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Inhibition of constitutive aryl hydrocarbon receptor (AhR) signaling attenuates androgen independent signaling and growth in (C4-2) prostate cancer cells

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Abstract

The aryl hydrocarbon receptor is a member of the basic-helix-loop-helix family of transcription factors. AhR mediates the biochemical and toxic effects of a number of polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). AhR is widely known for regulating the transcription of drug metabolizing enzymes involved in the xenobiotic metabolism of carcinogens and therapeutic agents, such as cytochrome P450-1B1 (CYP1B1). Additionally, AhR has also been reported to interact with multiple signaling pathways during prostate development. Here we investigate the effect of sustained AhR signaling on androgen receptor function in prostate cancer cells. Immunoblot analysis shows that AhR expression is increased in androgen independent (C4-2) prostate cancer cells when compared to androgen sensitive (LNCaP) cells. RT-PCR studies revealed constitutive AhR signaling in C4-2 cells without the ligand induced activation required in LNCaP cells. A reduction of AhR activity by short RNA mediated silencing in C4-2 cells reduced expression of both AhR and androgen responsive genes. The decrease in androgen responsive genes correlates to a decrease in phosphorylated androgen receptor and androgen receptor expression in the nucleus. Furthermore, the forced decrease in AhR expression resulted in a 50% decline in the growth rate of C4-2 cells. These data indicates that AhR is required to maintain hormone independent signaling and growth by the androgen receptor in C4-2 cells. Collectively, these data provide evidence of a direct role for AhR in androgen independent signaling and provides insight into the molecular mechanisms responsible for sustained androgen receptor signaling in hormone refractory prostate cancer.

Keywords

Dioxin; TCDD; AhR; Androgen receptor; Progression

1. Introduction

2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD) is a halogenated aromatic hydrocarbon reported to have tissue and species specific biological effects [1]. However, emerging evidence suggests that TCDD may inhibit prostate cancer progression. TCDD exposure significantly decreases serum testosterone levels in rats [2]. Furthermore, TCDD exposure

has been reported to decrease the risk of benign prostatic hyperplasia (BPH) in the general population [3]. These findings were further validated by a cohort study of Air Force Veterans which reported decreased incidence of BPH and serum testosterone with increasing levels of serum TCDD [4].

The aryl hydrocarbon receptor (AhR) mediates the biochemical effects of TCDD by direct binding to the receptor [5,6]. AhR is a basic helix-loop-helix (bHLH) protein located in the cytosol. The AhR is the only ligand-activated member of the bHLH family; it is activated by the binding of a wide range of environmental hydrocarbons [7]. While in the cytosol, AhR is found in a complex that consist of two molecules of HSP90, co-chaperone p23, immunophilin-like AhR interacting protein (AIP) and tyrosine kinase c-Src [8–11]. This protein complex is designed to maintain the inactive conformation and prevent nuclear translocation.

Upon binding by polyaromatic hydrocarbon (PAHs) or halogenated aromatic hydrocarbons (HAHs) of which 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) is the most potent HAH, AhR dissociates from its chaperone proteins and translocates to the nucleus where the receptor heterodimerizes with the AhR nuclear translocator protein (ARNT) [12]. The nuclear AhR complex interacts with xenobiotic response elements (XRE) in the promoter region of responsive genes, such as cytochrome P450-1A1 and 1B1 (CYP1A1 and CYP1B1) [13]. Subsequent recruitment of coactivators and general transcription factors results in transactivation [14,15]. Following the induction of AhR responsive genes, AhR signaling is immediately terminated by degradation or by binding to an inhibitor protein. In the latter, the AhR repressor (AhRR), an AhR responsive gene, competes with AhR to form a complex with ARNT [16,17]. Formation of the ARNT/AHRR heterodimer results in decreased transcriptional activity by AhR. Alternatively, AhR may undergo nuclear export, ubiquitination and subsequent degradation by the 26S proteasome [18].

Although extensively studied as a key regulator of xenobiotic metabolism, AhR has been shown to influence a number of cellular processes including differentiation, proliferation and cell cycle progression [19–24]. Activation of the receptor by exogenous ligands has been reported to antagonize androgen receptor signaling. For example, TCDD inhibited testosterone-dependent transcriptional activity and testosterone-regulated prostate specific antigen (PSA) expression in a dose dependent manner [25]. TCDD was also shown to block androgen dependent proliferation of prostate cancer cells [26]. Simultaneous activation of AhR and androgen receptor with TCDD and an androgen derivative respectively, decreased androgen receptor protein levels [27]. This observation has been contributed to the ability of AhR to promote the proteolysis of androgen receptor through assembling an ubiquitin ligase complex in which AhR acts as a substrate-recognition subunit to recruit the androgen receptor [28]. This action may explain the antiandrogenic actions of a number of PAHs and HAHs.

However, studies concerned with intrinsic functions of AhR have found that the receptor may promote carcinogenesis. AhR protein and mRNA expression is associated with phases of rapid proliferation and differentiation in certain tissues. AhR-defective cell lines demonstrate a reduced proliferation rate [29]. Ectopic over expression of AhR in immortalized normal mammary epithelial cells induced a malignant phenotype with increased growth and acquired invasive capabilities [30]. A separate study using a constitutively active AhR construct lacking a ligand binding domain revealed that AhR acts as a transcriptional co-regulator for the unliganded androgen receptor. These studies showed that the endogenous androgen receptor along with the constitutively active AhR was recruited to androgen-responsive elements to initiate signaling in an androgen depleted environment [31].

Androgens play a predominant role in male sexual development and growth of the prostate gland and early stage prostate cancers are dependent on androgens for growth [32]. Prostate cancer is the most commonly diagnosed cancer in men. It is estimated that 1 in 6 men will be diagnosed with prostate cancer within their lifetime and 1 in 36 will die from the disease. Prostate cancer is also the second leading cause of all cancer related deaths in men. The five year survival rate for men diagnosed with local or regional prostate cancer is 100%. However, men diagnosed with a distant metastasis have a five year survival rate of just 29%. Most men who die of prostate cancer present with hormone refractory prostate cancer (HRPC) [33]. Androgen deprivation therapy (ADT) is the predominant form of treatment for men diagnosed with regional prostate cancer. ADT suppresses testicular androgen synthesis and inhibits androgen receptor activation. However, most patients acquire resistance to ADT and develop hormone refractory prostate cancer (HRPC) following therapy [34]. This more advanced form of prostate cancer that is hormone refractory do not rely on the presence of androgens for growth [35]. Consequently, even in a hormone depleted environment, androgen receptor signaling continues to play a role in HRPC [36]. Androgen receptor signaling has been shown to be sustained by a variety of mechanisms including increased androgen uptake by prostate cancer cells, increased AR expression, AR gene mutation and activation by other transcription factors [37–40]. The objective of this study is to determine the ability of AhR to sustain androgen receptor signaling in prostate cancer cells that are not dependent on androgens for growth.

The molecular mechanism responsible for the emergence of these aggressive forms of prostate cancer remains largely unknown and as a result, there are no effective therapies against HRPC. A precise molecular mechanism is required to develop targeted therapeutic strategies. Therefore, studies are needed to identify all modulators of androgen receptor signaling that lead to the development and maintenance of HRPC. AhR may provide an additional target to ablate androgen-independent signaling in hormone refractory prostate cancer.

2. Materials and methods

2.1. Chemical and reagents

AhR agonist, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) was purchased from AccuStandard (New Haven, CT).

2.2. Cell culture

Adherent monolayer cultures of androgen dependent human prostate cancer cell line LNCaP (American Type Culture Collection) and androgen independent C4-2 human prostate cancer cell line (Dr. Valerie Odero-Marrah, Clark Atlanta University, Atlanta, GA) were maintained in RPMI 1640 medium supplemented with 5.0% FBS and 100 mmol/L each of penicillin and streptomycin. The subsequently derived AhR depleted cell line, C4-2(-AhR) and the scrambled vector control, C4-2SCR, were also maintained in RPMI 1640 supplemented media. Cells were grown at 37 °C with 5% CO₂ in humidified atmosphere, and media was replaced every third day. Cells were split (1:3), when they reached near confluence. Their response to androgens for growth and androgen receptor activity was monitored intermittently during the study.

2.3. RNA extraction, RT-PCR and qRT-PCR analysis

Total RNA was isolated from cell monolayers grown in 100 mm tissue culture dishes using RNeasy Mini Kit (Qiagen). 2 µg of the total RNA was reverse-transcribed using the Superscript II kit (Invitrogen), according to the manufacturer's recommendations. For RT-PCR analysis the cDNA served as a template in a 20 µl reaction mixture and was processed

using the following protocol: an initial denaturation at 94 °C for 5 min, followed by 30–33 amplification cycles (94 °C for 30 s, 55–60 °C for 45 s, and 72 °C for 1 min) and 72 °C for 10 min. The 20 µl PCR reaction mixture was mixed with 6× loading buffer and loaded onto a 1% agarose gel containing ethidium bromide. For qRT-PCR analysis, the cDNA served as a template in a 25 µl reaction mixture and was processed using the following protocol: an initial denaturation at 95 °C for 3 min, followed by 39 amplification cycles (95 °C for 10 s and 55–65 °C for 30 s), 95 °C for 10 s, 65 °C for 5 s and 95 °C for 50 s. The 25 µl qPCR reaction mixture was mixed with GoTaq qPCR Master Mix (Promega). Melt curve analyses were performed after each run to ensure a single product. Relative gene expression was determined using the $\Delta\Delta C_q$ calculation method. The forward (F) and reverse (R) primer sequences for AhR, CYP1B1, KLK2 and KLK3 are shown in Table 1. Primers to amplify the 470-bp cDNA fragment encoding L19 were used as an internal control.

2.4. Western blot analysis and quantification of protein

Protein samples were isolated using the Thermo Scientific NE-PER Extraction kit for cellular fractions or commercially available cell lysis buffer (Cell Signaling) for total protein. Protein samples were resolved by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was carried out with varying concentrations of primary antibody (see figure legends) in 5% milk. Blots were washed three times (15 min each) with TBST. The blots were then incubated in 1:2500 dilution of secondary antibody and washed three times (15 min each) with TBST, three times (10 min each) with TBS and once with ddH₂O (10 min). Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer. Multiple exposures of each set of samples were produced. The relative concentration of target protein was determined by computer analysis and normalized to an internal standard (topoisomerase, β -tubulin, β -actin).

2.5. Immunocytochemical staining and fluorescence microscopy

Cells grown on glass cover slips in 6-well plates were washed in cold PBS and fixed by incubation in a 1:1 methanol: acetone solution at 4 °C for 30 min and then air dried. Cells were rinsed and hydrated with Tris-buffered saline containing 0.05% Tween 20 (TBST) and transferred to a clean 6-well plate. The cells were incubated at room temperature for 1 h in 5% milk solution in TBST to block nonspecific binding, followed by incubation at room temperature with either affinity-purified rabbit anti-AhR polyclonal antibody at 1 µg/ml, or anti-AR monoclonal antibodies at 1:500 dilution in 4% milk solution in TBST. Cells were then washed three times (15 min each) with TBST. Cells were incubated with a 1:200 dilution of either fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies or tetra-methyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse (Jackson ImmunoResearch laboratories, West Grove, PA) in 4% milk at room temperature for 1 h. The cells were then washed three times (15 min each) with TBST, three times (10 min each) with TBS and once with ddH₂O (10 min). Cells were then mounted on slides using UltraCruz hard set mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI).

2.6. Development of stable (-AhR) cell line

C4-2 cells were plated at 2×10^6 per 6-cm plate and allowed to incubate overnight. AhR shRNA lentiviral particles (Santa Cruz) were mixed with culture media and added to the C4-2 cells in the presence of polybrene (4 µg/ml) with gentle and thorough mixing. Cells were incubated at 37 °C for 24 h. Infection medium was then removed and fresh medium was added to cells for an extra 24–48 h. Cells were then grown in puromycin supplemented media for 7 days, media was replaced every second day.

2.7. Cell proliferation assay

Growth of cells was assayed using the Promega CellTiter 96 Cell Proliferation Assay. Cells were resuspended to a final concentration of $1.0 \times 10^5 \text{ mL}^{-1}$ in RPMI. 50 μl of the cell suspension (5000 cells) was added to each well of the 96-well plate containing 50 μl of media with corresponding treatment resulting in a total volume of 100 μl . The Plates were incubated at 37 °C for 24–72 h in a humidified, 5% CO₂ atmosphere. Per manufacturers instructions, following incubation 15 μl of MTS solution was added to each well and incubated for 4 h. 100 μl of stop solution was added to each well and incubated for 1 h. Absorbances were read at 570 nm using the Synergy H1m multimode microplate reader.

2.8. Statistical analysis

Each experiment was carried out at least 3 times and all the values are expressed as mean + SEM. The differences between the groups were compared by *t*-test or ANOVA using InStat software (GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. AhR expression and localization

We chose to use the androgen sensitive LNCaP and androgen-independent C4-2 cell lines as a model system because the pair serve as an in vitro model of prostate cancer progression from hormone sensitive to hormone refractory. The androgen independent C4-2 cells were derived from a chimeric tumor induced by inoculating a castrated mouse with parental androgen sensitive LNCaP cells [41]. Comparing AhR mRNA and protein expression in the LNCaP cells to the C4-2 prostate cancer cell line revealed that AhR mRNA expression was increased in C4-2 cells by 3-fold (Fig. 1A and B). Subsequently, protein expression was also revealed to be increased 3-fold by immunoblotting of total protein (Fig. 1C).

Immunocytochemical staining revealed that the increased protein expression was accompanied by AhR protein expression in the cytoplasm and nucleus of androgen independent C4-2 cells while remaining in the cytosol of androgen sensitive LNCaP cells (Fig. 2A). Sub-cellular localization was confirmed by immunoblotting following cellular fractionation which revealed the majority of AhR protein in C4-2 cells is found in the nucleus. A 5-fold increase in AhR protein expression was seen in the nuclear fractions of C4-2 cells compared to LNCaP nuclear fractions (Fig. 2B).

3.2. Transcriptional activity of constitutively active AhR

To address the functional consequence of AhR nuclear localization in C4-2 prostate cancer cells, RT-PCR was utilized to determine mRNA expression of AhR responsive gene, CYP1B1. Furthermore, qRT-PCR was used to quantify CYP1B1 expression. We explored the transcriptional activity of AhR in both LNCaP and C4-2 prostate cancer cells. RT-PCR studies revealed there is intrinsic expression of CYP1B1 in C4-2 control cells. CYP1B1 expression is induced in LNCaP and further enhanced in C4-2 cells following exposure to 10 μM TCDD for 24 h (Fig. 2C). The overall fold change in CYP1B1 expression following TCDD exposure correlates to the overall AhR protein expression in each cell line. qRT-PCR revealed a 4-fold increase of CYP1B1 expression in C4-2 cells following treatment with TCDD compared to the 2-fold induction seen in LNCaP cells. The increase in CYP1B1 expression following TCDD exposure indicates that further induction can occur following ligand activation. The 2-fold induction of CYP1B1 in LNCaP cells was comparable in expression to the relative amount of CYP1B1 seen in C4-2 cells without ligand activation. CYP1B1 expression remained 4-fold higher in C4-2 cells following TCDD treatment when compared to the LNCaP treated cells (Fig. 2D). Decreasing AhR expression by shRNA resulted in a significant decrease in CYP1B1 mRNA expression in C4-2 cells (Fig. 3B).

Western blot analysis indicates a ~75% decrease in total AhR protein expression was sufficient to decrease CYP1B1 mRNA expression (Fig. 3A). Immunocytochemical staining revealed a consequential decrease in nuclear localization of AhR. Although not completely devoid of AhR expression, there is a noticeable decrease in AhR immunofluorescence in the nucleus of C4-2(-AhR) cells (Fig. 3D). Subcellular fractionation confirmed the decrease in AhR expression and further revealed a 50% decrease in AhR expression in the nucleus without a significant change in cytoplasmic AhR expression (Fig. 3E). These studies indicate that constitutive CYP1B1 expression in C4-2 cells is a direct result of ligand independent localization of AhR in the nucleus.

3.3. Expression of androgen receptor (AR) and androgen regulated genes

We investigated the effect of AhR expression and signaling on androgen receptor expression and signaling. Immunocytochemical staining revealed that depletion of AhR protein in C4-2(-AhR) cells resulted in reduced nuclear localization of androgen receptor (AR) (Fig. 4A). Additionally, subcellular fractions revealed an 80% decrease in androgen receptor protein expression in the nucleus while there was not a significant decrease in cytoplasmic androgen receptor expression (Fig. 4B). Western blot analysis confirmed an 80% decrease in phosphorylated androgen receptor, which is the active form of androgen receptor. The decrease in phosphorylated androgen receptor seen in the C4-2(-AhR) cells was sufficient to produce a significant decrease in overall androgen receptor protein expression (Fig. 4C). Furthermore, C4-2 (-AhR) cells also have a decrease in androgen responsive genes KLK2 and KLK3 (Fig. 4D). qRT-PCR revealed a 3-fold decrease in both KLK2 and KLK3 expression in C4-2(-AhR) cells compared to the scrambled control (Fig. 4E). These results indicate that androgen receptor signaling is in part sustained by constitutive signaling of AhR in C4-2 prostate cancer cells.

3.4. Effect of AhR depletion on proliferation

Next we examined the effects of shRNA-mediated inhibition of AhR signaling on the growth of C4-2 cells. C4-2(-AhR) cell growth under androgen depleted (CSS) conditions had a 50% decrease in overall growth rate compared to C4-2 (SCR) control cells in CSS media. Furthermore, growth of C4-2(-AhR) cells in CSS media is significantly inhibited compared to growth of C4-2(-AhR) cells under normal culture conditions (FBS) (Fig. 5A). Conversely, the growth rate of C4-2(SCR) cells is unaffected by androgen depleted (CSS) media. The effect of AhR expression on androgen independent growth was observed when C4-2 (SCR) and C4-2(-AhR) cells were grown in charcoal stripped media (CSS) for 24–72 h. C4-2(-AhR) cells were observed to have a decreased growth rate compared to C4-2(SCR) over time. The growth rate of C4-2(-AhR) was determined to be equivalent to the growth rate of androgen sensitive LNCaP cells (Fig. 5B). Together, these data show depletion of AhR expression in C4-2 cells reduces the growth rate under androgen depleted conditions.

4. Discussion and conclusion

A growing number of studies have reported increased expression of AhR within cancer cells and tumors. Tumors induced by exposure to known tumor initiator, 7,12-dimethylbenz[α]anthracene (DMBA) have significantly higher levels of AhR compared to normal mammary epithelial cells, which express very low levels [42]. AhR expression is also strongly correlated to cancer progression in pancreatic samples. Normal pancreatic tissue has extremely weak AhR expression compared to the still low expression seen in chronic pancreatitis tissues and strong expression in pancreatic cancer samples [43]. In addition to the increased expression of AhR seen in lymphoid malignancy, the receptor was shown to be active in the absence of exogenous ligands [44]. A few studies support the presence of the nuclear/activated form of AhR in the absence of an exogenous ligand. AhR

was seen in nuclear fractions of HeLa cells under normal culture conditions [45]. Furthermore, studies designed to mimic a constitutively active AhR have shown a role for the receptor in tumor progression. Following exposure to a hepatocarcinogen to induce tumors, one liver tumor was observed in AhR wild-type mice while 19 tumors were present in the 18 transgenic mice with constitutively active AhR [46]. In this present study, we show AhR mRNA and protein expression is increased in androgen independent C4-2 prostate cancer cell lines by 3-fold compared to the less aggressive androgen sensitive LNCaP prostate cancer cell line. AhR is also expressed in the nucleus of C4-2 cells without exogenous ligand activation. The LNCaP cells however, are virtually devoid of AhR expression in the nucleus, indicating that nuclear expression is acquired with androgen independent progression.

Further evidence that AhR is constitutively active in C4-2 cells is provided by the expression of AhR responsive genes under normal cell culture conditions and in the absence of exogenous ligands. CYP1B1 is expressed in C4-2 cells but require activation with TCDD to be expressed in LNCaP cells. Depletion of AhR expression in C4-2 cells reduced expression of CYP1B1. Reduction of overall AhR expression in C4-2 cells consequently reduced nuclear expression of AhR, confirming that CYP1B1 expression is due to the presence of activated, nuclear AhR. The data presented here supports previous reports that expression of CYP1B1 mRNA and protein were shown to be increased in advanced prostate cancer [47]. In addition, prostate cancer patients carrying specific CYP1B1 polymorphisms have an increased risk of developing more aggressive forms of prostate cancer [48]. Hormone refractory prostate cancer (HRPC) patients with CYP1B1 polymorphisms have a significantly lower response to docetaxel treatment [49]. Together, these results, along with previous reports, suggest that a constitutively active AhR plays a role in prostate cancer progression to a hormone refractory phenotype.

Increasing evidence suggest a role for AhR in the regulation of hormone signaling. AhR has been shown to have inhibitory as well as stimulatory-crosstalk with the estrogen and androgen receptor. The contradictory roles of AhR in androgen signaling have been contributed to cell specificity. However, the differences may be attributed to the receptor having distinct functions as a xenobiotic receptor that differs from its intrinsic functions. Activation of AhR with exogenous ligands has been shown to inhibit both E2 and DHT induced gene expression [50,51]. However, when the AhR is overexpressed in the absence of an exogenous ligand, the receptor appears to induce an estrogenic or androgenic response. Evidence that the intrinsic function of AhR is androgenic was provided by the ability of a constitutively active AhR to function as a transcriptional co-regulator for the unliganded androgen receptor. In addition to binding androgen responsive element, the AhR also induced activation of the unliganded androgen receptor [28]. Mammary epithelial cells overexpressing AhR exhibit several phenotypic changes indicative of increased cancer progression such as increased proliferation and invasion [52]. Our data show that shRNA mediated reduction of the constitutively active AhR found in C4-2 cells leads to a significant decrease in the phosphorylated/active form of the androgen receptor. There was also a reduction in androgen responsive genes indicating that the constitutively active AhR was responsible for the expression of androgen responsive genes.

A widely reported consequence of polyaromatic hydrocarbons exposure is inhibition of cell growth. As the most potent AhR agonist, TCDD has been shown to inhibit proliferation of a variety of cell types including neural precursor, renal proximal tubule, and hepatoma cells [53–55]. TCDD has also been shown to inhibit E2 stimulated cell proliferation in endometrial and breast cancer cells in a dose dependent manner [56,57]. In prostate cancer cells, TCDD inhibited androgen receptor mediated gene transcription through direct binding or competition for coregulators resulting in decreased cell growth [26]. Following the motif

of opposing intrinsic functions, overexpression of AhR protein enhances proliferation of cancer cells. Lung cancer cells overexpressing AhR grew at a faster rate than control cells. The rate of growth in the lung cancer cell clones was proportional to the increase in AhR expression [58]. Also, ectopic overexpression of AhR in immortalized normal mammary epithelial cells increase proliferation of clones based on the level of AhR expressed [30]. Compared to their wild-type counter-parts, hepatoma cells with reduced AhR expression have a decrease rate of proliferation that is increased with transfection of AhR cDNA [59]. In support of these findings, our own data show that shRNA mediated reduction of AhR expression causes a 50% decrease in the growth rate of androgen-independent C4-2 cells.

The decreased growth rate seen in C4-2(-AhR) may be due to decreased interactions between AhR and certain cell cycle proteins. TCDD exposure, which can result in decrease AhR protein expression, has been shown to inhibit cell cycle progression in a number of cell types. The report that TCDD activation induces a direct interaction between AhR and tumor suppressor retinoblastoma (Rb), suggest an additional mechanism through which AhR may suppress growth [60]. Phosphorylation of Rb by cyclin D: cyclin-dependent kinase (CDK) 4/6 complexes is required for progression from G1 to S phase. AhR prevents phosphorylation of Rb through direct binding to the tumor suppressor. In vitro studies revealed there are two binding sites for Rb within two distinct AhR domains and that association only occurs after AhR activation and translocation to the nucleus [61]. Also, TCDD has been shown to decrease levels of cyclin D to block cell cycle progression as well as interrupt the direct interaction between AhR and CKD4 [62]. Disruption of CDK4 interaction results in decreased phosphorylation of Rb and G1 cell cycle arrest [63]. Once again there are inverse reactions seen with ligand activation versus constitutive activity. Treatment with exogenous ligands induces cell cycle arrest while increased AhR expression promotes cell cycle progression. In lung cancer cells overexpressing AhR, the receptor serves as a co-activator of E2F-dependent transcription. When AhR was overexpressed the AhR:E2F interaction promotes cell cycle progression [64]. Conversely, siRNA depletion of AhR expression in keratinocytes lead to a reduction in protein levels of Rb and CDK2 resulting in decreased proliferation and cell cycle arrest [65].

In summary, the results of this study provide evidence that AhR helps to sustain androgen-independent growth of prostate cancer cells. AhR expression is upregulated in the androgen independent cell line and has constitutive activity. Attenuation of AhR activity reduces expression of phosphorylated androgen receptor and androgen responsive genes. The decrease in androgen signaling cause by AhR depletion decreases proliferation of androgen-independent cells in an androgen depleted environment. Together these data provide evidence that AhR plays a significant role in sustained androgen receptor signaling and growth. One mechanism AhR may use to activate androgen receptor is by phosphorylation of the receptor with Src kinase. Src kinase has been identified as a member of the inactive AhR protein complex and can transduce AhR signaling through the protein phosphorylation pathway [11]. Src was shown to mediate crosstalk between AhR and epidermal growth factor receptor in colon cancer cells [66]. Independently, studies have shown that Src kinase can induce androgen receptor transactivation in C4-2 cells. Inhibition of Src kinase function with a specific inhibitor resulted in decreased androgen receptor activation [67].

Additionally, AhR has been reported to directly interact with a number of nuclear proteins. The protein interactions range from transcription factors to coactivator and corepressor proteins. Reported interactions include, NFkB, COUP-TF1 and TFIIB [62–64,68–70]. AhR and androgen receptor share a number of coactivator proteins such as SRC1 and p300 [71,72]. Therefore, the precise molecular mechanism utilized by constitutive AhR signaling to activate androgen receptor signaling needs to be investigated further and could include induced activation via protein phosphorylation, direct heterodimerization and interacting via

coactivators. Considering, crosstalk of androgen receptor with other signaling pathways has been shown to cause its aberrant activation during androgen deprivation therapy in more advanced prostate cancers, AhR may serve as an additional therapeutic target in HRPC.

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References

1. Denison MS, Soshilov AA, He G, DeGroot DE, Zhao B. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol Sci.* 2011; 124:22–31.
2. Choi JS, Kim IW, Hwang SY, Shin BJ, Kim SK. Effect of 2,3,7,8-tetrachlorodi-benzo-p-dioxin on testicular spermatogenesis-related panels and serum sex hormone levels in rats. *BJU Int.* 2008; 101:5–250.
3. Gupta A, Schecter A, Aragaki CC, Roehrborn CG. Dioxin exposure and benign prostatic hyperplasia. *J Occup Environ Med.* 2006; 48:14–708.
4. Gupta A, Ketchum N, Roehrborn CG, Schecter A, Aragaki CC, Michalek JE. Serum dioxin, testosterone, and subsequent risk of benign prostatic hyperplasia: a prospective cohort study of Air Force veterans. *Environ Health Perspect.* 2006; 114:54–1649.
5. Poland A, Knutson JC. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol.* 1982; 22:54–517.
6. Xing Y, Nukaya M, Satyshur KA, Jiang L, Stanevich V, Korkmaz EN, et al. Identification of the ah-receptor structural determinants for ligand preferences. *Toxicol Sci.* 129:86–97. [PubMed: 22659362]
7. Safe S. Molecular biology of the Ah receptor and its role in carcinogenesis. *Toxicol Lett.* 2001; 120:7–1.
8. Perdew GH, Bradfield CA. Mapping the 90 kDa heat shock protein binding region of the Ah receptor. *Biochem Mol Biol Int.* 1996; 39:589–93. [PubMed: 8828811]
9. Kazlauskas A, Poellinger L, Pongratz I. Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor. *J Biol Chem.* 1999; 274:24–13519.
10. Meyer BK, Pray-Grant MG, Vanden Heuvel JP, Perdew GH. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Mol Cell Biol.* 1998; 18:88–978.
11. Enan E, Matsumura F. Identification of c-Src as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) through the protein phosphorylation pathway. *Biochem Pharmacol.* 1996; 52:612–1599.
12. Pollenz R. The aryl hydrocarbon receptor, but not the aryl hydrocarbon receptor translocator protein is rapidly depleted in hepatic and non-hepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-dioxin. *Mol Pharmacol.* 1995; 49:8–391.
13. Bacsí SG, Reisz-Porszasz S, Hankinson O. Orientation of the heterodimeric aryl hydrocarbon (dioxin) receptor complex on its asymmetric DNA recognition sequence. *Mol Pharmacol.* 1995; 47:8–432.
14. Whitlock JP Jr, Denison MS, Fisher JM, Shen ES. Induction of hepatic cytochrome P450 gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Mol Biol Med.* 1989; 6:78–169.
15. Alexander DL, Eltom SE, Jefcoate CR. Ah receptor regulation of CYP1B1 expression in primary mouse embryo-derived cells. *Cancer Res.* 1997; 57:506–4498.
16. Mimura J, Ema M, Sogawa K, Fujii-Kuriyama Y. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev.* 1999; 13:5–20.
17. Baba T, Mimura J, Gradin K, Kuroiwa A, Watanabe T, Matsuda Y, et al. Structure and expression of the Ah receptor repressor gene. *J Biol Chem.* 2001; 276:10–33101.

18. Prokipcak RD, Okey AB. Downregulation of the Ah receptor in mouse hepatoma cells treated in culture with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Can J Physiol Pharmacol.* 1991; 69:10–1204.
19. Hayashi S, Okabe-Kado J, Honma Y, Kawajiri K. Expression of Ah receptor (TCDD receptor) during human monocytic differentiation. *Carcinogenesis.* 1995; 16:1403–9. [PubMed: 7788861]
20. Bunaciu RP, Yen A. Activation of the aryl hydrocarbon receptor AhR Promotes retinoic acid-induced differentiation of myeloblastic leukemia cells by restricting expression of the stem cell transcription factor Oct4. *Cancer Res.* 2011; 71:80–2371.
21. Dever DP, Opanashuk LA. The aryl hydrocarbon receptor (AhR) contributes to the proliferation of human medulloblastoma cells. *Mol Pharmacol.* 2012
22. Hestermann EV, Stegeman JJ, Hahn ME. Relationships among the cell cycle, cell proliferation, and aryl hydrocarbon receptor expression in PLHC-1 cells. *Aquat Toxicol.* 2002; 58:13–201.
23. Hrubá E, Vondracek J, Libalova H, Topinka J, Bryja V, Soucek K, et al. Gene expression changes in human prostate carcinoma cells exposed to genotoxic and nongenotoxic aryl hydrocarbon receptor ligands. *Toxicol Lett.* 2011; 206:88–178.
24. Weiss C, Kolluri SK, Kiefer F, Gottlicher M. Complementation of Ah receptor deficiency in hepatoma cells: negative feedback regulation and cell cycle control by the Ah receptor. *Exp Cell Res.* 1996; 226:63–154.
25. Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C, Sone H. Cross-talk between 2,3,7,8-tetrachlorodibenzo-p-dioxin and testosterone signal transduction pathways in LNCaP prostate cancer cells. *Biochem Biophys Res Commun.* 1999; 256:8–462.
26. Barnes-Ellerbe S, Knudsen KE, Puga A. 2,3,7,8-Tetrachlorodibenzo-p-dioxin blocks androgen-dependent cell proliferation of LNCaP cells through modulation of pRB phosphorylation. *Mol Pharmacol.* 2004; 66:11–502. [PubMed: 15213291]
27. Morrow D, Qin C, Smith R 3rd, Safe S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced trans-activation. *J Steroid Biochem Mol Biol.* 2004; 88:36–27.
28. Ohtake F, Fujii-Kuriyama Y, Kato S. AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions. *Biochem Pharmacol.* 2009; 77:84–474.
29. Wang W, Smith R 3rd, Safe S. Aryl hydrocarbon receptor-mediated antiestrogenicity in MCF-7 cells: modulation of hormone-induced cell cycle enzymes. *Arch Biochem Biophys.* 1998; 356:48–239.
30. Brooks J, Eltom SE. Malignant transformation of mammary epithelial cells by ectopic overexpression of the aryl hydrocarbon receptor. *Curr Cancer Drug Targets.* 2011; 11:69–654.
31. Ohtake F, Baba A, Fujii-Kuriyama Y, Kato S. Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings. *Biochem Biophys Res Commun.* 2008; 370:6–541. [PubMed: 18334227]
32. Ryan CJ, Tindall DJ. Androgen receptor rediscovered: the new biology and targeting the androgen receptor therapeutically. *J Clin Oncol.* 2011; 29:8–3651.
33. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin.* 2012; 62:29–10.
34. Messing EM, Manola J, Sarosdy M, Wilding G, Crawford ED, Trump D. Immediate hormonal therapy compared with observation after radical prostatectomy and pelvic lymphadenectomy in men with node-positive prostate cancer. *N Engl J Med.* 1999; 341:8–1781. [PubMed: 10387936]
35. Saraon P, Jarvi K, Diamandis EP. Molecular alterations during progression of prostate cancer to androgen independence. *Clin Chem.* 2011; 57:75–1366.
36. Mao HL, Zhu ZQ, Chen CD. The androgen receptor in hormone-refractory prostate cancer. *Asian J Androl.* 2009; 11:73–69.
37. Leon CG, Locke JA, Adomat HH, Etinger SL, Twiddy AL, Neumann RD, et al. Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model. *Prostate.* 2010; 70:400–390.
38. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.* 2001; 61:5–3550.

39. Sun S, Sprenger CC, Vessella RL, Haug K, Soriano K, Mostaghel EA, et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. *J Clin Invest.* 2010; 120:30–2715.
40. Nadiminty N, Lou W, Sun M, Chen J, Yue J, Kung HJ, et al. Aberrant activation of the androgen receptor by NF-kappaB2/p52 in prostate cancer cells. *Cancer Res.* 2010; 70:19–3309.
41. Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int J Cancer.* 1994; 57:12–406.
42. Trombino AF, Near RI, Matulka RA, Yang S, Hafer LJ, Toselli PA, et al. Expression of the aryl hydrocarbon receptor/transcription factor (AhR) and AhR-regulated CYP1 gene transcripts in a rat model of mammary tumorigenesis. *Breast Cancer Res Treat.* 2000; 63:31–117. [PubMed: 11079157]
43. Koliopanos A, Kleeff J, Xiao Y, Safe S, Zimmermann A, Buchler MW, et al. Increased arylhydrocarbon receptor expression offers a potential therapeutic target for pancreatic cancer. *Oncogene.* 2002; 21:70–6059.
44. Hayashibara T, Yamada Y, Mori N, Harasawa H, Sugahara K, Miyanishi T, et al. Possible involvement of aryl hydrocarbon receptor (AhR) in adult T-cell leukemia (ATL) leukemogenesis: constitutive activation of AhR in ATL. *Biochem Biophys Res Commun.* 2003; 300:34–128.
45. Singh SS, Hord NG, Perdew GH. Characterization of the activated form of the aryl hydrocarbon receptor in the nucleus of HeLa cells in the absence of exogenous ligand. *Arch Biochem Biophys.* 1996; 329:55–47.
46. Moennikes O, Loeppen S, Buchmann A, Andersson P, Itrich C, Poellinger L, et al. A constitutively active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice. *Cancer Res.* 2004; 64:10–4707.
47. Tokizane T, Shiina H, Igawa M, Enokida H, Urakami S, Kawakami T, et al. Cytochrome P450 1B1 is overexpressed and regulated by hypomethylation in prostate cancer. *Clin Cancer Res.* 2005; 11:801–5793.
48. Cicek MS, Liu X, Casey G, Witte JS. Role of androgen metabolism genes CYP1B1, PSA/KLK3, and CYP11alpha in prostate cancer risk and aggressiveness. *Cancer Epidemiol Biomarkers Prev.* 2005; 14:7–2173.
49. Pastina I, Giovannetti E, Chioni A, Sissung TM, Crea F, Orlandini C, et al. Cytochrome 450 1B1 (CYP1B1) polymorphisms associated with response to docetaxel in Castration-Resistant Prostate Cancer (CRPC) patients. *BMC Cancer.* 2010; 10:511. [PubMed: 20875115]
50. Chen I, Hsieh T, Thomas T, Safe S. Identification of estrogen-induced genes downregulated by AhR agonists in MCF-7 breast cancer cells using suppression subtractive hybridization. *Gene.* 2001; 262:14–207.
51. Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C, Sone H. Role of estradiol receptor-alpha in differential expression of 2,3,7, 8-tetrachlorodibenzo-p-dioxin-inducible genes in the RL95-2 and KLE human endometrial cancer cell lines. *Arch Biochem Biophys.* 1999; 368:9–31.
52. Wong PS, Li W, Vogel CF, Matsumura F. Characterization of MCF mammary epithelial cells overexpressing the Arylhydrocarbon receptor (AhR). *BMC Cancer.* 2009; 9:234. [PubMed: 19604390]
53. Latchney SE, Lioy DT, Henry EC, Gasiewicz TA, Strathmann FG, Mayer-Proschel M, et al. Neural precursor cell proliferation is disrupted through activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Stem Cells Dev.* 2011; 20:26–313.
54. Han HJ, Lim MJ, Lee YJ, Kim EJ, Jeon YJ, Lee JH. Effects of TCDD and estradiol-17beta on the proliferation and Na+/glucose cotransporter in renal proximal tubule cells. *Toxicol In Vitro.* 2005; 19:30–21.
55. Kolluri SK, Weiss C, Koff A, Gottlicher M. p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells. *Genes Dev.* 1999; 13:53–1742.
56. Wormke M, Castro-Rivera E, Chen I, Safe S. Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. *J Steroid Biochem Mol Biol.* 2000; 72:207–197.

57. Oenga GN, Spink DC, Carpenter DO. TCDD and PCBs inhibit breast cancer cell proliferation in vitro. *Toxicol In Vitro*. 2004; 18:9–811.
58. Shimba S, Komiyama K, Moro I, Tezuka M. Overexpression of the aryl hydrocarbon receptor (AhR) accelerates the cell proliferation of A549 cells. *J Biochem*. 2002; 132:802–795.
59. Ma Q, Whitlock JP Jr. The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Mol Cell Biol*. 1996; 16:50–2144.
60. Ge NL, Elferink CJ. A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle. *J Biol Chem*. 1998; 273:13–22708. [PubMed: 9417038]
61. Puga A, Barnes SJ, Dalton TP, Chang C, Knudsen ES, Maier MA. Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. *J Biol Chem*. 2000; 275:50–2943.
62. Jung NK, Park JY, Park JH, Kim SY, Park JK, Chang WK, et al. Attenuation of cell cycle progression by 2,3,7,8-tetrachlorodibenzo-p-dioxin eliciting ovulatory blockade in gonadotropin-primed immature rats. *Endocr J*. 2010; 57:71–863.
63. Barhoover MA, Hall JM, Greenlee WF, Thomas RS. Aryl hydrocarbon receptor regulates cell cycle progression in human breast cancer cells via a functional interaction with cyclin-dependent kinase 4. *Mol Pharmacol*. 2010; 77:201–195.
64. Watabe Y, Nazuka N, Tezuka M, Shimba S. Aryl hydrocarbon receptor functions as a potent coactivator of E2F1-dependent transcription activity. *Biol Pharm Bull*. 2010; 33:97–389.
65. Kalmes M, Hennen J, Clemens J, Blomeke B. Impact of aryl hydrocarbon receptor (AhR) knockdown on cell cycle progression in human HaCaT keratinocytes. *Biol Chem*. 2011; 392:51–643.
66. Xie G, Peng Z, Raufman JP. Src-mediated aryl hydrocarbon and epidermal growth factor receptor cross-talk stimulates colon cancer cell proliferation. *Am J Physiol Gastrointest Liver Physiol*. 2012
67. Asim M, Siddiqui IA, Hafeez BB, Baniahmad A, Mukhtar H. Src kinase potentiates androgen receptor transactivation function and invasion of androgen-independent prostate cancer C4-2 cells. *Oncogene*. 2008; 27:604–3596. [PubMed: 17667942]
68. Tian Y, Ke S, Denison MS, Rabson AB, Gallo MA. Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J Biol Chem*. 1999; 274:5–510.
69. Klinge CM, Kaur K, Swanson HI. The aryl hydrocarbon receptor interacts with estrogen receptor alpha and orphan receptors COUP-TFI and ERRalpha1. *Arch Biochem Biophys*. 2000; 373:74–163.
70. Swanson HI, Yang JH. The aryl hydrocarbon receptor interacts with transcription factor IIB. *Mol Pharmacol*. 1998; 54:7–671.
71. Kumar MB, Perdew GH. Nuclear receptor coactivator SRC-1 interacts with the Q-rich subdomain of the AhR and modulates its transactivation potential. *Gene Expr*. 1999; 8:86–273.
72. Kobayashi A, Numayama-Tsuruta K, Sogawa K, Fujii-Kuriyama Y. CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *J Biochem*. 1997; 122:10–703.

Fig. 1.

Expression of AhR mRNA and protein in LNCaP and C4-2 prostate cancer cell lines. (A) Total RNAs were isolated and semi-quantitative RT-PCR was performed to determine the mRNA expression of AhR in LNCaP and C4-2 prostate cancer cell lines. mRNA levels were normalized using L-19 which serves as an internal control. (B) Quantitative real-time PCR was performed to quantify the expression level of AhR. The relative concentration of each PCR product was determined using the $\Delta\Delta C_q$ calculation method. L-19 was used as an internal control. Data are expressed as mean $\pm$ SEM ($n = 3$), and were analyzed by ANOVA. (*) denotes statistically significant differences ($*P < 0.05$) between cell lines. (C) Total cellular proteins were separated by SDS polyacrylamide gel electrophoresis and blotted using an anti-AhR antibody (1:1000 dilution). Anti- β -actin was used as a loading control. (Lower panel) Each bar represents mean $\pm$ SEM ($n = 3$) and were analyzed by Student's *t*-test. (*) denotes statistically significant differences ($*P < 0.05$) between cell lines.

Fig. 2.

Constitutive AhR signaling in androgen independent C4-2 prostate cancer cell lines. (A) Subcellular localization of AhR in LNCaP and C4-2 cell lines by immunocytochemical staining. AhR was visualized by staining with rabbit anti-AhR polyclonal antibodies followed by FITC-conjugated goat anti-rabbit antibody. The nuclei were counter-stained with DAPI fluorescence dye. Images from FITC and DAPI-fluorescence channels were merged. Images were captured on an Olympus wide fluorescence microscope (400 $\times$ magnification). (B) Subcellular localization of AhR in LNCaP and C4-2 prostate cancer cells by nuclear and cytoplasmic fractionation. The nuclear extracts (nuc) and cytoplasmic fractions (cyto) were analyzed by western blotting for AhR protein expression. The relative level of AhR was normalized by the respective β -tubulin or topoisomerase level. (Lower panel) Bars represent mean + SEM of the corrected values from three independent experiments and (*) denotes constitutive nuclear levels of AhR that are significantly different between cell lines. (C) RT-PCR analysis of CYP1B1 mRNA expression in prostate cancer cells. Cells were treated with 10 μ M TCDD or vehicle control (DMSO) for 24 h. Total RNAs were isolated and semi-quantitative RT-PCR was performed to determine the

mRNA expression of CYP1B1 in LNCaP and C4-2 prostate cancer cell lines. mRNA levels were normalized using L-19 which serves as an internal control. (D) Quantitative real-time PCR was performed to quantify the expression level of CYP1B1. The relative concentration of each PCR product was determined using the $\Delta\Delta C_q$ calculation method. L-19 was used as an internal control. Data are expressed as mean $\pm$ SEM ($n = 3$), and were analyzed by ANOVA. (*) denotes statistically significant differences ($*P < 0.05$) between cell lines.

Fig. 3.

CYP1B1 expression is regulated by constitutively active/nuclear AhR. (A) Confirmation of AhR protein expression in C4-2 cells in comparison to scrambled vector control (SCR) and shRNA lentivirus against AhR (-AhR) by western blotting. 20 μ g of protein isolated from C4-2 parental cells (C4-2), C4-2 cells infected with an scrambled sequence control (SCR) and C4-2 cells infected with an AhR shRNA lentivirus (-AhR) was analyzed by western blotting. Upper panel is a representative blot and lower panel is the densitometric quantitation of AhR protein. Each bar represents mean $\pm$ SEM of three independent cultures. Values from different experimental analyses were each normalized internally to the respective C4-2 control before the mean and SEM was calculated. (*) denotes significant lower value than that of SCR control ($p < 0.05$). (B) RT-PCR analysis of CYP1B1 mRNA expression in control (SCR) and AhR depleted (-AhR) clones. Semi-quantitative RT-PCR was performed to determine the mRNA expression of CYP1B1 in cell lines. mRNA levels were normalized using L-19 which serves as an internal control. (C) Quantitative real-time PCR was performed to quantify the expression level of CYP1B1. The relative concentration of each PCR product was determined using the $\Delta\Delta C_q$ calculation method. L-19 was used as an internal control. Data are expressed as mean $\pm$ SEM ($n = 3$), and were analyzed by ANOVA. (*) denotes statistically significant differences (* $P < 0.05$) between cell lines. (D)

Subcellular localization of AhR in scrambled control (SCR) and AhR depleted (-AhR) C4-2 cells by immunocytochemical staining. AhR was visualized by staining with rabbit anti-AhR polyclonal antibodies followed by FITC-conjugated goat anti-rabbit antibody. The nuclei were counter-stained with DAPI fluorescence dye. Images from FITC and DAPI-fluorescence channels were merged. Images were captured on an Olympus wide fluorescence microscope (400× magnification). (E) Subcellular localization of AhR in scrambled control (SCR) and AhR depleted (-AhR) C4-2 cells by nuclear and cytoplasmic fractionation. The nuclear extracts (nuc) and cytoplasmic fractions (cyto) were analyzed by western blotting for AhR protein expression. The relative level of AhR was normalized by the respective β -tubulin or topoisomerase level. (Lower right panel) Bars represent mean + SEM of the corrected values from three independent experiments and (*) denotes constitutive nuclear levels of AhR that are significantly lower than SCR control.

Fig. 4.

Constitutive AhR signaling regulates androgen receptor expression and activity. (A) Subcellular localization of androgen receptor (AR) in scrambled control (SCR) and AhR depleted (-AhR) C4-2 cells by immunocytochemical staining. AhR was visualized by staining with mouse anti-AR monoclonal antibodies followed by rhodamine-conjugated goat anti-rabbit antibody. The nuclei were counter-stained with DAPI fluorescence dye. Images from rhodamine and DAPI-fluorescence channels were merged. Images were captured on a Zeiss fluorescence-enabled microscope (400 $\times$ magnification). (B) Subcellular localization of AR in scrambled control (SCR) and AhR depleted (-AhR) C4-2 cells by nuclear and cytoplasmic fractionation. The nuclear extracts (nuc) and cytoplasmic fractions (cyto) were analyzed by western blotting for androgen receptor (AR) protein expression. The relative level of AR was normalized by the respective β -tubulin and topoisomerase level. (Lower right panel) Bars represent mean + SEM of the corrected values from three independent experiments and (*) denotes constitutive nuclear levels of AR that are significantly lower than SCR control. (C) Comparison of AR and phosphorylated androgen receptor (p-AR)

protein expression in C4-2 scrambled vector control (SCR) and AhR shRNA depleted cells (-AhR) by Western blotting. 20 μ g of protein isolated from C4-2 cells infected with a scrambled sequence control (SCR) and C4-2 cells infected with an AhR shRNA lentivirus (-AhR) was analyzed by western blotting. Upper panel is a representative blot and lower panel is the densitometric quantification of androgen receptor (AR) and phosphorylated androgen receptor (p-AR) protein levels. Values in the graph are mean + SEM of three independent cultures. Values from different experimental analyses were each normalized internally to the respective SCR control before the mean and SEM was calculated. (*) denotes significant lower value than that of SCR control ($p < 0.05$). (D) RT-PCR analysis of androgen responsive genes mRNA expression in control (SCR) and AhR depleted (-AhR) clones. Total RNAs were isolated and semi-quantitative RT-PCR was performed to determine the mRNA expression of KLK2 and KLK3 in cell lines. mRNA levels were normalized using L-19 which serves as an internal control. (E) Quantitative real-time PCR was performed to quantify the expression level of KLK2 and KLK3. The relative concentration of each PCR product was determined using the $\Delta\Delta C_q$ calculation method. L-19 was used as an internal control. Data are expressed as mean $\pm$ SEM ($n = 3$), and were analyzed by ANOVA. (*) denotes statistically significant differences (* $P < 0.05$) between cell lines.

Fig. 5. Constitutive AhR signaling regulates growth of androgen-independent C4-2 cells. (A) Comparison of C42 (SCR) and C4-2 (-AhR) growth in FBS vs. CSS media. Cell growth was measured using Promega CellTiter 96 Cell Proliferation Assay per manufactures instructions. Each bar represents mean + SEM ($n = 3$), $*p < .05$. (B) Growth of scrambled vector control (SCR) in comparison to AhR depleted (-AhR) and LNCaP cells. Cells were serum starved for 24 h and grown in charcoal stripped (CSS) media for an additional 24, 48 or 72 h. At each endpoint, cells were analyzed for DNA content using the cell proliferation assay as described in the materials and methods. Each data point represents mean + SEM ($n = 3$) and were analyzed by ANOVA, $*p < .05$.

Table 1

Primer sequences.

Gene	Primer	Sequence (RT-PCR)	Sequence (qRT-PCR)
L-19	Forward (5'-3')	GAAATCGCCAATGCCAACTC	TCCCAGGTTCAAGCGATTCTCCTT
	Reverse (5'-3')	TCTTAGACCTGCGAGCCTCA	TTGAGACCAGCCTGACCAACATGA
AhR	Forward (5'-3')	TCCACCTCAGTTGGCTTTGTTGC	TCCTTGGCTCTGAAGCTCAAGCTGT
	Reverse (5'-3')	ATTCGGATATGGGACTCGGCACAA	GCTGTGGACAATTGAAAGGCACGA
CYP1B1	Forward (5'-3')	AACGTCATGAGTGCCGTGTGT	TGCCTGTCACTATTCCTCATGCCA
	Reverse (5'-3')	GGCCGGTACGTTCTCCAAATC	TCTGCTGGTCAGGTCCTTGTTGAT
KLK2	Forward (5'-3')	CATCGAACCAGAGGAGTTCTTGC	TTAAGTCCACCTCACGTTCTGGCA
	Reverse (5'-3')	GAAGCACACCATTACAGACAAGTGG	TACACCTGTGTCTGCCCATTCTT
KLK3	Forward (5'-3')	AACCAGAGGAGTTCTTGACCCC	ACTTCAGTGTGTGGACCTCCATGT
	Reverse (5'-3')	GACGTGATACCTTGAAGCACACC	AGCACACAGCATGAACTTGGTCAC