# TCDD MODULATES SELECTED DEVELOPMENTAL SIGNALING PATHWAYS DURING MOUSE PROSTATE MORPHOGENESIS

Vezina CM[1], Hicks SM[2], Moore RW[1], Peterson RE[1, 2]

[1]School of Pharmacy and [2]Molecular and Environmental Toxicology Center, University of Wisconsin, 777 Highland Avenue, Madison, WI 53705 USA

**Abstract**
Exposure of male mice to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during gestation inhibits ventral prostatic bud formation from the fetal urogenital sinus (UGS) culminating in ventral prostate agenesis. We investigated interactions between TCDD-inducible aryl hydrocarbon receptor (AHR) signaling and three other signaling pathways implicated in mouse prostatic development: non-canonical wingless-related MMTV integration site 5A (WNT5A), all-*trans* retinoic acid (RA) and fibroblast growth factor 10 (FGF10). To determine whether these pathways stimulate or inhibit prostatic bud formation, UGS tissue from embryonic day (E) 14 male C57BL/6J mouse fetuses was cultured for 3 d in media containing vehicle (control), WNT5A recombinant protein, WNT5A-inhibitory antibody, RA or FGF10 recombinant protein. Prostatic budding was: unchanged by addition of WNT5A inhibitory antibody to culture media for 3 d, increased by addition of either RA or FGF10, and repressed by addition of either recombinant WNT5A or TCDD. Co-incubation of E14 UGS with WNT5A inhibitory antibody + TCDD rescued budding impairment by TCDD, incubation with FGF10 + TCDD abolished the bud-inhibiting effects of TCDD, and incubation with RA + TCDD was similar to TCDD alone. Thus, interference with RA and FGF10 signaling or inappropriate activation of WNT5A signaling by TCDD may impair prostatic bud formation.

**Introduction**
Prostate ductal development from the mouse UGS is induced by an androgen-dependent mesenchymal signal that initiates basal epithelial outgrowths, or buds, from underlying epithelium. Prostatic buds first appear at E16 and subsequently elongate, undergo branching morphogenesis and canalize between late gestation and early postnatal development to give rise to the ductal network of mature prostate. Prostate morphogenesis is complete by postnatal day 20 and secretory function is acquired during the pubertal surge shortly thereafter.

Initiation of prostatic buds is androgen-dependent. Prostatic bud initiation is almost completely inhibited by androgen ablation[1] or mutational inactivation of the androgen receptor in the UGS mesenchyme.[2] The mechanism of androgen-dependent prostatic bud initiation has not been completely resolved, but a number of developmental signaling pathways have been implicated in this process. These include epithelial growth stimuli, such as FGF10[3] and RA, and growth restrictive signals such as WNT5A,[4] which maintain proximal-distal guidance of elongating buds during prostate morphogenesis.

Mouse prostate development is impaired by *in utero* exposure to TCDD during late gestation. Fetal exposure to TCDD (5 μg/kg, maternal dose at E13) decreased ventral, anterior and dorsolateral prostate weights at postnatal day 35.[5] The effects of TCDD on mouse prostate development are likely caused by direct inhibition of prostatic bud formation. TCDD exposure on E13 completely inhibited the formation of ventral prostatic buds and significantly decreased the formation of dorsolateral prostatic buds as assessed on E18.[6] Inhibition of ventral prostatic bud formation, which results in complete agenesis of the ventral prostate,[7] requires functional AHR expression in the UGS mesenchyme[8] but downstream signaling pathways that mediate budding impairment by TCDD are not well understood. In this study, we begin to elucidate signaling pathways that are necessary for prostatic bud initiation and are inhibited by gestational exposure to TCDD.

**Materials and Methods**
*UGS Organ Culture.* UGSs were dissected from E14 male C57BL/6J mouse fetuses in phosphate-buffered saline and placed onto 0.4 μm Millicell-CM filters (Millipore, Billerica, MA) in tissue culture plates containing serum-free DMEM/F12 media supplemented with 2% insulin, transferrin, selenium, 25 μg/ml gentamicin, 0.25 μg/ml amphotericin B, and 10 nM 5α-dihydrotestosterone (DHT). Media were supplemented with TCDD (1

nM), WNT5A-inhibitory antibody (8 μg/ml, R&D Systems, Minneapolis, MN), RA (10 μM), FGF10 recombinant protein (300 ng/ml, R&D Systems), or WNT5A recombinant protein (2 μg/ml, R&D Systems), as indicated. Media were changed every 2 d. UGS specimens were prepared for scanning electron microscope (SEM) evaluation of prostatic budding after 3 d in culture.

*Scanning Electron Microscopy.* UGSs were prepared for SEM as previously described.[5] Briefly, UGSs were enzymatically digested in 1% trypsin (Difco, Sparks, MD) at 4 °C for 90 min. Enzyme activity was attenuated using 5% fetal bovine serum (Hyclone) and UGS mesenchyme (UGM) was removed from UGS epithelium (UGE) using fine forceps. UGEs were fixed in 2.5% glutaraldehyde (Ted Pella, Redding, CA) for 24 hr, dehydrated through a graded series of ethanol, and dried by the critical-point procedure using $CO_2$ prior to being mounted and coated with gold for analysis using the Hitachi S-570 SEM (Tokyo, Japan). Mounted samples were rotated within the SEM to generate at least four views of each sample. Images were examined, and total prostatic bud number was determined as the average total bud count of two individual blinded reviewers.

**Results and Discussion**

*WNT5A Decreases Prostatic Budding in the Absence of TCDD and Blockade of WNT5A Signaling Rescues Prostatic Budding Impairment in the Presence of TCDD.*

There is evidence that WNT5A regulates a developmental signaling pathway that is shared by multiple organs of the developing fetus. *Wnt5a*$^{-/-}$ (WNT5A KO) mice exhibit defects in craniofacial, ear and genital development.[9] Recently, *Wnt5a* expression was identified in UGS mesenchyme of male embryonic rats, implicating WNT5A in prostate development.[4] We assessed the role of WNT5A in prostatic budding using WNT5A KO mice and discovered that most WNT5A KO male fetuses exhibit defects in ventral prostatic bud formation, which partially phenocopies the effects of TCDD. Since WNT5A KO mice die perinatally, wild type (WT) and WNT5A KO E18 UGS tissues were grafted under the renal capsules of male immunodeficient mice to investigate their growth potential and determine whether failure to develop prostatic buds in male WNT5A KO UGS resulted in agenesis of ventral prostate. Grafted WNT5A KO male UGSs showed reduced ability to form ventral prostate, as determined by decreased lobe-specific secretory gene expression compared to WT controls.

Additional studies were performed to investigate the effects of WNT5A signaling on prostatic bud formation *in vitro* (Table 1). E14 WT UGSs were exposed *in vitro* to recombinant mouse WNT5A (2 μg/ml), WNT5A inhibitory antibody (8 μg/ml), TCDD + WNT5A, or TCDD + WNT5A inhibitory antibody. After 3 d, UGE was tryptically digested, mechanically separated from the overlying UGM, and visualized by SEM to quantify prostatic buds. Exposure to WNT5A significantly reduced UGS prostatic bud number, confirming previous reports that WNT5A is an inhibitor of prostate morphogenesis in rodents.[4] Exposure to WNT5A inhibitory antibody alone had no effect on prostatic budding, but protected the UGS from TCDD-induced reduction in prostatic bud number. Similarly, when cultured UGSs were grafted to the kidney capsules of male immunodeficient mice, those that had been exposed to WNT5A or TCDD showed reduced ability to form mature ventral prostate whereas those exposed to TCDD + WNT5A inhibitory antibody showed normal ventral prostate formation. These data support the hypothesis that TCDD inhibits ventral prostatic bud formation by increasing WNT5A signaling in the UGS.

*Retinoic Acid Induces Prostatic Budding in the Absence of TCDD but Cannot Reverse Budding Impairment in the Presence of TCDD.*

There is a wealth of information relating to the role of retinoic acid in later stages of prostate development and during adult prostate homeostasis but very little information about the role of retinoic acid in the initial stages of prostatic bud initiation and elongation. There are multiple lines of evidence supporting retinoid involvement in the prostatic budding process. First, retinoids actively participate in bud initiation in multiple organs. Second, vitamin A deficiency during rat embryogenesis prevents development of mature prostate, and prostate development can be recovered upon vitamin A supplementation between E10-15, shortly before rat prostatic bud initiation.[10,11] Third, gene deletion studies in transgenic mice implicate members of the retinoid signaling pathway in embryonic mouse prostate development.

We have confirmed retinoic acid receptor (RAR) α and γ and retinoid X receptor (RXR) α and γ protein expression in the UGSs of vehicle and TCDD-exposed male mouse fetuses. Using retinoic acid-responsive

reporter mice, we have also located retinoid activity in the UGS mesenchyme of male mouse fetuses. *In vitro* exposure of male mouse UGS specimens to all-*trans* retinoic acid almost doubled the amount of prostatic buds that formed after 3 d in organ culture, compared with vehicle-exposed control UGSs (Table 1). Induction of *Cyp26b1*, a known retinoid-responsive gene, was observed after 24 hr incubation with RA. Incubation of UGSs with RA + TCDD significantly decreased prostatic bud number compared with UGS tissues exposed to vehicle, RA or TCDD alone, suggesting that TCDD impairs RA signaling in the UGS.

*FGF10 Induces Prostatic Budding in the Presence and Absence of TCDD*

FGF10 is secreted by a discrete population of UGS mesenchyme cells, collectively referred to as the ventral mesenchymal pad. Secreted FGF10 binds to FGFR2 receptors on UGS epithelium and stimulates receptor tyrosine kinase-mediated mitogenic signaling. FGF10 is required for prostatic bud initiation in mice and rats. Male *Fgf10*-null mice possess only a rudimentary UGS deficient in prostatic buds.[12] Antagonism of FGFR by a specific chemical inhibitor blocks both mitogenic signaling and prostatic bud initiation.[13] *In utero* TCDD exposure on E15 also inhibits mitogenic signaling, as assessed by ERK1/2 phosphorylation on E16.

To determine if TCDD interferes with FGF10 signaling *in vitro*, E14 UGS were incubated in organ culture for 3 d in the presence of recombinant FGF10 (300 ng/ml), TCDD (1 nM), a combination of TCDD and FGF10, or vehicle (Table 1). FGF10 significantly increased prostatic budding compared to vehicle control. TCDD alone significantly decreased prostatic budding compared to vehicle control, but incubation with FGF10 + TCDD restored bud number to the same level as vehicle control. To determine if TCDD decreased *Fgf10* mRNA abundance in the UGS, male mouse fetuses were exposed *in utero* to vehicle (control) or TCDD (5 μg/kg, maternal dose) on E15 and *Fgf10* mRNA abundance and localization was assessed by *in-situ* hybridization. TCDD exposure did not significantly alter *Fgf10* mRNA abundance or substantially alter its localization in the UGS compared to vehicle control, although there was suggestive evidence that TCDD decreased the overall size of the *Fgf10* expression domain, the ventral mesenchymal pad, in whole-mount UGS samples probed for *Fgf10* by *in-situ* hybridization.

TCDD stimulates a paracrine signal, initiated by mesenchymal AHR, which inhibits prostatic bud formation from UGS epithelium. WNT5A, retinoid, and FGF10 signaling pathways represent three necessary components for prostatic bud formation in the male mouse UGS. While the individual factors that comprise each of these pathways are unique, they share an overarching theme of paracrine mesenchymal-epithelial signal transduction. The current study suggests that TCDD modulates these signaling pathways in the UGS to cause inhibition of prostatic bud formation.

**Acknowledgements**

Supported by NIH grants F32 ES014284, R37 ES01332, F31 HD049323.

**Table 1. Effects of TCDD exposure and WNT5A, retinoid or FGF10 signaling on the number of prostatic buds which form from the E14 male mouse UGS in 3 day organ culture [#]**

| Group | Number of Buds / UGS | |
|---|---|---|
| | Vehicle | TCDD |
| Vehicle | 27.5 ± 1.9 | 12.9 ± 1.9 * |
| WNT5A Blocking Antibody | 25.3 ± 2.9 | 27.9 ± 2.9 |
| Recombinant WNT5A Protein | 21.0 ± 2.3 * | 10.4 ± 2.1 * |
| Retinoic Acid | 49.4 ± 5.7 * | 15.5 ± 1.6 * |
| Recombinant FGF10 Protein | 41.2 ± 3.1 * | 23.3 ± 1.4 |

[#] UGSs from E14 male C57BL/6J mouse fetuses were incubated for 3 d in organ culture media containing DHT (10 nM) and either vehicle (control; 0.1% DMSO) or TCDD (1 nM) in the presence or absence of: WNT5A-inhibitory antibody (8 μg/ml), WNT5A recombinant protein (2 μg/ml), all-*trans* retinoic acid (10 μM), or FGF10 recombinant protein (300 ng/ml). At the end of the incubation, UGE was separated from UGM and

visualized by SEM. Prostatic buds were counted using micrographs from four separate angles to quantify budding over the entire UGS surface. Results are mean bud number per UGS ± SEM for $n \geq 6$ UGSs per group from at least 3 separate litters. Significant differences between means were determined using one-way ANOVA, followed by Fisher's LSD post-hoc test.

* Significantly different from vehicle ($p < 0.05$)

**References**


1. Lasnitzki I, Mizuno T. *Prostate* 1980; 1:147-56.
2. Lasnitzki I, Mizuno T. J. *Endocrinol.* 1980; 85:423-8.
3. Donjacour AA, Thomson AA and Cunha GR. *Dev. Biol.* 2003; 261: 39-54.
4. Pu Y, Huang L, Birch L, Prins GS. *Endocrinol.* 2007; 148:1697-706.
5. Lin T-M, Simanainen U, Moore RW, Peterson RE. *Toxicol. Sci.* 2002; 69:202-209
6. Lin T-M, Rasmussen NT, Moore RW, Albrecht RM, Peterson RE. *Tox. Sci.* 2003; 76:171-181.
7. Lin T-M, Simanainen U, Moore RW, Peterson RE. *Toxicol. Sci.* 2002; 69:202-209
8. Ko K, Moore RW, Peterson RE. *Toxicol. Appl. Pharmacol.* 2004; 196: 149-155.
9. Yamaguchi TP, Bradley A, McMahon AP, Jones S. *Development* 1999; 126:1211-23.
10. Wilson JG and Warkany J. *Am. J. Anat.* 1948; 88:357-407.
11. Wilson JG, Roth CB, Warkany J. *Am. J. Anat.* 1953; 92:189-217.
12. Donjacour AA, Thomson AA and Cunha GR. *Dev. Biol.* 2003; 261:39-54.
13. Kuslak SL, Marker PC. *Differentiation* 2007; In Press.