IN UTERO AND LACTATIONAL EXPOSURE TO TCDD INDUCES PERSISTENT HYPERMETHYLATION OF THE HOMEOBOX GENE *PITX1* IN MOUSE PROSTATE

Kurita, H[1,2], Lin, T-M[1], Tohyama, C[2], Peterson, RE[1]

[1]School of Pharmacy, University of Wisconsin, Madison, WI, USA; [2]Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Introduction

The goal of this research is to understand how activation of aryl hydrocarbon receptor (AhR) signaling by *in utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice affects prostate development, and later in life, susceptibility to prostate disease. The developing prostate is adversely affected by *in utero* and lactational exposure to TCDD[1]. Such exposure inhibits prostatic bud formation from the fetal urogenital sinus (UGS), completely prevents the ventral prostate lobe from forming, and permanently alters the structure and function of the other prostate lobes. β-catenin is essential for normal prostate development in mice and inhibition of β-catenin signaling is the key mechanism by which TCDD disrupts prostate development[2].

In utero and lactational exposure to TCDD also has a number of long-term effects on the prostate of male mice including: age-inappropriate androgen dependence in the dorsolateral prostate of senescent mice 500 days of age, increased incidence in 500 day old mice of hyperplastic lesions (cribiform structures) of the dorsolateral prostate, considered by some to be precancerous lesions, and increased incidence of prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) model[1,3]. Understanding the mechanism by which *in utero* and lactational exposure to TCDD affects prostate disease in aging mice, long after TCDD has been eliminated, is a long range goal of this work.

TCDD has been shown to increase DNA methyltransferase activity and methylation of *H19* and *Igf2* genes in pre-implantation mouse blastocysts[4]. Accordingly we sought to identify changes in the methylation status of genomic DNA in the UGS of mouse fetuses following *in utero* exposure to TCDD, and in the dorsolateral prostate of 21, 90, and 500 day old mice exposed *in utero* and via lactation to TCDD, but not in 500 day old, AhR knockout (AhRKO) mice.

The present study will show that the epigenetic signature discovered in the putative promoter region of the homeobox gene *Pitx1*[5] in the UGS of TCDD-exposed mouse fetuses is retained in the dorsolateral prostate of weanling, adult and senescent mice exposed perinatally to TCDD. This epigenetic signature, hypermethylation of the homeobox gene *Pitx1*, was found in the fetal UGS epithelium, which develops into the ductal network of the prostate, as well as in the epithelium of the dorsolateral prostate of mice up to 500 days of age. This is long after all TCDD to which these mice were exposed, transplacentally and via lactation, was excreted from the body. Furthermore, the TCDD-induced epigenetic alteration in this homeobox gene in the prostate was, not only apparently irreversible, but also AhR-dependent. This was evidenced by hypermethylation of *Pitx1* in dorsolateral prostate epithelial cells not being found in 500 day old AhRKO mice exposed to TCDD.

Materials and methods

Animals and experimental design

Heterozygous AhRKO ($Ahr^{+/-}$) mice were interbred to generate wild type ($Ahr^{+/+}$) and homozygous AhRKO ($Ahr^{-/-}$) male offspring. For *in utero* and lactational exposure, pregnant dams were administered a single, oral dose of either the corn oil vehicle (5 ml/kg, control) or TCDD (5 μg/kg dam) on embryonic day (E) 13.5. TCDD lactational exposure ended on postnatal day 21 when pups were weaned. Wild type ($Ahr^{+/+}$) and AhRKO ($Ahr^{-/-}$) male mice exposed *in utero* and via lactation to either corn oil (control) or TCDD were maintained until they reached senescence (500 days of age) at which time they were euthanized. The dorsolateral prostate was removed from each senescent male and digested with collagenase and DNase. The resulting supernatant

containing stromal cells was discarded and separated dorsolateral prostate epithelial cells were stored at -80°C for subsequent analyses.

An identical *in utero* and lactational vehicle and TCDD exposure protocol was used for C57BL/6J wild type male mice. These male mice, however, were euthanized at younger ages: male fetuses at E17.5 and the epithelial layer of the UGS was separated and collected, weanling males at postnatal day 21 and dorsolateral prostate collected, and adult males at postnatal day 90 and epithelial cells of the dorsolateral prostate collected. All UGS and prostate samples were digested with proteinase K and genomic DNA was purified for DNA methylation analyses.

Methylated DNA immunoprecipitation-microarray (MeDIP on chip)
The NimbleGen protocol for MeDIP on chip was followed. Dorsolateral prostate epithelial cell genomic DNA was sheared by *MseI* digestion and an aliquot was stored for input DNA. MeDIP was conducted using anti 5-methyl cytidine monoclonal antibodies. MeDIP-DNA and input DNA was amplified by using the WGA2 kit. Amplified MeDIP-DNA and input DNA was labeled with Cy3 and Cy5 dye using NimbleGen Dual-Color DNA Labeling Kits. The mixture of labeled MeDIP-DNA and input DNA were hybridized to a DNA microarray slide (NimbleGen Mouse DNA Methylation 2.1M Deluxe Promoter v2 Array) by using the NimbleGen Hybridization Kit. DNA microarray slide was scanned with MS 200 Microarray Scanner (NimbleGen). Data for the fold difference in hybridization chip intensity between treatment groups, expressed as $\log_2$ (TCDD – vehicle), were generated for 90 and 500 day old wild type mice and 500 day old AhRKO mice. The criterion for hypermethylation was greater than a two-fold increase in hybridization chip intensity between TCDD and vehicle (control) group [$\log_2$ (TCDD – vehicle) > 1.0] while the criterion for hypomethylation was the opposite, more than a two-fold decrease in hybridization chip intensity between TCDD and vehicle (control) group [$\log_2$ (TCDD – vehicle) < -1.0]. Methylation regions of interest were those altered by *in utero* and lactational TCDD exposure in both 90 and 500 day old mice but not in 500 day old AhRKO mice. SignalMap software was used for mapping and visualizing data. The same analysis was also performed using all peak data provided by NimbleScan software. Consistent changes in both analyses ($\log_2$ and peak) were selected. We also looked for clusters of more than one probe hit in a sequence ID region.

PCR confirmation of MeDIP on chip results
Genomic DNA was isolated from UGS epithelial cells of male fetuses at E17.5 or dorsolateral prostate of 21, 90 and 500 day old mice or 500 day old AhRKO mice. The isolated genomic DNA was sheared with MseI digestion. The methlylated DNA was then enriched by binding to Methyl-CpG-binding domain protein 2 (MBD2) according to the MethylMiner protocol (Invitrogen). MBD2 enriched DNA then was used to confirm the TCDD-induced DNA methylation alterations identified by MeDIP on chip analysis. DNA primers were designed according to targeted sequences provided by the chip manufacturer. Gene-specific methylation was measured by real-time PCR quantification comparing threshold point results obtained from the MBD2-captured portion and the input portion from the same dorsolateral prostate genomic DNA sample.

Results and discussion
To test the hypothesis that *in utero* TCDD exposure causes an AhR-dependent alteration in genomic DNA methylation in UGS basal epithelial cells and this epigenetic signature persists in epithelial cells of the dorsolateral prostate of TCDD-exposed mice as they age, we examined 90 and 500 day old wild type mice and 500 day old AhRKO mice. Mice in each group were exposed *in utero* and via lactation to either vehicle (control) or TCDD (5 µg/kg dam on E13.5). Genomic DNA methylation in the dorsolateral prostate epithelial cells isolated from these mice was evaluated for TCDD-induced changes in genomic DNA methylation by MeDIP on chip analysis (Table 1). Hypermethylation of DNA was found in two DNA sequence ID regions of chromosome 13 and one sequence ID region of chromosome 8 in both 90 and 500 day old wild type mice treated with TCDD. In addition, hypomethylation of genomic DNA was detected in dorsolateral prostate epithelial cells of the same two treatment groups, following *in utero* and lactational TCDD exposure. Specifically, hypomethylation of genomic DNA was observed in one sequence ID region of chromosome 3 and one sequence ID region of chromosome 9, respectively. Both TCDD-induced hypermethylation and hypomethylation of dorsolateral

prostate epithelium genomic DNA were AhR-dependent. This was demonstrated by neither effect being observed in dorsolateral prostate epithelial cells of 500 day old AhRKO mice exposed to TCDD (Table 1).

Probe set specific PCR primers were designed according to the sequences of the probe sets that detected TCDD-induced and AhR-dependent changes in genomic DNA methylation by MeDIP on chip analysis for confirmation of the hypermethylation and hypomethylation findings by methyl-CpG-binding domain protein 2 (MBD2)-mediated genomic DNA PCR. Of the five sequence ID regions that showed alterations in DNA methylation by MeDIP on chip analysis, one sequence, located on chromosome 13, covered the putative promoter region (55926839-8007) of the homeobox gene *Pitx1*. The other four sequence ID regions had no association with any gene in close vicinity. An important finding was that MBD2-mediated genomic DNA PCR results with the *Pitx1* probe set FS055926952 confirmed the MeDIP on chip finding of hypermethylation of the 5' region of *Pitx1*. All other PCR probe sets failed to confirm the hypermethylation and hypomethylation findings of MeDIP on chip analyses in Table 1.

Table 1
***In Utero* and Lactational TCDD Exposure Induced Alterations in Genomic DNA Methylation in the Dorsolateral Prostate Epithelium of Mice Assessed by MeDIP on Chip Analysis**

		Fold Difference in Hybridization Chip Intensity [log_2 (TCDD – Vehicle)]		
Age (Days) Genotype		90 Wild Type	500 Wild Type	500 AhRKO
DNA Sequence ID	Probe Set			
Hypermethylation				
chr13:55926839-8007[1]	CHR13FS055926952[2]	2.66	1.09	0.16
chr13:55926839-8007[1]	CHR13FS055927828	1.14	1.48	0.01
chr13:55929570-40786	CHR13FS055935008	1.27	1.85	0.06
chr13:55929570-40786	CHR13FS055935313	1.45	2.09	-0.02
chr13:55929570-40786	CHR13FS055936520	1.18	1.10	-0.32
chr13:55929570-40786	CHR13FS055938499	1.48	1.01	-0.32
chr8:87388009-99156	CHR08FS087394585	1.00	1.45	-0.13
chr8:87388009-99156	CHR08FS087394685	1.31	1.10	-0.36
Hypomethylation				
chr3:107690771-707131	CHR03FS107704566	-1.01	-1.09	0.07
chr3:107690771-707131	CHR03FS107704446	-1.30	-1.14	-0.47
chr9:121678724-89724	CHR09FS121684440	-1.02	-1.26	0.12
chr9:121678724-89724	CHR09FS121684530	-1.13	-1.17	-0.19

[1] Putative promoter region of *Pitx1*
[2] Probe set hypermethylation confirmed by MDB2-mediated PCR

The MBD2-mediated genomic DNA PCR results (Fig. 1) show hypermethylation of the 5' region of *Pitx1* in the prostate of TCDD-exposed mice of different ages. Hypermethylation of the 5' region of *Pitx1* was observed following *in utero* and lactational TCDD exposure in both 90 and 500 day old, wild type mice ($p \leq 0.05$) but not in 500 day old AhRKO mice ($p \geq 0.05$). Thus, hypermethylation of the putative promoter region of the homeobox gene Pitx1, caused by *in utero* and lactational TCDD exposure, is AhR-dependent.

The PCR results (Fig. 1) also revealed a trend towards hypermethylation of the 5' region of *Pitx1* in the UGS epithelium of mouse fetuses on E17.5. This is the same fetal tissue from which the dorsolateral prostate develops, and it is significant that an increase in methylation of the 5' region of *Pitx1* in the dorsolateral prostate of 21 day old mice, exposed *in utero* and via lactation to TCDD, was also observed ($p \leq 0.05$).

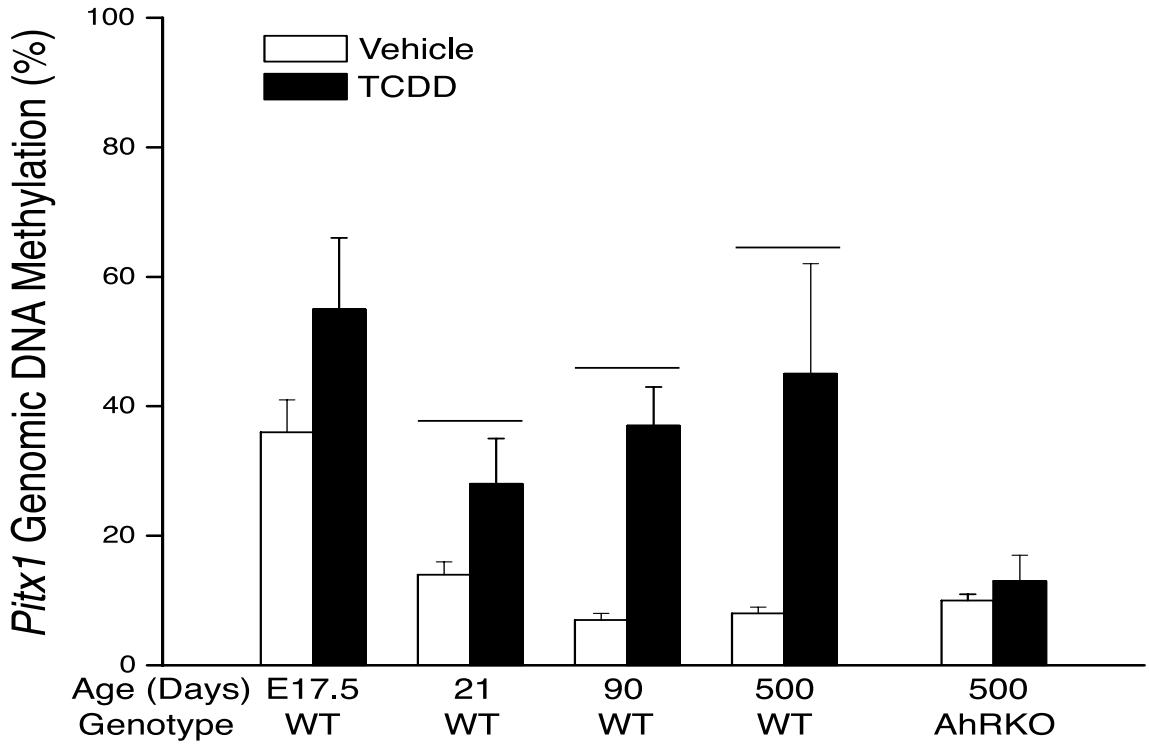

Figure 1. *In utero* and lactational TCDD exposure induced hypermethylation of the putative promoter region of the homeobox gene *Pitx1* in UGS and dorsolateral prostate of fetal, weanling, adult, and senescent mice. Results are mean±SE of 4-5 litter independent mice. Methylated genomic DNA in the UGS and dorsolateral prostate epithelium was enriched by MBD2 binding. *Pitx1* methylation was evaluated by PCR using primers (FS055926952) targeting the *Pitx1* putative promoter region specifically. Abbreviations are: embryonic day (E), wild type (WT) and AhR knockout (AhRKO). Horizontal lines indicate significant differences ($p \leq 0.05$).

In closing, *in utero* and lactational TCDD exposure causes an AhR-dependent, apparently irreversible hypermethylation of the putative promoter region of the homeobox gene *Pitx1* in the dorsolateral prostate of mice. Interestingly, *Pitx1* is reported to be a tumor suppressor gene[6]. Hypermethylation of DNA in this gene may be related to the progression of prostate cancer. Whether this TCDD-induced epigenetic signature disrupts the subsequent expression of *Pitx1* during prostate morphogenesis or prostate cancer progression remains to be investigated.

Acknowledgements
This study was supported by a Grant-in-Aid for JSPS Fellows (to HK) and by NIEHS grant ES001332 (to REP).

References

1. Vezina, CM, Lin, T-M, Peterson, RE. (2009); *Biochem. Pharmacol.* 77: 566-76
2. Branam, AM, Davis, NM, Moore, RW, Schneider, AJ, Vezina, CM, Peterson, RE. (2013); *Toxicol. Sci.* 133: 42-53
3. Fritz, WA, Lin, T-M, Moore, RW, Cooke, PS, Peterson, RE. (2005); *Toxicol. Sci*. 86: 387-95
4. Wu, Q, Ohsako, S, Ishimura, R, Suzuki, J S, Tohyama, C. (2004); *Biol Reprod* 70: 1790-7
5. Holland, PW. (2013); *WIREs Dev Biol* 2: 31-45
6. Kolfschoten , IG, van Leeuwen, B, Berns, K, Mullenders, J, Beijersbergen, RL, Bernards, R, Voorhoeve, PM, Agami, R. (2005); *Cell* 121: 849-58