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TCDD Inhibition of Canonical Wnt Signaling Disrupts Prostatic Bud Formation in Mouse Urogenital Sinus

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In mice, *in utero* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) reduces the number of dorsolateral prostatic buds resulting in a smaller dorsolateral prostate and prevents formation of ventral buds culminating in ventral prostate agenesis. The genes and signaling pathways affected by TCDD that are responsible for disrupting prostate development are largely unknown. Here we show that treatment of urogenital sinus (UGS) organ cultures with known inhibitors of canonical Wnt signaling also inhibits prostatic bud formation. In support of the hypothesis that TCDD decreases canonical Wnt signaling, we identify inhibitory effects of TCDD on multiple components of the canonical Wnt signaling pathway in the UGS that temporally coincide with the inhibitory effect of TCDD on prostatic bud formation: (1) expression of R-spondins (*Rspo2* and *Rspo3*) that promote canonical Wnt signaling is reduced; (2) expression of *Lef1*, *Tcf1*, and *Wif1*, established canonical Wnt target genes, is decreased; (3) expression of *Lgr5*, a RSPO receptor that activates canonical Wnt signaling, is reduced; and (4) expression of Dickkopfs (*Dkks*), inhibitors of canonical Wnt signaling, is not increased by TCDD. Thus, the TCDD-induced reduction in canonical Wnt signaling is associated with a decrease in activators (*Rspo2* and *Rspo3*) rather than an increase in inhibitors (*Dkk1* and *Dkk2*) of the pathway. This study focuses on determining whether treatment of TCDD-exposed UGS organ cultures with RSPO2 and/or RSPO3 is capable of rescuing the inhibitory effects of TCDD on canonical Wnt signaling and prostatic bud formation. We discovered that each RSPO alone or in combination partially rescues TCDD inhibition of both canonical Wnt signaling and prostatic bud formation.

Key Words: **prostate; urogenital sinus; TCDD; R-spondin; canonical Wnt; mouse; development; bud.**

Mouse prostate derives from the urogenital sinus (UGS), which is located between the bladder and pelvic urethra, and arises on embryonic day (E) 13.5. The UGS is composed of an epithelium surrounded by a dense mesenchymal layer. Prostate development is androgen dependent, requiring 5α-dihydrotestosterone (DHT) that binds to androgen receptors in the urogenital mesenchyme (UGM) and activates downstream signaling factors that interact with the urogenital epithelium (UGE) and initiate subsequent signaling events in the UGE, resulting in the formation of prostatic buds and later development of the mature prostate (Cunha and Chung, 1981).

Prostatic bud development is orchestrated through a series of timely events beginning with specification, where developmental signals determine the exact positioning of buds, followed by initiation, where the buds begin to arise from the basal epithelium (BE), and elongation, where the buds continue to grow out into the surrounding UGM. These events take place between E13.5 and E18.5, and by E18.5, all prostatic buds have formed. By puberty, the prostatic buds will give rise to ductal networks that constitute the four separate and distinct lobes of the mature prostate: anterior, dorsal, lateral, and ventral (Lin *et al*., 2003; Timms *et al*., 1994).

Treating pregnant C57BL/6J mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 μg/kg, po) on E13.5 results in severe prostatic budding defects in male fetuses on E18.5 including mispositioning and reduction in the number of dorsal and lateral buds and complete ablation of ventral prostatic buds (Lin *et al*., 2003). A time-course study determined that TCDD acts during the bud specification stage with the critical window for ventral bud inhibition occurring between E15.5 and E16.5 (Vezina *et al*., 2008b). Impairment of budding by TCDD is not a consequence of androgen insufficiency or a reduction in androgen receptor activity (Ko *et al*., 2004b). Rather it is most likely caused by misregulation of downstream signaling pathways triggered by TCDD activation of the aryl hydrocarbon receptor (AHR) in the UGM (Ko *et al*., 2004a). Likely candidates include paracrine factors that are secreted from UGM and stimulate prostatic bud formation by interacting with signaling pathways found in the basal UGE from which prostatic buds arise.

Growing evidence supports an essential role for canonical Wnt signaling during prostatic bud formation (Francis *et al*., 2013; Mehta *et al*., 2013). Recent findings showed canonical Wnt signaling to be critical for lineage commitment and bud outgrowth during early prostate development and have shown LEF1, a canonical Wnt target gene, to be a marker of prostatic buds (Simons *et al*., 2012; Wu *et al*., 2011). Other groups have shown canonical Wnt signaling to be important during later phases of prostate development such as in the regulation of branching morphogenesis, which is also disrupted by TCDD (Ko *et al*., 2002; Kudryavtseva *et al*., 2011). Wnt signaling is required for budding in multiple organs including breast, tooth, and hair follicle (Faraldo *et al*., 2006; Gat *et al*., 1998; Liu *et al*., 2008; Lo Celso *et al*., 2004) and WNT5A, a noncanonical Wnt agonist, inhibits prostatic bud formation *in vitro*. Importantly, treatment with a WNT5A antibody rescued TCDD inhibition of prostatic budding (Allgeier *et al*., 2008).

TCDD effects on canonical Wnt signaling during prostatic bud formation have not been extensively explored. R-spondins 1–4 (RSPOs) are activators of the canonical Wnt signaling pathway. The exact mechanisms by which RSPOs activate canonical Wnt signaling remain inconclusive. RSPOs appear to initiate Wnt signaling by several mechanisms including inhibition of Dickkofs (DKKs) that are extracellular antagonists of canonical Wnt signaling, synergism with canonical Wnts, activation of noncanonical Wnt signaling through heparin sulfate proteoglycan binding, and recently by activation of LGR receptors to enhance Wnt signaling (Carmon *et al*., 2011; de Lau *et al*., 2011; Glinka *et al*., 2011; Kim *et al*., 2008).

This study focuses on determining the effects of TCDD on canonical Wnt signaling in the UGS during the same time that it inhibits prostatic bud formation. We show that TCDD decreases *Rspo2* and *Rspo3* mRNA levels as well as Wnt target genes, *Lef1*, *Tcf1*, and *Wif1*, during prostatic bud development. We demonstrate that RSPOs enhance prostatic bud formation and partially rescue the inhibition of budding caused by TCDD. We demonstrate that stimulatory effects of RSPOs on budding are mediated through canonical Wnt signaling and explore mechanisms for TCDD inhibition of RSPOs by examining the expression of *Dkks* and *Lgrs*. We conclude that inhibition of canonical Wnt signaling significantly contributes to the reduction in prostatic bud formation in UGS organ cultures exposed to TCDD.

MATERIALS AND METHODS

Animals and treatments. C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained as previously described (Vezina *et al*., 2008b). All procedures were approved by the University of Wisconsin Animal Care and Use Committee. Timed-pregnant females were set up by pairing males with females overnight. The following day was considered E0.5. Pregnant females were administered a single dose of TCDD (5 μg/kg) or vehicle (corn oil, 5 ml/kg), po, on E15.5. Dams were euthanized via CO_2 asphyxiation when fetuses were at developmental stages E14.5, E16.5, E17.5, or E18.5.

UGS organ culture and prostatic bud counting. Male UGSs were harvested on E14.5 and grown in serum-free culture media containing 10nM DHT on a 0.4-μm Millicell-CM filter for 3 or 4 days. Media and supplements were replenished every 2 days. The following supplements were added, alone or in combination, to UGS organ culture media: 0.1% dimethyl sulfoxide (DMSO; vehicle control); 1nM TCDD (Cambridge Isotope Laboratories, Woburn, MA); recombinant mouse RSPO2 and RSPO3; DKK1 and DKK2 proteins (R&D Systems, Minneapolis, MN); and XAV-939 (Selleck Chemicals, Houston, TX). Some UGS tissues were harvested after 3 days and frozen in liquid nitrogen for real-time (RT) PCR analysis. Other UGS tissues were harvested following 4 days in culture and fixed in 4% paraformaldehyde (PF) overnight followed by graded dehydration into 100% MeOH in which the tissues were stored at −20°C until further analysis.

Whole-mount immunohistochemistry (IHC) was performed as described previously (Keil *et al*., 2012). Primary antibody against e-cadherin (1:500; Cell Signaling, Danvers, MA) was added overnight at 4°C. Following washes, samples were treated with anti-rabbit-AlexaFluor488 (Jackson ImmunoResearch, West Grove, PA [1:500]), washed, cleared with a series of glycerol dilutions, and mounted in antifade media (PBS containing 80% glycerol and 0.2% *n*-propyl gallate). Images were taken on an Olympus Fluoview FV1000 confocal microscope, and total bud number was assessed using a z series to visualize the entire UGS epithelium. Every third z series section was counted to obtain total bud number (see Supplementary fig. 1 for illustration of bud counting method). We counted all prostatic buds on the UGS and excluded the small, nonprostatic buds present on the pelvic urethra. Prostatic buds projected out from the UGE surface and were readily identified as buds. Whole UGS images in Figs. 1, 4, and 5 represent a compilation of the z series for a "representative" single UGS in each treatment group. Each representative image shows only a fraction of the prostatic buds that were counted in that particular UGS and is intended only as a visual aid for making gross comparisons between groups at the level of the whole UGS. Analyses were performed on at least four litter-independent samples.

Sectional and whole-mount* in situ *hybridization. Sectional *in situ* hybridization (ISH) and whole-mount ISH were performed according to protocols found at www.gudmap.org and described previously (Abler *et al*., 2011). Primer sequences for riboprobes are as follows: *Lgr5*F′ GCCTTAGAGCAGGAGAGCAT; *Lgr5*R′ CGATGTTAATACGACTCACTATAGGGTGTATAGTCCTGGCTGTCCT; *Lgr4*F′ GCTCATACCTTGAGCTGTCT; *Lgr4*R′ CGATGTTAATACGACTCAC TATAGGGACACTGAGAGGGGAATCACT. The reverse primers contained a synthetic T7 RNA polymerase recognition sequence. Riboprobes for *Rspo2* and *Rspo3* have been described previously (Mehta *et al*., 2011). The expression for each riboprobe was analyzed in at least three litter-independent UGSs per time and treatment. Vehicle- and TCDD-treated tissues were processed together to allow for comparisons to be made between biological replicates and treatment groups.

RNA isolation and RT PCR. UGS homogenization, RNA isolation, and reverse transcriptase PCR were previously described in Vezina *et al.* (2008a). RT PCR was performed as described by Lin *et al.* (2002, 2003) using the Roche LightCycler 1.5 (Roche Applied Science, Indianapolis, IN). Results are shown relative to *cyclophilin* mRNA abundance to normalize expression on a per cell basis. RT PCR primer sequences can be found in Supplementary table 1.

Sectional IHC. UGS tissues were fixed overnight in 4% PF, dehydrated into 100% MeOH, embedded in paraffin, and cut into 5-μm sagittal sections. Samples were rehydrated and boiled in 10mM sodium citrate for 20 min to unmask epitopes. Samples were washed with PBST (1× PBS, 0.1% Tween-20) and blocked in PBST containing 5% normal goat serum and 1% bovine serum albumin for 1 h. Primary antibody against LEF1 or TCF1 (1:100; Cell Signaling) was added in combination with antibody against cytokeratin 14 (KRT14) (1:100; Millipore, Billerica, MA) overnight at 4°C. Samples were washed and treated with anti-rabbit-AlexaFluor488 to detect LEF1 or TCF1 expression and anti-mouse-AlexaFluor546 (Sigma-Aldrich, St Louis, MO) for 1 h to detect KRT14 expression. Samples were washed and counterstained with DAPI and mounted in antifade media. Images were acquired on a confocal microscope. Percent of LEF1 or TCF1 positive BE cells in the UGS was determined by counting total number of BE cells positive for LEF1 or TCF1

staining in the entire BE, excluding the bladder, Wolffian and Mullerian ducts, and pelvic urethra and dividing this number by the total number of BE cells identified by positive KRT14 staining.

Litter independence and statistical analysis. *In vitro* UGS organ culture was performed on UGSs from four or more independent litters per group. Whole-mount e-cadherin staining and sectional IHC were performed on UGSs from three or more litters per treatment. RT PCR analysis was performed on at least four litter-independent UGSs from each treatment group. Error bars represent SE. ANOVA and Student's *t*-test were conducted on data that passed Levene's test for homogeneity of variance and appeared to be normally distributed.

RESULTS

Inhibitors of Canonical Wnt Signaling Reduce Prostatic Bud Formation

To determine whether inhibition of canonical Wnt signaling plays a role in TCDD inhibition of prostatic budding, UGS organ culture was performed in the presence of vehicle and TCDD. Prostatic bud formation with these treatments was compared with cultures treated with known inhibitors of canonical Wnt signaling, the DKKs and XAV-939, that antagonize by different mechanisms. DKK1 and DKK2 are extracellular antagonists of canonical Wnt signaling that inhibit by promoting internalization of LRP Wnt coreceptors, thereby decreasing canonical Wnt signaling (Niehrs, 2006). DKKs inhibit canonical Wnt signaling *in vitro* at concentrations above 100 ng/ml (Im and Quan, 2010). In contrast to the DKKs, the chemical canonical Wnt inhibitor, XAV-939, is less well studied. XAV-939 acts by preventing AXIN degradation, the rate-limiting step in the destruction of β-catenin.

Our goal was to determine whether treatment of UGS organ cultures with DKKs or XAV-939 would inhibit prostatic bud formation, similar to TCDD. However, because XAV-939 is less well established as a canonical Wnt inhibitor than the DKKs, we first sought to determine whether the concentration of XAV-939 used in our UGS organ culture experiments actually inhibited canonical Wnt signaling. UGSs were grown for 3 days in DHT-containing media. RT PCR was performed to determine mRNA levels of Wnt target genes, *Lef1* and *Lgr5* (Barker *et al*., 2007; Filali *et al*., 2002), in vehicle (control)- versus XAV-939-treated UGSs. We found that XAV-939 downregulated the expression of both target genes when added at a 10μM concentration (64% reduction of *Lef1* and 25% reduction of *Lgr5* compared with vehicle), showing that this XAV-939 concentration inhibited canonical Wnt pathway target gene expression as predicted (data not shown).

Next, we examined effects of TCDD and these two types of canonical Wnt inhibitors on prostatic budding (Fig. 1). Total bud numbers were compared among vehicle- (control), TCDD- (1nM), DKK1 + DKK2- (500 ng/ml each), and XAV-939- (10μM) treated UGSs by staining the UGE for e-cadherin and counting prostatic buds. All treatments decreased the total number of prostatic buds compared with control UGSs ($p < 0.05$, Fig. 1, bottom). A confocal image that is representative of the UGS for each treatment is also shown (Fig. 1, top). The arrowheads in each image point to prostatic buds, which are more plentiful in the vehicle (control) UGS than in the TCDD-, DKK1 + DKK2-, or XAV-939-treated UGS. Thus, both TCDD and the canonical Wnt inhibitors reduced prostatic bud formation (vehicle-25

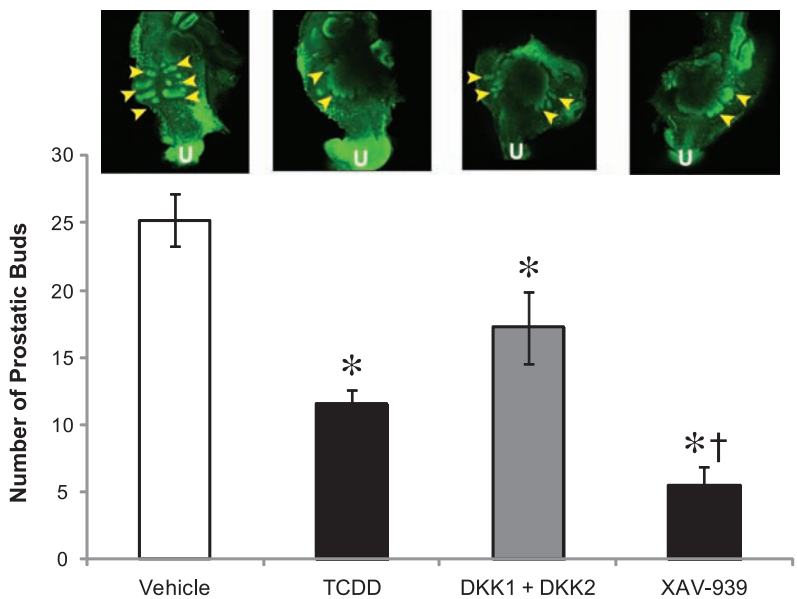

FIG. 1. Inhibitors of canonical Wnt signaling inhibit prostatic bud number similar to TCDD. E14.5 male UGSs were cultured for 4 days in media containing 10nM DHT with vehicle, 1nM TCDD, recombinant DKK1 + DKK2 (500 ng/ml each), or 10μM XAV-939 to inhibit canonical Wnt signaling. Buds were visualized by performing IHC specific for e-cadherin, an epithelium marker. The number of prostatic buds was determined by confocal microscopy. Arrowheads indicate areas where buds are present. U indicates urethra. Results are mean ± SE for at least four litter-independent samples per treatment. Asterisk indicates a significant decrease compared with control $p < 0.05$.

buds, TCDD-12 buds, DKK1 + DKK2-17 buds, and XAV-939-7 buds). These results provide a rationale for investigating inhibition of canonical Wnt signaling as a potential mechanism for disruption of prostatic bud formation by TCDD.

TCDD Decreases Canonical Wnt Signaling Target Gene Expression in the UGS

To explore whether TCDD is having an inhibitory effect on canonical Wnt signaling, we performed RT PCR using primers specific to confirmed Wnt signaling targets, including *Lef1*, *Tcf1*, and *Wif1* (Filali *et al*., 2002; Roose and Clevers, 1999; Yan *et al*., 2001). UGSs treated with either vehicle or TCDD at a concentration known to reduce prostatic bud formation were examined after 3 days in culture to determine whether TCDD altered the mRNA expression of Wnt target genes. Consistent with our hypothesis of TCDD downregulating canonical Wnt signaling, we found that *Lef1*, *Tcf1*, and *Wif1* mRNA levels were all significantly decreased following treatment with TCDD (Fig. 2). This finding supports a role for misregulation of the canonical Wnt signaling pathway in the reduction of prostatic bud number by TCDD.

TCDD Decreases Rspo2 and Rspo3 mRNA Expression in the UGS

Previous ISH results from our laboratory revealed at E16.5 that *Rspo2* and *Rspo3* mRNA levels were decreased in UGSs exposed to TCDD on E15.5 (5 µg/kg) compared with UGSs exposed to vehicle (5 ml/kg corn oil, po)(Moore *et al*., 2011). To determine whether these *in vivo* effects can be reproduced *in vitro*, a time-course study of *Rspo2* and *Rspo3* expression was performed in UGS organ culture. Male UGSs were harvested on E14.5 and treated in culture for 2, 3, or 4 days in DHT-containing media that contained either vehicle or TCDD (1nM).

Rspo2 mRNA expression in the UGS appears strongest after the 2-day culture and is prominently expressed in the ventral mesenchymal pad (VMP), a cell population known to mediate mesenchymal/epithelial interactions during prostatic bud development (Timms *et al*., 1995). *Rspo2* expression in TCDD-treated cultures was reduced in the VMP after 2 days (Fig. 3A, compare staining inside boxed area) consistent with our previous *in vivo* results (Moore *et al*., 2011). However, we did not detect a significant TCDD-induced change in *Rspo2* expression in 3- or 4-day cultures or in UGSs from E17.5 or E18.5 fetuses exposed *in utero* to vehicle or TCDD on E15.5 (results not shown).

Rspo3 expression overlaps with *Rspo2* expression in the VMP, and it is strongly expressed in Wolffian structures. The expression of *Rspo3* transcript remains robust throughout the entire time course, and no inhibition of *Rspo3* expression was observed after 2 days of exposure to TCDD (results not shown). However, there was a decrease in *Rspo3* levels in the VMP region of the UGS organ cultures after 3 and 4 days of exposure to TCDD compared with vehicle (Fig. 3A, compare staining inside boxed area). To assess expression of *Rspo3* in the UGS of mouse fetuses exposed *in vivo* to TCDD, a time-course study

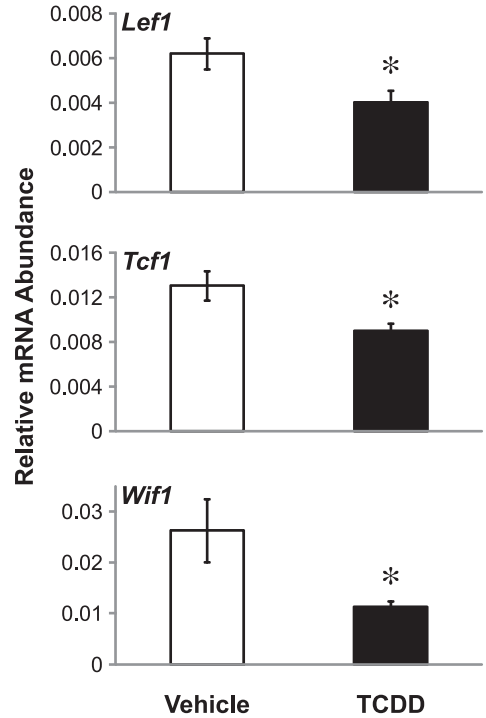

FIG. 2. TCDD downregulates canonical Wnt target genes in the mouse UGS. E14.5 male UGSs were cultured for 3 days in media containing 10nM DHT and either vehicle (0.1% dimethyl sulfoxide [DMSO]) or TCDD (1nM). RT PCR was performed to distinguish differences in mRNA expression of the canonical Wnt target genes, *Lef1*, *Tcf1*, and *Wif1*, between vehicle and TCDD-treated UGS samples. Results are mean ± SE for at least four litter-independent samples per treatment. Asterisk indicates a significant decrease compared with control $p < 0.05$.

was performed. E17.5 and E18.5 UGSs exposed to TCDD on E15.5 had less expression of *Rspo3* in the VMP than respective vehicle control UGSs (Fig. 3B, arrow). Thus, the *Rspo* findings demonstrate that TCDD causes a time- and region-specific reduction in *Rspo2* and *Rspo3* expression in the ventral UGM. This is the UGS tissue compartment where TCDD activation of AHR is required to ablate ventral prostatic bud formation (Ko *et al*., 2004a).

RSPOs Promote Prostatic Bud Formation

RSPOs induce canonical Wnt signaling in other systems (Kim *et al*., 2008; Nam *et al*., 2006). We hypothesized that RSPO2 and RSPO3 would enhance prostatic bud formation in the developing UGS by enhancing canonical Wnt signaling. UGSs were treated for 4 days with the following: vehicle; recombinant RSPO2 protein (100 ng/ml); recombinant RSPO3 protein (100 ng/ml); or the combination of RSPO2 and RSPO3 (100 ng/ml each). These concentrations have been shown to activate canonical Wnt signaling by other research groups (Kim *et al*., 2008). Whole-mount IHC was performed using an antibody specific to e-cadherin, and total prostatic bud number was assessed between treatment groups. RSPO2 and the combination of RSPO2 and RSPO3 significantly increased

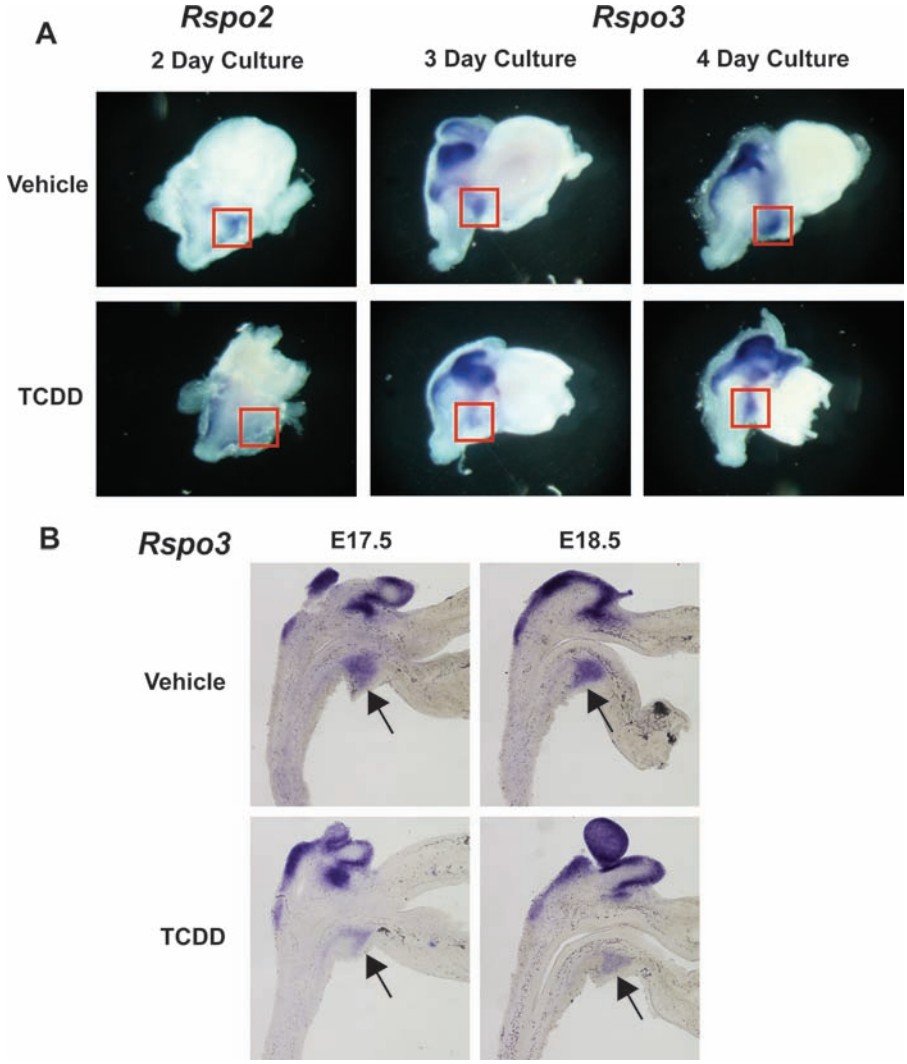

FIG. 3. *Rspo2* and *Rspo3* mRNA expression is inhibited by TCDD in the UGS. E14.5 male UGSs were cultured for 2, 3, or 4 days in the presence of 10nM DHT and either vehicle (0.1% DMSO) or TCDD (1nM). They were then subjected to ISH to visualize mRNA expression of *Rspo2* and *Rspo3* (A). Boxes indicate regions that differ in expression between vehicle- and TCDD-treated UGSs. Near mid-sagittal, 50µM sections of male UGS were processed by ISH to visualize mRNA expression of *Rspo3* on E17.5 and E18.5. Dams from which fetal UGSs were obtained were treated with either vehicle (5 ml/kg corn oil) or TCDD (5 µg/kg) po on E15.5 (B). Arrows indicate regions where expression differences exist between vehicle- and TCDD-treated UGSs. Results are representative of six litter-independent UGSs at each time.

mean total prostatic bud number compared with vehicle control (mean of 40 buds for RSPO2 and 43 buds for RSPO2 + RSPO3 compared with 25 buds for vehicle). The RSPO3 results were not statistically significant, but they did show a trend toward an increase in bud formation (Fig. 4). These results support the hypothesis that RSPOs can increase total bud number in UGSs by activating canonical Wnt signaling.

RSPOs Protect Against TCDD Inhibition of Prostatic Bud Number

Because TCDD reduces transcript abundance of both *Rspo2* and *Rspo3*, we sought to determine whether RSPO2 and/or RSPO3 could protect against the reduction in bud number caused by TCDD, by treating UGS organ cultures with these components in combination with TCDD. E14.5 male UGSs were grown for 4 days with the following supplements: vehicle, TCDD (1nM), RSPO2 (100 ng/ml), RSPO3 (100 ng/ml), RSPO2 + RSPO3 (100 ng/ml each), and all RSPO treatments combined with TCDD. TCDD treatment decreased the number of prostatic buds by over 50% compared with UGSs treated with vehicle, from a mean of 25 buds to 12 buds. Addition of RSPO2 and RSPO3 to TCDD-exposed UGSs partially rescued the total number of buds to 19 buds when added to the TCDD-containing culture media. The combination of RSPO2 and RSPO3 led to even greater rescue of bud number, from an average of 12 buds with TCDD alone to 24 buds, which is comparable with vehicle-treated UGSs (mean of 25 buds). Although RSPOs

were able to increase prostatic bud number in TCDD-treated UGSs to the vehicle level, they were not able to increase it to the number caused by RSPOs alone (19 buds and 24 buds in RSPO2 + TCDD and RSPO2 + RSPO3 + TCDD treatments compared with 40 and 43 buds in RSPO2 and RSPO2 + RSPO3 treatments) (Fig. 5). This could be due to RSPOs not being the sole mechanism responsible for TCDD inhibitory effects on budding.

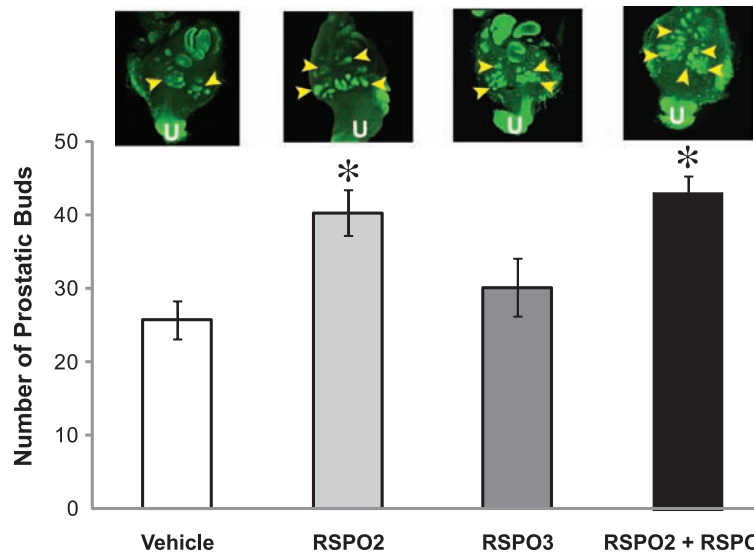

FIG. 4. Exogenous RSPO2 and RSPO3 increase prostatic bud number in control UGS *in vitro*. E14.5 male UGSs were cultured for 4 days in media containing DHT (10nM, control) with or without RSPO2 (100 ng/ml), RSPO3 (100 ng/ml), or RSPO2 + RSPO3 (100 ng/ml each). Prostatic buds were counted as described in the Figure 1 legend. Arrowheads indicate regions where buds are present. U indicates urethra. Results are mean ± SE of at least four litter-independent samples per treatment. Asterisk indicates a significant difference compared with vehicle ($p < 0.05$).

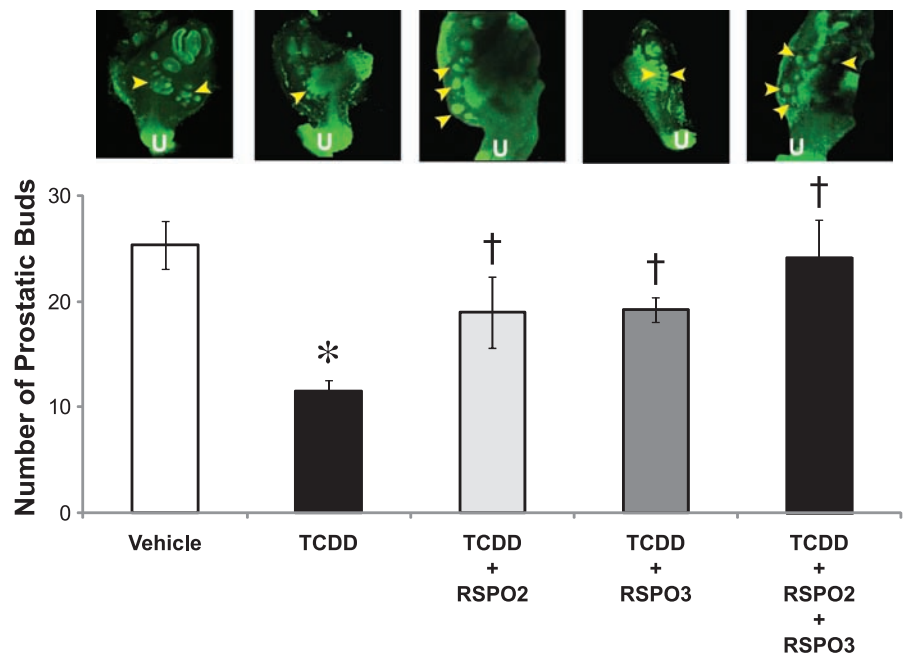

FIG. 5. Treatment with exogenous RSPO2 and RSPO3 partially protects against TCDD inhibition of prostatic bud development. E14.5 male UGSs were cultured for 4 days in DHT (10nM) containing media. Supplements to the media were either vehicle (0.1% DMSO) or TCDD (1nM) and RSPO2 and/or RSPO3 (100 ng/ml each). Treatment groups were vehicle, TCDD, RSPO2 + TCDD, RSPO3 + TCDD, and RSPO2 + RSPO3 + TCDD. Buds were visualized and quantified as described in the Figure 1 legend. Arrowheads represent areas where prostatic buds are present. U indicates urethra. Results are the mean ± SE of at least four litter-independent samples per treatment. Asterisk denotes a significant difference compared with vehicle, whereas a cross denotes a significant difference compared with TCDD ($p < 0.05$).

TCDD Inhibits Canonical Wnt Signaling and RSPOs Counteract the Effect

To further examine whether prostatic budding defects from TCDD exposure are due to disruption of the canonical Wnt signaling pathway, we examined how RSPOs and TCDD affected expression of downstream canonical WNT target proteins, LEF1 and TCF1, in cultured UGSs. *In vitro* UGS organ culture was performed for 4 days with media containing RSPO2 or RSPO3 alone or in combination with TCDD. Sectional IHC was performed using antibodies specific for LEF1 and TCF1. Sections were counterstained with KRT14 to mark BE and DAPI to mark nuclei. Percent of LEF1 or TCF1 positive BE cells in the UGS was determined by counting total number of BE cells positive for LEF1 or TCF1 staining in the entire UGS BE and dividing this number by the total number of BE cells identified by positive KRT14 staining. Our results show that both LEF1 and TCF1 are significantly reduced upon exposure to TCDD from 45 to 25% for LEF1 positive BE cells and from 60 to 25% for TCF1 positive BE cells (Figs. 6 and 7, compare high magnification insets). It should be noted that in the LEF1/KRT14 image, there is far less positive staining compared with vehicle control, and in the TCF1/KRT14 image, the inset shows no positive staining of TCF1, illustrating the inhibition of LEF1/TCF1 expression caused by addition of TCDD.

To determine whether RSPOs are able to protect against the inhibitory effects of TCDD on LEF1 and TCF1 expression, we performed sectional IHC on 4-day UGS cultures treated with RSPO2 (100 ng/ml), RSPO3 (100 ng/ml), or RSPO2 + RSPO3 (100 ng/ml each). RSPO2, RSPO3, and the combination of RSPOs were able to fully rescue the expression of LEF1 and TCF1 in the BE, demonstrating that RSPOs exert their effects through the canonical WNT signaling pathway and can overcome TCDD effects on Wnt signaling (Figs. 6 and 7). However, in vehicle-exposed UGS organ cultures, additions of RSPO2 and/or RSPO3 were not able to significantly induce LEF1 or TCF1 expression. Nevertheless, there was a nearly significant trend for induction of LEF1 in both the RSPO2 treatment and RSPO2 + RSPO3 treatment groups ($p = 0.07$ and $p = 0.08$, respectively; results not shown). These findings confirm that both transcript and protein levels of LEF1 and TCF1 are inhibited by TCDD in 3- and 4-day UGS cultures, respectively, and that RSPO2 and RSPO3 are able to reverse the inhibition.

Assessing Other Canonical Wnt Signaling Regulatory Genes for Misexpression by TCDD

Reduction in *Rspo*2 and *Rspo*3 by TCDD leads to a decrease in canonical Wnt signaling. There has been growing evidence that RSPOs activate canonical Wnt signaling by binding to LGRs (Carmon *et al*., 2011; de Lau *et al*., 2011; Glinka *et al*.,

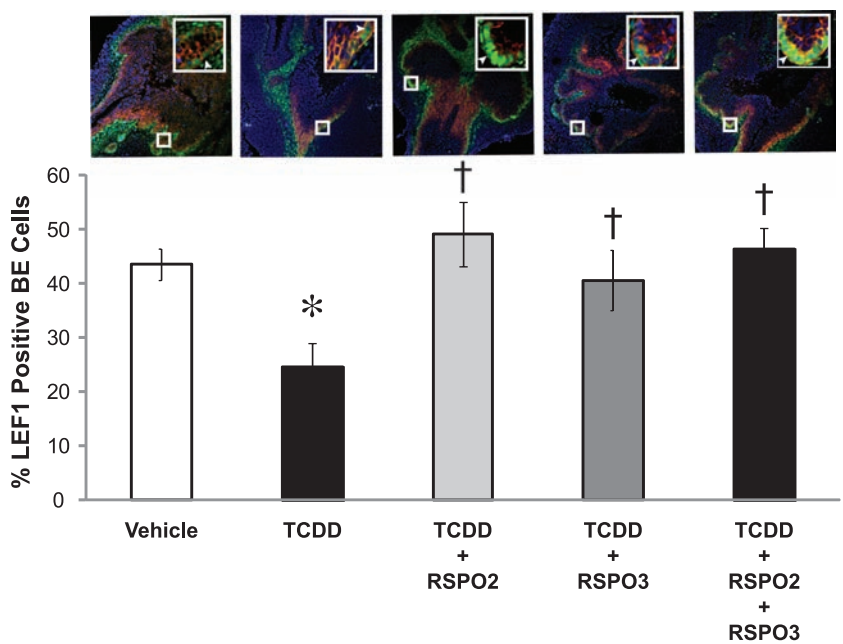

FIG. 6. TCDD inhibits the downstream canonical WNT target LEF1, and RSPOs fully restore LEF1. E14.5 male UGSs were cultured for 4 days in DHT (10nM) containing media. Supplements to the media were either vehicle (0.1% DMSO) or TCDD (1nM) and RSPO2 and/or RSPO3 (100 ng/ml each). Treatment groups were vehicle, TCDD, RSPO2 + TCDD, RSPO3 + TCDD, and RSPO2 + RSPO3 + TCDD. Near mid-sagittal sections (5 µm) were immunostained for LEF1 and counterstained for KRT14 to mark BE cells, which are the site of prostatic bud formation. Nuclei were stained with DAPI. Large boxes indicate enlarged images (200×) of small box areas in each sample to demonstrate colocalization of LEF1 and KRT14 in BE cells. Arrows indicate cells positive for LEF1/KRT14. Percent of BE cells positive for LEF1 was determined by counting the number of BE cells positive for LEF1 in the entire UGS and dividing that number by the total number of BE cells. Results are mean ± SE of at least four litter-independent UGSs per treatment. Asterisk denotes a significant difference compared with vehicle, whereas a cross indicates a significant difference compared with TCDD ($p < 0.05$).

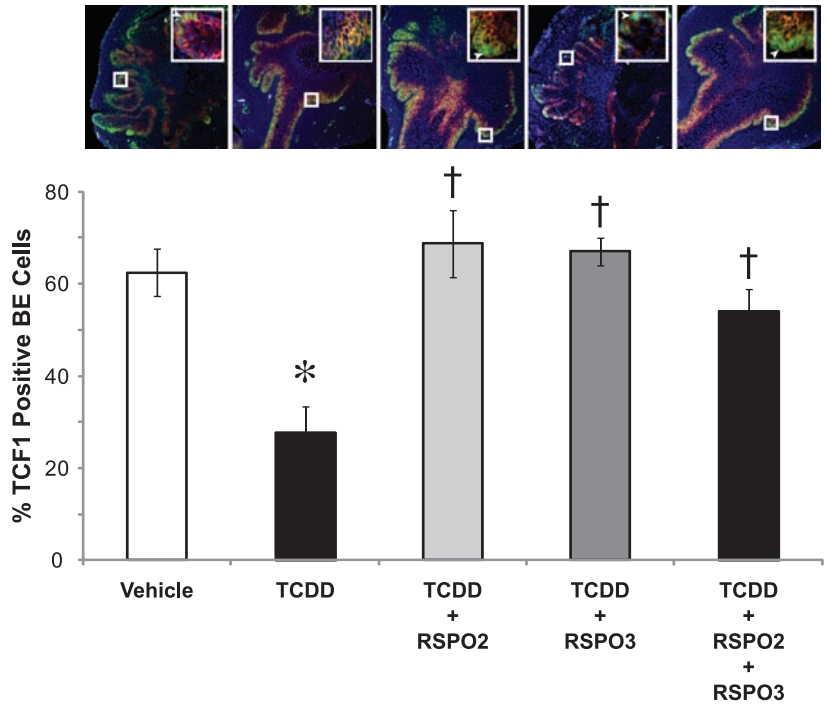

FIG. 7. TCDD exposure inhibits the downstream canonical WNT target, TCF1, and RSPOs fully restore TCF1 levels. E14.5 male UGSs were cultured for 4 days in DHT (10nM) containing media. Supplements to the media were either vehicle (0.1% DMSO) or TCDD (1nM) and RSPO2 and/or RSPO3 (100 ng/ml each). Treatment groups were vehicle, TCDD, RSPO2 + TCDD, RSPO3 + TCDD, and RSPO2 + RSPO3 + TCDD. Near mid-sagittal sections (5 µm) were immunostained for TCF1 and counterstained for KRT14 to mark BE cells, which are the site of prostatic bud formation. Nuclei were stained with DAPI. Large boxes indicate enlarged images (200×) of small box areas in each sample to demonstrate colocalization of TCF1 and KRT14 in BE cells. Arrows point to cells positively stained for TCF1/KRT14. There is no arrow present in the TCDD inset as no cells are positive for TCF1. Percent of BE cells positive for TCF1 was determined by counting the number of BE cells positive for TCF1 in the entire UGS and dividing that number by the total number of BE cells. Results are mean ± SE of at least three litter-independent UGSs per treatment. Asterisk denotes a significant difference compared with vehicle, whereas a cross indicates a significant difference compared with TCDD ($p < 0.05$).

2011). We found that TCDD significantly inhibits the mRNA expression of *Lgr5* but not *Lgr4* in UGSs from 3-day organ culture (Fig. 8A). To determine the expression of *Lgr5* and *Lgr4* in the developing UGS, we performed an *in vivo* time course using sectional ISH. *Lgr5* is not highly expressed at E16.5 (not shown), but at E17.5, *Lgr5* is expressed in both the UGE and the ventral UGM. The expression is depleted following TCDD exposure. *Lgr4* is strongly expressed in the UGE at both E16.5 (not shown) and E17.5, but its expression does not seem to be affected by TCDD (Fig. 8B). Thus, TCDD inhibits canonical Wnt signaling by at least two mechanisms: it reduces *Rspo2/3* abundance in UGM and reduces the abundance of their cognate receptor (*Lgr5*) in UGE.

Another way that TCDD could decrease canonical Wnt signaling in the UGE is by increasing DKK activity. We showed in Fig. 1 that DKKs, recognized inhibitors of canonical Wnt signaling, were capable of decreasing prostatic bud formation in mouse UGS organ cultures. This raised the possibility that TCDD might be decreasing canonical Wnt signaling not only by reducing the expression of activators of the pathway, *Rspo*2 and *Rspo*3, but also by increasing the expression of the inhibitors, *Dkk1* and *Dkk2*. This would lead to a decrease in canonical Wnt signaling and prostatic bud formation. Accordingly, UGSs were treated in culture for 3 days with either vehicle or TCDD followed by RT PCR analysis. We did not see either *Dkk1* or *Dkk2* transcript levels increased in the presence of TCDD (Supplementary fig. 2A). We also performed sectional ISH for *Dkk1* and *Dkk2* on the UGS of E17.5 fetuses exposed to vehicle (control) or TCDD on E15.5. Both *Dkks* were highly expressed in the UGM, but the expression of neither *Dkk* was affected by TCDD (Supplementary fig. 2B). We conclude that the TCDD-induced decrease in canonical Wnt signaling associated with a reduction in prostatic bud formation is not mediated by an increase in *Dkk1/2* expression.

DISCUSSION

Canonical Wnt Signaling Is Important for Prostatic Bud Formation and Is Inhibited by TCDD

We hypothesize that TCDD disrupts the balance between canonical and noncanonical Wnt signaling, and recent evidence from our laboratory and others supports this hypothesis. Previous data from our lab focused on the noncanonical pathway

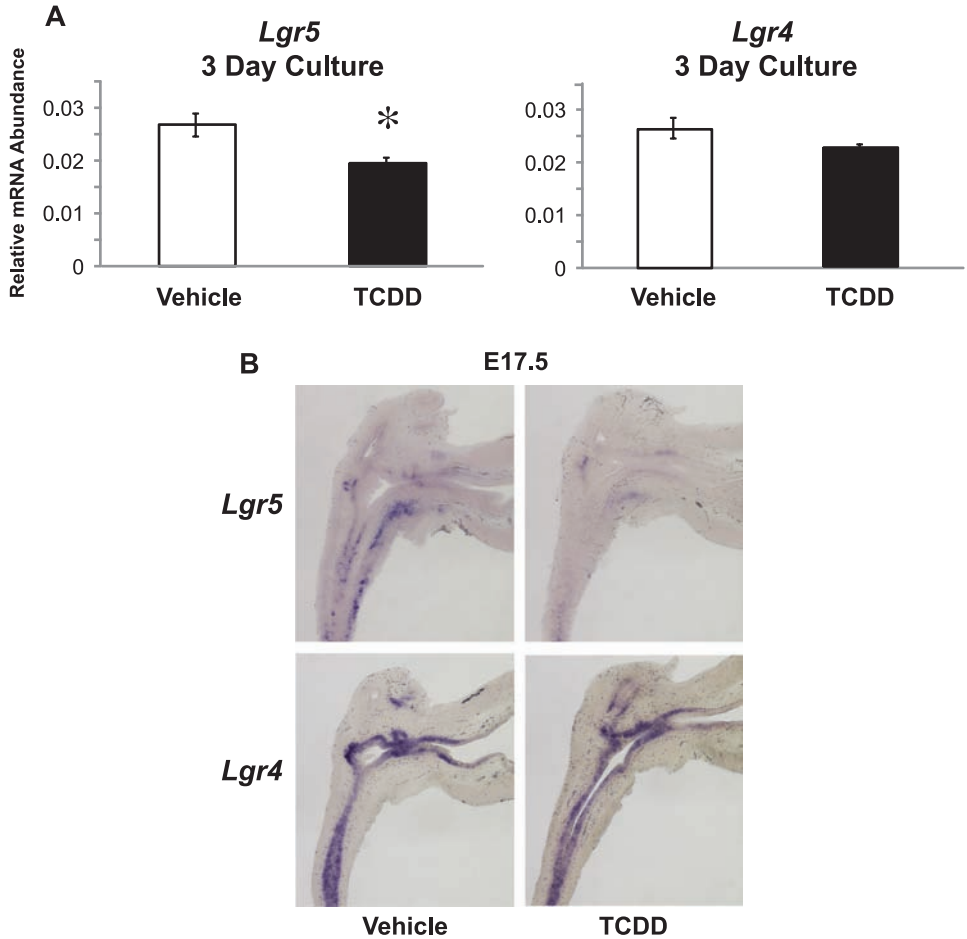

FIG. 8. TCDD downregulates *Lgr5*. E14.5 male UGSs were cultured for 3 days in DHT (10nM) containing media. RT PCR was performed to distinguish mRNA differences for *Lgr5* and *Lgr4* in vehicle- versus TCDD-treated samples (A). Results are mean ± SE of at least four litter-independent samples per treatment $p < 0.05$. Near mid-sagittal sections were cut from E17.5 fetal UGSs obtained from dams treated with either vehicle (5 ml/kg) or TCDD (5 µg/kg), po on E15.5 (B). Results represent at least three litter-independent samples per treatment.

and showed that TCDD enhanced noncanonical Wnt signaling as evidenced by addition of WNT5A, leading to inhibition of prostate budding, and by an antibody against WNT5A, fully rescuing the inhibitory budding effects of TCDD (Allgeier *et al.*, 2008). Noncanonical Wnt signaling also was shown to be upregulated following TCDD treatment in LNCaP prostate adenocarcinoma cells (Hrubá *et al.*, 2011). Evidence for canonical Wnt β-catenin signaling being downregulated by TCDD has been demonstrated both in liver progenitor cells and in the prevention of spheroid attachment to endometrial cells (Procházková *et al.*, 2011; Tsang *et al.*, 2012). In zebrafish, TCDD treatment inhibits fin regeneration secondary to upregulation of *Rspo1* and a morpholino against *Rspo1* protected against this effect of TCDD (Mathew *et al.*, 2008). Thus, there is a precedent for TCDD exposure adversely affecting development by causing misregulation of *Rspos*, activators of canonical Wnt signaling. Here we examined TCDD effects on canonical Wnt signaling and found the pathway to be inhibited and the expression of *Rspo2* and *Rspo3,* canonical Wnt agonists, to be downregulated.

Canonical Wnt β-catenin signaling is crucial for normal development. Signaling is tightly regulated and either too much or too little results in early developmental arrest (Kharaishvili *et al.*, 2011). Canonical Wnt signaling is critical in organs originating from a budding program including breast, tooth, and hair follicle (Faraldo *et al.*, 2006; Gat *et al.*, 1998; Liu *et al.*, 2008; Lo Celso *et al.*, 2004). Importantly, these organs are also all targets of TCDD toxicity (Keller *et al.*, 2007; La Merrill *et al.*, 2010; Panteleyev *et al.*, 1997). We provide evidence that canonical Wnt signaling is important for the formation of prostatic buds and that TCDD inhibits this pathway. Using the canonical Wnt inhibitor, XAV-939, and the extracellular antagonists of canonical Wnt signaling, DKK1 and DKK2, we showed that inhibition of canonical Wnt signaling reduced the formation of prostatic buds *in vitro* as did treatment with TCDD. Additionally, we were able to demonstrate downregulation of the canonical Wnt target genes *Lef1*, *Tcf1*, and *Wif1* in UGSs following *in utero* TCDD treatment, and reduced protein expression of LEF1 and TCF1

in UGSs following *in vitro* TCDD treatment. These genes are not only downstream targets of canonical Wnt β-catenin signaling but also major components of the pathway. *Lef1* and *Tcf1* are DNA binding partners of β-catenin and regulate transcription of β-catenin target genes. *Wif1* is an extracellular antagonist of Wnt signaling. When these components are collectively downregulated, this could lead to drastic consequences in UGS development.

Rspo2 and Rspo3 Are Downregulated by TCDD

Preliminary data from our lab showed that *Rspo2* and *Rspo3* were reduced in the VMP of E16.5 UGSs exposed to TCDD (Moore *et al*., 2011). The UGM is the site of TCDD action in the UGS (Ko *et al*., 2004a), and the VMP is a region of the UGS that secretes paracrine factors required for development of prostatic buds (Donjacour *et al*., 2003). We continued the investigation of TCDD effects on *Rspo2* and *Rspo3* by analyzing their expression *in vitro* using UGS organ culture analysis and also *in vivo* to explore later stages of prostatic bud development at E17.5 and E18.5 to look for differences between vehicle and TCDD-treated samples. We found that *in vitro*, Rspo2 levels are decreased following 2-day culture, consistent with previous *in vivo* results at E16.5, whereas at later stages of development *Rspo2* expression does not appear to be affected by TCDD. *Rspo3* expression does appear to be reduced in the VMP at both the E17.5 and E18.5 time points. This was also true in whole-mount *in vitro* samples after 3- and 4-day culture.

It is particularly significant that the TCDD-induced decrease in expression of the *Rspo2* and *Rspo3* was specific to the VMP region of the UGS. This is the same region of the UGS where TCDD exerts its inhibitory effect on ventral prostatic bud formation, resulting in ventral prostate agenesis. Like *Rspo2* and *Rspo3*, another inducer of prostatic bud formation in the mouse UGS, *Fgf10*, is expressed in the VMP. However, expression of *Fgf10* in the VMP is not affected by TCDD nor is activity of the FGF10 pathway in the UGS (Vezina *et al*., 2009). Prior to the discovery that TCDD downregulates *Rspo2* and *Rspo3* expression in a VMP region–specific manner, the identity of a gene or signaling pathway with expression localized to the ventral UGM that could potentially explain the TCDD impairment in ventral prostate development was unknown. Exactly how TCDD produces this ventral UGM region-specific effect on *Rspo* expression is not understood. It is not caused by the expression of AhR or ARNT in the UGS being confined to the VMP nor is it associated with a ventral UGS region-specific activation of AhR signaling by TCDD (Vezina *et al*., 2008b). AhR and ARNT are expressed ubiquitously throughout the UGS, not just in the VMP. We believe that reduction in both *Rspo2* and *Rspo3* contributes to impairment of prostatic bud development and downregulation of canonical Wnt signaling by TCDD.

RSPOs Promote Prostatic Bud Formation and Partially Protect Against Budding Inhibition by TCDD

Rspo2 and *Rspo3* are present in the VMP, and we predicted that they would promote prostatic bud formation by activating canonical Wnt signaling in the UGE. Supportive of this hypothesis, we found that addition of exogenous RSPO2 and RSPO3 in UGS organ culture media increases total prostatic buds formed. We also found that TCDD reduces *Rspo2* and *Rspo3* mRNA abundance, and we wanted to see whether restoring RSPO2 and RSPO3 activities could rescue the inhibitory budding effect of TCDD. We found that the addition of exogenous RSPO2 and RSPO3, alone or in combination, to TCDD-containing media restores prostatic bud numbers back to the level observed in UGSs grown in the absence of TCDD. We also found that RSPOs rescue the inhibition of LEF1 and TCF1 protein expression by TCDD, revealing that RSPOs are acting through the canonical Wnt signaling pathway to promote bud formation.

Differential Effects of TCDD on Expression of Other Canonical Wnt Signaling Regulatory Genes

Another possible mechanism of TCDD inhibition of *Rspo2* and *Rspo3* is through inhibition of LGR-induced canonical Wnt signaling. LGR4 and LGR5 enhance canonical Wnt signaling through binding to RSPOs (Carmon *et al*., 2011; de Lau *et al*., 2011; Glinka *et al*., 2011). RSPO binding to LGRs results in a synergistic response of canonical Wnt signaling, greater than achieved with Wnt ligands alone (Carmon *et al*., 2011). Consistent with this mechanism, we found that TCDD significantly reduced *Lgr5* transcript levels in 3-day UGS organ culture and inhibited *Lgr5* expression in ventral UGM and BE in E17.5 UGSs *in vivo*. We were not able to detect any expression differences in *Lgr4* between vehicle (control) and TCDD treatment groups; however, it was highly expressed in the entire UGE. We predict that RSPOs are secreted from the VMP and bind to LGRs in the BE to initiate prostatic bud formation. The fact that *Lgr5* is reduced, compiled with evidence for both *Rspo2* and *Rspo3* transcript reduction in the VMP by TCDD, could inhibit canonical Wnt signaling and bud formation. In contrast to *Rspos* and *Lgrs* contributing to the TCDD-induced decrease in canonical Wnt signaling, we found no evidence that a TCDD-induced increase in the expression of *Dkks* in the UGS occurs.

CONCLUSION

TCDD disrupts prostatic bud formation by altering the balance between noncanonical and canonical Wnt signaling. Previously, we showed that noncanonical Wnt signaling is increased by TCDD in the UGS, and we show in this study that canonical Wnt signaling is decreased. The decrease is

principally caused by a profound reduction in the expression of *Rspo2* and *Rspo3*, activators of canonical Wnt signaling. This results in downregulation of canonical Wnt target genes (*Lef1, Tcf1, Wif1*, and *Lgr5*) and a reduction in prostatic bud formation. Significantly, the TCDD-induced impairment in budding can be protected against by recombinant RSPO2 and RSPO3 proteins, which restore the balance in Wnt signaling.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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