AHR Regulates WT1 Genetic Programming during Murine Nephrogenesis

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Mounting evidence suggests that the blueprint of chronic renal disease is established during early development by environmental cues that dictate alterations in differentiation programming. Here we show that aryl hydrocarbon receptor (AHR), a ligand-activated basic helix-loop-helix-PAS homology domain transcription factor, disrupts murine renal differentiation by interfering with Wilms tumor suppressor gene (WT1) signaling in the developing kidney. Embryonic kidneys of C57BL/6J Ahr−/− mice at gestation d (GD) 14 showed reduced condensation in the nephrogenic zone and decreased numbers of differentiated structures compared with wild-type mice. These deficits correlated with increased expression of the (+) 17aa WT1 splice variant, decreased mRNA levels of Igf-1 rec., Wnt-4 and E-cadherin, and reduced levels of 52 kDa WT1 protein. AHR knockdown in wild-type embryonic kidney cells mimicked these alterations with notable increases in (+) 17aa WT1 mRNA, reduced levels of 52 kDa WT1 protein, and increased (+) 17aa 40-kDa protein. AHR downregulation also reduced Igf-1 rec., Wnt-4, secreted frizzled receptor binding protein-1 (sfrbp-1) and E-cadherin mRNAs. In the case of Igf-1 rec. and Wnt-4, genetic disruption was fully reversed upon restoration of cellular WT1 protein levels, confirming that functional interactions between AHR and WT1 represent a likely molecular target for renal developmental interference.

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or +KTS and –KTS alternative splice variants from splicing of exon 9. Biochemical and genetic evidence indicates that different Wt1 isoforms support different cellular functions, with ±KTS-regulating Wt1 DNA binding specificity (8). Changes in exon 5 splice variants also are associated with deficits in renal differentiation (9). Addition of 17 amino acids in exon 5 creates an mRNA isoform that regulates transactivation (10). N-terminal residues 1–182 encode a dimerization region implicated in the regulatory mechanism exerted by dominant negative mutants (11). The (+) KTS behaves as a transcription factor that regulates genes expressed during kidney development in vitro, including Igf-2, Pdgf, Egfr, Pax-2 and Wt1 itself, while the (+) KTS isoform regulates the splicing machinery of mammalian cells (12). Hammes (13) generated mouse strains that specifically lack the (+) KTS or (+) KTS Wt1 splice variants and showed that the (+) KTS isoform is important for maintenance of podocyte function. In fact, mice lacking the (+) KTS Wt1 variant are characterized by development of renal insufficiency with focal and segmental glomerular sclerosis and male-to-female sex reversal, similar to the human Frasier syndrome (14). Only preliminary results have been obtained regarding functional consequences of the presence or absence of the 17 amino acids encoded by exon 5 and containing a protein:protein interaction (15). At the cellular level, the balance between (+) exon 5 isoforms may be involved in regulation of proliferation, differentiation and apoptosis and prevention of tumor formation (16).

A functional link between AHR and WT1 was suggested by the previous finding that activation of AHR by benzo(a)pyrene, a hydrocarbon ligand of the receptor, induces the (+) KTS Wt1 isoform in cultured metanephric kidneys of Ahr+/– mice (5). Evidence is presented that AHR functions as a regulator of renal cell differentiation through mechanisms that involve changes in the relative abundance of WT1 splice variants and deregulation of downstream WT-1 signaling.

**MATERIALS AND METHODS**

**Animals/Metanephros Harvest**

C57Bl/6j wild type or Ahr knockout mice were purchased from Jackson Laboratory (Bar Harbor, MN, USA) and placed under standard housing conditions. The care, breeding and handling of animals were conducted in accord with NIH guidelines. At d 14 of gestation, mouse embryos were resected from C57Bl/6j wild-type or Ahr knockout pregnant dams; the developing kidneys were harvested and processed for further analysis in vivo as described before (5).

**Morphometric Analysis**

Embryonic kidneys were fixed by 4% paraformaldehyde. Hematoxylin and eosin (H&E) slides (5-μm sections) were examined by bright-field light microscopy. Computer-assisted analysis was performed on six to eight histological sections from embryonic developing kidneys of wild-type or Ahr−/− mice, respectively. Measurements were taken from serial sections of embryonic kidneys from three or more dams. The values shown represent the composite of serial sections with variance expressed as the difference between values for each kidney per mouse strain. Images were captured using an Olympus Vanox research microscope equipped with a 3-CCD Camera (Model Del-750) and analyzed with the “NIH-Image” v1.60 public domain program developed at the National Institutes of Health and available at http://zippy.nih.gov/pub/nih-image. The high quality of the sections used allowed for direct visualization and quantification of intermediate stages and also glomerulotubular structures of developing kidney throughout the entire length of the section.

**Cell Cultures/ siRNA Transfection**

The mK3 and mK4 cells were kindly provided by Stephen Potter (Children’s Hospital Medical Center, Cincinnati, OH, USA). Cells were seeded in 12-well plates and cultured Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. At approximately 50% confluence, siRNA duplexes were transfected into the cells with siPORT Lipid (Ambion, Austin, TX, USA). 6 μL of a 20 μmol/L stock solution of siRNA targets was transfected in each well to achieve a final concentration of 120 μmol/L. Total protein was harvested 36 to 48 h after transfection using M-PE Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA). The double stranded RNA (siRNA) used to target Ahr was: Sense: 5′-GGGCAAAGAG CUUUCUUGAAt-3′; and Antisense: 5′-UCAAAGAAGCUUUGGCCGt-3′. A silencing scrambled siRNA negative control (Ambion) was used in all experiments. In rescue experiments, mK4 cells were transfected with 2 μg of CB6″-Wt1 (−/−) plasmid encoding for 52 kDa Wt1 protein or empty vector 24 h after transfection with siRNA. After 48 h, protein and RNA were harvested for further analysis.

**Protein Harvesting**

Metanephroi were washed with PBS and homogenized in T-PER Tissue Extraction Reagent (Pierce Biotechnology). Total protein or diffractionated proteins (cytoplasmic and nuclear) were harvested using M-PER and NE-PER Extraction Reagents (Pierce Biotechnology), respectively, as described by the manufacturer. Protein concentration was measured by the method of Bradford. Cytoplasmic and nuclear fractions were separated using NE-PER (Pierce Biotechnology) as per manufacturer’s instructions with the addition of HALT protease inhibitor (Pierce Biotechnology). Protein concentrations were determined by Bradford using albumin standards created with the CERI and NER reagents.

**Western Analysis**

Protein samples were boiled for 2 min and applied to 4% to 12 % NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). Twenty micrograms of protein were loaded onto a 4% to 12% Bis-Tris gel (Invitrogen) and run in MOPS buffer under reduced conditions.
cytoplasmic protein HSP90

transcription factor Oct1 (SC-232) or the

membrane and probed for the nuclear

Proteins were transferred to a PVDF

oxidase conjugated secondary antibody.

were visualized with horseradish per-

Plymouth Meeting, PA, USA]. Signals

[BioMOL Research Laboratories Inc.,

Temecula, CA, USA] or Ahr antibody

H2-F6 [Chemicon International,

C19 [Santa Cruz Biotechnology] and

Secreted frizzled-related sequence

protein 1

Wilms tumor suppressor –KTS AGCTCAAAAGACACCAAAGGAG GGGCTTTTCACCTGTATGAG 125 bp 55ºC

Wilms tumor suppressor 17aa CCTGAGGACGCCCTACAGC C TGTGCCGTGGTTGCTCTGC 161 bp 55ºC

Wilms tumor suppressor +KTS AGCTAAGAACACACCAAGGAG GAAGGGCTTTTCACTTGTTTTAC 137 bp 55ºC

Wilms tumor suppressor –KTS AGCTAAAAGACACACCAAGGAG GGGCTTTTCACCTGTATGAG 125 bp 55ºC

After electrophoresis, proteins were

transferred to PVDF membranes and

probed with three different Wt1 antibo-

dies (Wt1 N-[180]: sc-846, Santa Cruz Biotechnology, Santa Cruz, CA, USA]; C19 [Santa Cruz Biotechnology] and H2-F6 [Chemicon International, Temecula, CA, USA] or Ahr antibody [BioMOL Research Laboratories Inc., Plymouth Meeting, PA, USA]). Signals were visualized with horseradish per-

oxidase conjugated secondary antibody. Proteins were transferred to a PVDF membrane and probed for the nuclear transcription factor Oct1 (SC-232) or the cytoplasmic protein HSP90a/β (SC-13119) (Santa Cruz Biotechnology). Signals were visualized with a correspond-

ing secondary antibody and chemiluminescence detection using ECL+ (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Membranes were developed using the enhanced chemiluminescent (SuperSignal West Dura) Western blotting system (Pierce Biotechnology). Three to five in-

dependent experiments were performed in all instances.

**RNA Isolation**

Total RNA was extracted from the cells using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to manufacturer’s specifications.

**RT-PCR**

Reverse transcription was performed with MuLV Reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and OligoDTs according to manu-

facturer’s specifications. PCR was per-

formed using DNA Taq Polymerase (Ap-

plied Biosystems) using specific primers and PCR thermoprofile sets for each

gene. Primer sequences and PCR condi-

tions for Cyp1a1 and E-cadherin were as published by Reiners et al. (17) and Hosono et al. (18). Primers used to detect specific sequences in the 5’ UTR region of Wt1 upstream of both AUGs are listed in Table 1. Owing to the high G-C rich content of the Wt1 5’ UTR region, PCR was performed using Advantage-GC 2 Polymerase (BD Biosciences, San Diego, CA, USA) according to manufacturer’s specifications. PCR reaction was carried out at 94°C for 3 min, 68°C for 30 s, and 72°C for 3 min for 35 cycles and the reaction incubated at 72°C for 5 min.

**Real-Time PCR Amplification**

Reverse transcription of RNA was car-

ried out using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA). Real-
time PCR amplification was performed using the iCycler Detection System (BIO-

RAD). For each run, 25 µL of 2 × SYBR Green Mix (BIO-RAD) and 300 nmol/L for both forward and reverse primers in a total volume of 50 µL were mixed. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, 50 cycles at 95°C for 15 s and 65°C for 1 min. All experiments were performed in triplicate. Primer sequences for mouse Wnt-4, Igf-1 rec. and sfrp-1 are also listed in Table 1. 18S rRNA was used as an internal control for all measurements. Quantification was performed using comparative (ΔCt) method as described by Peinnequin et al. (19).

**Statistical Analysis**

Statistical significance was determined by analysis of variance (ANOVA) fol-

lowed by LSD post hoc tests at the P < 0.05 level.

**RESULTS**

To examine the role of AHR during nephrogenesis in vivo, renal cell differentia-

tion was monitored in GD:14 embryos of Ahr+/+ and Ahr–/– mice. Renal blastema in the proliferation zone was less cond-

densed in developing kidneys from Ahr–/– embryos (Figure 1A). Morphomet-
Analysis revealed decreased numbers of glomeruli and tubuloepithelial structures in Ahr−/− mice after normalization by area, with equal numbers of comma and S-shaped bodies (Figure 1B). The embryonic kidneys of Ahr−/− mice expressed higher levels of the (+) 17aa Wt1 splice variant (Figure 1C), a change that correlated with lower levels of Igf-1 rec, Wnt-4 and E-cadherin mRNAs and reduced levels of 52 kDa Wt1 protein (Figures 1D, E). Immunological detection experiments established colocalization of the Wt1 and Ahr proteins in developing metanephric glomeruli, as well as in the adult glomeruli (Figures 2A–F).

A gene knockdown approach was employed to examine the role of AHR in genetic programming and MET in embryonic mK3 and mK4 cell lines, models of early uninduced and later-induced murine metanephric mesenchyme, respectively (20). The mK3 cells exhibited spindle-shaped morphologies with irregular cytoplasmic projections, while mK4 cells exhibited cobblestone morphologies and polygonal shapes (Figure 3A). The degree of cellular differentiation in these lines was monitored based on the expression of genes involved in MET. mK4 cells expressed higher levels of Igf-1 rec, Wnt-4 and E-cadherin mRNAs compared with mK3 cells, while transcript levels for Bf-2 and secreted sFrp-1 were higher in mK3 cells (Figure 3B). The pattern of Wt1 protein expression in these cell lines was striking, with the 52 kDa Wt1 protein isoform detected predominantly in mK4 cells; but higher levels of the two lower 40 kDa isoforms corresponding to the (+) exon 5 variants predominately the (+) 17 AA 40 kDa were noted in mK3 cells (Figure 3C). The specificity of the Wt1 40 kDa signal in Western blot experiments was verified using a H2-F6 Wt1 antibody that selectively recognizes the larger 52 and 62 kDa isoforms of Wt1 protein. The mK4 cells expressed higher cytoplasmic and nuclear levels of Ahr protein than mK3 cells (see Figure 3C), suggesting that expression of both Ahr and Wt1 proteins correlates with the degree of renal cell differentiation. The purity of fractions was confirmed using HSP90α/β and Oct1 as markers of cytoplasmic and nuclear identity, respectively (Figure 3D).

Ahr protein levels decreased by 50% to 70% in mK4 cells transfected with Ahr siRNA, and remained unchanged in cells treated with scrambled RNA (Figure 4A).
The efficiency of the Ahr knockdown was examined in mK4 cells treated for 16 h with benzo(a)pyrene, a hydrocarbon ligand of Ahr that activates Cyp1a1 via transcriptional mechanisms (21). Cyp1a1 mRNA levels increased two-fold in benzo(a)pyrene-treated cells, and knockdown of Ahr protein abolished this effect (Figure 4B, C). The influence of AHR on Wt1 mRNA was examined next by RT-PCR using a primer set that hybridizes to the 5′ UTR region upstream of both the 52 and 40 kDa isoform translation start sites, and that recognizes all major Wt1 variants. No alterations in total mRNA levels were observed following Ahr knockdown (Figure 5A). Next, the expression of Wt1 splice variants generated by both exon 5 and 9 splicing was examined by real time PCR. Four different set of primers were used to specifically recognize each of the splice variants including, (+) 17AA, (-) 17AA, (+) KTS, and (-) KTS. We conclude that the primer set used to amplify (+) 17AA only recognizes this splice variant and discriminates between (+) and (-) 17AA variants. Ahr knockdown caused a pronounced increase in the (+) 17aa splice variant in mK4 cells (Figure 5B), while other Wt1 splice variants did not change. A reduction of cellular Ahr protein levels caused a significant reduction in 52 kDa Wt1 protein levels, while the levels of (+) 17aa 40 kDa isoform increased which is consistent with the increases in the mRNA level for this Wt1 splice variant (Figure 5C). These finding were consistent with morphological shifts of mK4 cells toward the lesser differentiated mK3 phenotype (not shown). Together, these data implicate Ahr in posttranscriptional regulation of Wt1 in embryonic kidney cells.

To determine if posttranscriptional deregulation of Wt1 by AHR is of functional consequence during the course of renal cell differentiation, the expression of downstream effector genes in nephrogenesis was examined by RT-PCR. Marked decreases in Igfr-1 rec., Wnt-4 and sFrp-1 were observed following Ahr knockdown in mK4 cells (Figure 6). To confirm the role of WT1 in the regulation of the downstream targets, siRNA degradation of WT1 was examined. siRNA-induced degradation of WT1 downregulated markers of epithelial identity (Igf-1 rec, Igf-2 rec, Wnt-4 and E-cadherin), and either enhanced markers of mesenchymal identity such as srp1 (Figure 7A). No changes in Lhx1, a marker of renal vesicle and nephron progenitors, were observed with this treatment. Importantly, Wt1 silencing downregulated direct targets of Wt1 transcriptional control, confirming the usefulness of these markers to examine the differentiation state of developing kidneys (Figure 7B). To determine if these genes are regulated as a direct result of AHR-mediated interference with Wt1, mK4 cells were transfected for 24 h with CB6-Wt1, a plasmid encoding (+) 17aa / (-) KTS 52 kDa Wt1 protein, or empty vector, after downregulation of Ahr. Reduced Wt1 protein levels following Ahr knockdown were restored in mK4 cells transfected with Wt1 cDNA compared...
with empty vector, as determined by Western blot analysis (Figure 7C). Importantly, decreased Igf-1 rec and Wnt-4 mRNA levels following Ahr knockdown were restored by the Wt1 cDNA, indicating that deregulation of these targets lies downstream of Ahr and is mediated by posttranscriptional deregulation of Wt1. Wt1 did not restore sfrp-1 mRNA levels, indicating that regulation of this target is not directly mediated by Ahr:Wt1 interactions, at least under the experimental conditions examined (Figure 7D).

**DISCUSSION**

A developmentally-regulated pattern of Ahr expression in the kidney was first reported by Abbott et al. (22) who demonstrated that levels of Ahr protein are significantly higher in renal epithelial cells compared with mesenchymal cells, and downregulated by Ahr ligands. Bryant et al. (23) later showed that Ahr mRNA increases in metanephric tubules from GD:12–14, with continued expression throughout the life of the mouse. A casual link between Ahr and nephrogenesis was established in experiments showing that renal development is delayed in metanephric organ cultures of embryonic kidneys from Ahr–/– mice, or following ligand-induced downregulation of Ahr protein (5). Here we show that Ahr is involved in the regulation of developmentally-specific patterns of gene expression during MET in the kidney. Specifically, evidence is presented that: a) Ahr–/– embryos exhibit delayed epithelialization compared with wild-type counterparts; b) AHR and WT1 protein levels correlate with the degree of cellular differentiation in mK3 and mK4 cell lines; c) downregulation of Ahr protein disrupts gene expression profiles and differentiation programs in embryonic kidney cells lines.

The essential role of Wt1 in kidney development was unequivocally established in studies showing that homozygous Wt1 knockout mice fail to develop metanephric kidneys and die in utero (7).
In homozygous Wt1 knockout mice, the ureteric bud fails to grow out of the mesonephric duct and metanephric mesenchyme dies. Wt1 is expressed selectively in metanephric blastema, S-shaped bodies and glomerular epithelium during kidney development, and confined to the podocyte layer of the mature nephrons (24). Wt1 is mutated in a proportion of nephroblastomas, embryonic kidney tumors characterized histologically by incomplete epithelialization of the condensing renal mesenchyme. The evidence presented here indicates that Ahr plays a regulatory role during nephrogenesis and that this function is linked to regulation of the Wt1 signaling. The observed reduction in numbers of glomeruli in Ahr null mice may be explained by developmental delay and/or direct interference with metanephric epithelialization in Ahr null mice. It is interesting to note that although the molecular and cellular phenotypes elicited by BaP in the developing kidney are largely mediated by proteasome-mediated downregulation of AHR (25), BaP arrested metanephric differentiation at an earlier point than AHR null mice. These settled differences likely reflect the complexity of the biological response since disruption of metanephric differentiation by the carcinogen is known to involve AHR-dependent reactivation of repetitive sequences within the embryonic kidney genome (26,27).

Recent studies in this laboratory have shown that a coisogenic mouse strain expressing a functionally inactive D2N AhrD allele exhibits a 0.5- to 1-d delay in nephrogenesis compared with wild-type mice (25). Of note is that subtle renal deficits in renal cell differentiation associated with genetic loss of Ahr do not involve gross deficits in renal function, and only translate into reduced renal reserve capacity in Ahr null mice. Given that AhrD−/− embryos are viable at term, and do not exhibit overt deficits of renal structure, the pathogenetic consequences of delayed nephrogenesis seen in the absence of Ahr protein are likely coupled to deficits in renal function as mice continue to develop. Of note are reports showing that Ahr−/− mice exhibit cardiac hyperplasia and hypertrophy (28); pathobiological processes involving altered Wt1 functions.

RNA interference provided a complementary approach to gene ablation in vivo for the study of regulatory interactions between Ahr and Wt1 during the course of nephrogenesis. These experiments helped to differentiate renal developmental deficits that are associated directly with loss of Ahr protein from changes that are secondary to long-term adaptation. The efficiency of the siRNA knockdown approach was confirmed by the loss of Cyp1a1 inducibility in response to AHR activation by a hydrocarbon ligand in siRNA-treated cells. The downregulation of Ahr by siRNA altered the expression of (+) 17aa isoform in mK4 cells, suggesting that regulation of Wt1 by Ahr is mediated at the posttranscriptional level. This interpretation is in keeping with the finding that interference with Ahr signaling was without effect on total Wt1 mRNA levels. It should be noted that reciprocal interactions between Wt1 and Ahr may not be directly linked to phenotypic outcomes, and instead, impact nephrogenesis indirectly through the regulation of differentiation status. Interestingly, Ahr knockdown in mK3 cells, a line that exhibits a lesser degree of metanephric differentiation, did not affect the ratio of (±) exon 5 splice variants, and instead caused a shift in KTS splice variants toward the (−) KTS phenotype (not shown). This finding is consistent with previous observations that downregulation of Ahr protein following extended hydrocarbon ligand treatment in metanephric cultures shifted the (±) KTS ratio toward the (−) KTS phenotype in an Ahr-dependent manner (5).

The loss of Ahr protein in vivo correlated with decreased levels of 52 kDa Wt1 protein. Furthermore, expression of the
40 kDa Wt1 isoforms was correlated inversely with the degree of renal cell differentiation. A mechanistic link between Wt1 isoforms and Ahr is consistent with the finding that downregulation of Ahr protein in mK4 cells shifted the pattern of Wt1 expression from the mature 52 kDa isoform to the lower molecular weight isoforms. These findings suggest that these Wt1 isoforms support different cellular functions, an interpretation that is consistent with shifts in Wt1 protein expression profiles in mK3 cells compared with mK4 cells. Whether regulation of Wt1 splicing is a result of direct interactions of Ahr with the cellular splicing machinery, or involves exon skipping, or changes in mRNA stability is not known.

The relevance of functional Ahr/Wt1 interactions was monitored based on the expression of Bf-2, srrp-1, Igf-1rec, and Wnt-4. These genes play essential roles during MET in nephrogenesis, with Igf-1 rec, Wnt-4 and E-cadherin recognized as downstream targets of Wt1 during the course of renal cell differentiation and nephrogenesis (29,30). A role for Igf-1 rec in renal cell differentiation is well documented, with ubiquitous expression found to be developmentally regulated during metanephric maturation, and enhanced during tubulogenesis (31). Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney, and is expressed in pre-tubular mesenchymal cells shortly before aggregation and transformation into epithelial tubules (32). Wnt signaling is initiated by interaction of the Wnt protein with frizzled receptors (33). In addition to the membrane-bound forms of frizzled, several genes encoding soluble homologous proteins have been identified (34). These secreted frizzled-related proteins bind Wnt in the developing metanephros and function as modulators of Wnt signaling. E-cadherin is recognized as a Wt1 target gene (18), and a regulator of the epithelial phenotype. In keeping with their unique functions during the course of renal cell differentiation, mK4 cells were found to express higher levels of Igf-1 rec, Wnt-4 and E-cadherin than the lesser differentiated mK3 cells. In contrast, the expression of Bf-2 and sFrp-1 was higher in mK3 than mK4 cells. Both AHR and Wt1 increased as a function of renal cell differentiation status in vitro and in vivo, suggesting that these proteins are functionally linked in the regulation of MET.

AHR functions as a transregulator of gene expression during the course of mammalian cell differentiation (35). A role for AHR in posttranscriptional control of gene expression has not been recognized previously. Thus, the influence of AHR on developmentally-regulated expression of Wt1 splice variants during renal differentiation is intriguing. The relevance of these findings is emphasized by reports that differential splicing of exon 5 occurs in human kidneys and in Wilms tumor samples (9). While the ±KTS sequence is conserved throughout the species, and necessary for renal development, exon 5 is only present in placental mammals and is not required for nephrogenesis, implantation or lactation (36). Evidence for participation of exon 5 in transcriptional repression or Par4 binding suggests a possible role of this variant in the regulation of Wt1 expression in differentiated cells such as podocytes (37,15). Thus, future studies should investigate the role of AHR and exon 5 variants of Wt1 in the regulation of renal cell differentiation.

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involvement of AHR in the regulation of murine nephrogenesis raises important questions about its role in human kidney development and the linkage between prenatal exposures to AHR ligands and deficits in renal developmental program-

Figure 6. Ahr plays a regulatory role in programming of MET and differentiation of mK4 cells. Real time PCR was performed using specific primers for various molecular targets to determine the expression of effector genes essential in nephrogenesis. Ahr knockdown in mK4 caused a significant decrease in mRNA levels for Igf-1 rec. (A), Wnt-4 (B), and sfrp-1 (C) (*P < 0.05). One representative experiment out of three is shown. NT = no treatment; Scr = scrambled.

Figure 7. Interactions between Ahr and Wt1 during the course of renal cell differentiation. (A) Downregulation of markers of differentiation by Wt1 siRNA. mK4 cells were cultured in the presence of Wt1 siRNA for 3 d. qRT-PCR values were calculated to represent fold change normalized to 18S compared with nonspecific siRNA control. sfrp-1 and lim 1 homeobox gene were used as markers of mesenchymal identity, while Igf-1 rec, Igf-2 rec, Wnt4, and E-cadherin were used as markers of epithelial identity. (B) Derepression of Wt1 targets by Wt1 siRNA. mK4 cells were cultured in the presence of Wt1 siRNA for 3 d. qRT-PCR values were calculated to represent fold change normalized to 18S compared with nonspecific siRNA control. Syndecan1, paired box protein 2, EGF rec, retinoic acid receptor alpha, taurine transporter, and Wilms tumor transcription factor. (C) The mK4 cells were transfected for 24 h with the CB6-Wt1 (-) exon 5 (-) exon 9 plasmid encoding for 52 kDa Wt1 protein or empty vector after transfection with Ahr siRNA. Reduced Wt1 protein levels following Ahr knockdown were rescued in cells transfected with Wt1 cDNA compared with empty vector. (D) Real time PCR shows decreased mRNA levels for Igf-1 rec. and Wnt-4 following Ahr knockdown and effective rescue in cells transfected with Wt1 cDNA; however, decreases in sFrp-1 mRNA following Ahr knockdown were not restored in cells transfected with Wt1 cDNA. One representative experiment out of three is shown. NT = no treatment.

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DISCLOSURE
The authors declare that they have no competing interests as defined by Molecu-
lar Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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