

## SCREENING BY MICROARRAY ANALYSIS FOR GENES THAT ALTER PROSTATE DEVELOPMENT IN C57BL/6J MICE EXPOSED IN UTERO TO 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

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### Introduction

The administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to pregnant rats and mice leads to a disruption of prostate development in the male offspring (Mably *et al.*, 1992; Gray *et al.*, 1997; Faqi *et al.*, 1998; Lin *et al.*, 2003). Although it is not clear if this phenomenon occurs in human populations exposed to TCDD, the observed effect level is low among the various endpoints of TCDD developmental toxicity in animal studies. Clarification of the mechanism by which the effect is produced at the molecular level would help substantiate male reproductive toxicity caused by *in utero* TCDD exposure as a model for human health risk assessment. In both rats and mice, a critical window for TCDD disruption of prostate development in late pregnancy has been illustrated (Ohsako *et al.*, 2002; Lin *et al.*, 2002a). The primary alteration in gene expression that presumably causes this phenomenon depends on the fetal aryl hydrocarbon receptor gene (Lin *et al.*, 2002b) being expressed in the fetal urogenital sinus from which the outgrowth of prostatic buds occurs (Lin *et al.*, 2003; Ko *et al.*, 2004). In the male offspring of mice exposed to TCDD on gestation day 13 (GD 13), severe inhibitory developmental effects were found on ventral prostate development. These effects were significantly lower when *in utero* TCDD exposure occurred after GD 16 than GD 13 (Lin *et al.*, 2002a). Upon administration of TCDD to the dam on GD 13, cytochrome P450 1A1 (CYP1A1) and CYP1B1 were induced in the urogenital complex of the male offspring on postnatal day 14. Thus, "dioxin biomarker genes" are responsive to *in utero* and lactational TCDD exposure during the neonatal stage of development (Ohsako *et al.*, 2002). This suggests that key TCDD responsive genes involved in disrupting prostate development would be genes other than CYP1A1 and CYP1B1.

In the present study we administered a single dose of TCDD to mouse dams during the critical window (GD 13 or GD 14) for impairing prostate development, or later during a less TCDD sensitive period (GD 17). Microarray techniques were then used to compare gene expression profiles of the fetus versus the urogenital sinus in order to identify genes involved in disruption of prostate development caused by *in utero* TCDD exposure.

## Materials and Methods

### *Animals and administration*

Pregnant C57BL/6J mice were purchased from Charles River (Tokyo, Japan) and maintained in an air-conditioned isolated rack in the chemical hazard area at the National Institute for Environmental Studies. During the morning (10:00 to 11:00 am) on GD 13 or GD 17, mice were given a single oral dose of TCDD (10 µg/kg bw; 4% n-nonane in corn oil; 5 ml/kg) or an equivalent volume of vehicle, followed by sacrifice by cervical dislocation 24 hr post-administration. The fetuses were collected, and total fetal RNAs were isolated with Trizol Reagent (Invitrogen, CA, USA). In another set of experiments, the same dose of TCDD was administered to pregnant C57BL/6J mice on GD 14 or GD 17, followed by harvest of the fetuses 24 hr post-administration. Male fetuses were transferred and immersed in phosphate buffered saline, and their urogenital sinuses were dissected under a stereoscope. The urogenital sinuses of litters from one dam were combined and then subjected to RNeasy Micro Kit (QIAGEN, Hilden, Germany) to isolate total RNAs.

### *Microarray analysis*

Prior to microarray analysis, TCDD-exposed RNA samples were checked by reverse-transcription PCR. RNA specimens, obtained from fetuses whose CYP1A1 were well induced, were digested with DNase I, reverse transcribed and then labeled with Cy3 fluorescence. The probe was then hybridized to Atlas Glass Array Mouse 3.8 I (CLONTECH, CA, USA). The signals were scanned by a GenePix fluorescence scanner (Axon Instruments, CA, USA) and spot intensities were analyzed with ArrayGauge software (Fujifilm Co., Tokyo, Japan). cDNA's with a T7 promoter linked oligo dT primer, and biotin-labeled cRNAs were prepared from urogenital sinus RNA's according to the technical manual of the supplier. The cRNA samples were then hybridized to Murine Genome Mgu74Av2 GeneChip (Affymetrix, CA, USA). The GeneChip Operating Software (GCOS) supplied by Affymetrix was used to perform gene expression analysis.

## Results and Discussion

The Atlas Mouse Glass Array 3.8I is spotted with 3,756 gene probes made of 80-bp single strand oligonucleotides. A total of 822 gene spots resulted in significant signals on each of the 4 arrays used in this study. Of these, 132 genes and 239 genes were determined to be up-regulated (ratio > 1.5) and down-regulated (ratio < 0.67) in the GD13-TCDD exposed group, respectively. Among these gene populations, only 14 and 38 genes were also up-regulated or down-regulated in GD17-TCDD exposed mice, suggesting that the GD13 fetus is quite different from the GD17 fetus in terms of TCDD sensitivity. The genes altered only by GD13-TCDD exposure contain metabolic enzymes and cellular signal transducers.

The Mgu74Av2 microarray has a capacity to analyze 12,488 genes. As summarized in Table 1, GCOS software analysis distinguished several genes to be significantly altered ( $1 < \text{signal log ratio} < -1$ ) in the UGS by *in utero* TCDD exposure. Authentic biomarker genes for TCDD exposure, such as CYP1A1, CYP1B1, and aryl hydrocarbon receptor repressor (AhRR), were contained in the 15 genes discerned as up-regulated genes both by GD14- and GD17-TCDD treatments, whereas no down-regulated genes were found. The objective of this study was to screen for genes altered only during the critical window: that is, those altered by GD14-TCDD exposure more drastically

than by GD17-TCDD exposure. Several candidate genes that were up-regulated only by GD14-TCDD included 17 $\beta$ -hydroxysteroid dehydrogenase type II, tumor necrosis factor induced protein 2, and synaptotagmin III, while down-regulated genes included complement factor H or its related proteins. Complement factors have been well-documented to be down-regulated by TCDD exposure in liver with respect to serum enzymatic activity (White *et al.*, 1986). It is notable that the 15 genes down-regulated by GD14-TCDD treatment included 3 complement factor related proteins, with a structural similarity as secretory proteins and that these genes, established to be of hepatic origin, were expressed in UGS. Connexin-30, involucrin, and flavin-containing monooxygenase 5 were also distinguished as candidate genes, the expression of which was significantly up-regulated by GD14-TCDD. Involucrin is a keratinocyte differentiation marker, that has been reported to be up-regulated by TCDD exposure (Gaido and Maness, 1994). The observed up-regulation of involucrin in UGS suggests that the response of UGS seems to be similar to that in skin tissue. A sort of terminal differentiation of UGS epithelial cells might inhibit the outgrowth of prostatic buds from the UGS.

In order to clarify whether these candidate genes are physiologically or toxicologically relevant, further investigations to determine which cell type expresses these candidate genes in the UGS and an over-expression study using transgenic models might shed light on these important questions.

**Table 1. Summary of male fetal UGS genes whose expression levels were altered by *in utero* TCDD exposure.**

|                        | Up         | Down       | Total     |
|------------------------|------------|------------|-----------|
| <b>GD14</b>            | <b>39</b>  | <b>19</b>  | <b>58</b> |
| <b>GD17</b>            | <b>40</b>  | <b>8</b>   | <b>48</b> |
| <b>GD14 specific</b>   | <b>24*</b> | <b>19*</b> | <b>31</b> |
| <b>GD17 specific</b>   | <b>25</b>  | <b>8</b>   | <b>29</b> |
| <b>GD14 &amp; GD17</b> | <b>15</b>  | <b>0</b>   | <b>15</b> |

Numbers of genes was detected by GCOS software (Affymetrix) as significantly changed, more than 2-fold induction (signal log ratio > 1) or less than 1/2 reduction (signal log ratio < -1). \*: the genes significantly altered only by GD14-TCDD exposure.

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