The aryl hydrocarbon receptor inhibits prostate carcinogenesis in TRAMP mice

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The aryl hydrocarbon receptor (AhR) is a transcription factor that mediates the inhibitory effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on prostate growth and also modulates normal prostate development. This is evidenced by AhR null mice (Ahr−/−) having smaller dorsolateral and anterior prostates, even though all prostate lobes remain histologically normal. To test the hypothesis that loss of the AhR increases the rate of prostate carcinogenesis, the incidence of macroscopic prostate tumors was determined in Ahr+/+, Ahr+/− and Ahr−/− C57BL/6J transgenic adenocarcinoma of the mouse prostate (TRAMP) mice at 35, 70, 105, 140, 175 and 210 days of age. From 140 days, prostate tumor incidence was greater in Ahr+/− (60%) and Ahr+/− (43%) mice than in Ahr+/+ mice (16%). Allele quantification did not indicate a loss of the wild-type Ahr allele in heterozygous TRAMP tumors, suggesting that tumor formation in these mice was not due to a loss of Ahr heterozygosity. Prostatic SV40 large T antigen mRNA expression and protein localization were comparable in TRAMP mice of each Ahr genotype. Prostates from all mice of each Ahr genotype were histologically indistinguishable, exhibiting diffuse epithelial hyperplasia by 105 days of age. mRNA expression and protein localization for molecular markers of neuroendocrine differentiation, including chromogranin A and neuropilin-1, were elevated in prostate tumors compared to tumor-free ventral prostates, regardless of Ahr genotype or age. Taken together, these results demonstrate that the Ahr inhibits prostate carcinogenesis in C57BL/6J TRAMP mice by interfering with neuroendocrine differentiation.

Introduction

Male reproductive tract development is disrupted by in utero and lactational exposure to the aryl hydrocarbon receptor (AhR) agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 1,2). Ventral, dorsolateral and anterior prostate development in the mouse is inhibited in a lobe-dependent manner by in utero and lactational TCDD exposure (3–8). This response results from lobe-specific inhibition of both prenatal epithelial bud formation from the prostate anlage, the urogenital sinus (9) and postnatal ductal branching morphogenesis (8).

TCDD binds to the AhR, a basic helix-loop-helix/Per-Arnt-Sim ligand-activated transcription factor that is bound in the cytoplasm to two 90 kDa heat-shock proteins (HSP) and one AhR interacting protein (10,11). The ligand/AhR complex translocates to the nucleus where the chaperone proteins dissociate and the ligand-bound AhR dimerizes with the AhR nuclear translocator (ARNT) to enhance transcription of genes containing dioxin response elements. AhR and ARNT proteins have been identified in ventral and dorsolateral lobes of the rat prostate, and cytochrome P4501A1 is induced in both lobes in response to TCDD exposure (12).

The availability of mice lacking the AhR (Ahr−/−) has permitted AhR-mediated effects of TCDD to be identified and the role of the AhR in normal development to be determined (13–15). In AhR null mice, reduction in ventral, dorsolateral and anterior prostate weights caused by in utero and lactational TCDD exposure were AhR-dependent (6). Dorsolateral and anterior prostate weights in untreated AhR null mice were reduced compared with their wild-type littermates at various ages, suggesting that the AhR, in the absence of TCDD, is involved in normal development of these two prostate lobes. Serum testosterone concentrations were not altered in AhR null mice, and slight reductions in serum 5α-androstane-3α,17β-diol concentrations were unlikely responsible for the reduced dorsolateral and anterior prostate weights (6). Furthermore, prostate histology, androgen receptor (AR) mRNA levels, and androgen-dependent gene expression were not altered in AhR null mice, suggesting adequate androgen action (6). Impaired growth of the developing prostate by TCDD activation of AhR, or by absence of AhR from AhR null mice, raised the possibility that the AhR may regulate prostate carcinogenesis later, particularly if "reawakening" of early prostate growth regulatory signals is involved (16).

There have been a limited number of investigations on the potential role of AhR signaling in prostate cancer. Western blot analysis identified the AhR in epithelial and stromal cells of human fetal, benign hyperplastic and malignant prostate (17), suggesting that this organ could be a target of a TCDD response in men. Recent studies have demonstrated that Vietnam veterans exposed to TCDD-contaminated Agent Orange had a greater risk of developing prostate cancer (18), although some earlier studies failed to identify an association between TCDD exposure and prostate cancer risk (19). Recently, we found that in utero and lactational TCDD exposure increases the incidence of pre-cancerous prostatic lesions in senescent C57BL/6J mice, a mouse strain not naturally susceptible to prostate cancer (20). Such results raise the possibility that AhR activation by TCDD may increase prostate cancer risk, particularly in humans and animal models susceptible to prostate carcinogenesis.

Abbreviations: AhR, arylhydrocarbon receptor; AR, androgen receptor; ARNT, AhR nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
However, the role of AhR in prostate carcinogenesis in the absence of exogenous ligand remains to be determined. Development of prostate cancer in the TRAMP model provides an opportunity to investigate effects of Ahr genotype on distinct stages of mouse prostate carcinogenesis (21–25). The TRAMP model utilizes the rat probasin gene promoter to drive expression of simian virus 40 (SV40) large T and small t antigens. Transgene expression under control of the androgen-dependent probasin promoter is specific to prostatic epithelium and is hormonally and developmentally regulated. TRAMP mice express the T antigen oncoprotein by 8 weeks of age and develop distinct pathology in the prostate epithelium by 10 weeks of age. Advanced stages of tumor development are characteristically AR-negative and have greater localization for markers of neuroendocrine tumors (24). In the present study, we investigate the effect of Ahr genotype on prostate carcinogenesis in TRAMP mice. We report that prostate tumors develop with greater frequency in TRAMP mice lacking both (Ahr−/−) or one (Ahr+/−) Ahr allele compared with wild-type mice (Ahr+/+). Furthermore, we demonstrate that the tumors present in TRAMP mice of each Ahr genotype have evidence of a neuroendocrine phenotype, regardless of the age they first appear. These findings suggest that the Ahr inhibits onset of neuroendocrine prostate tumors in a gene–dosage-dependent manner in the TRAMP model.

Quantification of wild-type Ahr allele in heterozygous TRAMP prostate tumors
To determine if tumors in heterozygous TRAMP mice were due to loss of heterozygosity, specifically resulting from loss of the wild-type Ahr allele, prostate tumors from Ahr+/+, Ahr++ and Ahr−/− mice were processed as previously described for ear punch samples (26). Quantitative analysis of wild-type and knock out allele was determined by real-time (LightCycler) PCR. PCR reactions (6) using Ahr (AGTGAAGCCCATCCCCGGCT and ATCAAGAAGCCTTGCCGC, respectively) and neomycin for Ahr null (TTGGGTGGAGGGCATTTCG and AGGTGATGACAGGATGC, respectively), primers were denatured at 94°C for 30 s, then amplified for 40 three-step cycles (94°C for 0 s hold melting, 65°C annealing for 5 s, and 72°C for 10 s extension). Double-stranded fluorescent product was detected at the end of each cycle and abundance of each allele was derived from the respective crossing point using LightCycler software. Product specificity was determined by comparison of melting curves, and the ratio of wild-type Ahr allele relative to Ahr null allele was calculated for each sample.

Materials and methods

**Transgenic mice**
Animal care and use were in accordance with University of Wisconsin-Madison Research Animal Care and Use Committee guidelines and the NIH Guide for the Care and Use of Laboratory Animals. C57Bl/6-Tg(TRAMP)8247Ng/J mice (Stock number 003135) obtained from Dr. Christopher Bradfield (Department of Oncology, University of Wisconsin, Madison, WI) were backcrossed to C57BL/6J mice at Jackson Labs (Bar Harbor, ME) for more than 20 generations. Ahr null mice (Ahr−/−), obtained from Dr Christopher Bradfield (Department of Oncology, University of Wisconsin, Madison, WI), were backcrossed to C57BL/6J mice for more than 15 generations. Mice were housed in rooms maintained at 24 ± 1°C with a 12 h light–dark cycle. Feed (5015 Mouse Diet, PMI Nutrition International, Brentwood, MO) and tap water were available ad libitum.

Female TRAMP mice heterozygous for the probasin-driven SV40 T antigen were bred with C57BL/6J Ahr−/− males to prepare breeders necessary to generate experimental animals. Adult heterozygous F1 offspring (Ahr+/−) were interbred to obtain Ahr−/−, Ahr+/−, and Ahr+/+ mice. Experimental males that were TRAMP−/− and either Ahr−/−, Ahr+/−, or Ahr+/+ were generated by mating Ahr−/− × TRAMP−/− males with Ahr+/−, Ahr+/− × TRAMP−/− females.

**Ahr genotype was determined by PCR analysis of ear punch DNA from (26). TRAMP genotyping measured transgene DNA by quantitative real-time LightCycler (Roche Molecular Biochemicals, Indianapolis, IN) PCR using primers described previously (21) and cytokertatin 8 primers as the loading control (6).**

**Tissue preparation and histology**
TRAMP mice of each Ahr genotype were necropsied at 35, 70, 105, 140, 175 and 210 days of age to determine the incidence of macroscopic prostate tumors. Where tumor burden required euthanasia prior to scheduled termination, mice were included in the predetermined age group for analysis. A macroscopic tumor was characterized as any mass visible at the time of necropsy, and confirmed using a dissecting microscope, if necessary. To minimize genetic diversity, littermates of each Ahr genotype on each day were necropsied. Where tumor burden required euthanasia prior to scheduled termination, mice were included in the predetermined age group for analysis. A macroscopic tumor was characterized as any mass visible at the time of necropsy, and confirmed using a dissecting microscope, if necessary. To minimize genetic diversity, littermates of each Ahr genotype on each day were necropsied. To determine if tumors in heterozygous TRAMP mice were due to loss of heterozygosity, specifically resulting from loss of the wild-type Ahr allele, prostate tumors from Ahr+/+, Ahr++ and Ahr−/− mice were processed as previously described for ear punch samples (26). Quantitative analysis of wild-type and knock out allele was determined by real-time (LightCycler) PCR. PCR reactions (6) using Ahr (AGTGAAGCCCATCCCCGGCT and ATCAAGAAGCCTTGCCGC, respectively) and neomycin for Ahr null (TTGGGTGGAGGGCATTTCG and AGGTGATGACAGGATGC, respectively), primers were denatured at 94°C for 30 s, then amplified for 40 three-step cycles (94°C for 0 s hold melting, 65°C annealing for 5 s, and 72°C for 10 s extension). Double-stranded fluorescent product was detected at the end of each cycle and abundance of each allele was derived from the respective crossing point using LightCycler software. Product specificity was determined by comparison of melting curves, and the ratio of wild-type Ahr allele relative to Ahr null allele was calculated for each sample.

**Immunohistochemistry**
Large T antigen immunohistochemistry was carried out on deparaffinized and rehydrated tissue sections (5 μm). Boiling citric acid antigen retrieval preceded peroxide quenching of endogenous peroxidase activity. Mouse monoclonal SV40 large T antigen (Santa Cruz Biotechnology, Santa Cruz, CA) stained utilizing the M.O.M. kit (Vector Laboratories, Burlingame, CA). Chromogranin A (Zymed, San Francisco, CA) non-specific binding was blocked with non-immunized goat serum. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) preceded streptavidin-peroxidase incubation (Dako). Primary antibodies were incubated overnight at 4°C in a humidity chamber, and color developed using NOVA-Red chromagen (Vector Laboratories). Slides were photographed using a Nikon DS5 digital camera mounted on a Nikon Eclipse TE300 Microscope.

**Real-time reverse transcription PCR mRNA quantification**
MRNA expression was determined for dorsolateral prostate, ventral prostate, and prostate tumors collected during the time-course study. Chromogranin A and neuropilin-1 mRNA identified neuroendocrine differentiation and SV40 T antigen mRNA was determined to quantify transgene expression. PCR reagents and conditions were carried out as described for Ahr allele quantification, utilizing cDNA (6) and the following primer pairs: cyclophilin: ATACCCGCGATGACGCC, TCTCTCTGGATGAGACCT- CTCG, SV40 T antigen: AGAGCAAGGTGGCAGTG, TGGAGCTGT-GAATAATGCTG; chromogranin A: TTCAAGGCGCACTTGCG, AAT- AGTCAGAGTTCTCAGGC; neuropilin-1: TGGTTCTACGTGAACTCGG, TGAAGATGGCTACAGTGTGC, AR: AATCTGGATGTTGAGAGCCAGAG, AGAGAACAGACACTAGCAG. Serially-diluted standards of known concentrations (6) and unknown cDNA samples were amplified simultaneously using the same reaction mixture. mRNA abundance for each gene of interest was expressed relative to that of cyclophilin.

**Western blot analysis**
Frozen prostate or prostate tumors were homogenized in lysis buffer containing 0.1% Triton X-100 and protease inhibitors using a Tissumizer homogenizer (Tekmar, Cincinnati, OH). Protein concentrations were determined using the Pierce BCA kit (South San Francisco, CA). The extracts were boiled in sample buffer containing 10% β-mercaptoethanol and 10% SDS and 40 μg of protein from each sample was separated on SDS polyacrylamide gels. Proteins were transferred to a hybond membrane (Amer sham Biosciences, Piscataway, NJ) on a semi-drying blotting unit (Fisher Scientific, Pittsburgh, PA). Non-specific antibody binding was blocked by 5% non-fat dry milk in TBST. Antibodies for AR (Santa Cruz Biotechno logy, Inc, Santa Cruz, CA diluted 1:5000) and β-actin (Cell Signaling Technology, Beverly, MA) in TBST containing 3% non-fat dry milk were incubated overnight at 4°C. Membranes were washed with TBST prior to incubation with HRP-conjugated secondary antibody (Zymed, 1:1500) in blocking buffer. Membranes were washed with TBST, and bands detected using hyperfilm exposed to chemiluminescent substrate (Pierce). Band intensity was determined using ImageQuant TL software (Amer sham Biosciences). AR protein levels were expressed relative to β-actin protein levels for each sample.

**Statistical Analysis**
Statistical analysis was conducted with the litter as the experimental unit. Analysis of variance (ANOVA) was conducted on parametric data that passed Levene’s test for homogeneity of variance and were normally distributed. If a significant effect was found, the least significant difference test was used to determine which group(s) differed from the appropriate
**Effect of AhR on prostate carcinogenesis**

Microscopic analysis of tissue sections was utilized to more precisely characterize prostate histology at 105, 140 and 175 days of age in Ahr<sup>+/+</sup>, Ahr<sup>+</sup>− and Ahr<sup>-/-</sup> TRAMP mouse prostate histology. No regions of prostatic intraepithelial neoplasia were observed in TRAMP prostate at any age. However, at 105, 140 and 175 days of age, prostates from mice of all Ahr genotypes had diffuse epithelial hyperplasia characteristic of the TRAMP model, even Ahr<sup>+/+</sup> mice that did not typically develop macroscopic tumors. There were no differences in severity of the hyperplasia in the prostate among the three Ahr genotypes when no macroscopic tumors were present.

**Analysis of Ahr levels in Ahr<sup>+/+</sup> TRAMP tumors**

To determine if greater protection against prostate carcinogenesis in Ahr<sup>+/+</sup> than in Ahr<sup>+</sup>− TRAMP mice was due to either a gene–dosage effect or due to loss of Ahr heterozygosity, specifically loss of the remaining wild-type allele, we assessed wild-type Ahr allele levels relative to null allele levels in TRAMP prostates and prostate tumors. The relative level of the Ahr wild-type allele in Ahr<sup>−/-</sup> TRAMP tumors was 50% of Ahr<sup>+/+</sup> mouse prostates, indicating that they had not lost the Ahr allele (results not shown). This suggests that prostate tumors forming at a higher frequency in Ahr<sup>−/-</sup> TRAMP mice compared with Ahr<sup>+/+</sup> TRAMP mice were not due to loss of Ahr heterozygosity. Thus, inhibition of prostate carcinogenesis reflected a gene–dosage effect of the Ahr.

**Large T antigen expression in Ahr<sup>+/+</sup>, Ahr<sup>+</sup>− and Ahr<sup>-/-</sup> TRAMP mice**

Although a similar degree of diffuse hyperplasia was present in all TRAMP mice irrespective of Ahr genotype, it was necessary to exclude the possibility that greater tumor formation in Ahr<sup>+</sup>− and Ahr<sup>-/-</sup> TRAMP mice was caused by greater transgene expression. Large T antigen protein, detected by immunohistochemistry, was identified in nuclei of prostate epithelial cells lining glands with and without tumors (Figure 2A). Abundant large T antigen localization was also observed in small nodules, large tumors, and lymph node metastases (results not shown). Localization was similar in prostates of all Ahr genotypes at all stages of tumor development. Also, quantitative analysis of SV40 large T antigen mRNA abundance by RT–PCR did not indicate greater expression in dorsolateral prostates of Ahr<sup>+</sup>− and Ahr<sup>-/-</sup> TRAMP mice compared with Ahr<sup>+/+</sup> TRAMP mice at any age (Figure 2B). Taken together, these findings demonstrate that loss of AhR in Ahr<sup>+</sup>− and Ahr<sup>-/-</sup> TRAMP mice did not exacerbate prostatic SV40 large T antigen expression, or subsequent development of epithelial hyperplasia. Rather, it appears that greater prostate tumor incidence in mice lacking one or more Ahr allele is independent of these processes.

**Chromogranin A and neuropilin-1 mRNA expression: markers of neuroendocrine differentiation in prostate tumors**

To further characterize the prostate tumors in Ahr<sup>+/+</sup>, Ahr<sup>+</sup>− and Ahr<sup>-/-</sup> C57BL/6J TRAMP mice, we utilized real-time RT–PCR to quantitatively analyze mRNA expression of two genes indicative of neuroendocrine differentiation, chromogranin A and neuropilin-1, at 35-day intervals. Tumor-free ventral prostates had low chromogranin A mRNA expression at all ages investigated (Figure 3A). Chromogranin...
A expression was not altered over time in $Ahr^{+/+}$ or $Ahr^{-/-}$ C57BL/6J TRAMP mice when no tumors were present, but expression was greater in $Ahr^{-/-}$ C57BL/6J TRAMP mice at 105 than 35 days of age. Chromogranin A expression was not significantly different in $Ahr^{+/+}$, $Ahr^{-/-}$ and $Ahr^{-/-}$ C57BL/6J TRAMP ventral prostates without tumors at 35, 70 or 140 days of age. However, expression was significantly greater in $Ahr^{-/-}$ than in $Ahr^{+/+}$ C57BL/6J TRAMP mice at 105 days of age. For each $Ahr$ genotype, chromogranin A expression was significantly greater in prostate tumors than in ventral prostates without tumors at both 105 and 140 days of age. Gene expression in prostate tumors did not differ between 105 and 140 days of age, and was not altered as a function of $Ahr$ genotype. Thus, chromogranin A mRNA expression was elevated in prostate tumors compared with tumor-free ventral prostates, with the only exception being $Ahr^{-/-}$ TRAMP mice at 105 days of age which were not significantly altered.

Immunohistochemical localization of the neuroendocrine differentiation marker, chromogranin A, in $Ahr^{+/+}$, $Ahr^{-/-}$ and $Ahr^{-/-}$ TRAMP mice

Chromogranin A localization was investigated in paraffin-embedded prostate sections taken from 105, 140 and 175-day-old $Ahr^{+/+}$, $Ahr^{-/-}$ and $Ahr^{-/-}$ C57BL/6J TRAMP mice using standard immunohistochemical techniques. Figure 4A shows hematoxylin and eosin staining of a representative prostate section with diffuse hyperplasia. In the same tissue, chromogranin A immunostaining was not detected (Figure 4B). Chromogranin A positive cells were only rarely detected in prostates without tumors, regardless of $Ahr$ genotype.

Figure 4C shows a representative macroscopic prostate tumor with predominant absence of glandular structure and only a few glands maintaining glandular structure (arrows). Abundant chromogranin A staining was observed throughout the prostate tumor (Figure 4D, arrow heads). However, no staining was observed in tumor-free regions retaining glandular architecture (arrows). Chromogranin A staining was reflective of tumor morphology and independent of both mouse age and $Ahr$ genotype, as abundant staining was
present in all Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} C57BL/6J TRAMP mouse prostate tumors.

As observed for chromogranin A, immunohistochemical localization of neuroepithelial 1 was also detected in prostate tumors but not tumor-free prostates (results not shown). Thus, increased expression of neuroendocrine markers is confined to prostate tumors. This suggests that the Ahr regulates onset of neuroendocrine tumors in C57BL/6J TRAMP mice.

Quantification of AR mRNA and protein expression

To further characterize the prostate tumors in Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} C57BL/6J TRAMP mice, we compared AR status in prostate cancers with and without tumors. AR mRNA levels were not significantly different in Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} C57BL/6J TRAMP dorsolateral prostates at 35, 70, 105 or 140 days of age (Figure 5A). At 35 days, AR expression was significantly lower in Ahr\textsuperscript{−/−} C57BL/6J TRAMP mouse ventral prostates than Ahr\textsuperscript{+/+} TRAMP prostate (Figure 5A). At 105 days, AR mRNA expression was lower in Ahr\textsuperscript{+/+} and Ahr\textsuperscript{−/−} TRAMP mice compared with Ahr\textsuperscript{−/−} mice. AR mRNA expression did not differ in Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} TRAMP mouse ventral prostates without tumors at 70 or 140 days of age. AR expression was significantly reduced in 105 and 140 day prostate tumors from mice of all Ahr genotypes compared with tumor-free dorsolateral and ventral prostates.

At 105 and 140 days, AR protein levels were not significantly different in Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} C57BL/6J TRAMP mouse prostate tumors without prostate tumors (Figure 5B). However, similar to effects seen at the mRNA level, AR protein levels were significantly reduced in prostate tumors compared with tumor-free dorsolateral prostates in mice of each Ahr genotype.

Lymph node metastasis in Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} TRAMP mice

Lymph node metastases were not detected in any Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} or Ahr\textsuperscript{−/−} TRAMP mice at 35, 70 or 105 days of age (Table I). On the other hand, at least one Ahr\textsuperscript{+/+}, Ahr\textsuperscript{−/−} and Ahr\textsuperscript{−/−} TRAMP mouse had lymph node metastasis by 140 days, with differences in incidence between Ahr\textsuperscript{−/−} and Ahr\textsuperscript{+/+} TRAMP mice approaching statistical significance (P = 0.0527). By 175 days, a greater percentage of Ahr\textsuperscript{+/+} TRAMP mice had lymph node metastasis compared with Ahr\textsuperscript{+/+} TRAMP mice, while the slightly greater incidence in Ahr\textsuperscript{−/−} TRAMP mice approached statistical significance (P = 0.07). The incidence of lymph node metastasis at 210 days was significantly greater in Ahr\textsuperscript{−/−}, but not Ahr\textsuperscript{−/−} TRAMP mice compared with Ahr\textsuperscript{+/+} TRAMP mice. The percentage of lymph node
metastasis in only tumor-bearing mice did not differ as a function of Ahr genotype. Immunohistochemical localization of chromogranin A confirmed that all lymph node metastases were neuroendocrine in nature (results not shown).

Discussion

The major finding of the present study was that malignant prostate tumors rarely develop in Ahr+/+ TRAMP on a C57BL/6J background, but the incidence is greatly increased in Ahr+/− and Ahr−/− TRAMP mice. As mice age, all prostates developed diffuse hyperplasia characteristic of the TRAMP model, but Ahr+/− and Ahr−/− TRAMP mice develop malignant prostate tumors more frequently than Ahr+/+ mice (Ahr−/− > Ahr+/− > Ahr+/+). Evidence suggests that tumors have undergone neuroendocrine differentiation, a key aspect regulated by the Ahr.

The relatively low incidence of prostate tumors in wild-type (Ahr+/+) control mice at all ages investigated was below the incidence previously reported in TRAMP studies (21–23). TRAMP mice for chemoprevention studies typically cross C57BL/6J with FVB mice, greatly increasing tumor incidence. This raised the question of whether the Ahr would also inhibit prostate carcinogenesis in TRAMP mice of this genetic background. In C57BL/6J × FVB TRAMP mice, greater tumor incidence was observed in Ahr+/+ (67%) mice compared with Ahr+/− (42%) mice (W.A. Fritz, T.-M. Lin, S. Safe, R.W. Moore and R.E. Peterson, unpublished results). Unfortunately, Ahr−/− mice expected to have the greatest tumor incidence were not investigated as they were only available on a C57BL/6J background. Regardless, these results demonstrate that the Ahr also protects against prostate carcinogenesis in TRAMP mice on the more traditional genetic background (FVB × C57BL/6J) and is not a unique phenomenon in C57BL/6J TRAMP mice.

In the TRAMP model, prostate-specific transgene expression abrogates p53 and retinoblastoma protein (pRb) function, resulting in greater epithelial cell proliferation leading to prostate carcinogenesis. Despite the lower incidence of prostate tumors in Ahr+/+ C57BL/6J TRAMP mice, SV40 large T antigen mRNA abundance was not altered at any time investigated, and all mice expressed SV40 large T antigen protein in the prostate epithelium regardless of Ahr genotype. The mechanism through which the AhR inhibits the process of prostate carcinogenesis remains to be determined. Previous studies suggest that interaction between the AhR and pRb could inhibit SV40 T antigen-mediated reversal of cell cycle arrest (28). Thus, one plausible mechanism by which the AhR could inhibit prostate carcinogenesis in TRAMP mice is through direct interaction with pRb, thereby inhibiting transgene-induced circumvention of cell cycle arrest. However, interaction with pRb occurs preferentially when the AhR is ligand-bound (29). Thus, ligand exposure and subsequent nuclear translocation would be required for the AhR to inhibit cell cycle arrest through this mechanism. Furthermore, the AhR also reduced p53 transcriptional activity in human keratinocytes (30). If the AhR

Fig. 4. Representative immunohistochemical localization of chromogranin A in C57BL/6J TRAMP mouse prostate tumors. Localization of the neuroendocrine differentiation marker, chromogranin A, was assessed in paraffin-embedded sections of the prostate taken from 105, 140 and 175 day old Ahr+/+, Ahr+/- and Ahr−/− C57BL/6J TRAMP mice using standard immunohistochemical techniques. Histology of a representative C57BL/6J TRAMP mouse dorsolateral prostate (140 day old Ahr−/−) with diffuse hyperplasia is shown after hematoxylin and eosin staining (A). In an adjacent section of the same tissue, chromogranin A staining is absent, with only methyl green counterstain visible (B). A paraffin-embedded section of a representative prostate (140 day old Ahr−/−) tumor is shown after hematoxylin and eosin staining (C). Abundant chromogranin A immunolocalization (red staining) is shown in an adjacent section of the same prostate tumor (D). Note intense staining in tumor regions (D, arrow heads) and negative staining in epithelial cells of regions retaining glandular architecture (D, arrows). Magnification = 10x.
In and prostate tumors. Gene expression (relative to cyclophilin) was determined in C57BL/6J TRAMP mouse dorsolateral and ventral prostates, bands detected at 140 days of age. Values represent means ± SEM; *Significantly different than dorsolateral and ventral prostates without tumors; §significantly different than age-matched *Significantly different than ventral prostates. All differences are at $P < 0.05$. N.D. not determined.

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were to alter pRb or p53 regulation of cell cycle progression resulting in greater cell cycle arrest in $\text{Ahr}^{+/+}$ than in $\text{Ahr}^{-/-}$ TRAMP mice, then large T antigen expression would have been incapable of producing hyperplasia observed in TRAMP mice of each $\text{Ahr}$ genotype by 105 days of age. Thus, it appears that the AhR did not inhibit prostate tumor formation through interference with transgene expression or by inducing cell cycle arrest through direct interaction with pRb.

It has previously been demonstrated that the AhR is present in the stroma and epithelium of benign and malignant prostates (17). This suggests that the AhR could conceivably alter tumorigenesis in the TRAMP model through direct signaling regulation within the epithelium, or by indirect regulation of epithelial growth mediated by the stroma. However, it remains to be demonstrated if stromal or epithelial AhR are required for inhibition of prostate tumorigenesis, or if localization to both cell populations is essential.

Inactivation of AhR signaling in $\text{Ahr}^{-/-}$ mouse prostates was confirmed by demonstrating that they were no longer susceptible to ventral prostate agenesis and reduced dorsolateral prostate weights following TCDD exposure (6). However, there is limited evidence that could account for morphological alterations observed in untreated $\text{Ahr}^{-/-}$ mouse prostates (6). It was noted that macroscopic tumors in our TRAMP mice developed a neuroendocrine phenotype, and it is likely that this process was what was regulated by the AhR. Neuroendocrine differentiation has been previously identified in TRAMP mice (24) and other transgenic mouse models of prostate cancer (31), and it is believed that rapid onset of neuroendocrine tumors occurs in an androgen-insensitive stem cell population (32). This possibility is consistent with our observation that AR mRNA and protein expression were significantly reduced in the prostate tumors from each $\text{Ahr}$ genotype. Although tumors develop from stem cells following androgen deprivation, it is uncertain how this process would selectively occur in $\text{Ahr}^{-/-}$ TRAMP mice that have not been castrated. Future studies will be carried out to identify the mechanisms responsible for AhR-mediated regulation of neuroendocrine differentiation in the prostate as a whole, or selectively in specific stem cell populations in TRAMP mice.

The rapid growth of prostate tumors that have undergone neuroendocrine differentiation is believed to result from loss of androgen sensitivity coupled with production of growth factors that facilitate angiogenesis (33,34). An early angiogenic switch has been described in TRAMP mouse prostates as HIF-1$\alpha$ levels are elevated in hyperplastic lesions prior to tumor growth (25). Sufficient elevation of HIF-1$\alpha$ would presumably allow greater interaction with the dimerization partner, HIF-1$\beta$, otherwise known as ARNT, leading to greater VEGF production. Interestingly, ARNT is also a dimerization partner for the AhR (35), suggesting that competition for the same dimerization partner in mice expressing the AhR would reduce HIF-1$\alpha$ signaling. In this case, a greater proportion of ARNT would interact with the AhR, and would be less likely to dimerize with accumulating HIF-1$\alpha$, thereby reducing VEGF production and subsequent angiogenesis necessary to support prostate tumor growth. A similar phenomenon mediates cardiac hypertrophy in AhR null mice (36), where greater HIF-1$\alpha$ levels were associated with a subsequent increase in VEGF mRNA abundance. However, total HIF-1$\alpha$ protein levels did not differ in 105 or

Table I. Effect of $\text{Ahr}$ genotype on the percentage of male C57BL/6J TRAMP mice with pelvic lymph node metastasis as a function of age

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<th>Age (days)</th>
<th>Percent of mice with lymph node metastasis</th>
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<tr>
<td>$\text{Ahr}^{+/+}$</td>
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$^*$Number of mice per group is detailed in Figure 1. $^*$Significantly different from $\text{Ahr}^{+/+}$ TRAMP mice at the same age ($P < 0.05$).
140-day-old Ahr<sup>−/−</sup>, Ahr<sup>+/−</sup>, or Ahr<sup>+/+</sup> TRAMP prostates without tumors, even though HIF-1α activity, characterized by VEGF production, increased between these ages (W.A. Fritz, T.-M. Lin and R.E. Peterson, unpublished results). Despite similar HIF-1α protein levels at an age when tumors are becoming more frequent, it is possible that Ahr<sup>−/−</sup>-TRAMP mouse prostates are more susceptible to increasing HIF-1α activity than Ahr<sup>+/+</sup> TRAMP prostates, resulting in greater VEGF production that facilitates progression from proliferative lesions to larger tumors. However, this possible mechanism remains to be demonstrated.

In addition to cardiac hypertrophy, AhR null mice exhibit reduced liver weights (13) associated with altered vascular development due to a patent duc tus venosus (37), whereby closure of the portocaval shunt during early postnatal life fails to occur. AhR null mice also exhibit impaired immune development, evidenced by reduced lymphocyte accumulation in the spleen and lymph nodes (13). While it is uncertain how smaller livers could be causally associated with greater propensity for prostate carcinogenesis, heightened immune responses may be associated with prostate carcinogenesis (38). Because inflammatory cells commonly identified in the prostate produce oxidants capable of initiating cellular and genomic damage (39), it is unlikely that greater prostate tumor development would occur through this mechanism in AhR null mice, which would presumably have diminished immunity, rather than a heightened immune response. However, prostate-specific effects on immune responses remain to be investigated in AhR null TRAMP mice.

Alternatively, the AhR may affect prostate carcinogenesis through cross-talk with other signaling pathways, including the androgen signaling cascade (40). While castration (41) and anti-androgen (42) administration effectively reduce prostate carcinogenesis in TRAMP mice, it is unlikely that the AhR protects in this manner as circulating androgens are unaltered and androgen-dependent gene expression is maintained in AhR null mice (6). However, it remains possible that subtle perturbation of androgen signaling could have pronounced effects on prostate cancer given sufficient time to exert an effect. The AhR also can interact with estrogen receptors through cross-talk mechanisms (43,44). Although estrogenic/antiestrogenic compounds effectively inhibit prostate carcinogenesis in TRAMP mice (45,46), regulation of estrogen signaling by the AhR that would confer protection have yet to be identified in the TRAMP prostate.

In conclusion, using C57BL/6J TRAMP mice not treated with TCDD, we demonstrate that absence of AhR signaling in Ahr<sup>−/−</sup> mice results in greater prostate tumor incidence and that Ahr<sup>+/−</sup> mice rarely develop prostate tumors. We have shown that tumors forming in our TRAMP mice from all Ahr genotypes are neuroendocrine in nature. This inhibition of prostate tumor formation by the AhR would seem to contradict the association between AhR activation by TCDD and greater risk of prostate cancer in humans and induction of pre-cancerous lesions in mice exposed to TCDD (18,20). Yet, this reflects the seemingly contradictory effects observed in developing prostates, whereby both activation of AhR signaling by TCDD and absence of the AhR in Ahr<sup>−/−</sup> mice resulted in reduced dorsolateral and anterior prostate weights (6). Thus, the physiological role of the AhR appears to reflect an intricate balance where baseline signaling is required for normal prostate development, but greater activation of the AhR by potent ligands, particularly TCDD, disrupts normal prostate development and may increase prostate cancer risk. Future studies will investigate the mechanisms through which the AhR regulates prostatic carcinogenesis, and how the AhR inhibits onset of neuroendocrine prostate tumors.

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References


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