the head to the base of the tail.

Prostatic bud visualization and quantification. E18.5 UGSs were stained by ISH to visualize *Nkx3-1* mRNA, which marks prostatic buds. The stained tissues were then co-stained by IHC to visualize E-cadherin, which marks UGS epithelium. Prostate buds and UGS epithelium were visualized using a compound microscope and bud number for three samples per treatment group was quantified by three blinded individuals.

Statistical analyses. Levene's test was used to determine homogeneity of variance. One way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference *post hoc* test were used to detect mean differences between treatment groups ($p \leq 0.05$).

Results and discussion

Methyl donor enriched maternal diet increases litter size but not fetal growth. The main goal of this study was to examine effects of TCDD and folic acid on prostate development. Because prostate development is potentially impacted by embryonic growth rate, we first tested whether the folic acid enriched diet influenced embryonic growth in non-TCDD exposed mice. Dams were maintained on either control or methyl donor enriched (high folic acid) diet for two weeks. Mice were then timed mated and the resulting fetuses collected at E18.5 for morphometric analysis. We found a statistically significant increase in litter size from dams maintained on the methyl donor enriched diet compared to the control diet (8.67±0.33 vs 5.75±0.94 fetuses, p=0.05) but there was no significant difference in weight (1.14±0.04 vs 1.16±0.05 g, p=0.7) or crown to rump length (21.54±1.19 vs 22.62±0.39 mm, p=0.3) of fetuses from dams on the methyl donor diet compared to control diet. These results suggest that while a methyl donor enriched diet increases litter size it does not alter fetal growth.

Methyl donor enriched maternal diet does not protect against TCDD induced ventral prostate agenesis. We next tested the hypothesis that a folic acid enriched maternal diet protects against inhibition of fetal prostate development by TCDD. Dams were maintained on either control or methyl donor enriched (high folic acid) diet for two weeks. Mice were then timed mated and on E13.5 fetuses were exposed transplacentally to corn oil (5ml/kg dam, po) or TCDD (5µg/kg dam, po) administered to the pregnant dam. UGSs were removed from fetuses at E18.5 and stained by ISH to visualize and count prostatic buds (expressing *Nkx3-1* mRNA, purple) and also were stained by IHC to visualize the UGS epithelium (expressing E-cadherin, orange). Representative lateral images of stained UGSs are shown in Fig. 1A. The two representative UGSs from the corn oil (control) group were born to mothers fed a control diet (top left) or a high folic acid diet (top right). The two arrows, shown for each corn oil (control) UGS, point to ventral buds (left arrow) and dorsolateral buds (right arrow). They show that the number and pattern of ventral and dorsolateral prostatic buds in the UGS of a corn oil fetus, whose mother was fed a control diet (top left) or high folic acid diet (top right), were similar. Two representative

UGSs from male fetuses in the TCDD group whose mother was fed a control diet (bottom left) or high folic acid diet (bottom right) are also shown. Notice that no arrows are shown for the UGSs in the two TCDD groups. This is because the effect of TCDD on both groups was the same and significantly different from the control group. That is, *in utero* exposure to TCDD prevented the formation of ventral buds and reduced the number of dorsolateral buds in both TCDD treatment groups (bottom left and right) compared to the representative control UGSs where both types of buds are seen. Furthermore, TCDD inhibition of ventral and dorsolateral bud formation in the UGS of a fetus from a TCDD-exposed mother fed the control diet (bottom left) or high folic acid diet (bottom right) was similar.

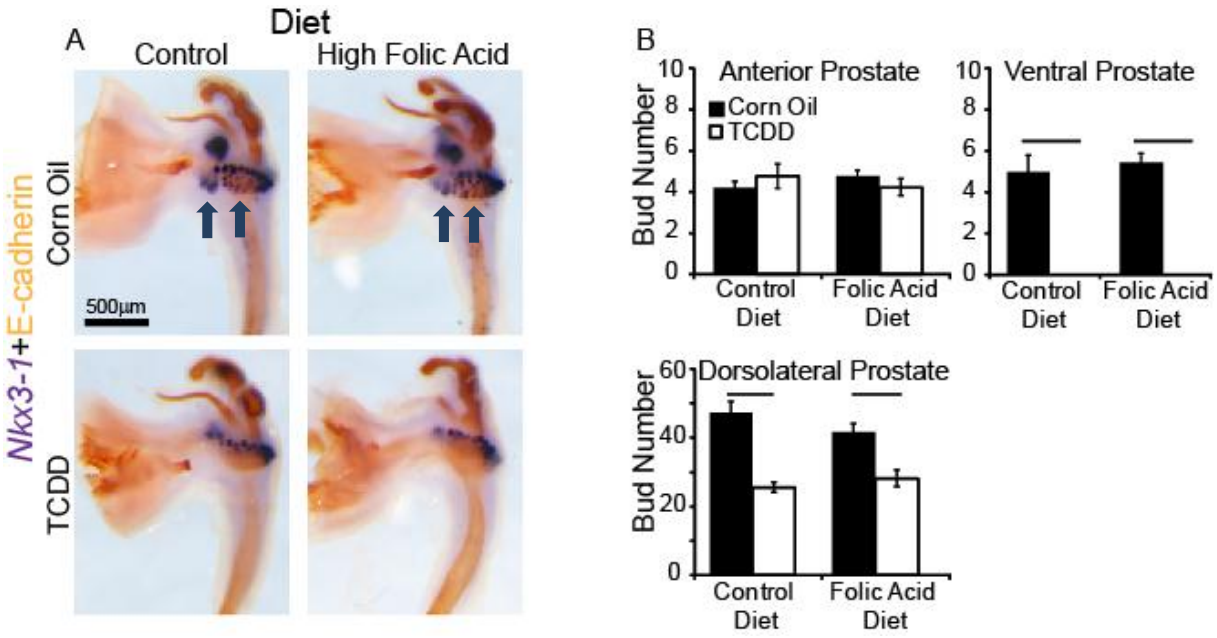

Figure 1. A methyl donor-enriched diet does not protect against TCDD induced changes in prostate development. Dams were maintained on either a control or a high folic acid diet and fetuses were exposed to corn oil (5ml/kg, po, maternal dose) alone or containing TCDD (5µg/kg, po, maternal dose) at embryonic day (E) 13.5. (A) UGSs were removed from fetuses five days later, at E18.5, and stained by ISH to visualize and count prostatic buds (expressing *Nkx3-1* mRNA, purple). Samples were also stained by IHC to visualize UGS epithelium (expressing E-cadherin, orange). The pair of vertical arrows in each panel of the corn oil (control) group indicate representative ventral (left) and dorsolateral (right) prostatic buds. (B) Prostatic bud counts are reported as mean ± SEM, n=3 UGSs/group, a horizontal line above a pair of bars indicates a significant difference between the corn oil control (solid bar) and TCDD (open bar) treatment groups $p \leq 0.05$.

A quantitative comparison of the control diet *versus* high folic acid diet on the actual number of prostatic buds in the UGS giving rise to the anterior, ventral, and dorsolateral prostate lobes of male fetuses exposed *in utero* to corn oil (control) or TCDD are also shown (Fig. 1B). Focusing first on prostatic bud formation in the anterior prostate, there was no effect of TCDD on anterior bud number and no effect of the high folic acid diet on anterior bud number in control or TCDD treatment groups.

The results, however, were strikingly different for ventral prostate bud formation. Here, as expected, *in utero* TCDD exposure blocked the formation of all ventral buds ($p \leq 0.05$) regardless of whether the mother had been fed a control diet or methyl donor enriched diet. In other words, the high folic acid diet did not prevent the inhibitory effect of TCDD on ventral prostate bud formation and this was also observed for the dorsolateral prostate (Fig. 1B). More specifically, TCDD reduced the number of dorsolateral buds in fetuses born to dams that were fed either a control diet or high folic acid diet when compared to the corn oil control group that was fed these same two diets ($p \leq 0.05$). The folic acid diet did not rescue dorsolateral buds from the inhibitory effect of TCDD. There also was no significant effect of the methyl donor enriched maternal diet alone on ventral or dorsolateral bud formation in control UGSs.

Thus, despite evidence that a methyl donor-enriched maternal diet can protect against DNA hypomethylation and developmental abnormalities such as cleft palate, caused by *in utero* TCDD exposure in the mouse fetus[8], maternal consumption of a folic acid enriched diet did not protect against disruption of fetal prostate development by TCDD. It is possible that TCDD's actions in the prostate are not prevented by a maternal methyl donor diet because the dominant effect of TCDD on DNA methylation status in the UGS is to cause hypermethylation of DNA. TCDD was recently found to cause hypermethylation of the putative promoter region of the homeobox gene *Pitx1*, in UGS epithelium of mouse fetuses and this epigenetic signature was retained in the dorsolateral prostate of mice up to 500 days of age. TCDD also increases DNA methyltransferase activity and methylation of *H19* and *Igf2* genes in pre-implantation mouse blastocysts[6]. So in the UGS it is possible that the key action of TCDD as it relates to prostate development may be to enhance DNA methylation in the same direction as a methyl donor diet - therefore precluding the possibility of a protective effect. Identifying changes in DNA hypermethylation of potentially important target genes such as *Pitx1* will be important in the future for determining whether TCDD acts via the epigenome to inhibit prostate development and/or alter susceptibility to adult prostate disease.

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