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The Developmentally-Regulated *Smoc2* Gene Is Repressed by Aryl-hydrocarbon Receptor (Ahr) Signaling

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Abstract

SPARC-Related Modular Calcium Binding Protein-2 (Smoc-2) is a broadly-expressed matricellular protein which contributes to mitogenesis via activation of Integrin-Linked Kinase (ILK). Here we show that expression of *Smoc2* is repressed in cultured cells following treatment with Aryl-hydrocarbon receptor (Ahr) ligands including the ubiquitous environmental pollutants Benzo[a] pyrene (B[a]P) and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). The *Smoc2* promoter contains two consensus putative Ahr-binding sites and *Smoc2* promoter-driven reporter genes are repressed in response to B[a]P in an Ahr-dependent manner in cultured cells. Using organ culture experiments we show that TCDD also represses *Smoc2* mRNA expression in testes from $Ahr^{+/+}$ but not $Ahr^{-/-}$ mice. Therefore, exposure to Ahr ligands is likely to affect *Smoc2* expression *in vivo*. Taken together our results indicate that *Smoc2* is a novel transcriptional target of activated Ahr. Perturbation of *Smoc2* expression may mediate the adverse developmental effects of environmental aryl-hydrocarbon exposure.

Keywords

Smoc2; Benzo[a]pyrene; Dioxin; Ahr; testes

1. Introduction

Polycyclic aromatic hydrocarbons (PAH), typified by Benzo[a]pyrene (B[a]P) are ubiquitous environmental pollutants that are generated during the combustion of carbon-containing fuels including coal, gasoline and tobacco smoke (Baum, 1978). PAH-metabolizing cytochrome P450 enzymes are transcriptionally induced by B[a]P and other aryl-hydrocarbons and this induction is largely dependent on a transcription factor termed the Aryl-hydrocarbon Receptor, or Ahr (Whitlock, 1999). PAH such as B[a]P, as well as non-genotoxic Halogenated Aromatic Hydrocarbons (HAH, including 2,3,7,8-Tetrachlorodibenzo-p-dioxin or TCDD) are ligands that bind to and activate Ahr (Mandal, 2005). Ligand binding causes the translocation of Ahr

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into the nucleus where it heterodimerizes with the Ahr Nuclear Transporter (ARNT). The ligand-bound Ahr/ARNT complex binds to specific promoter elements termed Xenobiotic Response Elements (XRE) that regulate the transcription of cytochrome P450 genes including *CYP1A1*, *CYP1A2*, and others (Whitlock, 1999). B[a]P is metabolized by cytochrome P450s, to generate the DNA-damaging species and 'ultimate carcinogen' benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) (Conney, 1982).

Ahr is also likely to influence cell growth and differentiation via direct mechanisms not requiring P450-mediated xenobiotic metabolism (Schmidt et al., 1996; Alexander et al., 1998; Lahvis et al., 2005). For example, studies with transgenic mice have demonstrated that Ahr and ARNT play a role in the developmental closure of a hepatic vascular shunt termed the ductus venosus (Lahvis et al., 2005). Microarray profiling experiments have shown that non-genotoxic Ahr ligands such as TCDD modulate the expression of many genes involved in signal transduction and cell cycle regulation (Hanlon et al., 2005). Thus Ahr activation by environmental PAH or HAH could result in crosstalk with developmental pathways, thereby accounting for some of the detrimental effects of aryl-hydrocarbon exposure (Puga et al., 2005).

To gain insight into possible mechanisms by which Ahr signaling influences gene expression, we performed global mRNA profiling of PAH-treated cells. Here we identify *Smoc2* (SPARC-related Modular Calcium-binding protein-2) as a novel aryl-hydrocarbon-regulated gene.

Smoc-2 belongs to a family of matricellular proteins that also includes BM40/osteonectin/SPARC (Secreted Protein Acidic and Rich in Cysteine), SC1/hevin/Sparcl, tsc36/Flik/Fatl1, and Testican-1/Spock1 (Bornstein and Sage, 2002). Matricellular proteins regulate cell-matrix interactions, cell adhesion, spreading, migration, wound repair and angiogenesis during development, disease, and in response to injury (Bornstein and Sage, 2002). These biological effects result from interactions between matricellular factors and growth factors, integrins and/or other extracellular matrix proteins. Smoc-2 potentiates responses to mitogenic and angiogenic factors including FGF and VEGF (Rocnik et al., 2006). Consistent with a role for Smoc-2 in growth control, activation of Integrin-Linked Kinase (ILK) and cyclin D1 expression are Smoc-2-dependent (Liu et al., 2007). Integrins αv, β1, and β6 mediate cell adhesion to the C-terminal EF-hand of Smoc-2, also suggesting a role for Smoc-2 in integrin activation (Maier et al., 2008). SNP profiling studies have identified polymorphisms in the human *SMOC2* gene with linkage to pulmonary function (Wilk et al., 2003; Wilk et al., 2007), indicating a possible role for SMOC-2 in normal growth and development. Here we demonstrate that Ahr ligands repress *Smoc2* both in cultured cells and in organ culture. Ahr-mediated repression of *Smoc2* expression provides a novel mechanism by which exposure to environmental agents might influence and perturb signal transduction events leading to defects in growth and development.

2. Material and Methods

2.1. Chemicals and antibodies

Benzo[a]pyrene was purchased from Sigma. TCDD was obtained from the Midwest Research Institute. All chemicals and antibodies were obtained from previously-described sources (Liu et al., 2007).

2.2. Cells and Culture

Swiss 3T3 cells were obtained from ATCC. *Ahr*$^{-/-}$ Mouse Embryonic Fibroblasts (MEFs) were provided by Dr. David Sherr (Boston University School of Medicine) and were cultured using a 3T3 protocol (Liu et al., 2007).

2.3. Microarray analysis

Quiescent Swiss 3T3 cells were stimulated with 10 % serum or 10 % serum + 1μM B[a]P for 17 hr. Total cellular RNA was harvested as described previously (Rocnik et al., 2006) and submitted to Genome Systems, Inc. (www.genomesystems.com) for labeling and hybridization to DNA chips containing 10,000 arrayed mouse expressed sequence tags (EST). Genome Systems provided a list of transcripts corresponding to arrayed EST clones that were differentially expressed between the two samples.

2.4. RNA blot analysis

20 μg samples of total RNA were electrophoresed on agarose gels, transferred to nitrocellulose filters and hybridized with random-primed ^{32}P-labelled cDNA probes exactly as described previously (Vaziri and Faller, 1995).

2.5. Isolation of the Smoc2 promoter

A DNA fragment containing 1087 bp of the 5′ region of the mouse *Smoc2* gene was amplified from 3T3 cell genomic DNA using the following primers: 5′-CGGGGTACCCCCCGTGTTGGGCTAGGGCAGGGTA-3′ (forward) and CTAGCTAGCGGTGACGCTGGAGGGGACCAAGCGA-3′ (reverse). The resulting PCR product was digested with *Kpn* I and *Nhe*I and ligated into the promoterless pGL2b luciferase vector (Promega).

2.6. Transfections and luciferase activity assays

Cells were seeded on 6-well plates and transfected with 4 μg of DNA using Lipofectamine 2000 (Invitrogen). Cells harvested for luciferase assays 48 hr post-transfection using a commercially available kit (Promega).

2.7. Extraction and analysis of mRNA from cultured cells

Total cellular RNA was extracted and analyzed by RT-PCR as described previously (Liu et al., 2007). The following primers were used for RT-PCR: 5′ CAGGTCCAGTGTCACAGCTACAC3′ (mouse Smoc-2 forward), 5′ GGTCTTGTTCTGCCGACTCTTAAC3′ (mouse Smoc-2 reverse), 5′ GGCTACAGCTTCACCACCACAGC 3′ (mouse β-actin forward), and 5′ CCACAGGATTCCATACCCAAGAAGG3′ (mouse β-actin reverse). The amplified products were separated on 1.0 % agarose gels and visualized under an UV transilluminator.

2.8. Preparation of whole cell extracts and Immunoblotting

Whole cell extracts were prepared and analyzed using SDS-PAGE and immunoblotting exactly as described previously (Liu et al., 2007).

2.9. Mice

CD-1 mice were purchased from Charles River Laboratories. *Ahr* mutant mice were a gift of Dr. D. H. Sherr (Boston University School of Medicine). *Ahr*−/− mice were generated by mating *Ahr*$^{+/-}$females to *Ahr*$^{-/-}$ males. Embryos were subsequently genotyped as previously described (Robles et al, 2000). Timed matings were used for all experiments where noon on the day of vaginal plug detection was designated as embryonic day (E) 0.5.

2.10. Extraction and analysis of RNA from cultured gonads

RNA was isolated using the RNeasy mini kit (Qiagen). cDNA was synthesized using oligo-dT primers and SuperScript II reverse transcriptase (RT) (Invitrogen). Quantitative real-time RT- PCR analysis was performed with an ABI PRISM 7900HT Sequence Detection System,

using *Power* SYBR Green PCR Master Mix (Applied Biosystems) and the following primers: *Smoc2* 5′ GACCCTCTTCCTCTTCTGG3′ and 5′ TCCTTCTTGCCAATGTCTCC3′; *Cyp1a1* 5′AGGATGTGTCTGGTTACTTTG3′ and 5′ AGAAACATGGACATGCAAG3′; *Hprt* (Bouma et al., 2004). All primer pairs produced single products of the expected size, without the formation of primer dimers. Validation experiments were performed according to Applied Biosystems guidelines (AppliedBiosystems, 2004). Fold change values were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Two-tailed Student's t-tests were performed to determine statistical significance.

2.11. Organ culture

Gonad/mesonephros complexes were dissected from E12.5 mice. One complex served as a control while the other was treated. TMTP Isopore membrane filters (5 μm, Millipore) were floated on 0.5 ml of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10 % fetal bovine serum and ampicillin (50μg/ml) in 4-well cell culture plates. Isolated complexes were placed on filters and incubated at 37°C and 5% CO_2. For drug treatments an appropriate volume of 1 mM TCDD stock solution in DMSO was added to give a final concentration of 1μM while an equivalent volume of DMSO was used as a control.

2.12. Wholemount in situ hybridization (WISH)

Digoxigenin (DIG)-labeled probes were generated by *in vitro* transcription of *Smoc2*- and *Cyp1a1*-containing plasmids using T7, SP6 or T3 RNA polymerase in the presence of DIG-labeled dUTP (Roche). Gonad-mesonephros complexes were dissected in PBS and fixed overnight at 4°C in 4 % paraformaldehyde/PBS. Whole-mount *in situ* hybridization was performed using standard protocols with minor modifications as described previously (Wilkinson, 1998).

3. Results

3.1. Identification of Smoc2 as a B[a]P-regulated mRNA

To gain insight into potential mechanisms by which PAH exposure affects gene expression we performed microarray experiments and determined global mRNA profiles of B[a]P-treated cells. Cultures of quiescent confluent Swiss 3T3 cells were treated with 10% serum in the presence or absence of 1μM B[a]P. Seventeen hours after serum treatment, replicate plates of control and B[a]P-treated cells were collected for analysis of RNA (by microarray and northern blotting), as described under 'Material and Methods'.

The microarray analysis identified fewer than 100 mRNA species whose expression changed by 3-fold or more as a result of B[a]P treatment. Therefore, B[a]P did not cause global perturbation of gene expression under our experimental conditions. According to the microarray analysis, cDNAs induced by B[a]P included *p21* and *Mdm2*, which we previously identified as B[a]P-inducible transcripts (Hsing et al., 2000). Genes encoding cyclin G (*Cdk5r1*), ornithine decarboxylase (*Odc*) and multi-drug resistant P-glycoprotein 1 (*Mdr1*), were also induced by B[a]P. The effect of B[a]P on *Mdr1* is consistent with a previous report showing that the *Mdr1* gene is Ahr-regulated (Mathieu et al., 2001). mRNAs whose abundance decreased after B[a]P treatment included T-Cadherin and IMAGE clone # 482198, which we subsequently identified as *Smoc2* as described under 'Materials and Methods'.

Several EST clones corresponding to B[a]P-responsive mRNAs (including *p21*, *Mdm2*, *Mdr1*, and *Smoc2*) were obtained and used as probes in northern blotting experiments to validate the microarray results. Fig. 1A shows the results of our RNA blotting experiments. For all ESTs tested, the changes in mRNA expression identified in the microarray experiment were reproduced using RNA blotting. Thus, B[a]P-induced increases in *p21*, *Mdm2*, *Mdr1*,

Odc and decreases in T-Cadherin, and *Smoc2* were readily detected, thereby validating the microarray analysis.

Because B[a]P is metabolized to genotoxic species such as BPDE in an Ahr-dependent manner in Swiss 3T3 cells (Vaziri and Faller, 1997), we considered the possibility that the changes in expression of *Smoc2* (and other mRNAs shown in Fig. 1A) occurred secondarily to a DNA damage response. Indeed, the induction of p53-regulated genes such as *p21* and *Mdm2* following B[a]P-treatment is due to acquisition of DNA damage (Vaziri and Faller, 1997). Moreover, since *Smoc2* expression is affected by cell cycle progression (Liu et al., 2007), and because PAH such as B[a]P perturb the cell cycle (Vaziri and Faller, 1997), it was possible that *Smoc2* levels were affected by BaP secondarily to cell cycle changes.

To distinguish between genotoxic and non-genotoxic mechanisms of gene repression we tested the effects of the non-genotoxic Ahr ligand TCDD on *Smoc2* mRNA levels. To eliminate possible effects of cell cycle status on *Smoc2* expression, we performed these experiments in cells which were growth-arrested in G0 by serum-starvation. As shown by the RT-PCR analyses in Fig. 1B, *Smoc2* mRNA levels were induced following serum-starvation as we reported previously (Liu et al., 2007). Interestingly, *Smoc2* mRNA levels were specifically reduced in response to B[a]P or TCDD (Fig. 1B). In other experiments, we found that treatment with BPDE (a product of B[a]P metabolism which elicits DNA damage but does not activate Ahr) did not affect *Smoc2* expression (P. L., data not shown). Therefore, the reduced expression of *Smoc2* in B[a]P or TCDD-treated cells cannot be attributed to a DNA damage response. Moreover, the decreased *Smoc2* expression following treatment with B[a]P or TCDD occured in quiescent (G0) cells and therefore was not due to Ahr ligand-induced changes in the cell cycle.

3.2. The Smoc2 promoter is B[a]P-responsive

To gain insight into possible mechanisms by which *Smoc2* mRNA levels are altered in response to B[a]P and TCDD we analyzed the sequence of the 5′ region of the *Smoc2* gene using PROMO search tool and the TRANSFAC 8.3 database (Messeguer et al., 2002). As shown in Fig. 2A, these analyses identified multiple core XRE (GCGTG) sequences (Swanson et al., 1995), a putative Hif1α(NVNGCACGT) consensus sequence, and several GC box-Sp1 (CCGCCC) sites upstream of the translational start site. For comparison, we also analyzed the corresponding region of the human *SMOC2* gene. Interestingly, putative XRE, HIF1α, and Sp1 sites were also present in the *SMOC2* gene (Fig. 2A). Therefore, *Smoc2* and *SMOC2* genes contain XREs, potentially accounting for the altered expression of *Smoc2* following treatment with Ahr ligands.

To test whether transcriptional mechanisms accounted for downregulation of *Smoc2* mRNA after B[a]P treatment we linked 1087 bp of the putative *Smoc2* promoter to a firefly luciferase cDNA. The resulting *Smoc2*-luciferase construct, as well as a promoter-less luciferase vector and a RSV-luciferase construct (as negative and positive controls for promoter activity, respectively) were transfected into 3T3 cells. As shown in Fig. 2B, the 1087 bp 5′ region of the *Smoc2* gene conferred a 180-fold increase in luciferase activity relative to ‘empty’ luciferase plasmid, thereby demonstrating that the *Smoc2* genomic fragment has promoter activity. We used reporter gene assays to determine the effect of B[a]P on *Smoc2* promoter activity. As shown in Fig. 2B, B[a]P-treatment resulted in a 65% decrease in *Smoc2*-dependent luciferase expression. The effect of B[a]P on the *Smoc2* promoter was specific since RSV-driven luciferase activity was unaffected by B[a]P treatment in a parallel experiment. These data demonstrate that transcriptional mechanisms can account for the effect of B[a]P on endogenous *Smoc2* mRNA levels.

3.3. Role of Ahr in regulation of Smoc2 expression

The repression of *Smoc2* by Ahr ligands, together with the presence of consensus core XREs in the *Smoc2* promoter (Fig. 2A) suggested a possible role for Ahr-ARNT in transcriptional regulation of this gene. Therefore, we tested the effect of over-expressed Ahr-ARNT on *Smoc2*-driven luciferase activity. As shown in Fig. 2B, co-transfection of CMV-Ahr and CMV-ARNT vectors repressed S*moc2*-dependent luciferase expression by 75%, even in the absence of B[a]P treatment. In CMV-Ahr/ARNT-transfected cells that additionally received B[a]P, *Smoc2*-dependent luciferase activity was reduced by 98%. RSV-driven luciferase activity was unaffected by co-transfected CMV-Ahr/ARNT and B[a]P. We also determined the effects of individual and combinatorial over-expression of Ahr and ARNT on *Smoc2*-Luciferase expression. In a representative experiment shown in Fig. 2D, ARNT and Ahr inhibited *Smoc2*-dependent luciferase activity by 45 % and 56 % respectively and combined expression of Ahr and ARNT led to a slight further increase in repression (61 % inhibition). Taken together, these data indicate that the *Smoc2* gene is a potential target of repression by Ahr-ARNT-containing complexes. However, given the potential for dimerization of Ahr and ARNT with other PAS family members, we do not exclude the possibility that *Smoc2* (or any other XRE-containing gene) is regulated by heterodimeric bHLH complexes containing additional PAS family members.

Since the results of Fig. 2 indicated a potential role for Ahr in regulating the *Smoc2* promoter we asked whether Ahr is specifically required for B[a]P-induced changes in *Smoc2* expression. Therefore, we compared the effect of B[a]P on *Smoc2* mRNA levels in $Ahr^{+/+}$ (3T3) cells and $Ahr^{-/-}$3T3 fibroblasts from *Ahr*-null mice. As shown in Fig. 3A, B[a]P-treatment decreased *Smoc2* mRNA levels in $Ahr^{+/+}$, but not $Ahr^{-/-}$ cells, suggesting that Ahr mediates down-regulation of *Smoc2* by B[a]P. To further test a role for Ahr in *Smoc2* regulation, we determined the effect of B[a]P on *Smoc2* promoter-driven luciferase expression in $Ahr^{-/-}$ cells. As shown in Fig. 3B, *Smoc2*-Luciferease activity was not significantly affected by B[a]P-treatment in $Ahr^{-/-}$ cells. However, when we reconstituted Ahr expression in $Ahr^{-/-}$ cells using CMV-Ahr, S*moc2*-Luciferase activity was repressed by 83%. Reconstitution of Ahr in $Ahr^{-/-}$ MEFS also conferred responsiveness to B[a]P (Fig. 3B). Taken together these data show that *Smoc2* expression is down-regulated in response to B[a]P via an Ahr-dependent mechanism.

As shown in Fig. 2A, the *Smoc2* promoter contains 2 consensus Ahr/ARNT sites. Deletion analyses of the *Smoc2* promoter showed that a minimal 424 bp promoter element containing two consensus Ahr/ARNT-binding sites retained responsiveness to B[a]P treatment (data not shown). Deletion of both Ahr/ARNT sequences resulted in abrogation of *Smoc2*-luciferase activity (data not shown) both basally and in B[a]P-treated cells. Therefore, we have not been able to determine whether the Ahr/ARNT sites are specifically required for repression of the *Smoc2* promoter in B[a]P-treated cells. Nevertheless, our data show that the *Smoc2* promoter is negatively regulated by B[a]P in an Ahr-dependent manner.

It was of interest to test whether the Ahr-mediated changes in *Smoc2*-luciferase expression and *Smoc2* mRNA were reflected by decreases in Smoc-2 protein levels. Therefore, we treated quiescent cells with B[a]P for varying times and performed immunoblot analysis. As shown in Fig. 3C, B[a]P-treatment decreased Smoc-2 levels in Ahr-expressing 3T3 cells (but not in $Ahr^{-/-}$ MEFs). In these immunoblotting experiments the reduced expression of Smoc-2 was not evident until 2 days post-B[a]P treatment, most likely reflecting the long half-life of Smoc-2 protein.

3.4. AhR-dependent repression of Smoc2 expression in an organ culture model

Potentially, changes in *Smoc2* expression could mediate some of the effects of exposure to environmental Ahr ligands. It is important therefore, to determine whether *Smoc2* expression

is sensitive to PAH/HAH in Ahr-expressing tissues. We have found that embryonic mouse testes express *Smoc2* in the interstitium, the region where the steroid-producing Leydig cells develop (Fig. 4A). Additionally, *Smoc2* is expressed in the mesonephros, in the region of the Müllerian and Wolffian ducts (Fig. 4A). In females, the Müllerian ducts develop into the oviducts, uteri, and upper vagina, while in males, the Wolffian ducts develop into the epididymides, vas deferentia, and seminal vesicles. Developmental and reproductive defects caused by *in utero* TCDD exposure are well documented, but the mechanisms of action are poorly understood. However, it was recently shown that pregnant female rats treated with TCDD on E11 produce male offspring with decreased testosterone levels on E19.5 due to decreased testicular steriodogenesis (Adamsson et al., 2008). This exposure tended to decrease the expression of StAR, p450scc, and 3β-HSD, which are expressed in Leydig cells. Since *Smoc2* is expressed in the testis, probably in Leydig cells, and because the testis is a target of TCDD action, we investigated the effects of TCDD exposure and *Ahr* status on *Smoc2* expression in this organ.

Gonad/mesonephros complexes were dissected from E12.5 CD-1 mouse embryos. This time point was chosen because it is coincident with the onset of *Smoc2* testicular expression. Isolated gonad/mesonephros complexes were cultured in the presence of 1 μM TCDD (or DMSO for controls) for 24 hours. Analysis of gene expression by quantitative real-time RT-PCR revealed a significant decrease of 24% ($n=11$, $p<0.01$) in *Smoc2* expression in testis/mesonephros complexes in response to TCDD (Fig. 4B). WISH analysis revealed that the suppression of *Smoc2* expression was not localized to a specific area (i.e. the testicular interstitium or mesonephros), although this finding does not preclude the possibility that *Smoc2* is downregulated in a specific cell type (Fig. 4B). Testis/mesonephros complexes express *Ahr* (data not shown) and as a control for Ahr activation in these experiments we examined expression of *Cyp1a1* (a known Ahr target gene) in parallel cultures of isolated gonads. As expected, TCDD induced an approximately 450-fold expression of *Cyp1a1* as determined by quantitative real-time PCR (Fig. 4B). Interestingly, *Cyp1a1* expression was induced in regions of the testis and the mesonephros that overlap with *Smoc2* expression (Fig. 4A), consistent with a role for Ahr in repression of *Smoc2* expression in the testes.

To test whether AhR mediates the repression of *Smoc2* in TCDD-treated gonads, testes harvested from E12.5 *Ahr*+/− and *Ahr*−/− mice were cultured in 1μM TCDD for 24 hours and analyzed for *Smoc2* expression. Similar to wild-type CD-1 mice, *Smoc2* expression was downregulated by 18% ($n=5$, $p<0.005$) in *Ahr*+/− mice in response to TCDD (Fig. 4C). However, *Smoc2* expression in *Ahr*−/− gonad/mesonephros complexes was unchanged ($n=7$, $p=0.48$). Similar to previous reports (Shimizu et al., 2000), *Cyp1a1* induction by TCDD only occurred in the AhR-expressing gonad/mesonephros complexes (Fig. 4C). We conclude that TCDD represses *Smoc2* mRNA levels in an *Ahr*-dependent manner in cultured embryonic testes.

4. Discussion

In this study we have identified *Smoc2* as an mRNA that is downregulated in response to Ahr ligands both *in vitro* and *in vivo*. Smoc-2 is a matricellular protein, which promotes cell cycle progression in mesenchymal, endothelial, and possibly other cell types (Rocnik et al., 2006; Liu et al., 2007), most likely by facilitating integrin-ILK-dependent signaling cascades (Liu et al., 2007). Our finding that *Smoc2* is repressed in response to Ahr ligands provides a novel link between environmental PAH/HAH exposure and growth control.

Increasingly, it appears likely that inappropriate Ahr activation by environmental agents may perturb growth and development via transcriptional regulation of genes involved in signal transduction and cell cycle regulation (Fisher et al., 2004; Hanlon et al., 2005; Thackaberry et

al., 2005). For example, Hanlon et al. identified glypican 1 as a TCDD-responsive gene in a recent microarray screen (Hanlon et al., 2005). Glypican is a membrane proteoglycan that affects growth factor signaling (Fransson, 2003). Poellinger and colleagues showed that the ECM component osteopontin is downregulated in mice expressing constitutively active form of Ahr (Kuznetsov et al., 2005). Our finding that Ahr regulates *Smoc2* expression provides an additional link between PAH exposure, Ahr activation and control of cell growth by the ECM. *Smoc2* shows a defined pattern of expression in the mouse embryo, suggestive of specific developmental roles (Liu et al., 2007). Ahr-mediated changes in expression of *Smoc2* (or other ECM components such as or glypican 1) could perturb growth factor signaling and cell cycle progression, thereby accounting for some of the detrimental consequences of of aryl-hydrocarbon exposure during development (Puga et al., 2005). Indeed we show that *Smoc2* is repressed in Ahr ligand-treated cultured embryonic mouse gonads.

Although our results show that Ahr mediates B[a]P-induced changes in *Smoc2* expression, further studies are necessary to determine whether the *Smoc2* promoter is directly repressed by Ahr. Potentially, ligand-activated Ahr could act directly on XREs or might regulate gene expression via indirect mechanisms. For example, Ahr appears to activate c-Ha-ras via a redox-sensitive mechanism (Enan et al., 1998; Kerzee and Ramos, 2000) and can also activate ERK, p38 MAP kinase (Park et al., 2005) and the src tyrosine kinase (Enan and Matsumura, 1996). Clearly, regulation of protein kinase cascades by Ahr could affect gene expression. Ahr also interacts physically with the Rb tumor suppressor and modulates E2F activity (Puga et al., 2000; Strobeck et al., 2000; Marlowe et al., 2004), thereby providing a link between Ahr and E2F-regulated cell cycle genes.

However, based on the presence of consensus Ahr/ARNT-binding sites in the *Smoc2* gene, we consider it likely that Ahr regulates *Smoc2* expression directly. Regulation of cellular genes such as *Cyp1A1* (encoding Cytochrome P-450 1A1) is one of the best-characterized responses to ligand-activated Ahr. *Cyp1A1* activation is mediated via direct binding of the ligand/Ahr/ARNT complex to XREs located in the 5′-flanking region of the gene. However, some XRE-containing genes are repressed by aryl-hydrocarbons, albeit via poorly-understood mechanisms. For example, the rat male-specific constitutive hepatic *Cyp2c11* gene is repressed by aryl-hydrocarbons at least in part via mechanisms involving changes in gene transcription (Lee and Riddick, 2000; Bhathena et al., 2002; Riddick et al., 2004).

Surprisingly, although Ahr binds to XREs in the *Cyp2c11* 5′-flanking region, *Cyp2c11* promoter-luciferase reporter constructs containing XREs were not repressed in response to treatment with Ahr ligands (Bhathena et al., 2002; Sawaya and Riddick, 2008). Therefore, Ahr ligands may down-regulate *Cyp2c11* by a negative transcriptional mechanism that is not solely due to Ahr binding to an identified XRE-like sequence (Bhathena et al., 2002). Clearly, transient transfections of luciferase-linked promoter fragments may not fully recapitulate the complexity of *Cyp2c11* regulation by Ahr *in vivo*.

In contrast with the *Cyp2c11* promoter, we have shown that *Smoc2*-luciferase constructs are repressed by B[a]P in an Ahr-dependent manner, thereby distinguishing the mechanisms of *Smoc2* and *CYP2C11* repression after aryl-hydrocarbon treatment. Resink and colleagues showed that the gene encoding T-cadherin (a glycosylphosphatidylinositol-modified cadherin subtype) contains a 5′ regulatory XRE and is repressed in an Ahr-dependent manner (Niermann et al., 2003). Interestingly, our microarray analysis also identified T-cadherin as a B[a]P-suppressed transcript. Based on the study by Resink and colleagues, it is likely that the downregulation of T-cadherin and *Smoc2* in B[a]P-treated mesenchymal cells occurs via Ahr-mediated repression.

Similar to Ahr, other ligand-activated nuclear receptors such as the estrogen receptor (ER) are able to induce or repress gene transcription. ER signaling is complex and subject to modulation by a large number of co-repressors or co-activators (Tremblay and Giguere, 2002). For example, AhR dissociates ERα-Sp1 interactions thereby inhibiting transcription of the CAD promoter (Khan et al., 2006). Therefore, co-regulator proteins and promoter context could determine whether ligand-activated Ahr induces or suppresses gene expression. There is mounting evidence that Ahr-mediated gene regulation involves co-activators and co-repressors. For instance, Puga and colleagues have shown that Ahr interacts with RB, a transcriptional repressor (Puga et al., 2000). Potentially, Ahr-mediated suppression of *Smoc2* expression could involve transcriptional co-repression.

Our data are consistent with repression of *Smoc2* by Ahr-ARNT complexes. However, as with any XRE-containing gene there exists the potential for other PAS proteins to heterodimerize with Ahr and/or ARNT to regulate *Smoc2*. As shown in Fig. 2A, *Smoc2* and *SMOC2* genes possess consensus HIF1αsites in addition to XREs. In unpublished experiments we have found that the endogenous *Smoc2* mRNA is downregulated in hypoxic cells and that *Smoc2* promoter-driven luciferase expression is repressed in response to both hypoxia and ectopically-expressed HIF1α(data not shown). Therefore, the *Smoc2* promoter is repressed by Ahr, HIF1α, ARNT, and perhaps bHLH complexes containing other PAS family members. Further experiments are underway to test roles for PAS family members and other transcriptional co-regulators in Ahr-mediated repression of the *Smoc2* promoter.

Specific elements that regulate the expression of the human *SMOC2* gene have yet to be demonstrated. However, the occurrence of six XREs in 575 bases immediately flanking the 5′ end of the coding sequence (Fig. 2A) provides circumstantial evidence that human *SMOC2* is regulated by the Ahr/ARNT heterodimer. These XREs are adjacent to or overlap GC-rich Sp1 sites (Fig. 2A), which are involved in the transcriptional regulation other Ahr/ARNT targets (Kobayashi et al., 1996;Wang et al., 1998). Environmentally-induced changes in SMOC-2 expression might impact human health. For example, a recent analysis of patient samples from the Framingham Heart Study demonstrated genetic associations between pulmonary function measures and human *SMOC2* (Wilk et al., 2007). Prior to the study of this patient group, the only genetic defect known to cause obstructive pulmonary disease involved the serine protease inhibitor alpha-1anti-trypsin (Tobin et al., 1983). SMOC-2 contains Kazal serine protease inhibitory motifs (Vannahme et al., 2003) and it is possible that putative SMOC-2-dependent protease inhibitory activity is similarly important for pulmonary function. Regardless of the mechanism by which SMOC-2 contributes to normal pulmonary function, repression of *SMOC2* provides a possible means by which environmental agents could perturb the normal physiology of the lung or other organs.

Our studies suggest that the testes are a target organ that may be adversely affected by environmental agents via AhR-dependent changes in Smoc2 expression. Several reports indicate that in utero exposure to dioxin is associated with abnormalities in reproductive tract development and reproductive functions including steroidogenesis (Cooke et al., 1998; Hurst et al., 2002)). We show here that Smoc2 is expressed in fetal testicular Leydig cells (steroid producing cells) and in the primordial reproductive tract. Moreover, we have demonstrated that the embryonic testis/mesonephros complex responds to dioxin exposure by reducing Smoc2 expression. The role of Smoc2 in embryonic gonad development has yet to be determined. However, our previous in vitro data suggests that SMOC2 is involved in mediating the mitogenic and angiogenic effects of growth factors such as VEGF, PDGF, and FGF (Rocnik et al., 2006; Liu et al., 2007). Intriguingly, members of these growth factors families are known to be essential for normal embryonic testis development (Colvin et al., 2001; Brennan et al., 2003; Bott et al., 2006). If Smoc2 does contribute to signaling by these growth factors in the testis, then its reduction in response to TCDD may compromise the development of this organ.

Clearly further studies are necessary to identify the developmental role(s) of *Smoc2* and the consequences of reduced *Smoc2* expression following aryl-hydrocarbon exposure in vivo.

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Abbreviations

SPARC
: secreted protein acidic and rich in cysteine

Smoc2
: SPARC-related modular calcium binding protein-2

ILK
: integrin-linked kinase

Ahr
: aryl-hydrocarbon receptor

B[a]P
: benzo[a]pyrene

TCDD
: 2,3,7,8-tetrachlorodibenzo-p-dioxin

PAH
: polycyclic aromatic hydrocarbons

HAH
: halogenated aromatic hydrocarbons

ARNT
: Ahr nuclear transporter

XRE
: xenobiotic response elements

BPDE
: benzo[a]pyrene-7,8-diol-9,10-epoxide

EST
: expressed sequence tags

WISH
: wholemount *in situ* hybridization

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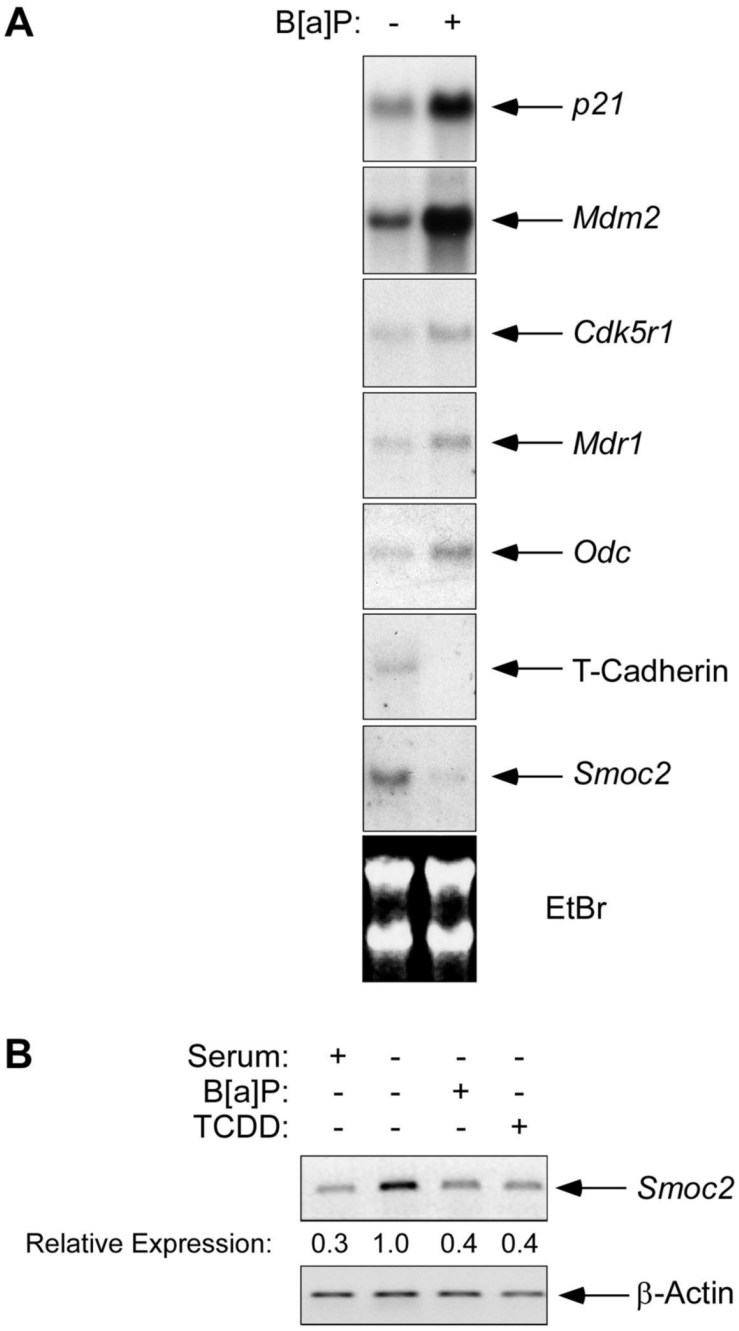

Fig. 1. *Smoc2* mRNA is repressed in response to Ahr ligands

(A) Samples of total RNA from control and 1 μM B[a]P-treated Swiss 3T3 cells were separated by electrophoresis on agarose gels (20μg/lane), transferred to nitrocellulose, and probed with various ^{32}P-labelled cDNAs as indicated.

(B) Quiescent Swiss 3T3 cells were treated with 1 μM B[a]P or 10 nM TCDD for 24 hr. RNA prepared from the resulting cells was analyzed by RT-PCR using primers specific for *Smoc2* and β-Actin as described under 'Materials and Methods'.

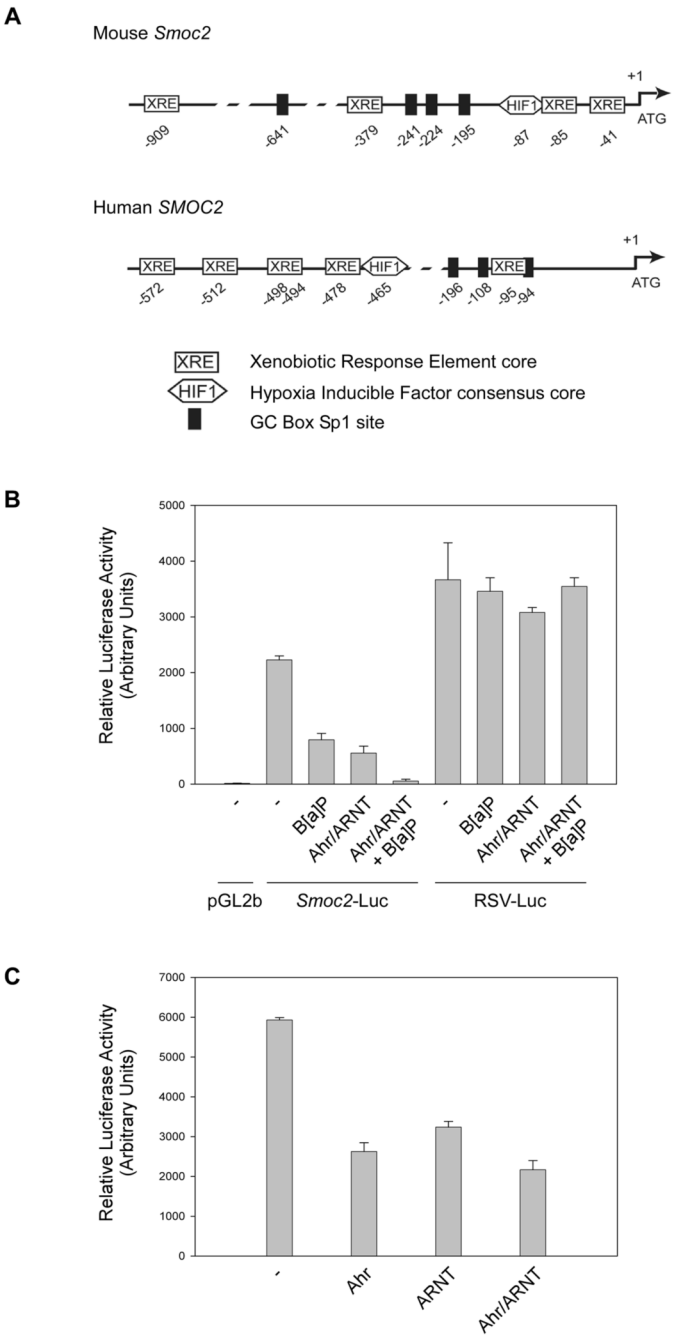

Fig. 2. The *Smoc2* promoter is responsive to B[a]P and Ahr/ARNT

(A) Putative Ahr/ARNT, Hif1α, and GC-box Sp1 binding sites identified in the mouse *Smoc2* and human *SMOC2* gene promoters using PROMO search tool and the TRANSFAC 8.3 database. Numbers indicate the position of core XRE (GCGTG), Hif1α(NVNGCACGT), and GC box-Sp1 (CCGCCC) elements relative to the translational start site.

(B) *Smoc2*-luciferase, a promoterless luciferase vector (pGL2b), and a strong constitutive RSV-luciferase reporter construct (RSV-Luc) were transiently transfected into 3T3 cells. In some transfections, Ahr and ARNT were co-expressed using CMV-driven expression plasmids. The total amount of DNA in each transfection was kept constant by including the appropriate amount of 'empty' pcDNA vector. 24 hr post-transfection, some cultures were treated with 1

μM B[a]P (or DMSO for controls) and incubated for an additional 24 hr prior to harvest for luciferase assays.

(C) CMV-Ahr and CMV-ARNT plasmids were transfected into 3T3 cells individually or in combination, together with *Smoc2*-luciferase. 48 hr post-transfection cells were harvested for luciferase assays.

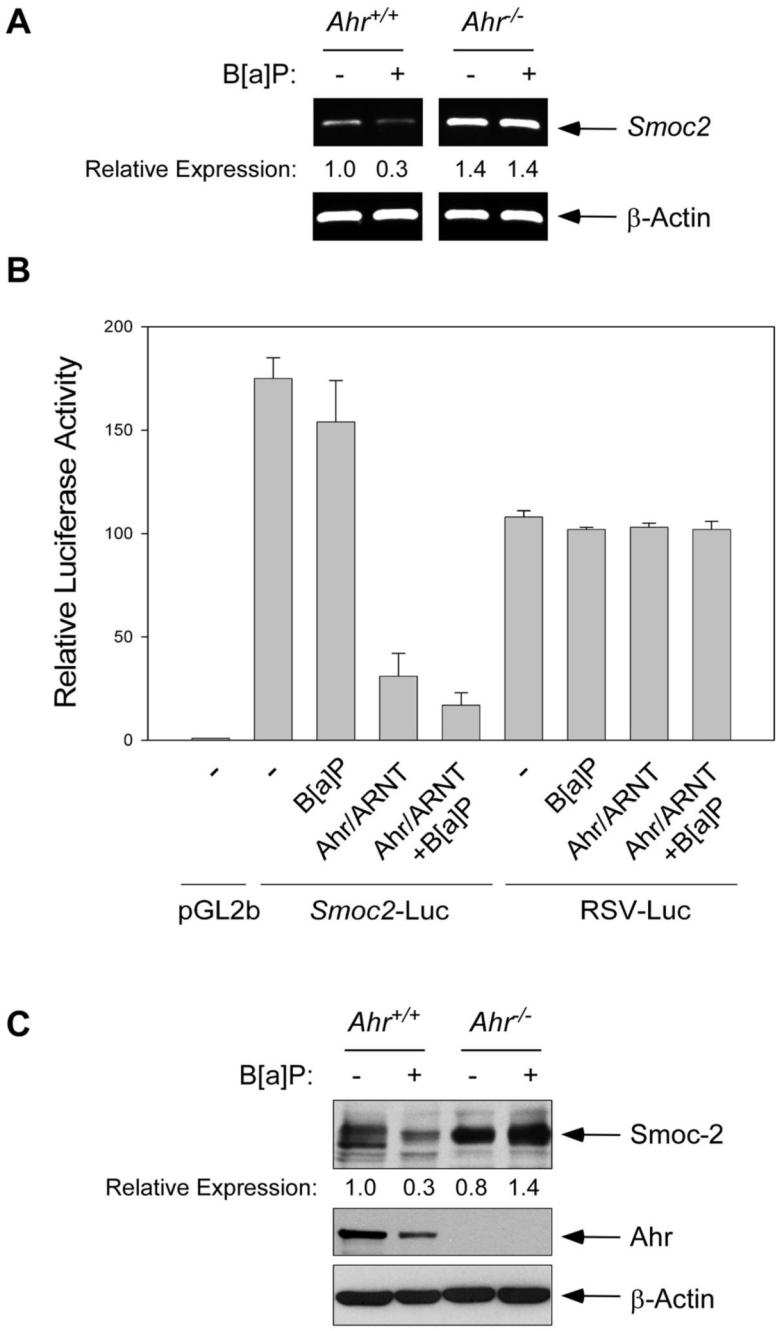

Fig. 3. Repression of *Smoc2* expression in B[a]P-treated cultured cells is Ahr-dependent

(A) Quiescent cultures of $Ahr^{+/+}$ and $Ahr^{-/-}$ fibroblasts were treated with 1 μM B[a]P. After 24 hr, total RNA was extracted and analyzed by RT-PCR using primers specific for *Smoc2* or β-Actin.

(B) $Ahr^{-/-}$ cells were transiently transfected with *Smoc2*-luciferase, pGl2b, RSV-Luc and CMV-Ahr + CMV-ARNT. 24 hr post-transfection, some cultures were treated with 1 μM B [a]P (or received DMSO for controls) and incubated for an additional 24 hr prior to harvest for luciferase assays.

(C) Quiescent $Ahr^{+/+}$ and $Ahr^{-/-}$ fibroblasts were treated with 1 μM B[a]P for 48 hr. Protein extracts were analyzed by SDS-PAGE and immunoblotting using antibodies against SMOC-2, Ahr, and β-Actin.

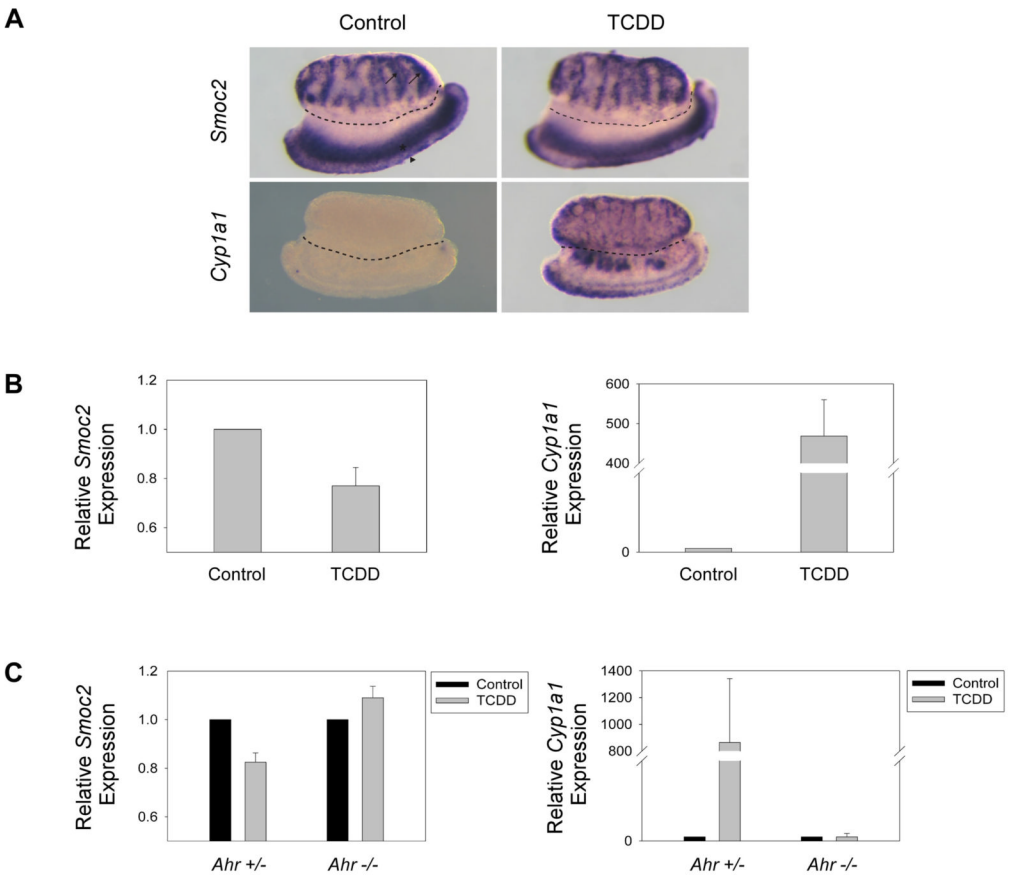

Fig. 4. *Smoc2* expression is downregulated in response to TCDD in testis/mesonephros complexes in an *Ahr*-dependent manner

(A) WISH analysis of *Smoc2* and *Cyp1a1* expression in gonad/mesonephros complexes treated with 1μM TCDD for 24 hours. Gonads are above the dotted line, mesonephroi are below. arrow=interstitium, arrowhead=Wolffian duct, asterisk=Mullerian duct.

(B) Real time RT-PCR analysis of *Smoc2* (left) and *Cyp1a1* (right) expression in wild-type gonad/mesonephros complexes treated with 1μM TCDD for 24 hours.

(C) Real time RT-PCR analysis of *Smoc2* (left) and *Cyp1a1* (right) expression in $Ahr^{+/-}$ and $Ahr^{-/-}$testis/mesonephros complexes treated with 1μM TCDD for 24 hours.