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Uncovering New Roles For The Aryl Hydrocarbon Receptor (AHR) In Breast Cancer

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UNCOVERING NEW ROLES FOR THE ARYL HYDROCARBON RECEPTOR (AHR) IN BREAST
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APPROVAL OF DISSERTATION

We, the faculty supervising the work of Justin Kirk Tomblin, affirm that the dissertation, *Uncovering New Roles for the Aryl Hydrocarbon Receptor (AHR) in Breast Cancer*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Travis Salisbury	Committee Chairperson	5/2/16 Date
Dr. Gary Rankin	Committee Member	5/3/16 Date
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DEDICATION

I dedicate this work to my wonderful girlfriend Caroline Hunter. You have helped keep me sane through this whole process, due in no small part to your love and support. You continue to believe in me even when I have no faith in myself. For this, I am forever grateful.

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Finally, thank you to my family and friends who have been so encouraging on this journey. To my parents, who have sacrificed so much to allow me to get to where I am today, I am eternally grateful. My only wish is to prove that your faith in me was not wasted.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that is responsive to many exogenous and endogenous ligands. AHR is of particular interest in cancer, and has been shown to play roles in tumor progression. As such, it has received growing attention as a possible chemotherapeutic target. Obesity increases the risk of breast cancer, complicates treatment of breast cancer, and stimulates the growth of larger, more aggressive mammary tumors. Many breast tumors in obese women are estrogen receptor (ER)-positive and, while targeting hormone receptors like ER is beneficial, many obese women see a recurrence of their cancer after standard chemotherapy regimens. Breast tumors also highly express AHR, which has made AHR targeting compounds (both agonists and antagonists) the subject of intense research in breast cancer models over the last decade. Our laboratory has uncovered several novel aspects of AHR signaling in response to cytokines, growth factors, and environmental toxicants, specifically the prototypical AHR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which underlie its role in tumorigenesis and tumor progression. We found that silencing AHR expression in breast tumor cells can block the growth response to adipokines (adipocyte-secreted factors), which are secreted into the breast tumor microenvironment by adipocytes. We have also shown AHR recruitment to the cyclin D1 (CCND1) gene promoter to increase the expression of this important oncogene involved in cell cycle progression upon insulin-like growth factor (IGF)-2 stimulus. AHR was also found to be necessary for basal and tumor necrosis factor (TNF) induced expression of superoxide dismutase 2 (SOD2), which encodes manganese superoxide dismutase (MnSOD), a crucial protein in the oxidative stress pathway. Finally, we have shown AHR is needed for the expression of solute carrier family 7 (amino acid transporter light chain, L system) member 5 (SLC7A5), which encodes L-type amino acid transporter 1 (LAT1) in breast tumor cells. The findings presented in this dissertation suggest

targeting the AHR with antagonists to treat breast cancer would be the most beneficial strategy, as AHR

has been implicated in several aspects of tumor initiation and progression.

CHAPTER I: THE ARYL HYDROCARBON RECEPTOR (AHR): A MULTI-FACETED

TRANSCRIPTION FACTOR.

1.1. INTRODUCTION AND BRIEF HISTORY OF AHR BIOLOGY.

Over the past 50 years, several advances in understanding the role of the aryl hydrocarbon

receptor (AHR) in cancerous and normal tissues have been made. The goal of this introductory section is

to provide some history of AHR research, before delving further into its complexity. During the

industrial revolution, the twentieth century experienced rapid growth which led to increased risk of

exposure to potentially hazardous environmental pollutants/toxicants. Chemical spills and accidental

exposure to halogenated or polycyclic aromatic hydrocarbons (HAH or PAHs) has unveiled a wide array

of detrimental health effects, which have been studied extensively by researchers [1]. Workers in

chemical factories face a particularly high risk for occupational exposure. One of the earliest PAH/HAH

chemical spills occurred in 1949 in Nitro, WV, not far from Marshall University, where workers were

exposed to toxic levels of the hallmark AHR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

TCDD is a toxic industrial byproduct found in herbicides like Agent Orange used during the Vietnam

War, as well as a byproduct of the paper bleaching process [2, 3]. Exposure to TCDD resulted in

chloracne, liver disease, leukemia, ischemic heart disease, and even death of some of the workers [4, 5].

However, the exact mechanism by which TCDD causes toxicity in humans is still unclear.

Some of the earliest research with PAHs resulted in the discovery of benzpyrene hydroxylase, an

enzyme named due to its ability to form hydroxylated products of 3,4-benzpyrene, but which would

quickly be renamed aryl hydrocarbon hydroxylase (AHH), as several PAHs induced its activity [6-8].

By the 1970s, numerous studies made it clear there was a direct correlation between induction of AHH

activity and increased cytochrome P450 1A1 (CYP1A1) activity, and that this response varied between

different mouse strains, specifically C57BL/6 and DBA/2 mice [9]. C57BL/6 mice were much more

responsive to 3-methylcholanthrene (3-MC)-stimulated increases in AHH activity than DBA/2 mice [10]. Researchers pondered whether diminished AHH activity in DBA/2 mice in response to 3-MC was due to these mice having a genetic defect, or whether they express a PAH/HAH receptor with reduced binding affinity [10]. Utilizing [^{3}H] TCDD, researchers found TCDD accumulated highly in the liver of C57BL/6 mice where it bound a receptor leading to induction of AHH activity [11]. In DBA/2 mice, the levels of radio-labeled TCDD in liver extracts were much lower, supporting the previous theory that they express a receptor with reduced binding affinity [11]. With these radio-labeled TCDD experiments, the Nebert lab christened this newly identified receptor, AHR [12]. During this time period, it was also determined that AHH and CYP1A1 were one and the same [13], and CYP1A1 will be used henceforth to refer to this important enzyme.

The hypothesis that DBA/2 mice expressed an altered AHR, while still speculation in the 1970's, was shown to be valid when the AHR was cloned in 1992 by the Bradfield group [14]. These studies revealed that reduced AHR ligand affinity was due to an altered AHR ligand-binding domain in these rodents [14]. Other studies found that mutations in the AHR ligand-binding domain were the reason for reduced AHR affinity for ligands in DBA/2 mice compared with C57BL/6 mice. When AHR coding sequences from the two strains were compared, two critical alterations were noted: 1) AHR had undergone a substitution (valine in place of alanine) at position 375 and 2) a T to C mutation in the stop codon was observed in the DBA/2 mouse strain [15]. The altered stop codon causes a lengthening of the carboxy-terminus in mature AHR protein in DBA/2 mice resulting in a ligand-binding domain with lowered ligand affinity [15]. The main advances in AHR biology after these initial findings were made in the uncovering of the canonical AHR signaling pathway, discussed in the next section.

1.2. CANONICAL AHR SIGNALING.

When bound by agonists such as TCDD, the AHR becomes an active transcription factor, moving into the nucleus to regulate expression of target genes. This section will go step by step through this well characterized process (summarized in Figure 1). In the cytoplasm, AHR is kept in an inactive state bound with two heat shock protein-90 (HSP90) proteins, which function as chaperone proteins for steroid hormone receptors as well (Figure 1) [16, 17]. While it was known that there was a third protein bound to AHR in its inactive state, its identity remained a mystery for some time. Practically simultaneously, three different research laboratories identified that this third protein that bound AHR in the cytoplasm was hepatitis B virus X-associated protein 2 (XAP2), however, its function still remains a mystery [18-20]. Later, p23 was also found to be a part of the cytoplasmic inactive AHR complex, and helps maintain AHR protein stability [21]. This complex keeps the nuclear localization signal of AHR concealed, preventing AHR from entering the nucleus in the absence of ligand [22, 23]. Upon TCDD binding, a conformational change occurs resulting in exposure of the nuclear localization signal and translocation of the now active complex into the nucleus (Figure 1). Once inside, XAP and p23 are released, and AHR binds via its basic helix-loop-helix (bHLH) motif to the aryl hydrocarbon nuclear translocator (ARNT), which is responsible for retaining the AHR in the nucleus (Figure 1) [24, 25]. Binding of ARNT results in the loss of HSP90 proteins [25], and this TCDD-AHR-ARNT complex can now actively drive transcription of genes.

Classically, the AHR mediates the expression of genes involved in phase I and phase II drug metabolism. These genes include those encoding monooxygenase enzymes such as the cytochrome P450s CYP1A1 and CYP1B1 [26]. This enzyme family is important for "first pass" metabolism in the liver, where a large portion of drugs and xenobiotics (meaning "foreign to the body") are bio-transformed [27]. CYP enzymes hydroxylate xenobiotics to aid in their elimination and excretion [27].
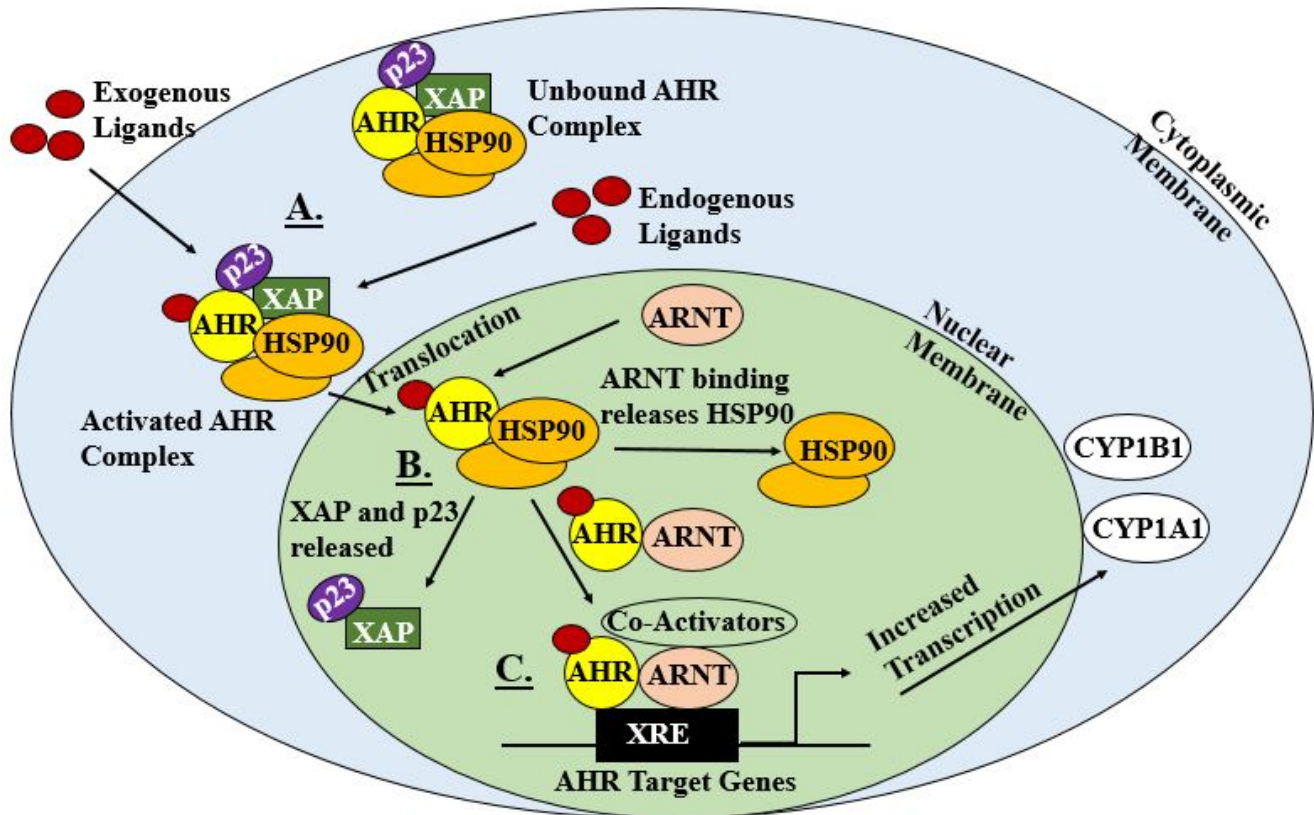
Figure 1. **The canonical AHR signaling pathway**. (A) Binding of agonist results in nuclear translocation of AHR. (B) Shedding of cytoplasmic chaperones and binding of ARNT occurs. (C) Active transcriptional complex binds at response elements to increase the expression of primary TCDD/AHR gene targets including CYP1A1 and CYP1B1. AHR = aryl hydrocarbon receptor, XAP = hepatitis B virus X-associated protein 2, HSP90 = heat-shock protein 90, ARNT = aryl hydrocarbon nuclear translocator, DRE/XRE = dioxin/xenobiotic response element, CYP1A1, CYP1B1 = cytochrome P450s 1A1 and 1B1. This figure created in Microsoft Powerpoint to summarize information found in references [16- 26].

Two enzymes in the family, CYP1A1 and CYP1B1, have been highly associated with cancer. CYP1A1 has been shown to regulate breast cancer proliferation and survival [28], in addition to cancer initiation via conversion of toxicants like benzo(α)pyrene (B(a)P) into genotoxic agents [29]. Silencing of CYP1A1 resulted in decreased colony formation, decreased proliferation, reductions in cyclin D1, and cell cycle arrest with increased apoptosis [28]. CYP1B1 has been touted as a "universal tumor marker," given its overexpression has been detected in many tumors, including lung, breast, and colorectal tumors [30]. CYP1B1 has been shown to convert estrogen into genotoxic metabolites like 4-hydroxyestradiol [31], one way it can initiate tumor formation. Glutathione-S-transferases (GSTs) are phase II enzymes whose expression is also induced by TCDD via AHR [32]. GSTs catalyze the conjugation of reduced glutathione (GSH) to xenobiotics to aid in detoxification [33]. Polymorphisms in the GST enzyme superfamily results in isozymes of various activity, which have been linked to chemotherapy resistance in tumors [34].

AHR and ARNT bind to specific regions in the DNA of target genes. These regions are termed AHR response elements (AHR-RE), often referred to in the literature as dioxin-response elements (DRE) or xenobiotic-response elements (XRE). The specific consensus DNA sequence for TCDD-AHR complex binding (5'-TGCGTGA-3') was first found in the CYP1A1 gene promoter [35], and lies upstream and proximal to the transcriptional start site (TSS) [35]. This site is recognized with high affinity by AHR-ARNT and confers transcriptional activity to TCDD-AHR target genes. The TCDD-AHR-ARNT complex recruits other co-activators of transcription such as the histone acetyltransferase p300, CREB-binding protein (CBP), and nuclear receptor co-activator 1 (NCOA-1) [36, 37]. CBP, NCOA-1, and p300 all activate transcription by acetylating histones. CBP and p300 interact, and when bound together, induce global acetylation of all sites on histones H2A and H2B, but preferentially acetylate lysines 14 and 18 of histone H3 and lysines 5 and 8 of histone H4 [38]. NCOA-1 preferentially

targets histones H3 and H4 for acetylation [39]. These proteins are followed by binding of the

chromatin-modifying protein, brahma-related gene 1 (BRG-1), a member of the ATP-dependent

SWI/SNF (SWItch/Sucrose Non-Fermentable)-like complex family, which utilizes ATP to facilitate

chromatin remodeling and the subsequent recruitment of basal transcriptional machinery to drive

expression of AHR target genes [40].

The discovery of AHR-RE/XRE was a landmark in AHR biology, and allowed for the

identification of other genes regulated by TCDD, such as ATP-binding cassette sub-family G member 2

(ABCG2) [41], which is involved in efflux drug transport in tumor cells, and aldehyde dehydrogenase

1A3 (ALDH1A3) [42], whose expression has been shown to be particularly high in breast cancer stem

cells (CSCs) [43]. Collectively, genes regulated by the canonical AHR pathway are highly associated

with tumors of various types, implicating AHR as an important transcription factor of merit when

studying any cancer, not just breast cancer. We identified and published ~140 TCDD regulated genes in

MCF-7 breast cancer cells (BCCs) using next generation RNA-sequencing (RNA-seq), which included

known TCDD targets CYP1A1, CYP1B1, ABCG2 and ALDH1A3 [44]. Using RNA-seq and comparing

our results with a previously published TCDD-AHR chromatin immunoprecipitation sequencing (ChIP-

seq) set, we identified and characterized for the first time TCDD/AHR regulation of solute carrier family

7 (amino acid transporter light chain, L system) member 5 (SLC7A5), which encodes L-type amino acid

transporter 1 (LAT1) in MCF-7 and MDA-MB-231 BCCs [44].

1.3. NON-CANONICAL AHR SIGNALING.

The AHR canonical signaling pathway, while very important, is only one way by which AHR

can alter gene expression. AHR can also "cross-talk" with other transcription factors. One of the earliest

reports suggesting AHR could interact with other proteins showed TCDD and epidermal growth factor

(EGF) worked cooperatively to suppress PPARγ activation and modulate focal adhesion complex

formation during adipogenesis [45]. In AHR-null mice, these effects on PPARγ and adipogenesis were reversed, revealing that TCDD inhibits triglyceride synthesis and adipocyte differentiation through an AHR-dependent mechanism [45]. Finding that Vietnam War veterans exhibited defects in thyroid function suggested that TCDD may also target the thyroid and its associated endocrine signaling [46]. AHR has also been shown to cross-talk with several transcription factors that regulate gene expression including the estrogen receptor (ER), androgen receptor (AR) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [47-49]. This section will summarize the ways in which AHR cross-talks with these important transcription factors.

Perhaps the most extensively studied of all is AHR-ER cross-talk (Figure 2). Experiments looking at long-term TCDD exposure in female Sprague Dawley rats were the first to suggest AHR and ER cross-talk, as TCDD inhibited the growth of estrogen-responsive mammary and uterine tumors [50]. Other reports established that TCDD inhibited 17β-estradiol (E2)-stimulated increases in the expression of ER target genes [51], however, the anti-estrogenic effects of AHR activation was not well understood for some time. The first hypothesis suggested that upon its induction by TCDD, CYP1A1 promoted the metabolism of E2 [52]. More recent reports have identified that TCDD-activated AHR can also induce the degradation of ER [53]and secondly, AHR can bind to inhibitory XREs found in ER target genes that are capable of suppressing gene expression [54]. Finally, TCDD and ER share common transcriptional coactivators, and TCDD, by stimulating AHR to bind such coactivators, induces a concomitant reduction in their binding to ER [55]. AHR also interacts with AR, and can play an important role in prostate development, as TCDD exposure either in utero or via breast milk has been shown to cause abnormal prostate growth [56].

AHR and NF-κB cross-talk has also been noted. In this regard, the expression of AHR is stimulated by NF-κB in dendritic cells treated with lipopolysaccharide (LPS) [49]. This study suggests
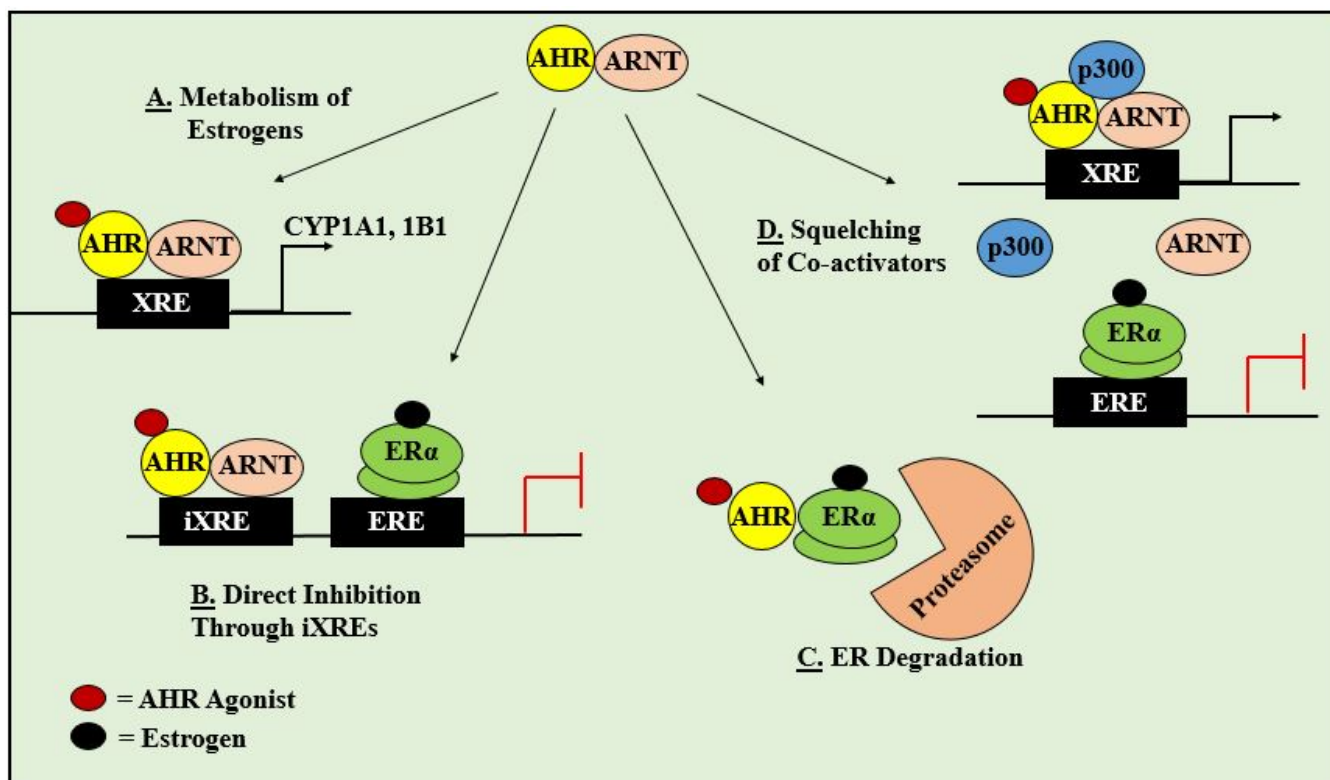
Figure 2. Mechanisms of AHR-ER crosstalk. (A) AHR upregulation of P450 enzymes leads to metabolism of estrogen, blunting its effects. (B) Agonist-bound AHR-ARNT complexes can bind to inhibitory response elements in ER target genes to silence expression. (C) Agonist-bound AHR targets ER for proteasomal degradation. (D) Activation of AHR squelches co-activator proteins preventing them from binding with ER transcriptional complexes. ERα = estrogen receptor alpha, AHR = aryl hydrocarbon receptor, ARNT = aryl hydrocarbon nuclear translocator, XRE = xenobiotic response element, ERE = estrogen response element, iXRE = inhibitory xenobiotic response element, CYP1A1, 1B1 = cytochrome P450s 1A1 and 1B1. This figure created in Microsoft Powerpoint summarizing information found in references [50-55].

inflammatory cytokines that activate NF-κB can induce AHR expression during immune cell activation

and differentiation. TCDD and NF-κB interact to promote robust increases in the expression of

interleukin (IL)-6 in MCF-7 breast cancer cells [57]. TCDD, in combination with IL-1β or phorbol 12-

myristate 13-acetate (PMA) treatment, resulted in synergistic increases in IL-6, which were decreased

by silencing either AHR or NF-κB with short interfering RNAs [57]. In another study, co-

immunoprecipitation (co-IP) experiments revealed AHR binds the RELA subunit of NF-κB [58]. TCDD

suppressed the binding of NF-κB to cognate NF-κB response elements in response to tumor necrosis

factor (TNF) and blocked NF-κB translocation to the nucleus in dendritic cells, with these effects

attributed to TCDD-induced AHR degradation (discussed in section 1.4) [58]. Our own data revealed

that AHR and NF-κB interact to regulate both basal and TNF-induced expression of superoxide

dismutase 2 (SOD2), which encodes manganese superoxide dismutase (MnSOD) in MCF-7 BCCs [59].

The importance of MnSOD in cancer is discussed in Chapter II. These results suggest an extensive

relationship between AHR and NF-κB, important for regulating the immune response as well as the

cytotoxic effects of TNF in tumor cells.

1.4. ATTENUATING AHR SIGNALING: TWO MECHANISMS.

This section will discuss the two main ways to "turn off" AHR signaling: 1) degrading AHR

through the ubiquitin-proteasome pathway (Figure 3) or 2) through transcriptional repression via the

AHR repressor (AHRR) (Figure 4). Several reports have demonstrated that TCDD-activated AHR

promotes the degradation of specific proteins, such as ER, by functioning as an E3 ubiquitin ligase [60,

61]. Consistent with its role as a ligand-activated E3 ubiquitin ligase, AHR becomes ubiquitinated [62],

and its levels are decreased in response to agonists like 3-MC and TCDD, via a proteasome-mediated

pathway (Figure 3) [60]. Once inside the nucleus, the ligand-activated AHR-ARNT complex interacts

with the ubiquitin ligase, cullin 4B (CUL4B) [61, 63]. Knockdown of CUL4B revealed that degradation
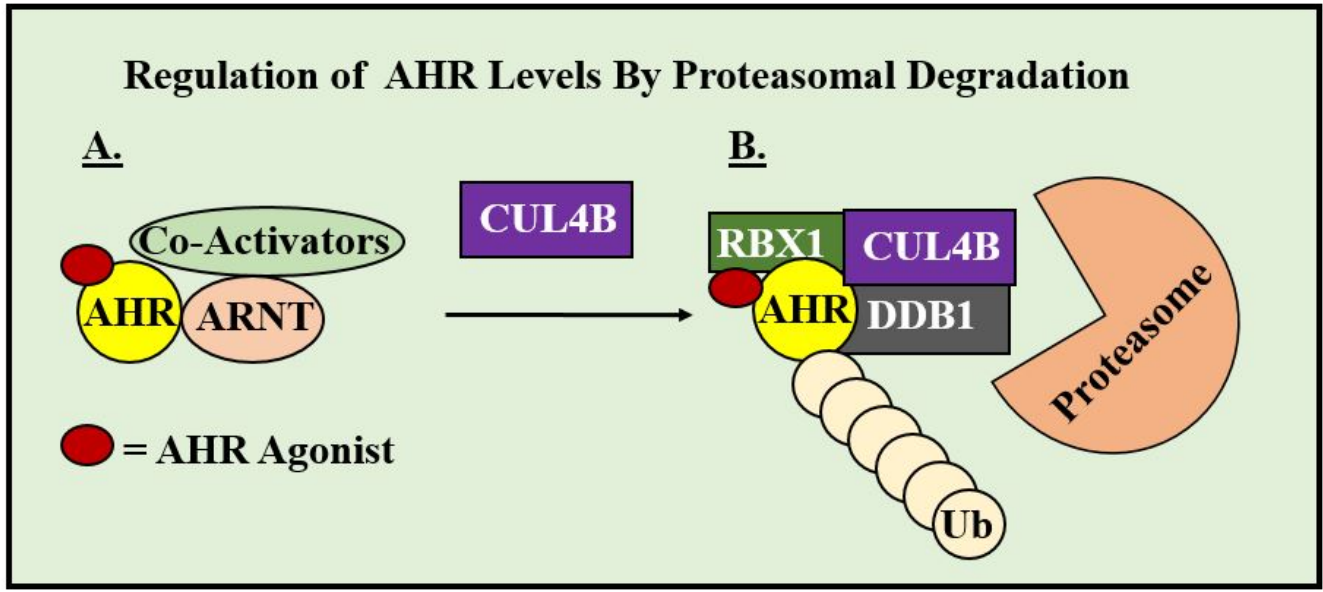
Figure 3. Degradation of agonist-bound AHR by proteasomes. (A) Agonist-bound AHR binds ARNT and co-activators forming an active transcriptional complex. (B) To shut off AHR signaling, CUL4B and other ubiquitinating enzymes poly-ubiquitinate AHR targeting it for degradation by proteasomes. AHR = aryl hydrocarbon receptor, ARNT = aryl hydrocarbon nuclear translocator, RBX1 = ring box-1, DDB1 = damaged DNA binding protein-1, Ub = ubiquitin, and CUL4B = cullin 4B. This figure created in Microsoft Powerpoint summarizing information found in references [60-65].
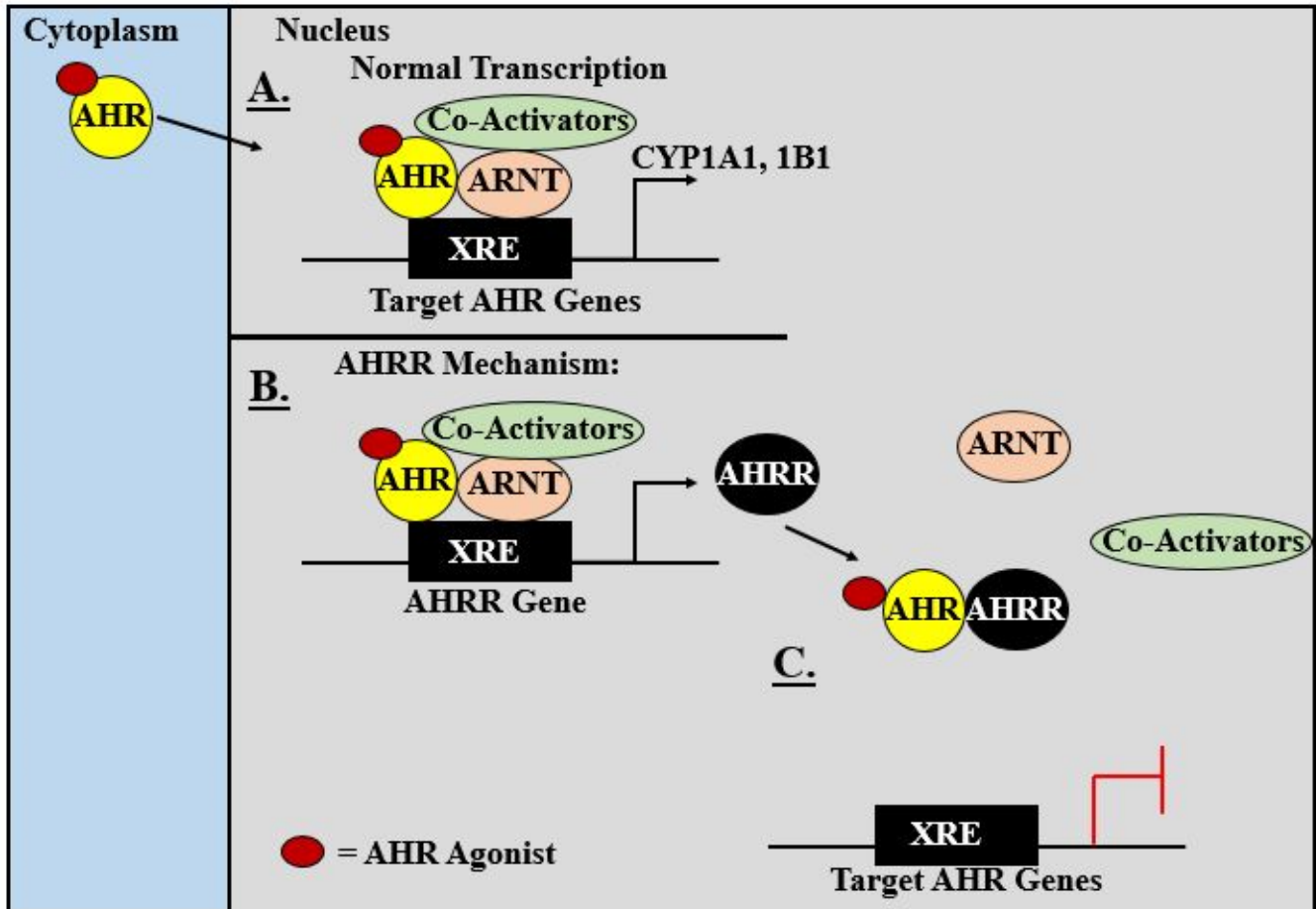
Figure 4. AHRR shuts down AHR signaling. (A) Agonist-bound AHR binds ARNT and co-activators to regulate expression of target genes such as CYP1A1 and CYP1B1. (B) Agonist-activated AHR also induces expression of AHRR. (C) AHRR protein competes with ARNT for binding AHR, which prevents active AHR complex formation and blocks binding at XREs to regulate gene expression. AHR = aryl hydrocarbon receptor, ARNT = aryl hydrocarbon nuclear translocator, XRE = xenobiotic response element, CYP1A1, 1B1 = cytochrome P450s 1A1 and 1B1, AHRR = AHR repressor. This figure created in Microsoft Powerpoint summarizing information found in references [66-69].

of AHR required expression of this critical enzyme, as it is needed for the recruitment of other enzymes, such as ring box-1 (RBX1) and damaged DNA binding protein-1 (DDB1), involved in the ubiquitination process [60]. The collective findings of three reports demonstrated that:1) immunoprecipitated AHR complex exhibits ubiquitinating activity [53, 64], 2) the binding of TCDD induces AHR to bind ER or AR, stimulating AHR, ER, and AR degradation (another example of AHR's anti-estrogenic and anti-androgenic properties) [53, 60], and 3) TCDD-bound AHR becomes ubiquitinated and is then degraded by proteasomes, as illustrated in Figure 3 [62, 65].

AHRR exhibits homology with AHR in the N-terminal domain, which coincidentally houses the already described bHLH motif critical for ARNT binding [66, 67]. Yet, the C-terminal domain of AHRR is quite unique [66, 67]. Two hypotheses were proposed to explain how AHRR inhibits AHR transcriptional activity. First, AHRR-ARNT heterodimers were speculated to interact with AHR-ARNT heterodimers to suppress AHR-ARNT transcription at gene promoters, and second, that AHRR competed for binding at XRE sites to silence AHR target gene expression [68, 69]. One seminal report by Evans et al. set out to determine which hypothesis was valid.

In this report, overexpression of ARNT failed to reverse AHRR inhibition of AHR signaling, which suggested squelching of ARNT was not the mechanism of AHRR induced repression [68]. An AHRR-mutant, which lacked ability to bind DNA, was still able to suppress AHR signaling, suggesting AHRR-ARNT heterodimers were not competing with AHR-ARNT heterodimers for binding at XRE sites to block AHR signaling [68]. Other experiments in this report found mutating AHRR C-terminal domain had no effect on repression either [68]. These results helped solidify the now accepted hypothesis of AHRR functioning, that AHRR binding to AHR via its N-terminal domain displaces ARNT from AHR which blunts AHR signaling. Our own RNA-seq data set found that AHR induced AHRR levels in MCF-7 BCCs [44], lending further credence to this model by which AHRR in response

to TCDD prevents excessive TCDD-AHR signaling by antagonizing AHR activity. Specifically, upregulation of AHRR expression results in higher AHRR levels which would then bind AHR protein to block its signaling via this negative-feedback mechanism (Figure 4).

1.5. TCDD AND AHR ROLES IN DEVELOPMENT, REPRODUCTION, AND IMMUNITY.

In the 1990's, three separate groups developed AHR-null (*Ahr-/-*) mice. This was carried out through deletion of either exon 1 or exon 2 of the AHR gene [70-72]. With these mice, endogenous roles for the AHR were uncovered, including roles in immunity, proper organ development, and reproduction. AHR-null mice exhibit decreased accumulation of lymphocytes in the spleen and lymph nodes, but not in the thymus, compared to wild-type, suggesting a role for the AHR in innate immunity [70]. This observation was built upon years later by others who uncovered that immune responses are modulated by AHR and the outcome is dependent on the AHR ligand. As an example, T regulatory cells (Tregs), which suppress excessive immune responses, are stimulated to differentiate in response to TCDD or kynurenine (kyn), leading to enhanced immunosuppression [73, 74]. Kyn is an endogenous tumor promoting ligand and product of tryptophan catabolism [75], as is 6-formylindolo [3,2-b] carbazole (FICZ) [76]. In contrast to TCDD or Kyn, FICZ enhances the differentiation of naïve T cells into proinflammatory T helper 17 (Th17) cells [77]. The dietary AHR ligands indolo [3,2-b] carbazole (ICZ) and 3,3-diidolylmethane (DIM) have been shown to play a role in the immune response as well. Cruciferous vegetable-derived indole-3-carbinol (I3C) is converted by stomach acid to the products, ICZ and DIM, which are high-affinity AHR ligands in the gut [78]. ICZ/DIM-activated AHR aids in the maintenance of gut microbiota, intraepithelial lymphocytes, IL-22 expression, and Th17 activity [78]. These studies helped establish that developmental and functional immunity is AHR-dependent.

The liver expresses higher levels of AHR than all other tissues [79]. Proper liver development has been shown to be AHR-dependent in rodents [71]. AHR-null mice exhibit smaller liver size as well

as thickening and fibrosis of the portal vein [71]. These mice also had moderate inflammatory changes in the bile ducts (cholangitis), which was postulated as a reason for their slowed and diminished growth compared to wild-type mice [80]. Indeed, in addition to growth, a role for AHR in the developing fetus has also been established, as AHR mediates the teratogenic effects of TCDD. Female Holtzman rats exposed to TCDD exhibited dramatic reductions in ovarian follicle number and size, revealing AHR activation effects follicle maturation [81]. Another report revealed TCDD exposure altered estrous cycling and blocked ovulation [82, 83]. TCDD has also been shown to induce the atypical release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in female Sprague Dawley rats [84]. TCDD exposure to pregnant rodents also induces cleft palate and kidney deficiencies in offspring [85]. When wild-type and AHR-null mice were gavaged with TCDD during pregnancy, only offspring from wild-type mice developed cleft palate and hydronephrosis [85].

Mouse studies have revealed roles for AHR in mammary development and function also, including pregnancy associated changes in the mouse mammary gland, and in utero mammary gland development. In this regard, detectable levels of AHR occur as early as day ten in the gestational period [86]. In addition to previously mentioned liver deficits, AHR-null mice have improper development of the mammary gland as well. Deletion of the AHR gene in mice leads to reductions in mammary gland size and fewer mammary gland terminal end buds, which are the proliferative structures found at the tips of ducts, compared with wild-type mice [87]. In utero TCDD exposure to normal mice caused defects in their mammary development, including altered lactogenesis, but this effect was variable and suggested critical periods of exposure during pregnancy [87, 88]. Mice exposed to TCDD during pregnancy also had reduced circulating levels of several hormones, including prolactin and E2, when compared to control mice [89]. Collectively, these reports underline an importance for AHR and its activation in

proper organ development, proper functioning of the reproductive system, and regulation of the immune system.

1.6. AHR AND CANCER.

AHR and cancer have been linked by numerous reports. One study found that after exposure to 7,12-dimethylbenz[a]anthracene (DMBA), a potent tumor promoter, AHR levels were dramatically higher in breast tumors relative to normal breast tissue in rats [90]. Aberrant high AHR expression and constitutively active AHR is common in many cancers including breast and prostate, even in the absence of exogenous AHR ligands, and contributes to increased invasiveness and metastasis [91-93]. AHRR has been shown to be a tumor suppressor in breast models [94], further linking AHR to development and progression of breast cancer. Endogenous AHR ligands like kyn and kynurenic acid have recently been established to promote tumor survival of breast tumors and gliomas while also suppressing the immune response through both autocrine and paracrine mechanisms [75, 95]. The Cancer Cell Line Encyclopedia has uncovered aberrantly high AHR expression in a number of malignant cell lines, including the neuroblastoma line CHP-212 and the endometrial cancer line HEC-151 [96]. Overexpressing AHR in a normal human mammary epithelial line caused these cells to exhibit traits of malignancy such as increased epithelial-to-mesenchymal transition (EMT), a process where cells lose cell-cell adhesion proteins and gain migratory properties leading to increased cell migration and invasion [97]. This report found that overexpressed AHR is sufficient to drive the transition from a normal breast epithelial cell to a malignant phenotype. The classic AHR gene target, CYP1B1, has also been found to be highly expressed in many malignant tumors, while the surrounding healthy tissue has minimal to no CYP1B1 expression [30, 98]. High CYP1B1 expression has been noted in cancers of the lung, colon, prostate and breast [30]. As mentioned previously, CYP1B1 metabolism of E2 to genotoxic metabolites like 4-hydroxyestradiol also promote cancer, independent of ER activation [31]. Elevated levels of CYP1B1 in

15

tumors is also believed to play a role in chemotherapeutic resistance. For instance, elevated CYP1B1 has been linked to docetaxel treatment failure, as the enzyme metabolizes the active drug to inactive metabolites and reduces docetaxel efficacy [99].

AHR promotes cancer via transcriptional mechanisms and by functioning as a scaffold protein for cell cycle promoting proteins. In regards to transcriptional mechanisms, TCDD-activated AHR regulates the expression of Slug, an important transcription factor that is one of the main drivers of EMT [100]. ChIP analysis revealed AHR binds to XRE in the Slug gene promoter to regulate expression [100]. Slug then regulates the expression of E-cadherin, with loss of E-cadherin a classic mark of EMT, as cadherin loss results in less cell-cell adhesion and promotes metastasis [101]. In this regard, DMBA and TCDD have also been shown to suppress the transcription of T-cadherin via AHR [102]. AHR activation also increases the levels of matrix metalloproteinase (MMP)-9 and MMP-1 which aid in degradation of extracellular matrix and are crucial enzymes for metastasis to occur [103, 104]. AHR can also function as a scaffold protein for regulating the phosphorylation of retinoblastoma protein (Rb), a key player in cell cycle progression in tumor cells [105]. When Rb is hypophosphorylated, it prevents expression of G1 to S phase target genes regulated by E2F transcription factor 4 (E2F4) by binding and sequestering E2F4 [106]. AHR was revealed to be needed for the hyperphosphorylation of Rb and subsequent release of E2F4 to occur in BCCs [105].

Two important papers from the Eltom lab have characterized AHR levels in the increased aggressiveness of triple-negative breast cancer (TNBC). One report utilizing the TNBC model cell line, MDA-MB-231, illustrated that knockdown of AHR resulted in reduced expression of many important genes relevant in cancer, including ABCG2, mucin-1 (MUC1) and IL-8, which are associated with chemotherapy resistance, tumor survival and poor prognosis in patients [107]. Another report showed AHR knockdown in TNBC cells reduced anchorage-independent growth, as well as growth of

16

xenografts and lung metastasis in mice, again using MDA-MB-231 cells [108]. An important recent study has linked the kyn-AHR pathway in TNBC cells to play a role in anoikis resistance [109]. Anoikis is a form of programmed cell death which normal epithelial cells undergo if detached from the basement membrane [110]. Inhibition or silencing of AHR or tryptophan dioxygenase 2 (TDO2), the enzyme which produces kyn, decreased anchorage-independent growth and made SUM-159pt cells more sensitive to anoikis [109]. Recently, we have published that knockdown of AHR and AHR regulation of SLC7A5 expression was important for MDA-MB-231 BCC growth also [44]. Collectively, researchers have linked AHR to tumor initiation, tumor invasion, metastasis, and EMT, revealing the complexity by which AHR functions in tumors (Table 1).

Our laboratory has been focused over the past four years in further uncovering tumor promoting roles for AHR (Figure 5). We found that AHR is necessary for the proliferative response to adipocyte-secreted factors (termed adipokines) in breast tumor cells (Chapter IV) [111]. This growth response is especially important in the context of obesity, where obese women have a greater incidence for breast cancer, are more resistant to cancer therapy, and have a higher rate of breast cancer recurrence than lean women with breast cancer [112, 113]. We also found that induction of cyclin D1 (CCND1) by the adipokine insulin-like growth factor (IGF)-2 is mediated by AHR (Chapter V) [114]. CCND1 is an important cell-cycle regulator important for actively dividing tumor cells to move through G1-S phase of cell division [106]. CCND1 is also an oncogene that is overexpressed in breast tumors compared with normal breast tissue [115]. Thus, our finding that AHR increases CCND1 expression in BCCs could have important clinical implications. We then found that regulation of SOD2 by TNF also required AHR (Chapter VI) [59]. Considering that SOD2 encodes manganese superoxide dismutase (MnSOD), a mitochondrial protein that reduces reactive oxygen species (ROS) [116], our findings suggest a novel mechanism by which AHR, by increasing MnSOD, lowers ROS in breast cancer cells stimulated

AHR in Cancer		
Processes	**Specific Role**	**References**
Cell Cycle Progression	Functions as scaffold protein for regulating Rb phosphorylation	[105]
Epithelial to Mesenchymal Transition	Regulates transcription of Slug, Downregulates cadherins	[100-102]
Metastasis	Regulates expression of matrix metalloproteinases (MMPs)	[103,104]
Anoikis	Endogenous AHR ligands linked to resistance to anoikis in triple-negative breast cancer	[109]

Table 1. Characterized roles for the AHR in cancer.
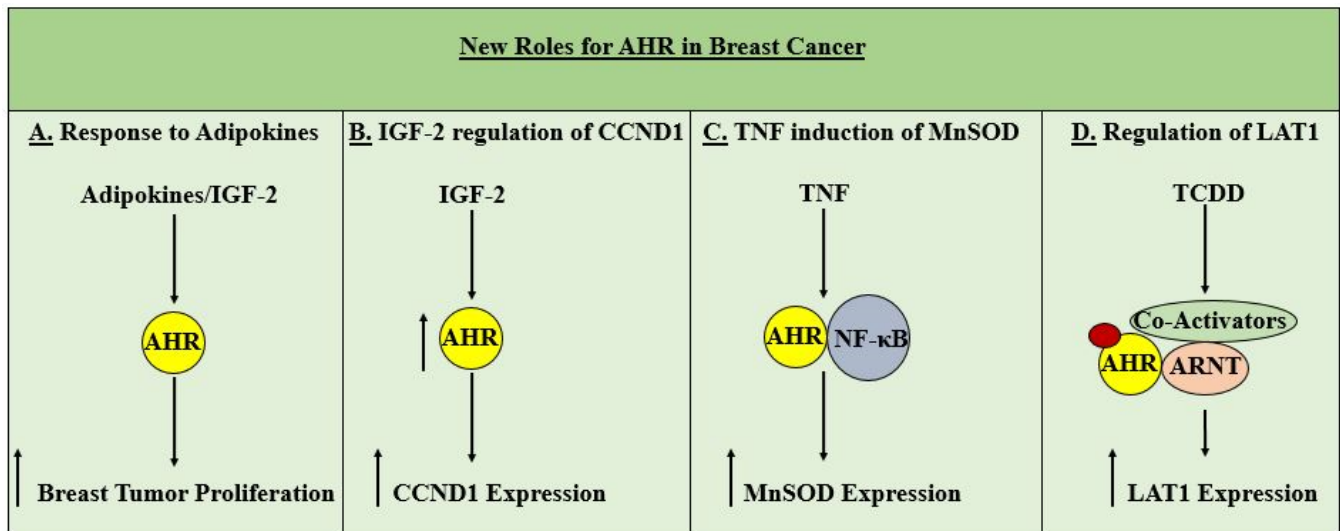
Figure 5. New roles for AHR in breast cancer. (A) Adipokines, including IGF-2, increase the proliferation of breast tumor cells, which we found to be AHR-dependent. (B) IGF-2 increases of AHR, which then binds to the promoter of CCND1 at two sites to induce CCND1 expression. (C) TNF regulation of SOD2, which encodes MnSOD, requires interactions between AHR and NF-κB. (D) TCDD and endogenous signaling stimulates AHR regulation of LAT1. AHR = aryl hydrocarbon receptor, IGF-2 = insulin-like growth factor 2, TNF = tumor necrosis factor, NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells, MnSOD = manganese superoxide dismutase, TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, LAT1 = L-type amino acid transporter 1. This figure summarizes the important findings of my research and was made in Microsoft Powerpoint.

with TNF. Indeed, we demonstrated that AHR knockdown BCCs are more sensitive to TNF-induced cell death compared with AHR expressing BCCs [59]. This is an important finding as it is suggests a role for AHR in reactive oxygen species maintenance and TNF-induced tumor cell death. Finally, we have shown that AHR is needed for regulation of solute carrier family 7 (amino acid transporter light chain, L system) member 5 (SLC7A5) which encodes L-type amino acid transporter 1 (LAT1) (Chapter VII) [44]. LAT1 is an important transporter which brings in amino acids such as leucine and tryptophan to drive protein synthesis in tumor cells, helping to maintain tumor proliferation [117]. To better understand these findings, the next chapter summarizes the roles of each of these important genes in cancer, as well as the effects of various secreted factors by adipose tissue important in obesity-driven cancers.

CHAPTER II: OBESITY, ADIPOKINES, AND GENES OF INTEREST IN BREAST CANCER.

2.1. OBESITY AND BREAST CANCER.

Epidemiological data has revealed that the prevalence of obesity has steadily increased over the past 25 years [118], continuing to be a major health concern in the United States. Adipose tissue is a potent endocrine gland and larger amounts of adipose tissue contributes to the chronic low grade inflammation seen in obese patients compared with lean patients [119]. Adipose tissue secretes a variety of peptide hormones, termed adipokines [120]. This cocktail of factors can influence multiple processes, including regulation of energy homeostasis (leptin, adiponectin, resistin), insulin sensitivity (adiponectin), vascular growth (vascular endothelial growth factor (VEGF)), and the immune response (interleukin (IL)-6 and tumor necrosis factor (TNF)) [120]. In the literature, there is evidence that obesity is a risk factor for certain cancers, including post-menopausal breast cancer [112, 113]. In pre-menopausal women, the ovaries are the primary source of circulating estrogen [121]. In contrast, adipose tissue, not the ovaries, is the major source of estrogen in obese post-menopausal women [122]. Estrogen can cause tumor formation, and supports the growth of established ER-positive breast tumors [121]. Estrogen production by adipose tissue after menopause, via aromatization of androstenedione in adipose tissue to estrone and estradiol, is speculated to be a main component of obesity-driven increases in post-menopausal breast cancer [122]. While estrogen is certainly a dominant factor, it was postulated that the lower levels of adiponectin coupled with higher levels of leptin seen in obese women could increase breast cancer incidence and stimulate the growth of larger, more aggressive breast tumors in obese women compared with lean women [123]. In addition to high levels of leptin, additional adipokines that stimulate proliferation of ER-positive breast cancer include collagen VI, VEGF, insulin-like growth factor (IGF)-1, and IGF-2 [124-126]. In the following sections, the effects and mechanisms of these cancer promoting adipokines will be discussed (Figure 6).
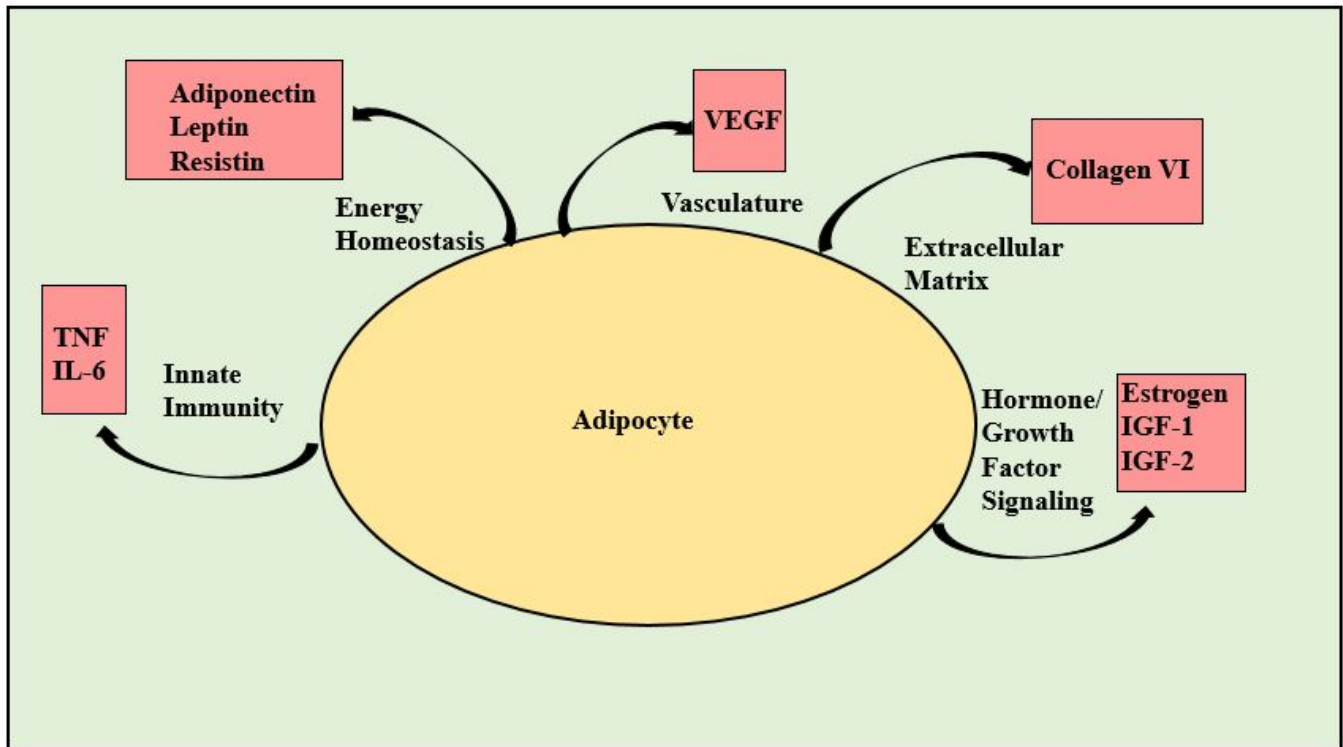
Figure 6. Important secreted adipokines and their roles in the body. Adiponectin, leptin, and resistin play roles in regulating energy homeostasis. Vascular endothelial growth factor (VEGF) is important in angiogenesis. Collagen VI is an extracellular matrix protein also important for proper angiogenesis to occur. Estrogen and insulin-like growth factors (IGFs)-1 and 2 are potent endocrine signaling proteins important in growth and changing gene expression in target tissues. Tumor necrosis factor (TNF) and interleukin (IL)-6 are involved in maintaining proper immune system responses.

2.2. ADIPONECTIN AND CANCER.

Adiponectin plays roles in cancer, vascularization, and maintains insulin sensitivity [127]. Adiponectin, often referred to as ACPR30, exists in serum in three forms: as a trimer, a hexamer, and as a high molecular weight isoform [128]. Epidemiological studies have revealed that low circulating levels of adiponectin are associated with increases in breast cancer risk [129]. For instance, low serum adiponectin concentrations were associated with larger, more aggressive breast tumors [129]. Adiponectin suppresses the growth of MDA-MB-231 and MCF-7 BCCs, an effect mediated by binding cognate adiponectin receptors AdipoR1 and AdipoR2 [130, 131]. These two receptors exert different effects upon binding, as determined from *in vivo* studies. AdipoR1- and AdipoR2-deficient mice were bred and used to distinguish important aspects of these two receptors [132, 133]. Strikingly, AdipoR1-deficient mice exhibited severe metabolic dysfunction and diet-induced weight gain compared to wild-type mice, whereas AdipoR2-deficient mice were protected from perturbations in metabolism, suggesting AdipoR1 activation suppresses metabolic dysfunction in the context of a high fat diet [132, 133]. AdipoR2 was shown to be more important in mediating revascularization effects of adiponectin [133]. Adiponectin is anti-angiogenic as well, inducing apoptosis of endothelial cells *in vivo* [134]. Adiponectin also promotes insulin signaling, through binding its cognate receptors and activating the adenosine monophosphate dependent kinase (AMPK) pathway [135]. AMPK activation by adiponectin is the main driver of its insulin sensitizing effects [135]. The diabetes drug, metformin, has also been shown to activate AMPK which explains its insulin sensitizing properties as well [136]. Low serum adiponectin levels result in chronic insulin resistance, and subsequent hyperinsulinemia [137]. Breast tumors express the insulin receptor (IR), and insulin binding to IR on BCCs stimulates their proliferation and inhibits apoptosis [138, 139]. Thus, hyperinsulinemia in obesity is postulated to promote the growth of larger, more aggressive breast tumors in obese women compared with lean women. Therefore, there

23

are three ways that low adiponectin levels in obesity can contribute to cancer growth: less adiponectin to bind its cognate receptors to inhibit cancer proliferation [130, 131], hyperinsulinemia which can drive tumor growth [138], and increases in angiogenesis [134].

2.3. LEPTIN AND CANCER.

The obesity-associated increases in leptin have been attributed to the increased number of adipocytes and increased leptin expression by adipose tissue in obese women compared with lean women [140]. Leptin binds its cognate receptor, leptin receptor (LEPR), which activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to elicit changes in the expression of genes that are important in cancer including the important cell cycle regulator CCND1, discussed in depth in section 2.9 [141]. The action of leptin is not limited to breast cancer, as it also regulates thermogenesis, inhibits appetite, and stimulates the metabolism of fat to maintain a normal body weight [142]. In obesity, studies suggest high serum leptin enables it to act as a potent breast tumor signaling hormone, as breast tumor cells express LEPR [143]. Case-control studies have provided evidence for this, as high serum leptin levels correlated with increased breast cancer incidence in several reports [144-146]. Dramatic evidence for leptin in breast cancer was provided by studies performed in obese mice with defects in the leptin receptor isoform B (LEPR-b), considered the major active isoform of LEPR [147]. Mice with non-functional LEPR-b were crossbred with transforming growth factor alpha (TGFα) oncogene-expressing mice (MMTV-TGFα mice) [147]. Female offspring with non-functional LEPR-b did not develop oncogene-driven breast tumors, even with circulating leptin levels 12-20-fold higher than in lean mice, whereas female offspring with functional LEPR-b had an 80% incidence rate [147]. Leptin triggers LEPR-positive cancer stem cell (CSC) self-renewal through JAK/STAT pathway activation [148]. CSCs are cells within a tumor that have a high capacity to initiate tumor formation [149]. Importantly, silencing LEPR in MDA-MB-231 BCCs inhibited the expression of stem cell self-

24

renewal transcription factors NANOG, SOX2, and OCT4, due to loss of JAK/STAT activation [150]. LEPR-null MDA-MB-231 cells also lost many of their metastatic properties and underwent a mesenchymal-to-epithelial transition, with a gain of E-cadherin, an adhesion protein needed for cell-cell interactions, and loss of vimentin, an intermediate filament protein highly expressed in mesenchymal cells which helps anchor organelles [150].

2.4. RESISTIN AND CANCER.

Resistin is secreted from monocytes and adipocytes and is known to play roles in atherosclerosis, stroke, and many cancers [151-153]. These cancers include colorectal, endometrial, and prostatic cancers, in addition to breast cancer [154-158]. One case-control study found that serum resistin levels were much higher in women with breast cancer when compared with women without breast cancer [156]. Another study found that breast tumors express resistin, and that resistin expression was correlated with a poor prognosis [159]. Resistin expression in breast tumors is likely mediated by estrogen, as a prior report noted estrogen induced resistin expression in 3T3-L1 adipocytes, which required ER and extracellular regulated kinase (ERK) activation [160]. Resistin secretion by adipocytes in the obese state could also be due to higher circulating levels of estrogen noted in obese patients [161]. Resistin also likely contributes to the pro-inflammatory environment noted in obesity, as numerous reports have linked resistin to inflammatory processes [162-164].

2.5. IGFS, INSULIN, AND CANCER.

We found that adipocytes in cell culture secrete high levels of IGF-1 and IGF-2 [111]. The insulin and IGF family of proteins have been linked extensively to cancer, and exert their effects through binding IR-A, IR-B, the IGF-1 receptor (IGF1-R), or the hybrid IGF1-R/IR receptor, resulting in activation of various signaling cascades (Figure 7) [165, 166]. IGF1-R can be activated by both IGF-1
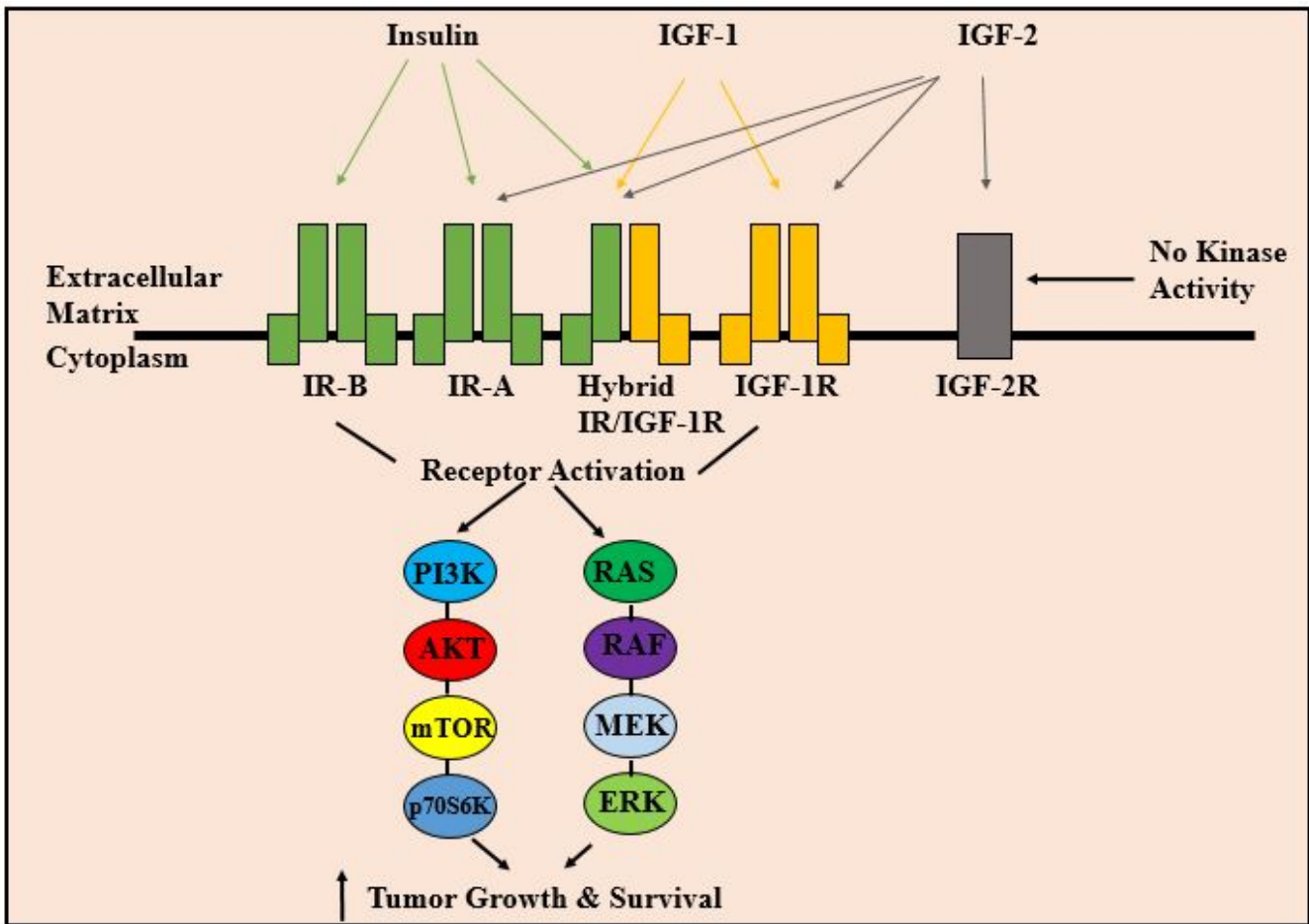
Figure 7. The insulin and IGF family signaling pathways. Insulin, insulin-like growth factor (IGF)-1, and IGF-2 can activate various receptors. IGF-1 can activate its cognate receptor, the IGF-1 receptor (IGF1-R) or the hybrid IGF1-R/insulin receptor (IR) receptor. Insulin can activate IR-A, IR-B, or the hybrid IGF-1/IR receptor. IGF-2 can bind its own cognate receptor, the IGF-2 receptor (IGF-2R), IGF1-R, IGF1-R/IR, or IR-A. IGF-2R has no kinase activity, yet activation of the other receptors leads to activation of downstream signaling cascades as noted in the figure. PI3K = phosphatidylinositol-3-kinase, AKT = protein kinase B, mTOR = mammalian target of rapamycin, S6K = ribosomal S6 kinase, MEK = mitogen activated protein kinase kinase, ERK = extracellular regulated kinase. This figure made in Microsoft Powerpoint to summarize information in references [165, 166].

and IGF-2, with IGF-1 having higher affinity [166]. IGF1-R/IR preferentially binds IGF-1 and IGF-2, with lower affinity for insulin [166]. IR exists in two isoforms: IR-B, which are the traditional receptors which insulin binds, and IR-A, a fetal form of IR expressed on certain tumors, including breast tumors, which preferentially binds IGF-2 [166]. IGF-2R is a non-signaling receptor with no kinase activity, and acts to internalize and degrade circulating IGF-2 [166].

Among insulin/IGF receptors, IGF1-R has received the greatest attention in cancer [165]. Some of the earliest reports linking IGF1-R to cancer found that transformation of mouse embryo fibroblasts (MEFs) by oncogenes such as SV40 large T antigen and c-Src required an intact, functional IGF1-R gene [167, 168]. Overexpression of the oncogene K-Ras in mouse mammary glands stimulated formation and growth of IGF1-R-overexpressing mammary tumors [169]. These tumors resembled human basal-like breast tumors, which are resistant to current cancer treatment options [169]. K-Ras overexpressing mammary tumors in these mice were shown to require IGF1-R for growth, as gene deletion experiments targeting the IGF1-R gene delayed the growth of these tumors [169]. Treatment with the IGF1-R inhibitor picropodophyllin (PPP) slowed the growth of K-Ras overexpressing mammary tumors, as well as MDA-MB-231 breast cancer xenografts in mice [169]. Other prior reports identified that breast tumors grew faster in wild-type mice than in IGF-1-deficient mice [170]. Exogenous IGF-1 administered to mice increased the growth and metastasis of colon cancer [171]. Acromegaly, a condition characterized by growth hormone excess, is associated with higher incidence of colon cancer, attributed to overproduction of IGF-1 [172]. In contrast, Laron-type dwarfism is associated with low serum IGF-1 levels and less risk for developing tumors [173]. Collectively, these reports have revealed that the IGF signaling axis promotes both the transformation and the progression of many cancers in both rodents and humans.

As noted, insulin resistance and subsequent hyperinsulinemia occurs frequently in obesity [112, 113]. Breast tumors are responsive to the mitogenic and anti-apoptotic effects of insulin, as breast tumors express IR [138, 139]. Researchers have worked extensively to uncover the role of insulin and IR in cancer, with the majority of this work done by the LeRoith group. They developed a transgenic mouse model which expresses a dominant-negative IGF1-R specifically in skeletal muscle [174]. The formation of hybrid receptors between the mutant and endogenous IGF1-R and IR occurred, resulting in ligands failing to bind and activate these receptors [174]. These mice, called MRK mice, therefore developed insulin resistance and hyperinsulinemia [174]. Breast tumor cells grafted into the mammary fat pad of MRK mice grew faster than in wild-type mice, attributed to higher circulating insulin in MRK mice [139]. Breast tumors from MRK mice exhibited higher levels of IR signaling activation than control mice, including the AKT and mammalian target of rapamycin (mTOR) pathways [175]. The PI3K/AKT/mTOR pathway is a crucial signaling node in tumor cells, and 70% of breast tumors exhibit hyperactivation of this pathway [176]. Dosing with the PI3K inhibitor NVP-BKM120 or the dual PI3K/mTOR inhibitor BEZ235 reduced breast tumor size in MRK mice [175]. High levels of insulin in these mice specifically activated IR on tumor cells, not IGF1-R [175]. When mice were given the insulin analog AspB10, which selectively targets IR, they developed larger mammary tumors than mice administered vehicle control [175]. In all, these reports reveal hyperinsulinemia is sufficient to drive breast tumor growth.

2.6. TNF, IL-6, AND CANCER.

There is now a sufficient body of evidence that chronic inflammation is conducive to tumor development and tumor progression [177, 178]. Two important cytokines involved in inflammation and the immune response, TNF and IL-6, are also secreted by adipocytes and will be reviewed in this section [179, 180]. TNF is a complex cytokine that regulates cancer and high serum TNF levels have been

shown in cancer patients [181]. Unlike normal tissue, it has been revealed that tumors of various types, including those of skin, ovarian, and pancreatic cancers, produce TNF [182]. In ovarian cancer, TNF has been shown to promote angiogenesis and leukocyte infiltration in the ovarian cancer microenvironment [183]. In a lung cancer model, TNF produced by malignant cells contributed to increased permeability of the vasculature leading to malignant pleural effusion (MPE), where fluid buildup occurs between the thin layers of tissue lining the outer lung and chest cavity [184]. TNF promoted the adhesion and migration of MDA-MB-231 BCCs through induction of lectin-like oxidized-lowdensity lipoprotein (oxLDL) receptor-1 (LOX-1) in endothelial cells, which promotes metastasis [185]. Blocking TNF in a mouse model was shown to reduce the development of colorectal cancer resulting from chronic colitis [186]. Macrophage-derived TNF in the microenvironment was shown to activate Wnt signaling in gastric cancer as well [187]. Indeed, given adipocytes secrete TNF into the microenvironment [188]; adipocyte-derived TNF illustrates another way obesity can contribute to cancer growth.

High serum IL-6 levels have been shown to be a good prognostic marker, as patients with endometrial, ovarian, and hormone-refractory prostate cancers exhibit increased IL-6 levels [189-191]. In breast tumors, IL-6 mRNA levels were found to correlate with the aggressiveness of the cancer, as highest IL-6 mRNA was noted in the highly aggressive and metastatic basal-like subtype [192]. Another study linked human epidermal growth factor 2 receptor (HER2) signaling with IL-6 expression in breast cancer [193]. HER2 is an oncogene upregulated in a subset of breast tumors, and HER2 signaling through PI3K/AKT/mTOR and ERK pathway activation contributes to tumor proliferation [194]. This study found that HER2 overexpression was associated with increased IL-6 production and secretion which then acted on breast tumor cells to activate STAT3 through an autocrine loop, forming a HER2-IL-6-STAT3 axis [193]. In pancreatic cancer patients, high IL-6 levels were linked with advanced cancer stage as well as development of cachexia [195]. Another clinical study found that high IL-6

levels correlated with a diminished response to chemo and hormone-based cancer therapies in metastatic breast cancer patients [196]. Lastly, IL-6 was shown to increase expression of VEGF and promote angiogenesis in gastric tumors, another way it can contribute to cancer growth [197]. Collectively, these reports reveal a role for TNF and IL-6 in tumor progression, independent of their actions on the immune system.

2.7. COLLAGEN VI AND CANCER.

Collagen VI is an extracellular matrix protein highly expressed in cancer [198]. Collagen VI is typically found in the tumor stroma, near blood vessels, and helps facilitate the process of angiogenesis [198]. Collagen VI, by activating the NG2/chondroitin sulfate proteoglycan (CSP) receptor, activates β-catenin signaling [124]. Upon its activation, β-catenin translocates from the cell membrane to the nucleus, promoting its binding to T-cell factor (TCF) transcription factors that stimulate the expression of genes important in cancer, like CCND1 [199]. Collagen VI induced CCND1 expression in MCF-7 BCCs via NG2/CSP receptor binding, stimulating proliferation [124]. Collagen VI also induced expression of IL-8 and VEG-F in MCF-7 BCCs, further evidence of its tumor promoting effects [124]. Collagen VI production by macrophages facilitates their recruitment and adhesion at the tumor site resulting in increased inflammation in the tumor microenvironment [200]. While correlated with tumor invasiveness in breast cancer, high collagen VI expression has also been noted in ovarian cancer, melanoma, and glioblastomas [198]. Upregulation of metallothioneins by collagen VI has been shown [124] which could contribute to chemotherapy resistance in tumors, as these small trace metal and free radical scavenging proteins have been linked to cisplatin treatment failure [201]. One of the lesser researched adipokines, uncovering the mechanisms of collagen VI in cancer is still a growing area.

2.8. VEGF AND CANCER.

The role of VEGF in vascular permeability and angiogenesis has been well characterized [202]. Tumor cells also express VEGF receptors (VEGFRs) VEGFR1, VEGFR2, and VEGFR3, and are VEGF responsive [203]. VEGFRs are receptor tyrosine kinases [202] and belong to the same receptor class as IR and IGF1-R. Neuropilins (NRPs) are transmembrane proteins which function as co-receptors for other receptor classes, including VEGFRs [204]. NRPs complex with VEGFRs to increase VEGF affinity, in addition to functioning as VEGF receptors themselves [203, 204]. VEGF can modulate various steps in tumorigenesis, including tumor initiation and progression as well as modulating CSC functioning. VEGFR1 activation has been shown to increase invasion and migration of colorectal cancer through ERK activation and translocation of the RELA subunit of NF-κB into the nucleus to activate target gene expression [205]. Other reports have characterized the ability of NRP-mediated VEGF signaling to promote breast tumor survival via AKT pathway activation [206]. In terms of CSCs, two reports have shown VEGFR2 and NRPs appear to help sustain stem cell proliferation in both skin and brain tumors [207, 208]. Targeting VEGF has received continued interest, due to promising work done with the anti-VEGF antibody, bevacizumab. In patients with advanced stage breast cancer, bevacizumab treatment increased apoptosis of breast tumor cells [209]. In conclusion, the role of VEGF in cancer is more complex than initially thought, as not only does it facilitate increased blood supply to the tumor, it also acts directly on tumor cells to promote their proliferation and survival.

2.9. CCND1 AND CANCER.

The following sections will discuss genes involved in cancer that we have found to be dependent on AHR regulation. CCND1 was the first gene we identified whose expression was dependent on AHR [114]. CCND1, one of several D-type cyclins, is an important cell-cycle regulatory protein that promotes cellular division [115]. CCND1 exerts its effects through binding its partners, cyclin-dependent kinases

4 and 6 (CDK4/CDK6) [115]. The activation of these CCND1-CDK complexes phosphorylate

retinoblastoma (Rb) protein. Increases in the phosphorylation of Rb inhibits its activity, and decreases in

Rb activity is necessary for cell proliferation (Figure 8) [115]. CCND1 is an oncogene, and is

overexpressed in a variety of tumor types, commonly through alterations in gene copy number [210-

212]. In breast cancer, 50-70% of all breast tumors overexpress CCND1 [213, 214]. Compounds that

inhibit CCND1/CDK signaling have been investigated as potential anticancer agents. For instance, the

pan-CDK inhibitor flavopiridol showed promising preclinical antitumor activity, however, its efficacy in

clinical trials was poor [215-219]. Flavopiridol treatment failure has been attributed to dosing during the

study which was not optimal and/or poor pharmacokinetics [215, 216]. Second generation CDK

inhibitors, including palbociclib, abemaciclib, and ribociclib, which are specific for CDK4 and CDK6,

have been developed and are currently in phase 3 clinical trials [220]. Other options include inhibiting

CCND1 translation into protein or increasing CCND1 protein degradation. Translation of CCND1

mRNA is dependent on mTOR, thus, mTOR inhibitors are being investigated in CCND1 overexpressing

cancers, including mantle-cell lymphoma (MCL) [221]. Silencing expression of the deubiquitinating

enzyme USP2 increased cyclin D1 degradation and inhibited the growth of tumor cells which

overexpressed cyclin D1, but not control fibroblast cells, suggesting increasing cyclin D1 degradation

could be a novel therapy which targets only tumor cells [222]. Based on our work showing that AHR

induces CCND1 expression in MCF-7 BCCs [114], we postulate that AHR antagonists could be a new

mechanism to reduce CCND1 expression in breast cancer.

2.10. SOD2 AND CANCER.

Superoxide dismutase 2 (SOD2), encodes manganese superoxide dismutase (MnSOD), an

enzyme that neutralizes reactive oxygen species (ROS) [223]. By reducing ROS, MnSOD inhibits cell

death, as high levels of ROS induce oxidative damage to cellular macromolecules in the mitochondria
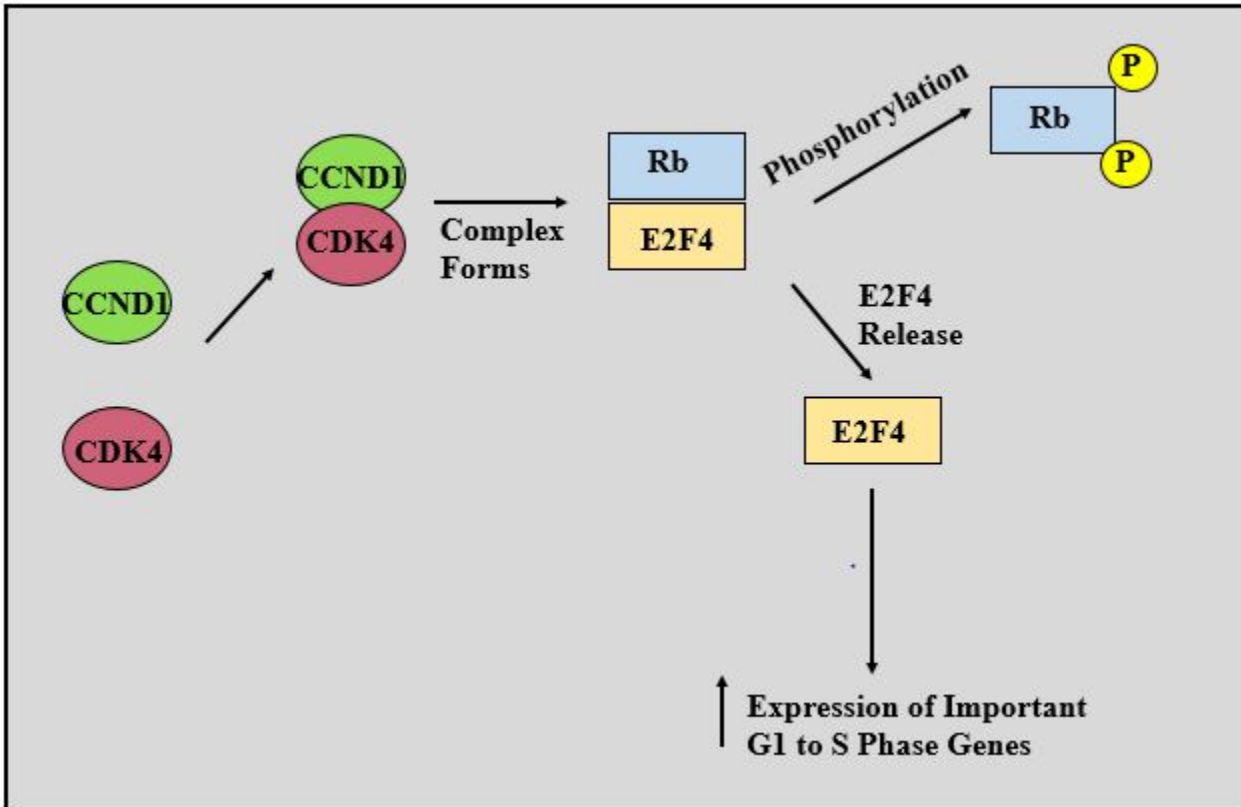
Figure 8. Role of CCND1 in cell cycle progression. CCND1 and CDK4 interact to form a stable complex. This complex then phosphorylated Rb, removing its inhibitory effects on E2F4. E2F4 can then move into the nucleus and stimulate gene expression. CCND1 = cyclin D1, CDK4 = cyclin-dependent kinase 4, Rb = retinoblastoma protein, E2F4 = E2F transcription factor 4. This figure was made in Microsoft Powerpoint to summarize information from reference [115].

[223]. While two other genes (SOD1 and SOD3) also encode superoxide dismutases [224], only SOD2 is essential for life, as knockout of SOD2 has been shown to cause embryonic and neonatal lethality in mouse models [225, 226]. MnSOD inactivates ROS by catalyzing the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), which is then further converted to less toxic water and oxygen by glutathione peroxidase, preventing mitochondrial damage (Figure 9) [223, 227]. The SOD2 gene is expressed in the nucleus and its product, MnSOD, translocates to the mitochondria via a targeting sequence [228]. MnSOD is a TNF target gene, and its upregulation by TNF reduces ROS production in cancer cells [228, 229]. Prior reports have shown that TNF-stimulated increases in SOD2 transcription are mediated by the transcription factor NF-κB [228]. As detailed in Chapter VI, and in our prior report [59], we identified that TNF regulation of MnSOD in MCF7 BCCs is mediated by AHR. AHR and NF-κB were both recruited to NF-κB-RE in the SOD2 gene upon TNF stimulus, and knockdown of AHR blocked TNF-stimulated increases in MnSOD [59]. Silencing AHR also sensitized BCCs to TNF cytotoxicity by preventing upregulation of MnSOD [59]. Considering that our work shows AHR promotes MnSOD expression, and that MnSOD promotes the survival of breast tumor cells, we postulate that AHR antagonists may inhibit breast tumor survival by suppressing MnSOD expression.

2.11. LAT1 AND CANCER.

We have most recently published that SLC7A5, which encodes the protein L-Type Amino Acid Transporter 1 (LAT1), is a primary AHR gene target in MCF-7 and MDA-MB-231 BCCs [44]. LAT1 facilitates the uptake of large neutral amino acids including leucine, arginine, phenylalanine, and tryptophan [230-232]. LAT1 is overexpressed in a variety of tumor types, including breast, colorectal, and prostate, compared with surrounding normal tissue [233]. Upregulation of LAT1 is postulated to promote cancer growth by facilitating the uptake of leucine by tumor cells. Increases in intracellular leucine is necessary for the activation of mTOR signaling, specifically mTORC1 [234, 235].
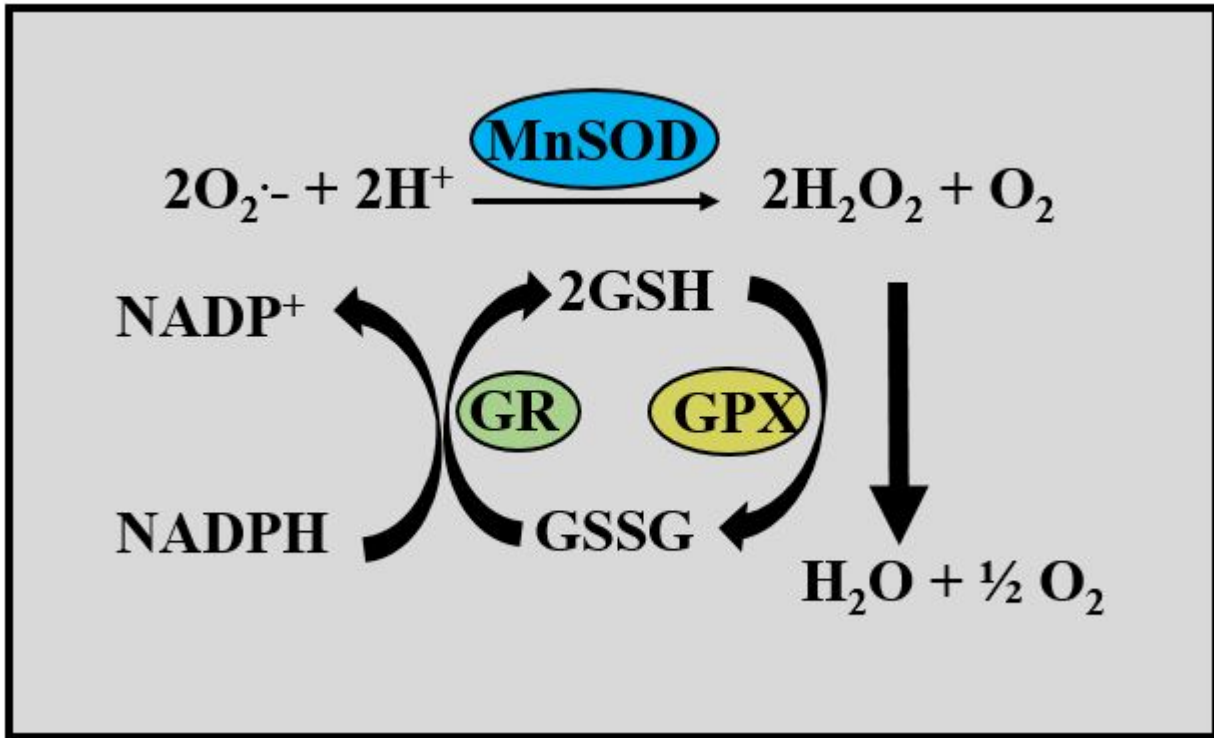
Figure 9. Role of MnSOD in mitochondrial oxidative stress. MnSOD converts superoxide anion $(O_2^{\cdot-})$ radical into $H_2O_2 + O_2$. H_2O_2 is then further converted to H_2O by GPX using GSH as electron donor, forming GSSG in the process. GSSG is then converted back to GSH by GR using NADPH as electron donor to complete the cycle. MnSOD = manganese superoxide dismutase, GPX = glutathione peroxidase, GR = glutathione reductase, GSSG = glutathione disulfide, GSH = reduced glutathione, NADPH = nicotinamide adenine dinucleotide phosphate. This figure made in Microsoft Powerpoint and summarizes information found in references [223, 224].

Increases in the activity of mTORC1 promotes the phosphorylation and activation of mTORC1 downstream targets such as ribosomal S6 kinase (p70S6K), eukaryotic initiation factor (eIF), and 4E-binding proteins (4E-BPs) which promote protein synthesis and cell survival (Figure 10) [234]. Hyperactivation of mTOR and its downstream constituents have been associated with many tumor types, including melanoma, late-stage head and neck cancers, and ductal cell breast cancers [235, 236]. Importantly, prior reports have demonstrated that breast tumors that express high levels of LAT1 were more resistant to tamoxifen treatment than breast tumors that expressed low levels of LAT1 [237, 238]. We recently published the first data indicating that LAT1 is a primary AHR gene target in MCF-7 and MDA-MB-231 BCCs [44]. The finding that AHR promotes LAT1 expression and function, suggests that AHR antagonists may offer a new way to overcome tamoxifen resistance by downregulating LAT1. Indeed, my recent work from our laboratory has revealed that CCND1, MnSOD and LAT1 are primary AHR target genes in human BCCs, suggesting that the inhibition of AHR may be useful in the treatment of breast tumor growth and survival.
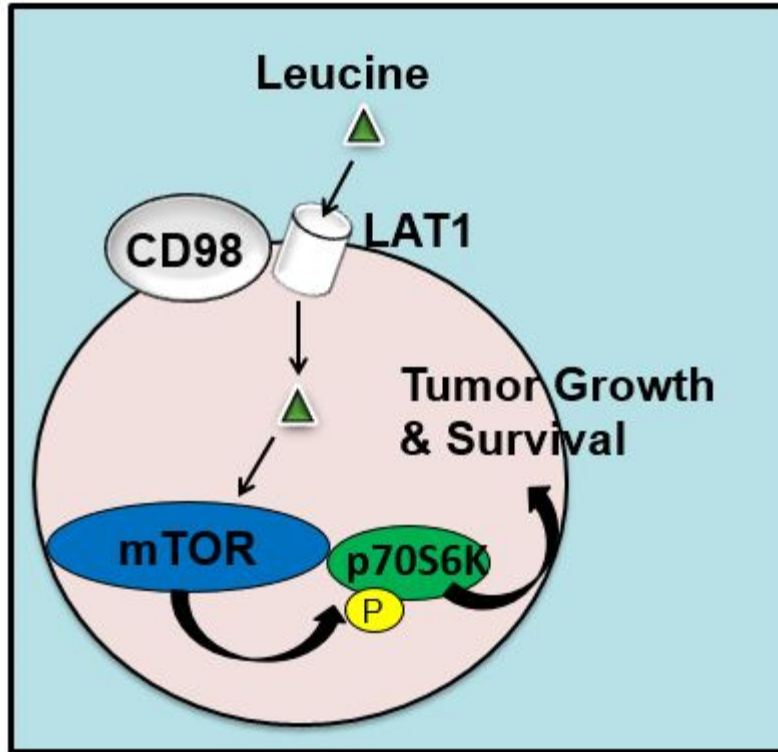
Figure 10. Role of LAT1 in tumor cell survival. LAT1 facilitates the uptake of amino acids like leucine through interactions with chaperone CD98. Leucine activates mTOR signaling which activates downstream targets, such as p70S6K, that are important for increasing protein synthesis and maintaining tumor survival. LAT1 = L-type amino acid transporter 1, CD98 = 4F2 heavy chain antigen, mTOR = mammalian target of rapamycin, p70S6K = ribosomal s6 kinase 1. This figure made in Microsoft Powerpoint to summarize information found in references [230-235].

CHAPTER III: METHODS TO STUDY AHR SIGNALING.

3.1 MATERIALS

The purpose of this introductory methods sections is to provide a brief overview of the methods that were used in this dissertation (Table 2). MCF-7, T-47D, and MDA-MB-231 breast cancer cells and 3T3-L1 fibroblast cells were purchased from ATCC (Manassas, Va). Dulbecco's Modified Eagle Medium/High glucose (DMEM) with L-glutamine and sodium pyruvate, phenol red-free DMEM, phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Sodium dodecyl sulfate (SDS), 30 % acrylamide/bis solution, ammonium persulfate, Tween-20, and 2-mercaptoethanol were obtained from Bio-RAD (Hercules, CA). Trypsin-EDTA was purchased from Thermo Fisher Scientific (Pittsburgh, PA). DMSO, SU5416, CH-223191, 3-isobutyl-1-methylxanthine (IBMX), insulin, dexamethasone, L-leucine, and all designed mRNA and ChIP primers were purchased from Sigma Aldrich (St. Louis, MO). TCDD was purchased from Cambridge Isotopes Laboratory (Andover, MA). Recombinant human IGF-2, IGF-2 blocking antibody, and recombinant human TNF were purchased from R & D Systems (Minneapolis, MN). All short interfering RNAs (siRNAs) were purchased from GE Dharmacon (Lafayette, CO). The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX): AHR (cat # H-211), NF-κB RELA (cat # sc-372), ARNT (cat # sc-17812), p300 (cat # sc-584) and control random IgG (cat # 2027). The following antibodies were purchased from Millipore (Temecula, CA): Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cat # MAB374) and cyclin D1 (cat # 04-1151). The following antibodies were purchased from Cell Signaling Technology (Santa Cruz, CA): LAT1 (cat # 5347), acetyl-histone H3 (K9) (cat # 9649), and acetyl-histone H3 (K14) (cat # D4B9). MnSOD antibody purchased from Abcam (Cambridge, UK) (cat # ab13533).

ChIP	mRNA	Western	Function
Gene Targets	**Gene Targets**	**Proteins**	
	AHR	AHR	Gene Regulation
CCND1	CCND1	CCND1	Cell Proliferation
SOD2	SOD2	MnSOD	ROS Reduction
LAT1	LAT1	LAT1	Leucine Uptake
CYP1A1, 1B1	CYP1A1		Metabolism
	GAPDH	GAPDH	Loading Control

Table 2. Methods and targets to study AHR signaling.

3.2. GENE BINDING: CHROMATIN IMMUNOPRECIPITATION.

Chromatin immunoprecipitation (ChIP) assays were conducted to investigate the binding of transcription factors, transcriptional coactivators and changes in histone H3 acetylation within chromatin corresponding to CYP1A1, CYP1B1, CCND1, SOD2 and LAT1 genes. To this end, 500,000 BCCs were plated into 60 mm culture plates in DMEM supplemented with 10% FBS for twenty four hours prior to treatment with vehicle, IGF-2 (100 ng/mL, three hr), TNF (10 ng/mL, twleve hr) or TCDD (10 nM, forty five min). Post treatment, formaldehyde (1% final concentration) was added to cell culture medium for ten min, and then cells were incubated in glycine (final concentration 0.5 M) for five min in order to quench the crosslinking reaction. Cells were then rinsed with PBS, scraped and collected in PBS, and pelleted by brief low speed centrifugation (800 RPM). Cell pellets were then lysed in 300 μL of lysis buffer (1% SDS; 5 mM EDTA; 50 mM Tris–HCl, pH 8) per 60 mm plate plus protease inhibitors (Thermo Scientific) for 15 min on ice.

Sonication (Fisher Sonic Dismembrator Model 500 at 15% amplitude) was utilized to shear chromatin to an average length of 500 bp. Cellular extracts containing sheared chromatin were then diluted 1:10 in dilution buffer (16.7 mM Tris–HCl, pH 8; 167 mM NaCl; 1.2 mM EDTA; 0.01% SDS; 1.1% Triton X-100) and rotated overnight at 4°C with 1 μg of non-specific IgG or antibodies recognizing specific transcription factors, the transcriptional coactivator p300 or acetylated histone H3 at lysine residues 9 and 14 (Table 3). Antibody-chromatin complexes were collected using 5 μL of magnetic protein A or G beads (Dynabeads, Invitrogen) with rotation at 4° C for 90 min. Using magnetic separation (Life Technologies; part # 49-2025), beads were washed sequentially with buffer 1 (20 mM Tris–HCl, pH 8; 150 mM NaCl; 2.0 mM EDTA; 0.1% SDS), buffer 2 (20 mM Tris–HCl, pH 8; 500 mM NaCl; 2.0 mM EDTA; 0.1% SDS), buffer 3 (10 mM Tris–HCl (pH 8); 0.25 M LiCl; 1 mM EDTA; 1% NP-40; 1% deoxycholate), and then 1× TE buffer for five mins each, and incubated at

ChIP Antibodies			
Protein	**Catalog #**	**Concentration**	**Company**
AHR	H-211	1 μg	Santa Cruz Biotech
ARNT	sc-17812	1 μg	Santa Cruz Biotech
p300	sc-584	1 μg	Santa Cruz Biotech
Acetyl-histone H3 (K9)	9649	1 μg	Cell Signaling
Acetyl-histone H3 (K14)	D4B9	1 μg	Cell Signaling
NF-κB RELA	sc-372	1 μg	Santa Cruz Biotech
Control IgG	2027	1 μg	Santa Cruz Biotech

Table 3. List of antibodies used for ChIP experiments

65° C for four to six hours in elution buffer (1% SDS, 0.1 M NaHCO$_3$) containing 1 µL proteinase K (10 mg/ml stock) per ChIP reaction to elute off beads and cleave peptide bonds to aid in DNA purification. DNA was purified (Qiagen; cat # 28204) and analyzed using real time quantitative PCR (detailed in section 3.4.). Primers spanning AHR-response elements (AHR-RE) in the promoter regions of CYP1A1, CYP1B1, intron 3 of the LAT1 gene, NF-κB-RE in the SOD2 gene, or the activator protein 1 (AP1)-RE or T-cell factor (TCF)-RE in CCND1 were used to investigate the binding of AHR, ARNT, NF-κB, p300 or increases in the acetylation in histone H3 in these gene targets (Table 4). ChIP data was expressed as % input, in which signals obtained from the ChIP are divided by signals obtained from an input sample.

3.3. SHORT INTERFERING RNA: GENE KNOCKDOWN EXPERIMENTS.

Gene specific short interfering RNAs (siRNA) were used to knockdown the expression of specific genes in order to evaluate their function. 200,000 cells in 1 mL of DMEM + 10% FBS were mixed directly with 100 nM of short interfering RNA (siRNA) that was either non-targeting (control), AHR targeting (AHR-siRNA), NF-κB RELA targeting, or LAT1 targeting (LAT1-siRNA) and 3 µl of transfection reagent (Lipofectamine RNAi Max, Life Technologies), and then plated into 35 mm tissue culture plates. After thirty six hours, cells were treated with vehicle, IGF-2 (100 ng/mL, three hr), TNF (10 ng/mL, twelve hr) or TCDD (10 nM, six hr, or sixteen hr). Treatments were removed, and total cellular RNA or protein was extracted with RNA-purification kits (Qiagen RNeasy) or 2× Laemmli sample lysis buffer, respectively. In some experiments, total RNA was isolated using TRI-reagent in accordance with protocols provided by the manufacturer (Sigma Aldrich). For cell growth studies, treatments were prolonged and are described in section 3.7.

ChIP DNA Primers for PCR		
Gene Element	**Forward Primer**	**Reverse Primer**
CCND1 AP1-RE	5′-GGCAGAGGGGACTAATATTTCCAGCA-3′	5′-GAATGGAAAGCTGAGAAACAGTGATCTCC-3′
CCND1 TCF-RE	5′-GCTCCCATTCTCTGCCGG-3′	5′-CGGAGCGTGCGGACTCTG-3′
CYP1A1 AHR-RE	5′-ACGCAGACCTAGACCCTTTGC-3′	5′-CGGGTGCGCGATTGAA-3′
CYP1B1 AHR-RE	5′-GTGCGCACGGAGGTGGCGATA-3′	5′-GCTCCTCCCGCGCTTCTCAC-3′
LAT1 AHR-RE	5′-GCACGTACCTGTAGGGGTTG-3′	5′-ATGCTCTCTCCCCGGTGATT-3′
SOD2 NF-κB-RE	5'-GGAAAAGGCCCCGTGATTT-3'	5'-TCCTGGTGTCAGATGTTGCC-3'

Table 4. ChIP primers used to analyze gene regulation by transcription factors.

3.4. MESSENGER RNA: REAL TIME QUANTITATIVE PCR.

Total cellular RNA was isolated with TRI-Reagent or RNEasy Purification Kits (Qiagen) and quantitated by Nanodrop spectrophotometry. RNA at 100-300 ng starting concentration was reverse transcribed to complementary DNA (cDNA) (Verso cDNA kit; Thermo Fisher Scientific; cat # AB-1453/B). The resulting cDNAs were subjected to quantitative real-time PCR (RT-qPCR) using gene specific primers (300 nM per reaction) and 40 cycles of PCR in accordance with Absolute Blue SYBR Green Rox Mix (Thermo Fisher Scientific; cat # AB-4162/B) protocols (Table 5 contains all mRNA primer sets). Relative gene expression between control and treated cells was calculated using the formula $2^{-\Delta\Delta CT}$, as described by Livak and Schmittgen [239]. GAPDH mRNA levels served as the internal control. The Harvard Primer Bank http://pga.mgh.harvard.edu/primerbank/ and NIH primer blast search engines http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome were used to design primers. Primer specificity was verified with melt curve analysis during each real time PCR run.

3.5. PROTEIN: WESTERN BLOTTING.

To assay changes in protein levels, 200,000 cells were plated in 35 mm plates for 24 h prior to specific treatments. Once treatments were carried out, total cellular extract was isolated in 200 μL of Laemmli sample buffer and approximately 12.5 μg of protein was subjected to SDS PAGE analysis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-RAD; Hercules, CA). Membranes were blocked in PBS, .01% Tween 20 (PBS-T), 5% (wt/vol) low-fat powdered milk for one hr and incubated overnight with primary antibody at 4° C with gentle mixing. Primary antibodies used, their dilutions, and where they were obtained is provided in Table 6. Membranes were rinsed five times (five minutes each wash) with PBS-T and then incubated with an appropriate HRP-labeled secondary

mRNA Primer Sets

Gene Targets	Forward Primer	Reverse Primer
AHR	5′-ACATCACCTACGCCAGTGG-3′	5′-CTCTATGCCGCTTGGAAGGAT-3′
CCND1	5′-CCGCAATGACCCCGCACGAT-3′	5′-AGGGCAACGAAGGTCTGCGC-3′
SOD2	5′-GGAAGCCATCAAACGTGACTT-3′	5′-CCCGTTCCTTATTGAAACCAAGC-3′
CYP1A1	5′-CTTCACCCTCATCAGTAATGGTC-3′	5′-AGGCTGGGTCAGAGGCAAT′-3'
CYP1B1	5′-CTGCACTCGAGTCTGCACAT-3′	5′-TATCACTGACATCTTCGGCG-3′
HMGCS2	5′-CAATGCCTGCTACGGTGGTA-3′	5′-GACGGCAATGTCTCCACAGA-3′
OAS1	5′-CAGACGATGAGACCGACGAT-3′	5′-CCTGGAGTGTGCTGGGTCTA-3′
PLA2G2	5′-ACCAGACGTACCGAGAGGAG-3′	5′-CGCTGGGGATTGGTGACTG-3′
ABCG2	5′-ACGAACGGATTAACAGGGTCA-3′	5′-CTCCAGACACACCACGGAT-3′
NRF2	5′-TCCAGTCAGAAACCAGTGGAT-3′	5′-GAATGTCTGCGCCAAAAGCTG-3′
ALOX5	5′-CTCAAGCAACACCGACGTAAA-3′	5′-CCTTGTGGCATTTGGCATCG-3′
ALDH3A1	5′-TGTTCTCCAGCAACGACAAGG-3′	5′-AGGGCAGAGAGTGCAAGGT-3′
PKD1L1	5′-CGCCTCTGGATTGTGATAACAG-3′	5′-CGGTCCCAGTAGCACACAG-3′
PYDC1	5′-CACACGTATAGCTACCGGCG-3′	5′-CGCGTAAGACAACAGCAGTG-3′
PGR	5′-TTATGGTGTCCTTACCTGTGGG-3′	5′-GCGGATTTTATCAACGATGCAG-3′
MGP	5′-TCCGAGAACGCTCTAAGCCT-3′	5′-GCAAAGTCTGTAGTCATCACAGG-3′
SERPIN3A	5′-TGCCAGCGCACTCTTCATC-3′	5′-TGTCGTTCAGGTTATAGTCCCTC-3′
CREB3L	5′-CCTCCCGAAGCCTCCTATTCT-3′	5′-GGGGTTGATTTCCCAGCCA-3′
SERPIN5A	5′-ATGCCCTTTTCACCGACCTG-3′	5′-TGCAGAGTCCCTAAAGTTGGTAG-3′
ADORA	5′-CCACAGACCTACTTCCACACC-3′	5′-TACCGGAGAGGGATCTTGACC-3′
GAPDH	5′-CATGAGAAGTATGACAACAGCCT-3′	5′-AGTCCTTCCACGATACCAAAGT-3′
LAT1	5′-CCGAGGAGAAGGAAGAGGC-3′	5′-GAAGATGCCCGAGCCGATAA-3′
RELA	5′- TCCAGACCAACAACAACCCC-3′	5′-GATCTTGAGCTCGGCAGTGT-3'

Table 5. Primer sets for mRNA studies.

Western Antibodies			
Protein	**Cat #**	**Conc. & Incubation**	**Company**
AHR	H-211	1 : 2000, overnight, 4C	Santa Cruz Biotech
CCND1	04-1151	1 : 20000, overnight, 4C	Millipore
GAPDH	MAB374	1 : 10000, 1 h, room temp.	Millipore
LAT1	5347	1 : 2000, overnight, 4C	Cell Signaling
MnSOD	ab13533	1 : 5000, overnight, 4C	Abcam

Table 6. Antibodies used for western blotting.

antibody (diluted 1:10,000 in PBS, .01% tween-20, 5% milk) (Thermo Scientific, Pierce) for one hr, followed with rinsing five times (five mins each wash) in PBS-T. Membranes were developed with enhanced chemiluminescent substrate (Millipore, Immobilon ECL substrate) and exposure to X-ray film (Midwest Scientific). GAPDH western blots were performed as a loading control for all western blot experiments herein to confirm equal protein loading. Normalized levels of proteins of interest were expressed as a ratio relative to GAPDH levels. Densitometry was calculated with ImageJ PC-based software (National Institute of Health).

3.6. FUNCTION: LEUCINE UPTAKE.

Leucine uptake experiments were performed in MCF-7 cells grown to confluence on 24 well plates. The cells were first washed twice with Na-free buffer (130 mM TMACl, 4.7 mM KCl, 1 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20 mM HEPES; pH 7.4) and incubated with the same for ten min at room temperature. The uptake was then initiated by incubating the cells for thirty sec with Na-HEPES buffer (130 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20mM HEPES; pH 7.4) with 10 µCi of 3H-L-Leucine (PerkinElmer; Waltham, MA) and 10 µM L-Leucine (Sigma). The reaction was stopped with ice cold Na-HEPES buffer after which the cells were washed twice with the same ice-cold buffer. The cells were then lysed in 500 µl of 1 N NaOH followed by incubation for 20 min at 70° C. The lysed contents of each well was collected in a 7 ml scintillation tube and mixed with 5 ml Ecoscint A (National Diagnostics; Atlanta, GA). Leucine uptake experiments were conducted using chemicals obtained from Sigma–Aldrich. The vials were kept in the dark for forty eight hours and the radioactivity was determined in a Beckman 6500 scintillation counter. Data expressed as leucine uptake pmol/mg protein.

3.7. FUNCTION: CANCER PROLIFERATION.

For cellular proliferation studies, two methods were used: manual cell counting via hemocytometers or commercially available colorimetric proliferation assay kits. For specific treatments, refer to methods and results sections found in the subsequent chapters of this document. Generally, for manual cell counting, breast cancer cells were seeded into six well plates at a density of 80,000 cells per well. Once specific treatments were applied, cells were maintained in culture for three days to observe discernable changes in cell number. After three days, treated cells were rinsed with PBS solution, subjected to trypsinization, and then suspended in complete medium (DMEM, 10% FBS, and 1% penicillin/streptomycin (P/S)) at a final volume of 1mL cell suspension per well. Cells were diluted 1:1 with trypan blue to determine the number of viable cells, as dead cells take up the compound and exhibit a blue color and can be excluded from counts [240]. 12.5 µL of each cell suspension was loaded onto hemocytometers and counted. Three replicates were performed per treatment group and these numbers were averaged. Control untreated cells were assigned a value of 1, and fold change in cell number for treated cells was determined and expressed graphically.

Proliferation was assayed using commercially available Aqueous One Solution Cell Proliferation Assay kits (Promega, Madison, WI). Cells were plated into 96 well plates at a density of 1,000 to 2,000 cells per well, depending on the cell line. After three days, cell proliferation was assayed in accordance with the manufacturer's protocol.

3.8. STATISTICAL ANALYSIS.

Two-tailed, paired t tests with confidence intervals of 95% were used to determine statistically significant differences between two groups. The Student Newman–Keuls (SNK) post hoc test was used to determine statistically significant differences among groups following one-way analysis of variance

(ANOVA). "*" denotes statistical significance reached at P < 0.05, "**" denotes statistical significance reached at P < 0.01, and "***" denotes statistical significance reached at P < 0.001.

CHAPTER IV: ARYL HYDROCARBON RECEPTOR LIGANDS INHIBIT IGF-II AND ADIPOKINE STIMULATED BREAST CANCER CELL PROLIFERATION.

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ABSTRACT

Obesity increases human cancer risk and the risk for cancer recurrence. Adipocytes secrete paracrine factors termed adipokines that stimulate signaling in cancer cells that induce proliferation. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that plays roles in tumorigenesis, is regulated by exogenous lipophilic chemicals, and has been explored as a therapeutic target for cancer therapy. Whether exogenous AHR ligands modulate adipokine stimulated breast cancer cell proliferation has not been investigated. We provide evidence that adipocytes secrete insulin-like growth factor 2 (IGF-2) at levels that stimulate the proliferation of human estrogen receptor (ER) positive breast cancer cells. Using highly specific AHR ligands and AHR short interfering RNA (AHR-siRNA), we show that specific ligand-activated AHR inhibits adipocyte secretome and IGF-2-stimulated breast cancer cell proliferation. We also report that a highly specific AHR agonist significantly ($P < 0.05$) inhibits the expression of E2F1, CCND1 (known as Cyclin D1), MYB, SRC, JAK2, and JUND in breast cancer cells. Collectively, these data suggest that drugs that target the AHR may be useful for treating cancer in human obesity.

Keywords: AHR, IGF-2, Adipokines, Obesity, Breast Cancer

4.1. INTRODUCTION

Human obesity is common and has been linked with increases in breast cancer risk and breast cancer recurrence [112, 113, 241]. Although the underlying links between obesity and cancer are not completely clear, adipocytes themselves are postulated to play a role [112, 113, 124, 241-246]. Adipocytes secrete multiple paracrine factors termed adipokines that stimulate signaling in human cancer cells that stimulate proliferation [124, 242-244, 246]. Specific adipokines that stimulate the proliferation of human estrogen receptor (ER) positive breast cancer cells are leptin, collagen VI, and members of the insulin-like growth factor (IGF) family of proteins [124, 242, 243, 245]. In ER positive breast cancer cells, leptin through its cognate membrane spanning cytokine leptin receptor activates the JAK/STAT pathway [244, 247]. Collagen VI by activating the NG2/chondroitin sulfate proteoglycan receptor activates AKT and β-catenin signaling [59, 124, 243]. IGF-1 and IGF-2 signal through specific cell surface tyrosine kinase receptors, IGF-1 receptor (IGF1-R) and insulin receptor isoform A (IR-A)), that are highly expressed on human ER expressing breast cancer cells [248, 249]. The critical pathway by which IGF-1 and IGF-2 stimulate breast cancer cell proliferation is the PI3K pathway that leads to increases in AKT activity [248, 249]. Leptin, collagen VI, and IGF proteins have all been reported to stimulate increases in the transcription and expression of CCND1 (also known as Cyclin D1) in ER expressing breast cancer cells [124, 243, 244, 249]. CCND1 is a regulatory protein that activates the cell cycle, increases cell proliferation, and has been implicated as a promoter of breast tumorigenesis [115]. New drugs and drug targets that inhibit adipokine stimulated breast cancer cell proliferation could be particularly relevant to reducing the higher rates of breast cancer risk and breast cancer recurrence that are observed in obese women compared to women of normal weight. However, there are currently no specific therapies for

reducing breast cancer risk and recurrence in obesity.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that has been explored as a therapeutic target for cancer [250, 251]. Ligand-activated AHR inhibits the growth of some human cancer cell lines [250, 251]. The AHR is stimulated by lipophilic chemicals that function as AHR agonists including the environmental toxicant 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and the experimental drug 3-(3,5-dimethyl-1H-pyrrol-2-yl methylene)-1,3-dihydro-indol-2-one (SU5416) [250, 252]. In the absence of an exogenous AHR agonist, the AHR is located in the cytoplasm bound to p23, HSP90, and XAP2 chaperon proteins [253]. Upon activation by an agonist, the AHR dissociates from p23, HSP90, and XAP2, translocates into the nucleus, and stimulates transcription by binding to sequence specific response elements termed AHR response elements (AHR-RE) in enhancers of genes that are stimulated by AHR ligands [253]. CYP1A1 is a prototypical AHR regulated gene target that has been used to study AHR signaling [253]. Whether specific ligand-activated AHR inhibits adipokine stimulated breast cancer cell proliferation and the potential mechanisms by which this could occur have not been investigated.

Obese women with ER positive breast tumors have worse clinical outcomes and have a higher risk for breast cancer recurrence than obese women with ER negative breast tumors [254]. This suggests that ER positive breast cancer cells could be more sensitive to the proliferative effects of mitogenic adipokines than ER negative breast cancer cells. The human ER positive MCF-7 breast cancer cell line has been used extensively as a model to investigate mitogenic adipokine signaling in human breast cancer cells and MCF-7 cells, express leptin, collagen VI, and IGF receptors [124, 243, 244, 249]. The human T-47D breast cancer cell line expresses ER and IGF receptors, and fewer reports have used this cell line to investigate adipokine signaling in

human breast cancer cells [250]. Given these prior reports, the purpose of this study was to examine the possibility that specific ligand-activated AHR inhibits mitogenic adipokine signaling in human MCF-7 breast cancer cells and to provide preliminary insights into the mechanism by which this occurs. Primary findings were also validated in T-47D cells.

4.2. MATERIALS AND METHODS

4.2.1. Conditioned Media

A previously published standard adipocyte differentiation protocol was used to differentiate murine 3T3-L1 preadipocyte fibroblasts into fully differentiated adipocytes [243]. Specifically, confluent 3T3-L1 fibroblasts were treated with Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 160 nM insulin, 250 nM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)) for 3 days, followed by 10% fetal bovine serum (FBS) and 160 nM insulin for 3 days and then DMEM 10% FBS for an additional 6 days, with a medium change every three days. In order to examine the effects of adipocyte secreted adipokines in the absence of confounding factors in FBS, medium was removed from fully differentiated adipocytes, followed by rinsing twice with phosphate buffered saline (PBS), and adipocytes were then incubated in phenol red-free, serum-free DMEM for an additional 24 hr. This serum-free adipocyte conditioned medium (adipo-CM) was centrifuged and stored at −80°C prior to being applied to breast cancer cells in cell culture. Phenol red-free, serum-free DMEM conditioned by 3T3-L1 fibroblasts for 24 hrs was also isolated. Fibroblast conditioned medium (fibro-CM) was applied to breast cancer cells as a control media not conditioned by an adipocyte. DMEM, FBS, P/S, and PBS were purchased from Thermo Fisher Scientific (HyClone Labs, Logan, UT). IBMX, insulin, and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2. Breast Cancer Cell Growth Experiments

Prior to specific growth experiments, MCF-7 and T-47D cells purchased from ATCC (Manassas, VA) were maintained in DMEM, 10% FBS, and 1% penicillin/streptomycin (P/S). To explore whether adipo-CM stimulated cancer proliferation more than fibro-CM, phenol red-free, serum-free DMEM (unconditioned medium), fibro-CM, or adipo-CM was applied to MCF-7 or

T-47D cells for three days in culture, after which cells were collected and total live cell number was determined using a hemocytometer and manual cell counting. Preliminary experiments were conducted to determine the optimal dose of TCDD to use in proliferation experiments. In our preliminary experiments, we found that MCF-7 cells were more sensitive to the antiproliferative effects of TCDD than, T-47D cells (data not shown; n= 3). Thus, in all remaining experiments, MCF-7 cells were treated with 10 nM TCDD and T-47D cells were treated with 100 nM TCDD. The 100 nM dose of SU5416 was selected based on our preliminary data showing that SU5416 at this dose is a strong AHR agonist based on its ability to stimulate increases in CYP1A1 gene expression (data not shown; n= 3). Fibro-CM or adipo-CM supplemented with DMSO vehicle, TCDD, or SU5416 was applied to overnight serum-starved (phenol red-free DMEM) MCF-7 or T-47D cells for three days in culture, after which cells were collected and trypan blue and manual cell counting were used to determine live cell number. In other experiments, overnight serum-starved (phenol red-free DMEM) MCF-7 or T-47D cells were stimulated with PBS vehicle or IGF-2 (100 ng/mL; R & D Systems) supplemented with DMSO vehicle, TCDD, or SU5416 for three days in culture, after which cells were collected and live cell was determined with trypan blue. The IGF-2 dose was based on the work of Worster et al. showing that IGF-1 (100 ng/mL) induced the proliferation of human breast epithelial cells [255].

4.2.3. SiRNA Experiments

In order to show that TCDD and SU5416 inhibition of IGF-2 requires the AHR, breast cancer cells were plated in DMEM, 10% FBS, and P/S (80,000 cells per well of a six-well plate) for 24 hr, then transiently transfected with 50 nM of a single short interfering RNA (siRNA) that specifically targets the AHR (AHR-siRNA) or with a nontargeting control siRNA (con-siRNA) with 2 μL of DharmaFECT reagent 1 for 24 hr. Following removal of transient transfection

reagent, cells were serum-starved overnight in phenol red-free DMEM and then treated with

IGF-2 (100 ng/mL) supplemented with DMSO vehicle or TCDD (MCF-7 (10 nM), T-47D

(100 nM)) or SU5416 (MCF-7 100 nM) for three days in culture, after which cells were collected

and trypan blue was used to determine the number of live cells. Con-siRNA (cat number D-

001810-01-20), AHR-siRNA (J-004990-05), and DharmaFECT transfection reagent number 1

were purchased from Thermo Scientific, Dharmacon. DMSO and SU5416 were purchased from

Sigma-Aldrich (St. Louis, MO). TCDD was purchased from Cambridge Isotopes Laboratory

(Andover, MA) (cat number ED-901-B).

4.2.4. Western Blot Experiments

To validate AHR-siRNA mediated knockdown of the AHR, breast cancer cells (300,000

cells per 35 mm plate) were transfected with con-siRNA or AHR-siRNA for 36 hr, followed by

isolation of total cellular extract in 250 μL of 2X sample lysis buffer (Bio-RAD; cat number 161-

0737). Total cellular extract (~12.5 μg of protein) was subjected to SDS PAGE in Mini-

PROTEAN TGX 4–12% Precast Gels (Bio-Rad; Hercules, CA) and transferred to

polyvinylidene difluoride (PVDF) membranes (Bio-Rad; Hercules, CA). Membranes were

blocked in PBS, 0.05% Tween 20 (PBS-T), and 5% (wt/vol) low-fat powdered milk for one hr,

followed by overnight incubation at 4°C with rocking with an appropriate primary antibody.

Membranes were rinsed five times (five minutes each wash) with PBS-T and then incubated with

an appropriate HRP-labeled secondary antibody (Thermo Scientific, Pierce) for 1 h, followed by

rinsing five times (five minutes each wash) in PBS-T followed by the application of enhanced

chemiluminescent substrate (Thermo Scientific, Millipore) and exposure to X-ray film (Midwest

Scientific). Equal protein loading was confirmed by glyceraldehyde 3-phosphate dehydrogenase

(abbreviated as GAPDH) western blots. AHR antibody was purchased from Santa Cruz

Biotechnology, cat number: sc-5579, and diluted 1 : 5,000 in PBS, 0.01% tween-20, and 5% powdered milk. GAPDH was purchased from Millipore, cat #: MAB374, and diluted 1 : 20,000 dilution in PBS, .01% tween-20, and 5% powdered milk.

4.2.5. RT-qPCR Experiments

A RT2 Profiler PCR Array (PAHS-502ZC) (Superarray Bioscience Corporation, Qiagen) was used to compare gene expression between MCF-7 cells treated with IGF-2 (100 ng/mL) plus DMSO vehicle and MCF-7 cells treated with IGF-2 (100 ng/mL) plus TCDD (10 nM) for 48 hrs. The PAHS-502ZC PCR array allows for the integration of 84 genes that are prooncogenes or tumor suppressors and thus are key genes in tumorigenesis. Treatments were stopped, total RNA was isolated (RNeasy Mini Kit, Qiagen), and 1 μg of RNA was converted into cDNA (RT2 First Strand Kit, Qiagen). cDNA was combined with RT2 SYBER Green ROX qPCR Master Mix (Qiagen), and changes in gene expression were analyzed by RT2 Profiler PCR Array (PAHS-502ZC). Statistically significantly differentially expressed genes between groups were calculated by RT2 Profiler PCR Array Data Analysis software package that calculates ΔΔ CT calculated fold changes and uses Student's t-test to calculate two-tail, equal variance P values. Experiments were performed three times (n= 3).

4.2.6. IGF-2 Blocking Antibody Experiments

In order to explore whether adipocytes secreted levels of IGF-2 are sufficient to stimulate the proliferation of breast cancer cells, a control mouse IgG (5 μg/mL) (R & D systems, cat number MAB002) or human IGF-2 blocking antibody (5 μg/mL) (R & D Systems, cat number MAB292) was added to adipo-CM prior to being added to human breast cancer cells for three days in culture, after which total live cell number was determined using trypan blue. This particular IGF-II blocking antibody was selected because it has been shown to specifically

58

neutralize human IGF-2 in MCF-7 proliferation assays and exhibits 100% cross-reactivity with mouse IGF-2 (R & D Systems product sheet, cat number MAB292).

4.2.7. Mouse Adipokine Array Kit

Protein adipokine arrays were purchased from R&D systems (Minneapolis, MN, cat number ARY-013) and conducted in accordance with the manufactured protocols. Normalized adipokine levels were calculated as the density of a specific adipokine divided by the density of an internal loading control on each array. Densitometry was calculated with ImageJ PC-based software (National Institute of Health).

4.2.8. Statistical Analysis

All experiments were performed for a minimum of three times. One way analysis of variance (ANOVA), followed by Student-Newman-Keuls Post-Hoc Tests, was performed to determine statistically significant differences between multiple groups. Student's t-test was used to determine statistically significant differences between two groups. All statistical tests were run at a 95% confidence interval, and significance was denoted as $P < 0.05$.

4.3. RESULTS AND DISCUSSION

4.3.1. Adipocytes Secrete IGF-2 at Levels That Stimulate Breast Cancer Cell Proliferation

The application of adipocyte conditioned medium (adipo-CM) to MCF-7 or T-47D breast cancer cells for three days in culture significantly ($P < 0.0001$ and $P < 0.005$, resp.) increased proliferation more than the application of fibroblast conditioned medium (fibro-CM) or unconditioned medium (uncond-M) (Figures 11 (A) and (B)). Adipokine protein arrays revealed that the levels of several adipokines in adipo-CM were significantly higher than in fibro-CM, including insulin-like growth factor-2 (IGF-2) (by approximately three-fold; ($P < 0.05$)) (Figures 11 (C) and (D)). Since the levels of IGF-2 were higher in adipo-CM than in fibro-CM (Figures 11 (C) and (D)), and MCF-7 and T-47D cells overly express IGF1-R and IR-A [248, 249], we questioned the role of IGF-2 in adipo-CM. The addition of a specific IGF-2 blocking antibody to adipo-CM significantly reduced MCF-7 ($P < 0.0003$) and T-47D ($P < 0.05$) proliferation compared to control cells incubated in adipo-CM containing a nonspecific control IgG (Figures 11 (E) and (F)). This result suggests that adipocyte secreted IGF-2 contributes to the proliferation of ER expressing breast cancer cells.

4.3.2. Ligand-Activated AHR Inhibits Adipo-CM and IGF-2 Stimulated Breast Cancer Cell Proliferation

TCDD is a highly specific AHR agonist [253]. The application of adipo-CM plus TCDD to MCF-7 and T-47D cells significantly ($P < 0.0001$ and $P < 0.002$, resp.) reduced proliferation compared to that observed in control cells stimulated with adipo-CM in the presence of vehicle DMSO (Figures 12 (A) and (B)). Conversely, MCF-7 and T-47D proliferation in fibro-CM was not inhibited by TCDD (Figures 12 (A) and (B)). These data suggest that activation of the AHR

(a)

(b)

(c)

(d)
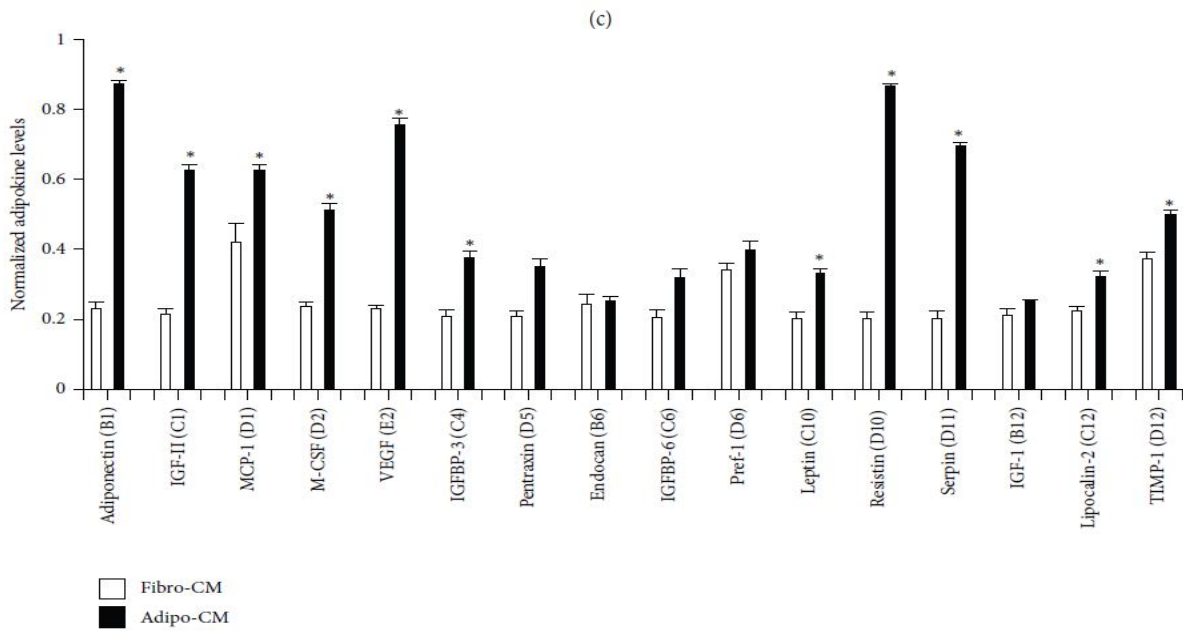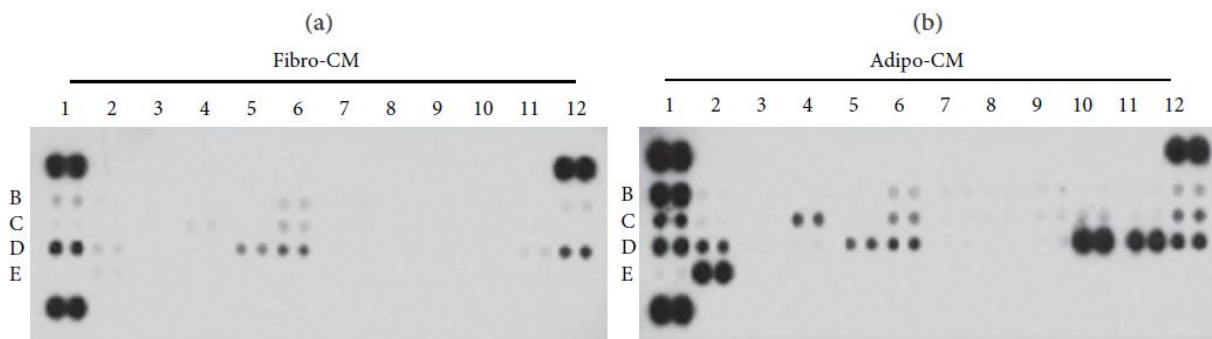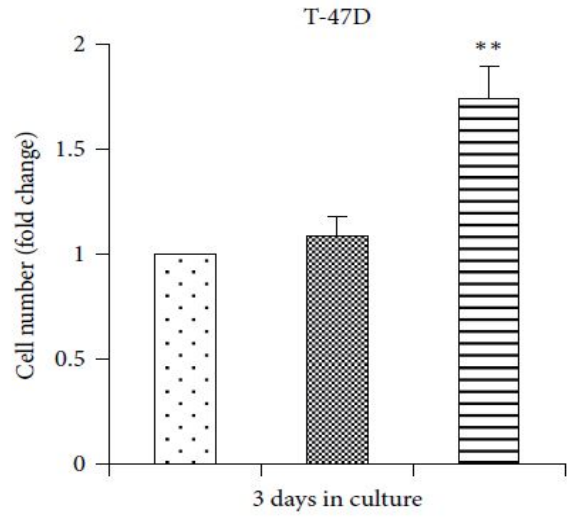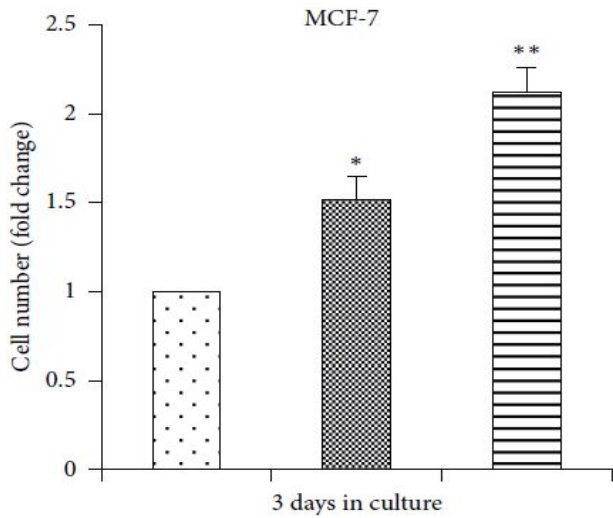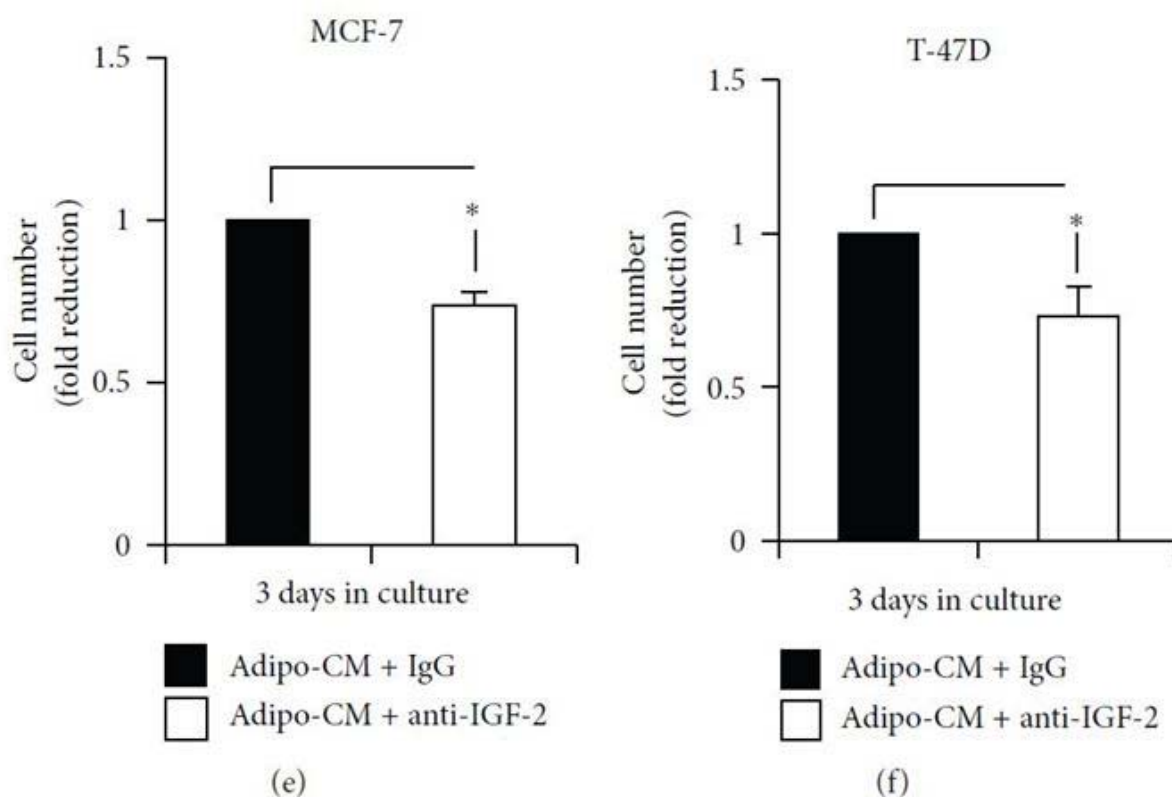
Figure 11. Adipocytes secrete levels of IGF-2 that contribute to adipo-CM stimulated breast cancer cell proliferation. (A, B)The total number of live MCF-7 (A) and T-47D (B) cells grown in unconditioned (uncond-M), fibroblast (fibro-CM), or adipocyte (adipo-CM) conditioned medium for three days in culture was determined and is shown relative to the number of live cells in the uncond-M group, which was arbitrarily assigned a value of 1. Data shown are the means ± S.E. for three replicate experiments, and significant (P < 0.05) induction of cell number by fibro-CM (∗) compared to the uncond-M group or by adipo-CM (∗∗) compared to the uncond-M and fibro-CM groups is shown. (C, D) Adipokine protein arrays were used to determine the relative levels of adipokines in adipo-CM and fibro-CM. (D) Data shown are the means ± SE for three experiments, and significantly (P < 0.05) higher levels of an adipokine in adipo-CM (∗) compared to fibro-CM are shown. Normalized adipokine levels were calculated as the densitometry of an adipokine normalized to the densitometry of an internal loading control. (E, F) The total number of live MCF-7 (E) and T-47D (F) cells treated with nonspecific IgG (5 μg/mL) or a specific IGF-2 blocking antibody (5 μg/mL) in adipo-CM for three days in culture was determined and is displayed relative to the number of live cells in the fibro-CM nonspecific IgG group, which was arbitrarily assigned a value of 1. Data shown are the means ± S.E. for three replicate experiments. A significant (P < 0.05) decrease in cell number by IGF-2 antibody (∗) is shown.
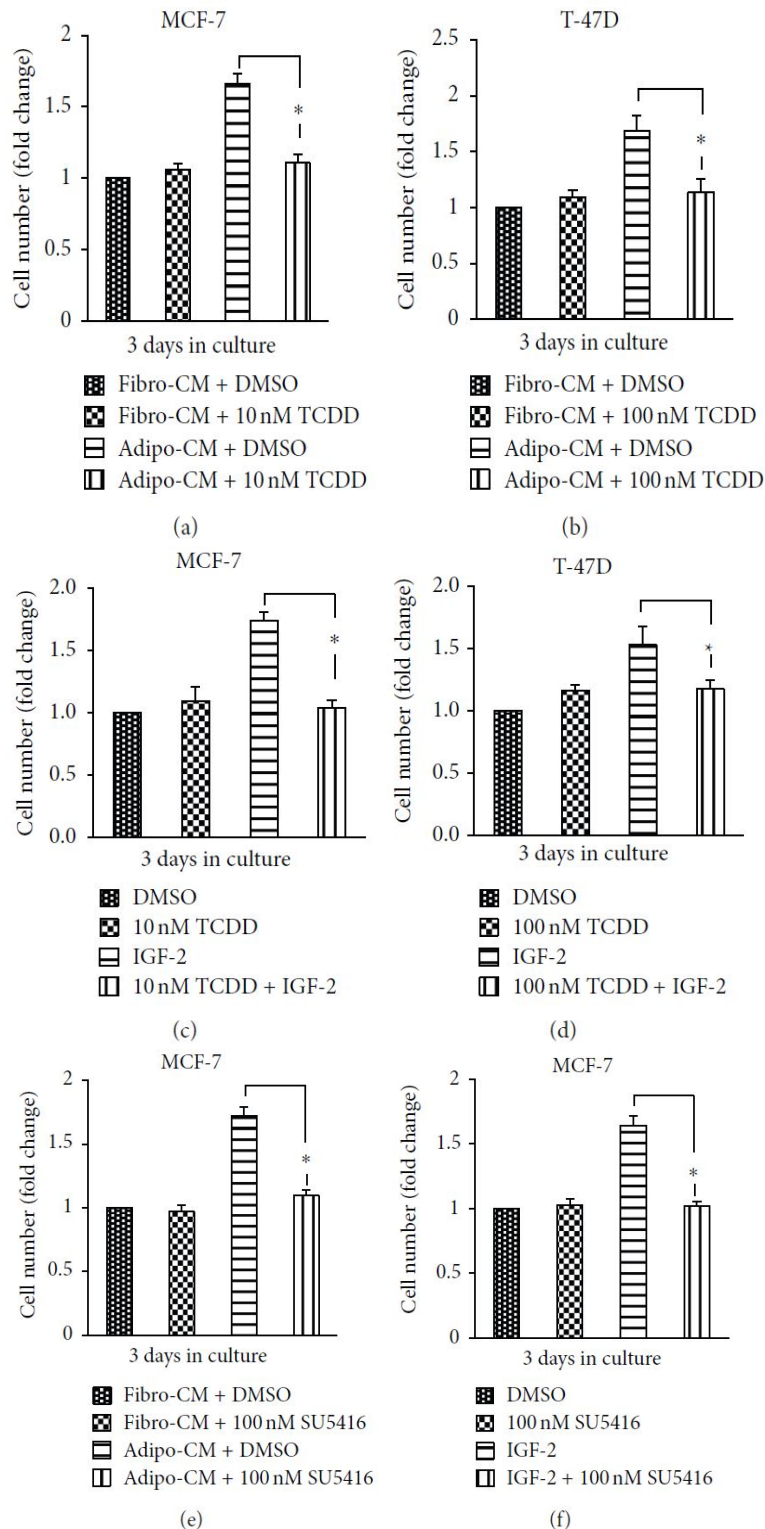
Figure 12. AHR ligands inhibit adipo-CM and IGF-2 stimulated breast cancer cell growth. (A, B) The total number of live MCF-7 (A) and T-47D (B) cells treated with DMSO vehicle or TCDD in fibro-CM or adipo-CM for three days in culture was determined and is displayed relative to the number of live cells in the fibro-CM DMSO control group, which was arbitrarily assigned a value of 1. Data shown are the means ± S.E. for three experiments, and a significant (P < 0.05) decrease in cell number by TCDD (*) is indicated. (C, D) The total number of MCF-7 (C) and T47D (D) cells treated with DMSO or TCDD alone or plus IGF-2 (100 ng/mL) for three days in culture was determined and is displayed relative to the number of live cells in the DMSO group, which was assigned a value of 1. Data shown are the means ± S.E. for three experiments, and a significant (P < 0.05) decrease in cell number by TCDD (*) is indicated. (E, F) The total number of MCF-7 cells stimulated with DMSO or SU5416 in fibro-CM or adipo-CM (E) or with DMSO or SU5416 alone or plus IGF-2 (100 ng/mL) (F) for three days in culture was determined and is displayed relative to the number of live cells in the DMSO group, which was assigned a value of 1. Data shown are the means ± S.E. for three experiments, and a significant (P < 0.05) decrease in cell number by TCDD (*) is indicated.

63

with a highly specific AHR ligand specifically inhibits the mitogenic effects of adipokines that are present in the adipo-CM. Next, we conducted experiments to determine whether TCDD inhibits IGF-2 stimulated breast cancer cell proliferation. Treating MCF-7 and T-47D cells with IGF-2 (100 ng/mL) plus TCDD significantly ($P < 0.0001$ and $P < 0.0034$, resp.) inhibited proliferation compared to that observed in control cells stimulated with IGF-2 plus DMSO vehicle (Figures 12 (C) and (D)). This result indicates that activation of the AHR by a highly specific AHR agonist blocks IGF-2 stimulated breast cancer cell proliferation.

To provide further evidence that ligand-activated AHR inhibits the proliferation effects of adipokines, we tested a second AHR agonist, SU5416 [252]. The application of adipo-CM with SU5416 significantly ($P < 0.0001$) inhibited MCF-7 cell proliferation compared to that observed in adipo-CM plus DMSO vehicle group (Figure 12 (E)). SU5416 did not reduce breast cancer cell number in the presence of fibro-CM (Figure 12 (E)). Further, SU5416 significantly ($P < 0.001$) blocked IGF-2 stimulated MCF-7 cell proliferation (Figure 12 (F)). Collectively, these data indicate that exogenous AHR ligands inhibit the proliferative effects of mitogenic adipokines (including IGF-2) in human ER expressing breast cancer cells.

4.3.3. AHR Ligand-Stimulated Inhibition of IGF-2 Requires the AHR

Next, experiments were conducted to provide evidence that exogenous AHR ligands inhibit the proliferative effects of adipokines through a mechanism that is dependent on the AHR. To this end, MCF-7 and T-47D cells were transiently transfected with a short interfering RNA that specifically targets the AHR (AHR-siRNA) or with a nontargeting control siRNA (con-siRNA) prior to treatment with IGF-2 (100 ng/mL) supplemented with DMSO vehicle or TCDD. The level of AHR protein in AHR-siRNA transfected MCF-7 and T-47D cells was significantly ($P < 0.0002$ and $P < 0.0001$, resp.) lower than in con-siRNA transfected cells (Figures 13 (A) and

(B)). In the presence of con-siRNA, TCDD significantly ($P < 0.001$) inhibited IGF-2 stimulated

MCF-7 proliferation (Figure 13 (B)). Reducing the AHR in the presence of IGF-2 significantly

($P < 0.001$) inhibited proliferation relative to control cells treated with con-siRNA plus IGF-2

(Figure 13 (B)). This result suggests that in the absence of exogenous AHR ligands, the AHR

itself plays an endogenous role in MCF-7 cells that is required for maximal proliferation in the

presence of IGF-2. Importantly, as shown in Figure 13 (B), reducing AHR prevented TCDD

inhibition of IGF-2 (Figure 13 (B); compare last two right bars). This latter result provides

evidence that TCDD inhibits IGF-2 by activating the AHR, because TCDD does not inhibit IGF-

2 in cells with reduced expression of the AHR (Figure 13 (B)). The TCDD-stimulated inhibition

of IGF-2 by AHR was further confirmed in T-47D cells, which showed the TCDD

antiproliferative effects towards IGF-2 were reversed upon AHR knockdown (Figures 13 (C) and

(D)). To provide evidence that a different AHR ligand also inhibits IGF-2 by specifically

activating the AHR, AHR-siRNA experiments were repeated with SU5416. As shown in Figure

13 (E), under con-siRNA conditions, SU5416 significantly ($P < 0.0001$) inhibited IGF-2

stimulated MCF-7 cell proliferation. In accordance with our prior result, reducing the AHR itself

inhibited MCF-7 cell proliferation in the presence of IGF-2 compared to control cells transfected

with con-siRNA and stimulated with IGF-2 (Figure 13 (E)). AHR-siRNA blocked SU5416

inhibition of IGF-2 (Figure 13 (E); compare last two right bars). Collectively, these data provide

mechanistic evidence that upon activation by exogenous AHR agonists TCDD and SU5416, the

AHR inhibits IGF-2 stimulated MCF-7 and T-47D proliferation.
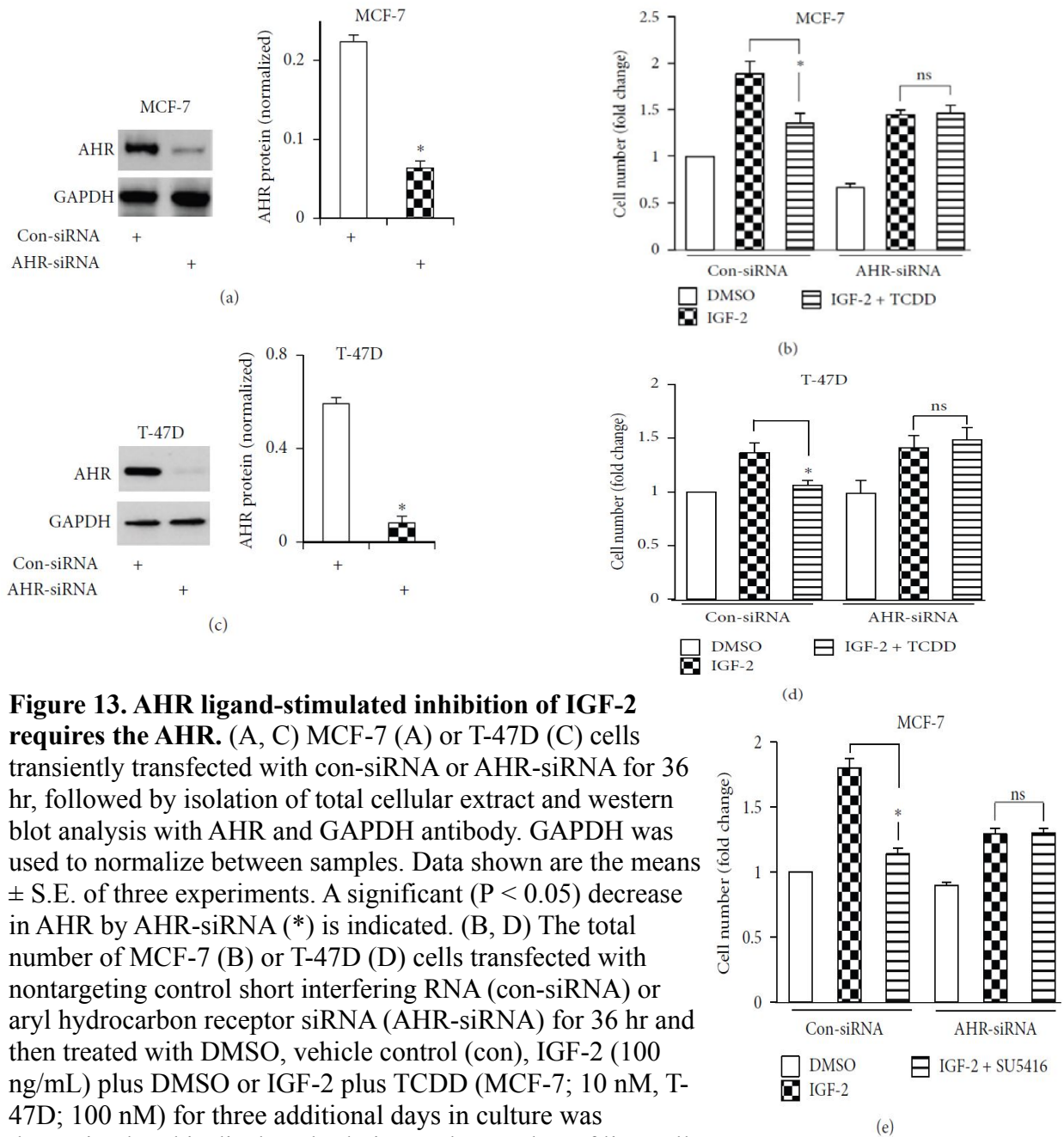
Figure 13. AHR ligand-stimulated inhibition of IGF-2 requires the AHR. (A, C) MCF-7 (A) or T-47D (C) cells transiently transfected with con-siRNA or AHR-siRNA for 36 hr, followed by isolation of total cellular extract and western blot analysis with AHR and GAPDH antibody. GAPDH was used to normalize between samples. Data shown are the means ± S.E. of three experiments. A significant ($P < 0.05$) decrease in AHR by AHR-siRNA (*) is indicated. (B, D) The total number of MCF-7 (B) or T-47D (D) cells transfected with nontargeting control short interfering RNA (con-siRNA) or aryl hydrocarbon receptor siRNA (AHR-siRNA) for 36 hr and then treated with DMSO, vehicle control (con), IGF-2 (100 ng/mL) plus DMSO or IGF-2 plus TCDD (MCF-7; 10 nM, T-47D; 100 nM) for three additional days in culture was determined and is displayed relative to the number of live cells in the DMSO con-siRNA group, which was assigned a value of 1. Data shown are the means ± S.E. of three experiments. (E) The total number of MCF-7 cells transfected with con-siRNA or AHR-siRNA for 36 hr and then treated with DMSO vehicle control (con), IGF-2 (100 ng/mL) plus DMSO, or IGF-2 plus SU5416 (100 nM) for three additional days in culture was determined and is displayed relative to the number of live cells in the DMSO con-siRNA group, which was assigned a value of 1. Data shown are the means ± S.E. of three experiments.

66

Symbol	Description	P value	Fold regulation
E2F1	E2F transcription factor 1	0.042684	−4.0169
CCND1	Cyclin D1	0.007724	−3.1767
MYB	V-myb myeloblastosis viral oncogene homolog (avian)	0.026054	−3.1760
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	0.027237	−2.5269
JAK2	Janus kinase 2	0.028474	−2.0015
JUND	Jun D proto-oncogene	0.026855	−2.0066

Table 7. Reduced expression of proto-oncogenes in MCF-7 cells stimulated with IGF-2 plus TCDD compared to cells stimulated with IGF-2 at 48hrs.

4.3.4. TCDD Stimulates Reductions in E2F1, CCND1, MYB, SRC, JAK2, and JUND Gene Expression

To begin to investigate the mechanism by which ligand-activated AHR inhibits proliferation, experiments were conducted to determine whether TCDD reduces the expression of genes that could be important for breast cancer cell proliferation in the presence of IGF-2. To this end, we used a commercially available RT2 Profiler PCR Array that is capable of integrating 84 genes that are protooncogenes or tumor suppressors and thus key regulators of tumorigenesis. We focused on comparison between two groups, MCF-7 cells stimulated with IGF-2 and MCF-7 cells cotreated with IGF-2 plus TCDD for 48 hrs. This analysis revealed that the expression of E2F1, CCND1, MYB, SRC, JAK2, and JUND was significantly reduced in cells treated with IGF-2 plus TCDD compared to that observed in cells stimulated with IGF-2 (Table 7; n= 3).

TCDD inhibition of these specific gene targets could be one mechanism by which ligand-activated AHR inhibits mitogenic adipokine signaling, because the observed downregulated genes in TCDD treated cells play important roles in breast cancer cell proliferation. E2F1 is a transcription factor that binds retinoblastoma protein, enhances the proliferation of human cancer cells, and stimulates increases in the transcription and expression of CCND1 [256]. The tyrosine kinase JAK2 upon activation by cytokine receptor induced signaling to stimulate the phosphorylation and activation of STAT3 [247]. When activated by JAK2, STAT3 promotes the transcription and expression of CCND1 [244]. The basic leucine zipper (bZIP) transcription factor JUND by function as a component of AP-1 enhances the transcription and expression of CCND1 [257]. MYP is a 17 beta-estradiol regulated transcription factor that has been reported to be important for the proliferation of ER expressing human breast cancer cells, including T-47D and MCF-7 cells [257].

4.4. CONCLUSION

In summary, we provide evidence that adipocyte secreted IGF-2 is sufficient to contribute to the proliferation of human ER expressing breast cancer cells. This result suggests that adipocyte secretion of IGF-2 could play a role in promoting breast cancer in the context of obesity. Adipocytes have also been reported to secrete IGF-1 in the presence of high glucose and exogenously added fatty acids [245]. Both IGF-1 and IGF-2 enhance the proliferation of human cancer cells through IGF1-R and IR-A [248, 249]. Using highly specific AHR agonists and AHR-siRNA, we provide the first evidence suggesting that specific ligand activation of the AHR inhibits adipo-CM and IGF-2-stimulated proliferation of ER positive human breast cancer cells. It is important to note that E2F1, SCR, JAK2, and JUND are critical upstream transcriptional regulators of CCND1 [244, 247, 256, 258]. Thus, their downregulation by TCDD (Table 7) could be one mechanism by which TCDD downregulates CCND1 expression. CCND1 is also a critical mediator of the proliferative effects of IGF proteins and other adipokines like leptin [244, 249]. Therefore, TCDD inhibition of CCND1 expression (Table 7) is likely to be one of the major mechanisms that inhibit mitogenic adipokine signaling in breast cancer cells. The results of this study provide the impetus for future study investigating the transcriptional mechanisms by which ligand-activated AHR by regulating the expression of prooncogenes modulates mitogenic adipokine signaling in human breast cancer cells. Collectively, this report provides evidence that drugs that target the AHR may reduce breast cancer risk in the context of human obesity.

4.5. CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

4.6. ACKNOWLEDGEMENTS

CHAPTER V: INSULIN LIKE GROWTH FACTOR 2 REGULATION OF ARYL HYDROCARBON RECEPTOR IN MCF-7 BREAST CANCER CELLS.

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ABSTRACT

Insulin like growth factor (IGF)-1 and IGF-2 stimulate normal growth, development and breast cancer cell proliferation. Cyclin D1 (CCND1) promotes cell cycle by inhibiting retinoblastoma protein (Rb). The aryl hydrocarbon receptor (AHR) is a major xenobiotic receptor that also regulates cell cycle. The purpose of this study was to investigate whether IGF-2 promotes MCF-7 breast cancer proliferation by inducing AHR. Western blot and quantitative real time PCR (RT-qPCR) analysis revealed that IGF-2 induced an approximately two-fold increase ($P < .001$) in the expression of AHR and CCND1. Chromatin immunoprecipitation (ChIP), followed by qPCR indicated that IGF-2 promoted ($P < .001$) a seven-fold increase in AHR binding on the CCND1 promoter. AHR knockdown significantly ($P < .001$) inhibited IGF-2 stimulated increases in CCND1 mRNA and protein. AHR knockdown cells were less ($P < .001$) responsive to the proliferative effects of IGF-2 than control cells. Collectively, our findings have revealed a new regulatory mechanism by which IGF-2 induction of AHR promotes the expression of CCND1 and the proliferation of MCF-7 cells. This previously uncharacterized pathway could be important for the proliferation of IGF responsive cancer cells that also express AHR.

Keywords: Aryl hydrocarbon Receptor, IGF-2, CCND1, breast cancer cells

5.1. INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor whose activity is regulated by lipid soluble environmental toxicants [253]. 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) is a prototypical AHR agonist which is found in Agent Orange [253]. The binding of TCDD to AHR stimulates the AHR to translocate into the nucleus and stimulate transcription through specific xenobiotic response elements (XREs) in enhancers and promoters of TCDD stimulated genes [253, 259]. TCDD through AHR induces the expression of a "battery" of phase I and phase II drug metabolizing enzymes including the prototype TCDD-AHR gene target cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) [253, 259].

The AHR also regulates cell cycle in part by binding with Cyclin D1 (CCND1) and cyclin dependent kinase 4 (CDK4) [105, 260]. CDK4 phosphorylates retinoblastoma protein (Rb), which inhibits Rb-mediated repression of E2F transcription factors [261-263]. The activation of E2F induces the expression of E2F target genes that are important for DNA synthesis and cell cycle advance [261-263]. Mitogens promote CDK4 activity by increasing the levels of cyclin proteins including CCND1 [261-263]. By functioning as a regulatory subunit on CDK holoenzymes, CCND1 promotes the phosphorylation and inhibition of RB1 to promote cell cycle advance and proliferation [261-263]. The AHR binds to CDK4 during advance through the cell cycle in human MCF-7 breast cancer cells [105]. TCDD binding to AHR attenuates AHR binding with CDK4, which correlated with cell cycle arrest and reductions in RB1 phosphorylation in MCF-7 cells [105]. CCND1 was also present in CDK4-AHR complexes [105].

Insulin like growth factor (IGF)-1 and IGF-2 stimulate growth, development and the proliferation of human cancer cells including breast cancer cells [248, 249]. MCF-7 breast cancer

73

cells have been reported to express high levels of IGF-1 receptor (IGF1-R) and insulin receptor subtype A receptor (IR-A) [248, 249]. IGF-R1 and IR-A mediate the proliferative effects of IGFs on human breast cancer cells by inducing the phosphoinositide 3-kinase (PI3K)/AKT (protein kinase B) pathway and the mitogen-activated protein kinase (MAPK) pathway [248, 249, 264]. IGF-1 and IGF-2 have also been reported to increase levels of CCND1 to induce proliferation [248, 249, 262]. CCND1 promoter activity is regulated through multiple enhancers including activator protein-1 (AP-1) and T-cell factor (TCF)/lymphoid enhancing factor (LEF) sites [265-268]. The transcription factors Jun and Fos bind to the AP-1 response elements [265, 266]. The transcriptional co-activator β-catenin confers transcriptional activity to TCF/LEF transcription factors bound to TCF/LEF elements in the CCND1 promoter [267, 268].

We have recently shown that adipocytes secrete levels of IGF-2 that are sufficient to stimulate the proliferation of MCF-7 and T-47D breast cancer cells [111]. We also found that AHR knockdown MCF-7 cells were less responsive to the proliferative effects of IGF-2 [111]. The purpose of this study was to investigate if: 1) IGF-2 signaling regulates the AHR and 2) IGF-2 induction of CCND1 requires AHR. We provide evidence that IGF-2 signaling activates AHR and that AHR is important for inducing the expression of CCND1 and MCF-7 proliferation. This is a new link between IGF-2 signaling and AHR.

5.2. MATERIALS AND METHODS

5.2.1. Materials and MCF-7 cell culture

Dulbecco's Modified Eagle Medium/High glucose (DMEM) with L-glutamine and sodium pyruvate, 10% fetal bovine serum, penicillin, and streptomycin (100μg/mL) and phosphate buffered saline (PBS) were purchased from Fisher Scientific. Non-specific control RNA (cRNAi) (cat # D-001810-01-20), short interfering RNA against the AHR (AHRi) (J-004990-08-0010) and Dharmafect transfection reagent (#1) were purchased from Thermo Scientific. MCF-7 human breast cancer cells were purchased from ATCC (Manassas) and maintained in DMEM, 10% FBS, with penicillin (100U/mL) and streptomycin (100 μg/mL) and .01 μg/mL bovine insulin (Cell Applications, Inc.) Insulin like growth factor 2 (IGF-2) was purchased from R & D systems and reconstituted in phosphate buffered solution.

5.2.2. Western blot analysis to determine IGF-2 induction of AHR and CCND1

200,000 MCF-7 cells plated in 35 mm plates (50% confluent) were serum starved overnight in phenol red-free DMEM and then treated with PBS vehicle or IGF-2 (100 ng/mL) for three hrs. This time point was selected based on our preliminary time course experiments showing that IGF-2 induction of CCND1 mRNA is maximal at three hr post IGF-2 (data not shown). Total cellular extract was isolated in 200 μL of 2X sample lysis buffer (Bio-RAD; cat #161-0737) and approximately 12.5 μg of protein was subjected to SDS PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad; Hercules, CA). Membranes were blocked in PBS, .01% Tween 20 (Bio-Rad; Hercules, CA) (PBS-T), 5% (wt/vol) lowfat powdered milk for one hr and incubated overnight with primary antibody at 4° C with gentle mixing. Membranes were rinsed five times (five mins each wash) with PBS-T and then incubated with an appropriate HRP-labeled secondary antibody (diluted 1:10,000 in PBS, .01% tween-20,

5% milk) (Thermo Scientific, Pierce) for one hr, followed with rinsing five times (five mins each wash) in PBS-T. Membranes were developed with enhanced chemiluminescent substrate (Millipore, Immobilon ECL substrate) and exposure to X-ray film (Midwest Scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Millipore (cat # MAB374), AHR antibody from Santa Cruz (Cat # H-211) and CCND1 antibody from Millipore (cat # 04-1151). Equal protein loading was confirmed by GAPDH western blots. Normalized levels of AHR, and CCND1 were expressed as a ratio of AHR/GAPDH and CCND1/GAPDH. Densitometry was calculated with ImageJ PC-based software (National Institute of Health).

5.2.3. qPCR analysis to determine IGF-2 induction of AHR, CYP1A1, and CCND1

200,000 MCF-7 cells plated in 35 mm plates were serum starved overnight in phenol red-free DMEM, and then treated with PBS vehicle or IGF-2 (100 ng/mL) for three hrs. Total RNA was isolated in TRI-Reagent and quantitated by Nanodrop spectrophotometry. RNA was reverse transcribed to complementary DNA (cDNA) (Verso cDNA kit; Thermo Fisher Scientific; cat # AB-1453/B). Resulting cDNAs were subjected to quantitative real-time PCR (Q-PCR) using gene specific primers (300 nM per reaction) and 40 cycles of PCR in accordance with Absolute Blue SYBR Green Rox Mix (Thermo Fisher Scientific; cat # AB-4162/B) protocols. Relative gene expression between controls and IGF-2 treated cells was calculated using the formula 2-$\Delta\Delta$CT, as described by Livak and Schmittgen [239]. Glyceraldehyde-3-phosphate (GAPDH) mRNA levels served as the internal control. Primer sequences for GAPDH, AHR, CCND1 and CYP1A1 were: GAPDH (forward, 5′-CATGAGAAGTATGACAACAGCCT-3′; reverse, 5′-AGTCCTTCCACGATACCAAAGT-3′), AHR (forward, 5′-ACATCACCTACGCCAGTGG-3′; reverse, 5′-CTCTATGCCGCTTGGAAGGAT-3′), CCND1 (forward, 5′-

CCGCAATGACCCCGCACGAT-3′; reverse, 5′-AGGGCAACGAAGGTCTGCGC-3′) and

CYP1A1 (forward, 5′-CTTCACCCTCATCAGTAATGGTC-3′; reverse, 5′-

AGGCTGGGTCAGAGGCAAT′-3). The Harvard Primer Bank

http://pga.mgh.harvard.edu/primerbank/was used to design primers. Primer specificity was

verified with melt curve analysis and NIH primer blast search engines

http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

5.2.4. Chromatin immunoprecipitation

MCF-7 cells (500,000 per 60 mm plate) were serum starved overnight in phenol red-free

DMEM, and then treated with PBS vehicle or IGF-2 (100 ng/mL) for three hr. Formaldehyde

(1%) was then added to medium for ten mins, followed by Glycine (.5M) for five mins. Cells

were rinsed with PBS, collected in PBS, pelleted by centrifugation, and lysed in 300 μL of lysis

buffer (1% SDS; 5 mM EDTA; 50 mM Tris-HCl, pH 8) per 60 mm plate plus protease inhibitors

(Thermo Scientific) for fifteen mins on ice. Cell extracts were sonicated (five times, each time

ten seconds) and diluted 1:10 in dilution buffer (16.7 mM Tris-HCl, pH 8; 167 mM NaCl; 1.2

mM EDTA; 0.01% SDS; 1.1% Triton X-100), rotated overnight at 4C with 1 μg of non-specific

IgG (Santa Cruz; cat # 2027) or anti-AHR antibody (Santa cruz; cat # H-211). Antibody-

chromatin complexes were collected using 5 μL of magnetic protein A beads (life technologies;

cat # 100.01D) with rotation at 4° C for 90 min. Using magnetic separation (life-technologies;

part # 49-2025), beads were washed sequentially with buffer 1 (20 mM Tris-HCl, pH 8; 150 mM

NaCl; 2.0 mM EDTA; 0.1% SDS), buffer 2 (20 mM Tris-HCl, pH 8; 500 mM NaCl; 2.0 mM

EDTA; 0.1% SDS), buffer 3 (10 mM Tris-HCl (pH 8); 0.25M LiCl; 1mM EDTA; 1% NP-40; 1%

deoxycholate), and then 1X TE buffer for five mins each, and incubated at 65° C for four to six

hrs in elution buffer (1% SDS, 0.1 M NaHCO3) with proteinase K. DNA was purified (Qiagen;

cat # 28204) and analyzed using real time PCR. Primers spanning AP-1 and TCF/LEF response elements in the CCND1 promoter and xenobiotic response elements (XREs) in the CYP1A1 promoter were used: AP-1 (forward, 5′-GGCAGAGGGGACTAATATTTCCAGCA-3′; reverse, 5′-GAATGGAAAGCTGAGAAACAGTGATCTCC-3′) [269], TCF/LEF (forward, 5′-GCTCCCATTCTCTGCCGG-3′; reverse, 5′-CGGAGCGTGCGGACTCTG-3′) [270] and XRE (forward, 5′-ACGCAGACCTAGACCCTTTGC-3′, reverse, 5′-CGGGTGCGCGATTGAA-3′) [271]. ChiP data was expressed as % input, in which signals obtained from the ChIP are divided by signals obtained from an input sample.

5.2.5. AHR knockdown experiments

MCF-7 cells were reverse transfected using methods we have used previously to selectively target genes for knockdown [272]. Briefly, 200,000 MCF-7 cells were plated in phenol red-free DMEM, 5% charcoal treated FBS, 50 nM cRNAi or AHRi, 2 μL of Dharmafect #1 per well of a six well plate for twelve hr and then new media was applied for twenty four hrs. Cells were then serum starved overnight in phenol red-free DMEM, followed by treatment with PBS vehicle or IGF-2 (100 ng/mL) for three hr. Treatments were stopped and cellular protein or mRNA was isolated for western blot and Q-PCR analysis, respectively, as detailed in sections 2.2 and 2.3.

5.2.6. Cell growth experiments

MCF-7 cells were reverse transfected with 50 nM cRNAi or AHRi as detailed in 2.4 for 36 hr and then serum starved overnight in phenol red-free DMEM. Cells were treated with PBS vehicle or IGF-2 (100 ng/mL) for three additional days. Cells were collected in trypsin and manual cell counting using a hemocytometer and trypan blue was used to determine live cell number.

5.2.7. Statistics

Two-tailed, paired t tests with confidence intervals of 95% were used to determine statistically significant differences between two groups (vehicle versus IGF-2 treatment) in Figure 14. The Newman–Keuls (SNK) post-hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA) in Figure 15, Figure 16, and Figure 17. Specific P values are indicated in the results sections.

5.3. RESULTS

5.3.1. IGF-2 increases AHR expression

To investigate whether IGF-2 stimulates AHR expression, overnight serum starved MCF-7 cells were treated with vehicle or IGF-2 (100 ng/mL) for three hrs. Western blot analysis revealed that IGF-2 induced 1.7- and 1.5-fold increases ($P < .01$) in AHR and CCND1 protein, respectively, compared with vehicle (Figure 14 (A)). The observed increases in AHR and CCND1 protein correlated with significant increases ($P < .01$) in AHR mRNA (1.9-fold) and CCND1 mRNA (2-fold) in IGF-2 treated cells compared with control cells (Figure 14 (B)). The levels of CYP1A1 mRNA, which is a TCDD-AHR gene target, were not induced by IGF-2 treatment (Figure 14 (B)). This result suggests that IGF-2 signaling does not induce rapid increases in the levels of a lipophilic endogenous AHR ligand capable of inducing CYP1A1 expression.

5.3.2. IGF-2 stimulates AHR binding to CYP1A1 and CCND1 gene promoters

To determine if IGF-2 promotes AHR binding to gene promoters, we performed chromatin immunoprecipitation experiments followed by real-time quantitative PCR (Q-PCR) (ChIP-qPCR). We first focused on AHR binding XREs in the CYP1A1 promoter [271]. AHR-ChIP-qPCR experiments revealed that AHR binding to the XREs on the CYP1A1 promoter in vehicle treated cells was low and not significantly higher than non-specific IgG (Figure 15 (A)). In contrast, IGF-2 promoted a 5-fold increase ($P < .0001$) in AHR binding to the XREs on the CYP1A1 promoter compared to vehicle and this was significantly higher than non-specific IgG (Figure 15 (A)).
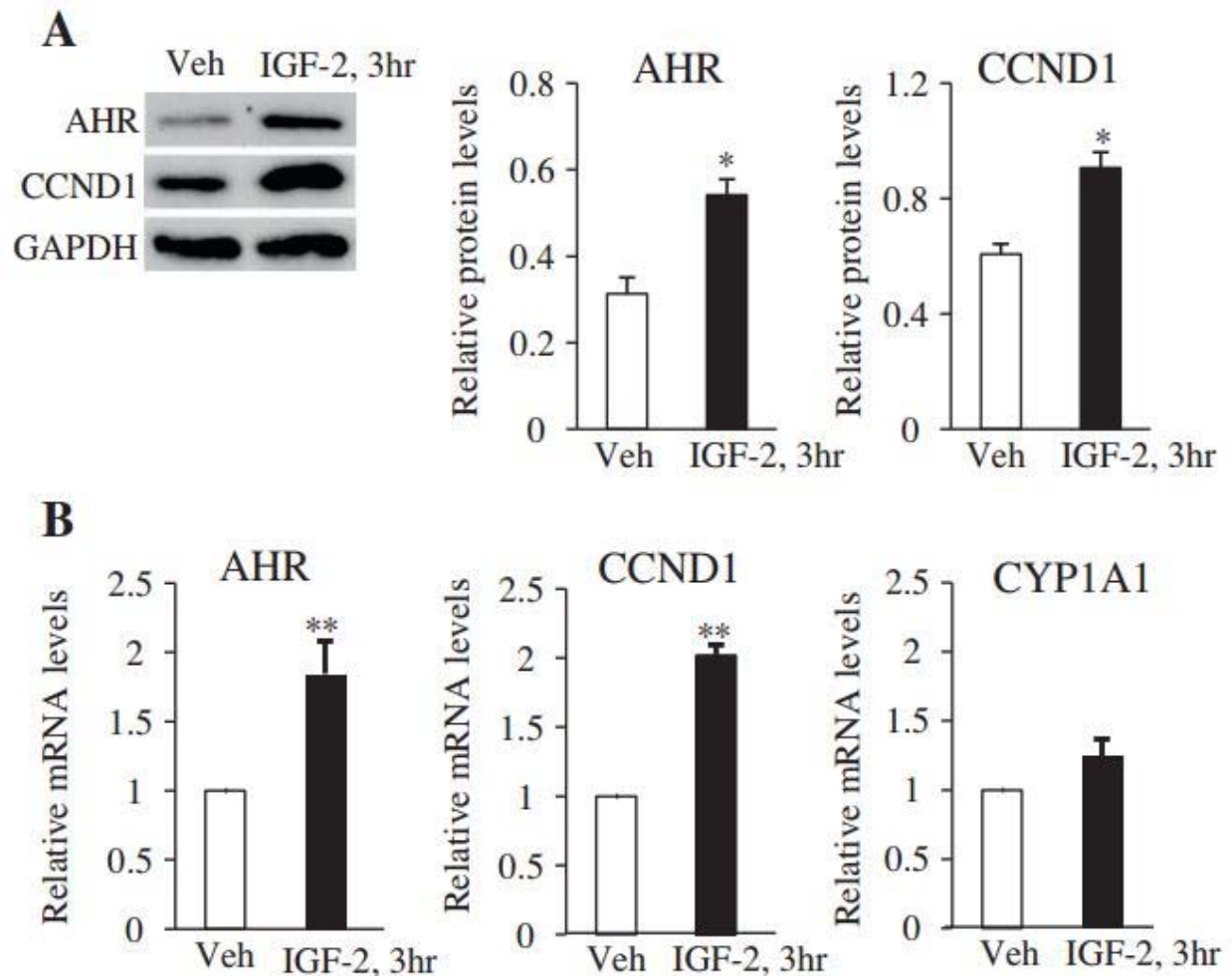
Figure 14. IGF-2 stimulates AHR. MCF-7 cells were treated with vehicle (Veh) or IGF-2 (100 ng/mL) for 3 h. (A) Total cellular protein was then isolated and subjected to Western blot analysis. The blot was then probed with the indicated antibodies. Relative level of AHR and CCND1 protein was expressed as a ratio of AHR/GAPDH and CCND1/GAPDH. Significant ($P < .01$) increases in AHR, CCND1 protein by IGF-2 are indicated (*). (B) Quantitative real-time quantitative polymerase chain reaction (Q-PCR) analysis of relative mRNA levels of CCND1, AHR and CYP1A1, with normalization to GAPDH internal control. The value in vehicle treated cells was set to 1. Significant ($P < .01$) increases in AHR and CCND1 mRNA by IGF-2 are indicated (**). CYP1A1 mRNA was not significantly induced by IGF-2. Data shown are the means ± S.E. of (A) three and (B) four independent experiments.
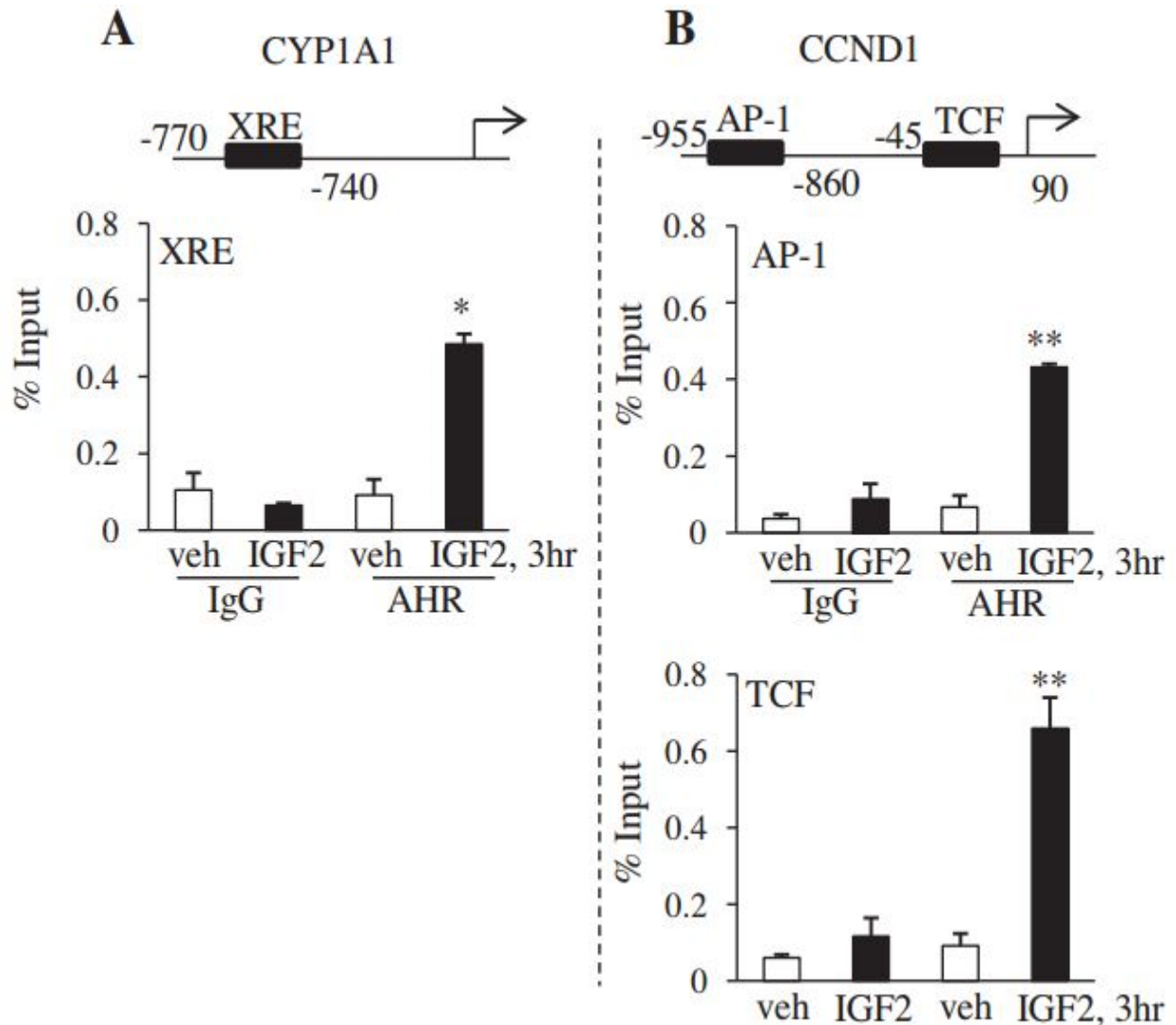
Figure 15. IGF-2 stimulates AHR binding on CYP1A1 and CCND1 gene promoters. MCF-7 cells were treated with vehicle (Veh) or IGF-2 (100 ng/mL) for 3 h and chromatin immunoprecipitation (ChIP) experiments were conducted, followed by Q-PCR. (A) A significant ($P < .0001$) increase in AHR binding to the XRE on the CYP1A1 promoter induced by IGF-2 is indicated (*). (B) A significant ($P < .0001$) increase in AHR binding to the AP-1 and TCF/LEF site induced by IGF-2 is indicated as (**). Data shown are the means ± S.E. of three independent experiments.

To determine if IGF-2 stimulates the AHR to bind to the promoter of an induced gene, we examined AHR binding to the AP-1 and TCF/LEF response elements on the CCND1 promoter. These transcription factor binding sites were selected because AP-1 and TCF/LEF sites promote CCND1 transcription [265, 267, 268]. The binding of AHR to AP-1 and TCF/LEF sites on the CCND1 promoter was minimal in vehicle treated cells and not significantly different than non-specific IgG (Figure 15 (B)). In contrast, the binding of AHR to AP-1 and TCF/LEF response elements on the CCND1 promoter was substantially increased (approximately seven-fold) by IGF-2 and this was significantly ($P < .0001$) greater than non-specific IgG (Figure 15 (B)). Collectively, these results indicate that IGF-2 promotes AHR binding to CYP1A1 and CCND1 gene promoters.

5.3.3. AHR knockdown inhibits IGF-2 induction of CCND1

Next, experiments were conducted to investigate if IGF-2 induction of CCND1 requires AHR. Overnight serum starved control and AHR knockdown MCF-7 cells were treated with vehicle or IGF-2 (100 ng/mL) for three hrs. As shown in Figure 16 (A), AHR protein levels were lower ($P < .0001$) in AHR knockdown cells (by ten-fold) than control cells. IGF-2 stimulated ($P < .001$) approximately two-fold increases in AHR and CCND1 protein in control cells, which was completely abrogated in AHR knockdown cells (Figure 16 (A)). Analysis of mRNA revealed that IGF-2 stimulated a 1.5-fold increase in CCND1 mRNA, which was reduced ($P < .01$) to 1.3-fold in AHR knockdown cells (Figure 16 (B)). These findings indicate that AHR knockdown cells are less responsive to IGF-2-stimulated increases in CCND1 mRNA and CCND1 protein.

5.3.4. AHR knockdown compromises MCF-7 proliferation

To determine the role of AHR in cell proliferation, MCF-7 control and AHR knockdown cells were treated with vehicle or IGF-2 (100 ng/mL) for three days. IGF-2 stimulated a 2.3-fold
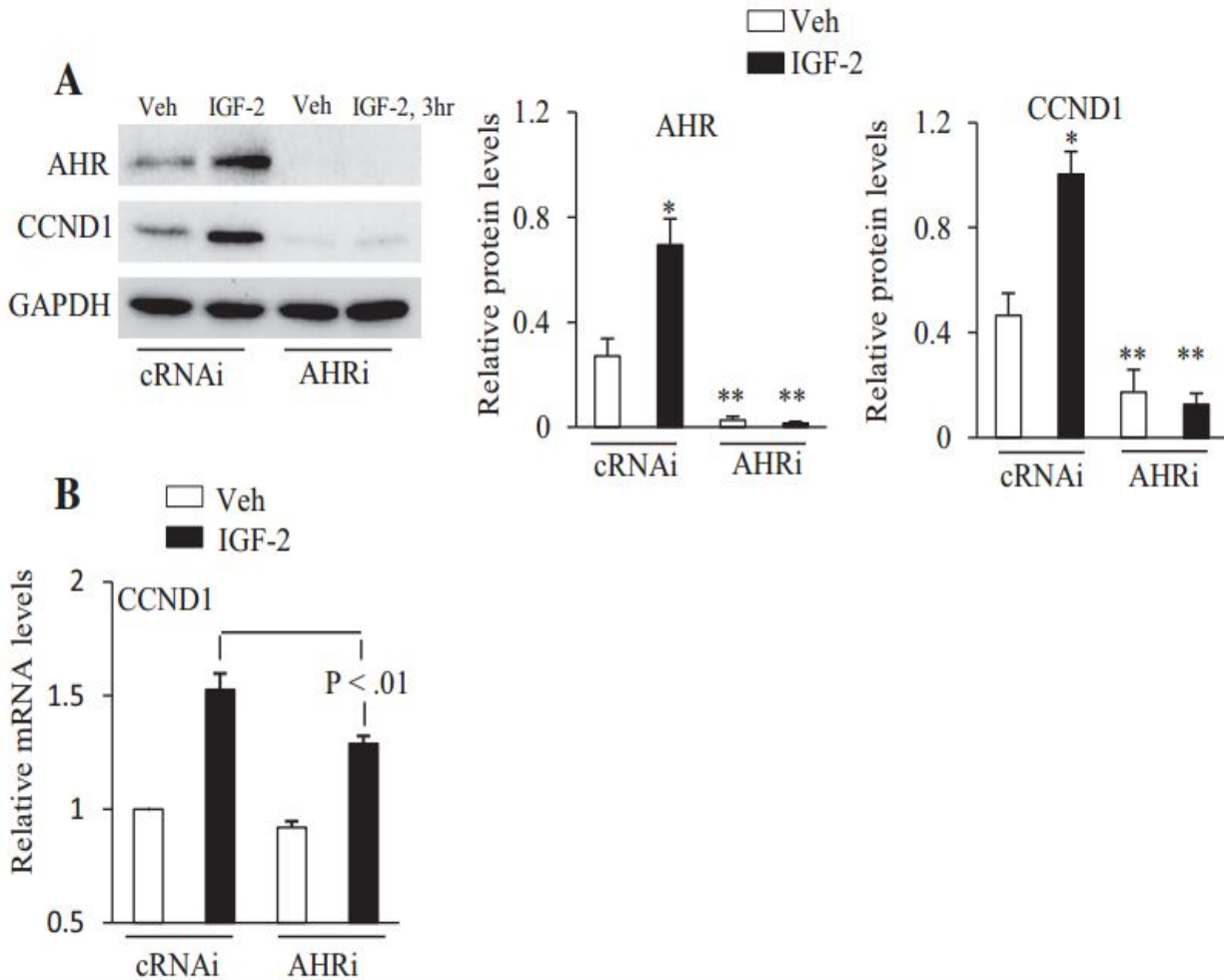
Figure 16. IGF-2 induction of CCND1 requires AHR. MCF-7 cells were reverse transfected (see Section 2 for details) with cRNAi or AHRi prior to treatment with vehicle (Veh) or IGF-2 (100 ng/mL) for 3 h. (A) Total cellular protein was then isolated and subjected to Western blot analysis. The blot was then probed with the indicated antibodies. Relative level of CCND1 and AHR protein was expressed as a ratio of AHR/GAPDH and CCND1/GAPDH. A significant (P < .001) increase in AHR and CCND1 by IGF-2 is indicated by (*). A significant (P < .001) decrease in AHR and CCND1 by AHRi is indicated by (**). (B) Q-PCR analyses of relative levels of CCND1 mRNA, expressed normalized to GAPDH. A significant decrease in CCND1 by AHRi is indicated by (P < .01). Data shown are the means ± S.E. of three independent experiments.
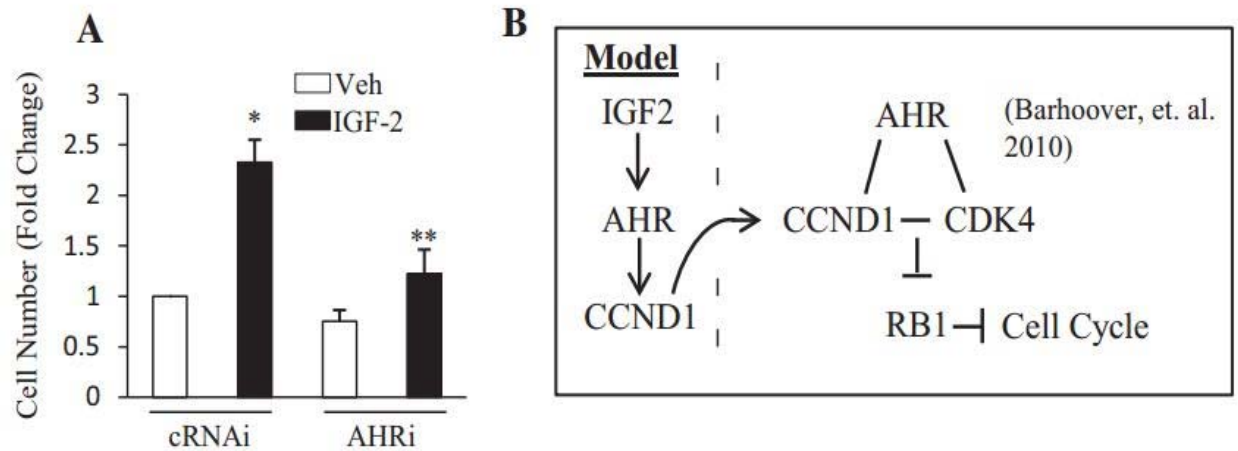
Figure 17. IGF-2 induction of MCF-7 proliferation requires AHR. (A) MCF-7 cells were reverse transfected (see Section 2 for details) with cRNAi or AHRi prior to treatment with vehicle (Veh) or IGF-2 (100 ng/mL). After three days in culture, cells were collected and live cell number was determined. Cell number (Fold change) was expressed relative to the number of live cells in the cRNAi plus Veh group, which was arbitrarily assigned a value of 1. (B) Model of AHR roles in IGF-2 signaling. Dotted lines distinguish our data herein and data from Barhoover et al. 2010. IGF-2 signaling increases AHR, increases in AHR stimulate increases in CCND1 protein. The increased levels of AHR in IGF-2 treated cells functions as a scaffold that bridges CCND1 and CDK4, which in turn stimulates the phosphorylation and inhibition of RB1. Inhibition of RB1 promotes cell cycle and MCF- 7 proliferation

increase in the number of live MCF-7 cells, which was reduced ($P < .0001$) to 1.2-fold by AHR

knockdown (Figure 17 (A)). Reduced proliferation is consistent with observed reductions in

CCND1 levels in AHR knockdown cells compared with control cells (Figure 16 (A)), given that

CCND1 induces cell cycle progression.

5.4. DISCUSSION

Our data indicates that IGF-2 signaling increases the levels of AHR mRNA and protein (Figure 14) as well as the binding of AHR to AP-1 and TCF/LEF response elements on the CCND1 promoter (Figure 15). We propose that the observed increase in AHR binding to the CCND1 promoter is important for the induction of CCND1 expression. Indeed, we found that AHR knockdown completely abrogated IGF-2 stimulated increases in CCND1 protein and significantly inhibited the induction of CCND1 mRNA compared to control cells (Figure 16).

Modeled in Figure 17 (B) are our findings herein and the findings of Barhoover et al 2010. We show that IGF-2 increases AHR, which in turn stimulates increases in CCND1 protein (Figure 9-11). Based on the results of Barhoover et al 2010 [105], the observed increases in AHR and CCND1 would bind to CDK4, which would promote the phosphorylation and inhibition of RB1 to promote cell cycle advance and MCF-7 proliferation (Figure 17 (B)). Our results showing that AHR knockdown cells are significantly less responsive to the proliferative effects of IGF-2 (Figure 17 (A)) support a requirement of AHR for the induction of CCND1 and CDK4-induced phosphorylation of RB1 in MCF-7 cells [105]. Collectively, our results and the findings of Barhoover et al 2010 [105], provide two different but complementary mechanisms of action by which AHR may mediate the proliferative effects of IGF proteins and perhaps other mitogens that induce CCND1. These new findings suggest that human cancer cells that are highly responsive to IGF growth factors may require the AHR for maximal proliferation.

Specific transcriptional proteins have been reported to stimulate the expression of AHR. Nuclear Factor, Erythroid 2-Like 2 (NRF-2) through the activation of an antioxidant response element (ARE) in the promoter of AHR, induces AHR transcription [273]. Overexpression of constitutively active β-catenin stimulated increases in AHR mRNA and AHR protein in human

colon cancer cells [274]. A prior report showed that the application of medium containing 10% calf serum, platelet-derived growth factor (PDFG) or basic fibroblast growth factor (bFGF) to overnight serum starved murine 3T3 fibroblasts stimulated increases in the levels of AHR protein and the activity of a murine AHR promoter reporter construct, which correlated with the onset of DNA synthesis [275]. Our finding that IGF-2 induced increases in AHR in MCF-7 cells further links growth factor signaling with endogenous AHR regulation. Our result showing that IGF-2 induction of AHR is important for the induction of CCND1 provides insight as to the mechanism by which endogenous AHR regulation stimulates proliferation.

In conclusion, we provide evidence that IGF-2 induction of AHR is important for the induction of CCND1 and MCF-7 proliferation. Barhoover et al 2010 have shown that TCDD inhibits MCF-7 cell cycle by disrupting interactions between CDK4 and AHR in MCF-7 cells [105]. We postulate that TCDD and other exogenous AHR ligands may impact cell proliferation by interfering with AHR-protein interactions on the CCND1 promoter.

5.5. ACKNOWLEDGMENTS

CHAPTER VI: ENDOGENOUS ARYL HYDROCARBON RECEPTOR PROMOTES BASAL AND INDUCIBLE EXPRESSION OF TUMOR NECROSIS FACTOR TARGET GENES IN MCF-7 CANCER CELLS.

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that upon

activation by the toxicant 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) stimulates gene

expression and toxicity. AHR is also important for normal mouse physiology and may play a role

in cancer progression in the absence of environmental toxicants. The objective of this report was

to identify AHR-dependent genes (ADGs) whose expression is regulated by AHR in the absence

of toxicants. RNA-Seq analysis revealed that AHR regulated the expression of over 600 genes at

an FDR<10% in MCF-7 breast cancer cells upon knockdown with short interfering RNA.

Pathway analysis revealed that a significant number of ADGs were components of TCDD and

tumor necrosis factor (TNF) pathways. We also demonstrated that siRNA knockdown of AHR

modulated TNF induction of MNSOD and cytotoxicity in MCF-7 cells. Collectively, the major

new findings of this report are: (1) endogenous AHR promotes the expression of xenobiotic

metabolizing enzymes even in the absence of toxicants and drugs, (2) AHR by modulating the

basal expression of a large fraction of TNF target genes may prime them for TNF stimulation and

(3) AHR is required for TNF induction of MNSOD and the cellular response to cytotoxicity in

MCF-7 cells. This latter result provides a potentially new role for AHR in MCF-7 cancer

progression as a mediator of TNF and antioxidant responses.

Keywords: Aryl hydrocarbon receptor (AHR); Gene expression; Breast cancer; Xenobiotics;
Tumor necrosis factor

GRAPHICAL ABSTRACT

Endogenous AHR target genes and pathways in MCF-7 cancer cells

AHR ⟶ Biofunctions
1. Cell movement
2. Cell growth proliferation
3. Cell morphology
4. Amino Acid metabolism
5. Drug metabolism

TNF targets

Xenobiotic metabolizing enzymes
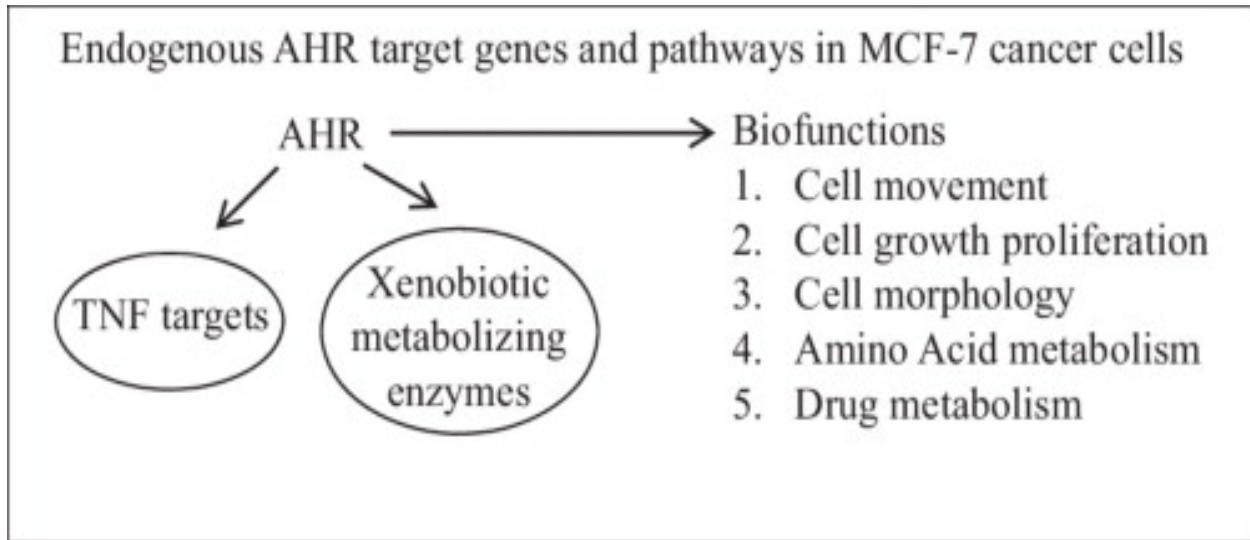
6.1. INTRODUCTION

The environmental toxicant TCDD acts through a ligand-activated transcription factor, the aryl hydrocarbon receptor (AHR), to regulate gene expression and induce toxicity [253]. In the absence of TCDD, AHR localizes to the cytoplasm and is physically associated with heat shock protein 90 (HSP90), AHR interacting protein (AIP) and protein p23 in a protein complex [253]. TCDD stimulates AHR to undergo a conformational change that stimulates its translocation to the nucleus and dissociation away from HSP90, AIP and p23 [253]. Upon entering the nucleus, AHR physically interacts with AHR nuclear translocator (ARNT) to activate canonical TCDD target genes containing dioxin response elements (DREs), including CYP1A1, CYP1B1, NRF2 and AHR Repressor (AHRR) [253]. Prior pathway analyses have shown that TCDD regulated gene sets that are associated with metabolism of xenobiotics by cytochrome P450's, xenobiotic metabolism signaling, and fatty acid and lipid metabolism pathways; these findings are consistent with induction of phase I and phase II drug metabolizing enzymes [276, 277].

Several studies have shown that AHR inhibits and stimulates gene expression in the absence of TCDD [278-281]. For instance, Boutros et al. reported that knockdown of AHR in liver and kidney of mice disrupted the expression of 417 and 379 genes, respectively [278]. Adenoviral-mediated knockdown of AHR in primary mouse hepatocytes *in vitro* induced significant changes in the expression of 97 genes at 12 h and 246 genes at 24 h [279]. Chang et al. reported that AHR knockdown altered the expression of 1133 genes in mouse embryonic fibroblasts [280]. Mouse hepatoma cells (Hepa-1) express an AHR that binds DREs, while a variant line, Hepa-1 C35, harbors a dysfunctional mutant AHR that fails to bind DREs [281]. Consistent with AHR being an endogenous regulator of gene expression, the Hepa-C35

93

transcriptome is dramatically disrupted compared to parent Hepa-1 cells [282]. The findings that AHR knockout mice are less fertile, exhibit higher rates of intestinal cancers, and have developmental and vascular defects suggests that AHR regulation of gene expression in rodent models is physiologically important [283-287].

AHR has been reported to play roles in breast tumorigenesis. Knockdown of AHR in breast cancer cells (BCCs) inhibits mitogen-induced proliferation (MCF-7 cell line), invasion/migration (MDA-MB-231 cell line) and xenograft tumorigenicity (rodent mammary fibroblasts) [108, 111, 114, 288]. Further, rat mammary tumors have been shown to express higher levels of AHR than normal mammary tissue [90]. The mechanism(s) of AHR action in breast tumorigenesis is not clear. We reasoned that defining AHR-dependent genes (ADGs) in MCF-7 BCCs would identify pathways downstream of AHR that are important in cancer. To this end, we performed expression profiling via RNA-Seq on control and AHR knockdown MCF-7 cells in the absence of external stimuli. Pathway analysis of ADGs revealed new roles for AHR. First, MCF-7 cells maintain expression of xenobiotic metabolizing enzymes in the absence of toxicants. Second, AHR promotes basal expression of a large fraction of TNF target genes in MCF-7 cells. Finally, knockdown of AHR inhibited TNF-induced increases in MnSOD and promoted the cytotoxic response in MCF-7 cells. This latter result provides a potential new role for AHR in cancer as a mediator of MnSOD induction and the antioxidant cytoprotective response to TNF.

6.2. METHODS

6.2.1. Materials and MCF-7 cell culture

Dulbecco's Modified Eagle Medium/High glucose (DMEM) with l-glutamine and sodium pyruvate, phenol red-free DMEM, phosphate buffered saline (PBS), fetal bovine serum (FBS), charcoal-treated FBS, penicillin, and streptomycin were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Sodium dodecyl sulfate (SDS), 30% acrylamide/bis solution, ammonium persulfate, Tween-20, and 2-mercaptoethanol was obtained from Bio-RAD (Hercules, CA). Non-specific control RNA (cRNAi) (cat no. D-001810-01-20), short interfering RNA (siRNA) against AHR (AHR-siRNA, cat no. J-004990-08-0010), RELA (RELA-siRNA, cat no. J-003533-06-0010) and DharmaFECT 1 Transfection Reagent (no.1) were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotopes Laboratory (Andover, MA). MCF-7 human breast cancer cells were purchased from ATCC (Manassas, VA) and maintained in DMEM, 10% FBS, with penicillin (100 IU/mL) and streptomycin 100 (μg/mL).

6.2.2. AHR knockdown for RNA-Seq

To knockdown AHR for RNA-Seq analysis, 200,000 MCF-7 cells in six-well tissue culture plates were transfected with 50 nM AHR-siRNA in phenol red-free DMEM, 10% charcoal-treated FBS and DharmaFECT 1 Transfection Reagent following the manufacturer's protocols. After 36 h, cells were serum starved overnight in phenol-red free DMEM. Control cells in 6-well tissue culture plates were transfected with 50 nM control-siRNA using the same methods used to knockdown AHR.

6.2.3. Whole transcriptome expression profiling via RNA-Seq

Total RNA was isolated from overnight serum starved control (five replicates) and AHR knockdown MCF-7 (six replicates) using RNA purification columns (Qiagen, Valencia, CA) with DNase treatment. DNase was purchased from Qiagen. RNA sample quality was assessed using Bioanalyzer RNA Nano chips (Agilent); all RNA samples had an RNA Integrity Number greater than or equal to eight. RNA-Seq libraries were prepared from 1 μg of total RNA using a TruSeq RNA Prep Kit (Illumina Inc., San Diego, CA).

6.2.4. RNA-Seq analysis

RNA-Seq on AHR knockdown and control MCF-7 cells was performed using an Illumina HiSeq1000 in a 2 × 100 base paired end design yielding a minimum of 50 million reads per sample. Demultiplexing of samples was performed using CASAVA 1.8.2 (Illumina). Reads were aligned to the human reference genome (hg19/GRCh37) using TopHat 2.0.6. [289]. TopHat was configured to use BowTie 0.12.8 [290] and SAMtools 0.1.18. [291]. Aligned reads were then mapped to genes from the ensemble database using Bioconductor [292] packages Rsamtools and biomaRt [293]. Data were then analyzed using the DESeq Bioconductor package [294] as follows. Counts were normalized to account for differences in sequencing depth between samples. Samples were clustered using the top 30 expressing genes. One control sample, which did not cluster with the remaining control samples, was removed from further analysis. In order to mitigate the loss of statistical power from multiple hypothesis correction, we removed the lowest 40% of genes by total read count across all samples and performed differential expression analysis on the remaining 60%. Following standard practice (for example, [294]), genes statistically significant at a false discovery rate of 10% were reported, irrespective of fold change. To validate the low-expression filtering step, we repeated the analysis without removing

the 40% of genes that were low expressers (data not shown). None of the filtered genes were identified as statistically significant in this analysis, while the loss of statistical power resulted in 126 of the unfiltered genes losing significance. Sequencing data were deposited in the Gene Expression Omnibus (GEO) database maintained by the National Center for Biotechnology Information (NCBI) and are accessible with accession number GSE52036s.

6.2.5. Ingenuity pathway analysis (IPA)

Differentially expressed genes (FDR < 10%) were expressed as a ratio of AHR knock-down/control level and loaded into Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, CA) in order to perform an IPA Core Analysis under default settings. Of the 634 RNAs, 496 were mapped to known functions and pathways by IPA. In IPA, a biological function is a process or disease with a pre-defined set of molecules (genes). IPA was used to compute significant associations between biological functions and our ADG set. Specifically, we ran a Core Analysis in IPA which used Fisher's Exact Test to assign levels of statistical significance to associations between biological functions and our gene set. We configured the core analysis to report Benjamini–Hochberg corrected p-values. We also used the Upstream Regulator Analysis function to identify candidate regulators of ADG pathways.

6.2.6. Validation of RNA-Seq by qRT-PCR

Real-time reverse-transcription PCR (RT-qPCR) analysis from control and AHR knockdown MCF-7 cells (five replicates) was carried out to validate RNA-Seq (AHR knockdown detailed in Section 2.2.). Total RNA was isolated using Qiagen RNA purification columns and DNase treated. Reverse transcription was performed with 100 ng of total RNA using Verso cDNA kit (Thermo Fisher Scientific; cat no. AB-1453/B). PCR of cDNA was conducted with SYBER GREEN and ROX qPCR mix (Qiagen) with a five min denaturing step

at 95° C, followed by 40 cycles of 15 s at 95° C, 30s at 60° C, 30s at 72° C. Relative gene

expression was calculated using the formula 2∆∆CT, as described by Livak and Schmittgen

[239]. Glyceraldehyde-3-phosphate (GAPDH) mRNA levels served as the internal control.

Primer sequences GAPDH [forward 5′-catgagaagtatgacaacagcct 3′ and reverse 5′-

agtccttccacgataccaaagt-3′], OAS1 [forward 5′-cagacgatgagaccgacgat-3′ and reverse 5′-

cctggagtgtgctgggtcta-3′], PKD1L1 [forward 5′-cgcctctggattgtgataacag-3′ and reverse 5′-

cggtcccagtagcacacag-3′], PLA2G2 [forward 5′-accagacgtaccgagaggag-3′ and reverse 5′-

cgctggggattggtgactg-3′], SERPIN5A [forward 5′-atgccctttttcaccgacctg-3′ and reverse 5′-

tgcagagtccctaaagttggtag-3′], PYDC1 [forward 5′-cacacgtatagctaccggcg-3′ and reverse 5′-

cgcgtaagacaacagcagtg-3′], HMGCS2 [forward 5′-caatgcctgctacggtggta-3′ and reverse 5′-

gacggcaatgtctccacaga-3′], SERPIN3A [forward 5′-tgccagcgcactcttcatc and reverse 5′-

tgtcgttcaggttatagtccctc-3′], CYP1A1 [forward 5′-cttcaccctcatcagtaatggtc-3′ and reverse 5′-

aggctgggtcagaggcaat-3′], CYP1B1 [forward 5′-ctgcactcgagtctgcacat-3′ and reverse 5′-

tatcactgacatcttcggcg-3′], NRF2 [forward 5′-tccagtcagaaaccagtggat-3′ and reverse 5′-

gaatgtctgcgccaaaagctg-3′], PGR [forward 5′-ttatggtgtccttacctgtggg-3′ and reverse 5′-

gcggattttatcaacgatgcag-3′], MGP [forward 5′-tccgagaacgctctaagcct-3′ and reverse 5′-

gcaaagtctgtagtcatcacagg-3′], ADORA [forward 5′-ccacagacctacttccacacc-3′ and reverse 5′-

taccggagagggatcttgacc-3′], CREB3L [forward 5′-cctccccgaagcctcctattct-3′ and reverse 5′-

ggggttgatttcccagcca-3′], AHR [forward 5′-acatcacctacgccagtgg-3′ and reverse 5′-

ctctatgccgcttggaaggat-3′], ALOX5 [forward 5′-ctcaagcaacaccgacgtaaa-3′ and reverse 5′-

ccttgtggcatttggcatcg-3′], ALDH3A1 [forward 5′-tgttctccagcaacgacaagg-3′ and reverse 5′-

agggcagagagtgcaaggt-3′], RELA [forward 5′- tccagaccaacaacaacccc-3′ and reverse 5′-

gatcttgagctcggcagtgt] and ABCG2 [forward 5′-acgaacggattaacagggtca-3′ and reverse 5′-

ctccagacacaccacggat-3′]. The Harvard Primer Bank http://pga.mgh.harvard.edu/primerbank/was used to design primers above. The primer sequences for the UGTA isoforms have been published [295]. Primers were purchased from Sigma (St. Louis, MO). Primer specificity was verified with melt curve analysis and NIH primer blast search engines located at http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome. Two-tailed, paired t tests with confidence intervals of 95% were used to determine statistically significant differences between controls and AHR knockdown cells.

6.2.7. Western blot analysis determination of MnSOD

AHR knockdown prior to western blot analysis was carried as detailed in Tomblin and Salisbury [114]. Briefly, MCF-7 cells (200,000) were mixed directly with siRNA (50 nM control or AHR-siRNA) and DharmaFECT 1 Transfection reagent (2 μL-per well), added to phenol red-free DMEM, 10% charcoal treated FBS in 6-well tissue culture plates and cultured for twenty four hrs. Following serum starvation in phenol red-free DMEM for sixteen hrs, cells were treated with either H_2O vehicle or human recombinant TNF (10 ng/mL) (R & D Systems) for twelve hrs. Treatments were removed, adherent and detached cells were collected and total cellular extract was isolated in 250 microliter of 2× sample lysis buffer (Bio-RAD; cat no.161-0737) and approximately 10 microgram of protein was subjected to SDS PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in PBS, 0.01% Tween 20 (PBS-T), 5% (wt/vol) low fat powdered milk for one hr and incubated overnight with primary antibody at 4° C with gentle mixing. Membranes were rinsed five times (five min each wash) with PBS-T and then incubated with an appropriate HRP-labeled secondary antibody (Thermo Fisher Scientific) (diluted 1:10,000 in PBS-T, 5% milk) for one hr, followed with rinsing five times (five min each wash) in PBS-T. Membranes were developed with

enhanced chemiluminescent substrate (Millipore Corporation, Billerica, MA) and exposure to X-ray film (MidSci, St. Louis, MO). Antibodies were purchased from the following vendors: (1) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody from Millipore (cat no. MAB374), (2) AHR antibody from Santa Cruz (Santa Cruz, CA, Cat no. H-211) and (3) MnSOD antibody from Abcam (Cambridge, MA, cat no.: ab13533). GADPH was diluted 1:10,000, while AHR and MNSOD were diluted 1:2000 in PBS, 0.01% Tween-20, 5% powdered milk. Densitometry was calculated with ImageJ PC-based software (National Institute of Health). The Student–Newman–Keuls (SNK) post-hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA).

6.2.8. qRT-PCR analysis TNF induction of SOD2

MCF-7 cells were reverse transfected in six-well tissue culture plates as detailed in Section 2.6 and then treated with H2O vehicle or TNF (10 ng/mL) (R&D Systems) for twelve hrs. Treatments were removed, adherent and detached cells were collected and total RNA was isolated in TRI-Reagent (Sigma-Aldrich, St. Louis, MO) and quantitated by NanoDrop spectrophotometry. RNA was reverse transcribed to cDNA (Verso cDNA kit; Thermo Fisher Scientific; cat no. AB-1453/B). Resulting cDNAs were subjected to qRT-PCR with SYBR Green Rox Mix (Qiagen) using PCR reaction conditions detailed in Section 2.6. Relative gene expression among groups was calculated using the formula 2ΔΔCT, as described by Livak and Schmittgen [239]. Primer sequences for SOD2 mRNA were (forward, 5′-GGAAGCCATCAAACGTGACTT-3′; reverse, 5′-CCCGTTCCTTATTGAAACCAAGC-3′). The SNK post-hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA).

6.2.9. qRT-PCR analysis of TCDD treated cells

MCF-7 cells plated in 35 mm tissue culture plates (200,000 cells/mL) were serum starved overnight in phenol red-free DMEM. For TCDD stimulation, either 0.1% (v/v) Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) or TCDD (10 nM at the final concentration) was added directly to media along with either H2O vehicle or TNF (10 ng/mL) (R&D Systems) for twelve hrs. Treatments were stopped and cells were rinsed once with PBS. Total RNA was isolated using TRI reagent (Sigma-Aldrich) and SOD2 mRNA was measured using real time RT-qPCR analysis.

6.2.10. Chromatin immunoprecipitation followed by qPCR (ChiP-qPCR)

For ChIP, one 80% confluent 150 mm plate of MCF-7 cells was serum starved in phenol red-free DMEM and then treated with H2O vehicle or TNF (10 ng/mL) for one h or twelve hrs. Post treatment, cells were cross-linked with formaldehyde (Sigma-Aldrich) (0.75% v/v) for ten min at room temp, followed by the application of glycine (0.125 M) (Sigma-Aldrich) for five min. Cells were rinsed with cold PBS, pelleted by centrifugation, and cell pellets were lysed in 1 mL Lysis Buffer (50 mM Tris–HCl pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS plus protease inhibitors (Thermo Scientific no. 78410). After fifteen min, extracts were sonicated (five times, each time ten s) and diluted 1:10 in dilution buffer (1% Triton X-100, 2 mM EDTA pH8, 20 mM Tris–HCl pH 8, 150 mM NaCl plus protease inhibitors), rotated overnight at 4° C with 5 μg of non-specific IgG (Santa Cruz; cat no. sc-2027), 5 μg of anti-AHR antibody (Santa Cruz; cat no. H-211) or 5 μg of anti-p65 NFKB antibody (Santa Cruz; cat no. sc-372). Antibody-chromatin complexes were collected using ten microliters of magnetic protein A beads (Invitrogen; cat no. 100.01D) with rotation at 4° C for 90 min. Using magnetic separation (Life-Technologies; part no. 49-2025), beads were washed three times

(ten min each wash) with wash buffer (20 mM Tris–HCl, pH 8; 150 mM NaCl; 2.0 mM EDTA; 0.1% SDS) and once with final wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 500 mM NaCl) and incubated at 65° C for four to six hrs in elution buffer (1% SDS, 0.1 M NaHCO3) with proteinase K (20 mg/mL) (Invitrogen Life-Technologies., Carlsbad, CA). DNA was purified with phenol-chloroform extraction followed by isopropanol precipitation and analyzed using real time PCR. Phenol, chloroform and isopropanol were purchased from Sigma-Aldrich. Primers spanning NFκB response elements in intron 2 of SOD2 were: [forward 5′-GGAAAAGGCCCCGTGATTT-3′ and reverse 5-TCCTGGTGTCAGATGTTGCC-3′] [296]. ChiP data was expressed as % input, in which signals obtained from the ChIP are divided by signals obtained from an input sample. Statistical differences among groups were determined by the SNK post-hoc test following one-way analysis of variance (ANOVA).

6.2.11. Cell viability

MCF-7 cells (200,000/mL) were mixed directly with 50 nM siRNA (either control or AHR-siRNA) and Dharmafect no. 1 transfection reagent (2 μL/mL), added to phenol red-free DMEM, 10% charcoal treated FBS and plated into 60 mm tissue culture plates (3 mL per plate) and cultured for 24 h. Following serum starvation in phenol red-free DMEM for 16 h, cells were treated with either H2O vehicle or human recombinant TNF (10 ng/mL) (R&D Systems) for 12 h. Cell viability was measured with trypan blue stain (Thermo Fisher Scientific). The percentage of non-viable cells were calculated as: non-viable cell (%) = (total number of non-viable cells/total number of cells) multiplied by 100. The SNK post-hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA).

6.3. RESULTS

6.3.1. Effect of AHR knockdown on MCF-7 gene expression

Expression profiling on control and AHR knockdown MCF-7 cells was conducted to identify a set of ADGs in the absence of stimuli. AHR knockdown inhibited the expression of 380 genes and promoted the expression of 254 genes at FDR < 10%, with all reported fold changes being at least 1.2 fold; we refer to the combined group of 634 genes as the ADG set. A full list of these genes is included as a supplemental file with NCBI GEO data deposit (accession number GSE52036). Real-Time qRT-PCR was used to validate RNA-Seq expression in a set of 30 genes. The rational for selecting validation genes listed in Table 8 is that they were among either the top downregulated (CYP1A1, HMGCS2, OAS1, PLA2G2, ALDH3A1, PKD1L1), the top upregulated (CREB3L1, PYDC1, MGP, ADORA1, PGR, SERPIN3A, and SERPIN5A) ADGs or known TCDD gene targets (CYP1A1, CYP1B1, ABCG2, ALDH3A1, NRF2 and UDP-glucuronosyltransferases (UGTAs). ALOX5 was selected for validation because it is the rate limiting gene in leukotriene synthesis [297].

In general, there is a good concordance between the RNA-Seq and qRT-PCR measurements. Levels of AHR mRNA were lower in knockdown MCF-7 cells than controls as measured by RNA-Seq (~four-fold) and qRT-PCR (~seven-fold) from independent experiments (Table 8). Expression of known TCDD-target genes (CYP1A1 [298], CYP1B1 [298] and ALDH3A1 [299]) was lower in AHR knockdown MCF-7 cells by both RNA-Seq and qRT-PCR measurements (Table 8). Prior reports have shown that TCDD stimulates increased expression of UGTAs in mouse liver [259]. RNA-Seq and qRT-PCR assays revealed that UGT1A1, UGT1A3,

Gene ID	AHR-RNA-Seq	qRT-PCR
AHR	0.24585	0.14400 ± 0.040
CYP1A1	0.04738	0.43253 ± 0.215
CYP1B1	0.44897	0.36685 ± 0.137
HMGCS2	0.12976	0.53902 ± 0.114
OAS1	0.13104	0.62920 ± 0.054
PLA2G2	0.11346	0.42180 ± 0.073
ABCG2	0.35987	0.51580 ± 0.146
NRF2	No difference	0.55050 ± 0.003
ALOX5	0.49086	0.47342 ± 0.024
ALDH3A1	0.05942	0.17310 ± 0.067
UGT1A1	0.31011	0.50581 ± 0.069
UGT1A3	0.30741	0.43706 ± 0.037
UGT1A4	0.32062	0.60735 ± 0.066
UGT1A5	0.32055	0.60620 ± 0.079
UGT1A6	0.32404	0.61148 ± 0.085
UGT1A7	0.33345	0.53582 ± 0.058
UGT1A8	0.33597	No difference
UGT1A9	0.33357	No difference
UGT1A10	0.33502	No difference
PKD1L1	0.16430	0.29732 ± 0.031
PYDC1	2.50200	No difference
PGR	1.87529	1.40625 ± 0.103
MGP	2.39768	1.2750 ± 0.034
SERPIN3A	1.92517	1.52785 ± 0.031
CREB3L	2.67290	1.40500 ± 0.018
SERPIN5A	1.88800	1.7890 ± 0.233
ADORA	2.03342	1.61912 ± 0.209

Column 2 is expressed as RNA-Seq ratio of AHR knockdown/control level ($FDR < 10\%$). Column 3 is expressed as real-time qRT-PCR ratio of AHR knockdown/control normalized to GAPDH expression ($P < 0.05$).

Table 8. Fold change expression value from AHR knockdown MCF-7 compared to controls.

UGT1A4, UGT1A5, UGT1A6, UGT1A7 mRNAs were lower in AHR knockdown cells than controls (Table 8). UGT1A8, UGT1A9, and UGT1A10 were not differently regulated by qRT-PCR, but their levels were lower (~three-fold) in AHR knockdown cells compared with controls based on RNA-Seq measurements (Table 8). The drug transporter, ABCG2, has been reported to be induced by TCDD in human cells (breast, colon and liver), but not in rodent cells [41]. ABCG2 mRNA was ~three fold lower in AHR knockdown MCF-7 cells than controls in both RNA-Seq and qRT-PCR data sets (Table 8). NRF2 is a transcription factor that stimulates the expression of anti-oxidant enzymes [259]. Prior reports have shown that NRF2 is a TCDD gene target [300, 301]. NRF2 expression was not differentially expressed by RNA-Seq, but its levels were lower (~50%) in AHR knockdown cells compared with controls when assayed by qRT-PCR (Table 8). The levels of the PLA2G2 and ALOX5 were lower in AHR knockdown MCF-7 cells than controls by RNA-Seq and qRT-PCR (Table 8).

AHR knockdown had modest stimulatory effects on the expression of several genes. As measured by RNA-Seq, CREB3L was the most upregulated gene (by 2.67290) in AHR knockdown MCF-7 cells compared with controls (Table 8). The expression of PGR, MGP, SERPIN3A, CREB3L, SERPIN5A, and ADORA were increased in AHR knockdown cells compared with controls by RNA-Seq and qRT-PCR (Table 8). Observed expression levels of IGHG2, IGHA1 and RNF128 were reduced by AHR knockdown by RNA-Seq analysis (GEO submission GSE52036), but not by qRT-PCR (data not shown). This discrepancy could be attributed to IGHG2, IGHA1 and RNF128 transcript levels that were below qRT-PCR detection limits (Ct values higher than 35; data not shown). We note that RNA-Seq fold changes were greater than qRT-PCR fold changes for several genes including: CYP1A1, HMGCS2, OAS1,

PLA2G2, ALDH3A1, MGP, CREB3L, UGTAs and ADORA; however the direction of expression changes were the same (Table 8).

6.3.2. Pathway analysis of AHR-dependent genes

In order to determine functions and pathways regulated by ADGs, we analyzed the ADG set using the ingenuity pathway analysis (IPA) core analysis tool which finds gene sets that are over-represented in defined, canonical cellular pathways and molecular functions. Of the 634 genes, 496 were mapped to known functions and pathways by IPA. These ADGs were significantly associated with cancer-related pathways including: cellular movement, cell cycle, cellular growth and proliferation, cell death and survival, cellular development and cellular morphology (Table 9). In addition, significant numbers of ADGs were over-represented in pathways involved in post-translational modification and in the metabolism of drugs, amino acids and small molecules (Table 9).

We refined the pathway analysis by applying the IPA Upstream Regulator Analysis tool to determine if the ADGs are connected through a common upstream regulator. This analysis revealed that ADGs were enriched among the following IPA canonical regulatory pathways: beta-estradiol (endogenous hormone), tumor necrosis factor (TNF) (cytokine), tumor protein 53 (TP53) (transcriptional regulator), lipopolysaccharide (chemical drug), decitabine (chemical drug), calcitriol (chemical ligand), dexamethasone (glucocorticoid receptor), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) (kinase), cyclin-dependent kinase inhibitor 1A (CDKN1A) (kinase), TGF-β (growth factor) and TCDD (toxicant) (Table 10). Specifically, IPA reveals that 74 of 171 TNF pathway target genes are ADGs (Table 10). Of the 74 ADGs in the TNF pathway, 44 exhibited patterns of expression consistent with inhibition of TNF activity (Table 10). The finding that IPA revealed 87 of 197 beta-estradiol target genes are

Category	*B–H P-value	Target molecules in dataset
Cellular movement	1.37E – 06–4.32E – 02	101
Cell cycle	1.96E – 06–5.67E – 02	90
Cellular growth and proliferation	2.38E – 06–5.67E – 02	149
Cell death and survival	4.46E – 06–5.67E – 02	150
Amino acid metabolism	2.24E – 05–4.32E – 02	18
Drug metabolism	2.24E – 05–4.32E – 02	12
Post-translational modification	2.24E – 05–3.04E – 02	19
Small molecule biochemistry	2.24E – 05–5.67E – 02	73
Cell morphology	2.53E – 04–5.13E – 02	85
Cellular development	3.74E – 04–5.67E – 02	138

* P-values are calculated by Fishers exact test and corrected for multiple testing by the Benjamini–Hochberger P-values (B–H) method (B–H P-value). Column 2 shows the range of B–H corrected P-values for the biofunctions in a given category. Target molecules in dataset are the number of RNA-Seq ADGs in a given biofunction.

Table 9. IPA cellular and molecular functions associated with RNA-Seq ADGs.

ADGs is not surprising, considering that AHR and the estrogen receptor (ER) have been reported to interact extensively (Table 10) [51, 302]. Finally, ADGs were found to be significantly enriched within the TCDD pathway (23 of 125 TCDD pathway genes were ADGs) (Table 10). The IPA-predicted inhibition of TCDD activity (Table 10) was based in part on the observed inhibition of conical TCDD target genes including: CYP1A1, CYP1B1 and ALDH3A1 in AHR knockdown cells compared with controls.

6.3.3. Comparison of AHR-dependent gene set with known TCDD and AHR effects

TCDD is a strong exogenous AHR ligand that is resistant to degradation [253]. TCDD has been reported to regulate the expression of 104 genes in MCF-7 cells [277]. To identify ADG genes that are induced by TCDD in MCF-7 cells, we overlapped published TCDD microarray data and AHR knockdown RNA-Seq expression profiles [277]. While the majority of ADGs (621) did not overlap with reported TCDD-regulated genes, there were 13 genes in both sets (Figure 18). Common genes included CYP1A1, CYP1B1 and ALDH3A1, which are important in lipid metabolism, small molecule biochemistry, and drug metabolism (Figure 18).

Lo and Matthews identified TCDD-induced binding sites in MCF-7 cells using ChIP-Seq technology [277]. Since these should represent AHR binding sites, we compared the TCDD-ChIP-Seq gene set with ADG set and found that approximately 15% of ADGs have a TCDD-AHR binding site. This finding suggests that the remaining 85% could be indirect AHR gene targets. The 80 specific TCDD-ChIP-Seq genes that overlap with the ADG set are shown in Figure 19. Common target genes included ABCG2, CYP1A1 and CYP1B1 which are known TCDD-AHR target genes [41, 277].

IPA-upstream regulator	IPA-upstream regulator activity prediction	*P-value of overlap	Target molecules in dataset	Target molecules in IPA-upstream regulator pathway
Beta-estradiol		5.64E – 19	87	197
TNF	Inhibited	1.79E – 13	74	171
TP53	Inhibited	4.15E – 13	66	137
Lipopolysaccharide		6.29E – 12	73	187
Decitabine		1.46E – 11	34	160
Calcitriol	Inhibited	3.16E – 11	33	159
Dexamethasone		4.26E – 11	70	170
ERBB2		2.66E – 10	38	170
CDKN1A		3.07E – 10	22	123
TGFβ		4.11E – 09	66	198
TCDD	Inhibited	5.13E – 07	23	125

An IPA-upstream regulator regulates a IPA-defined set of target molecules (genes). Column 2 reveals IPA upstream regulator activity determined by comparing reported gene responses to a given upstream regulator to observed expression changes in AHR knockdown cells compared to controls. Column 4 shows the number of target molecules in the RNA-seq AHR knockdown dataset that are in a given IPA-upstream regulator pathway. Column 5 indicates that total number of target molecules that are in a given IPA-upstream regulator pathway.

* P-value of overlap are calculated by Fisher exact test.

Table 10. IPA upstream regulators associated with RNA-Seq ADGs.
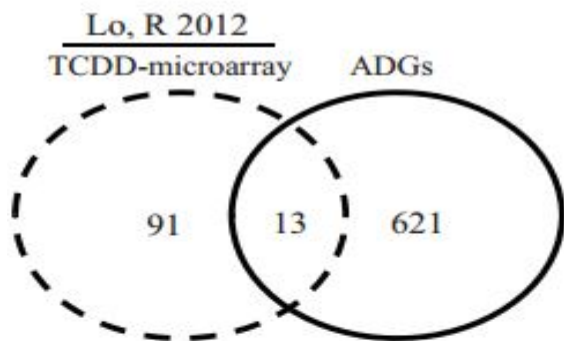
Figure 18. Genes in common between AHR knockdown RNA-Seq expression profiles and TCDD-microarray data. Analysis of reported TCDD-microarray data demonstrated that 13 of 634 ADGs were TCDD-regulated genes. The specific 13 ADGs that overlapped with TCDD-microarray genes in MCF-7 cells are shown in the table.

Lo, 2012
TCDD-ChIP-Seq   ADGs

1679   80   554
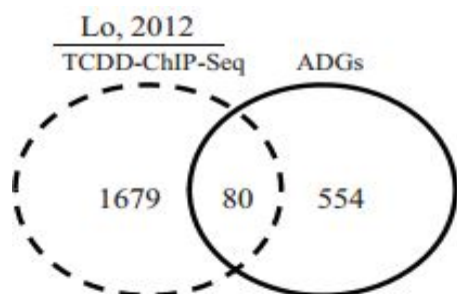
80 ADGs that overlap with reported TCDD-CHIP-seq genes.

GENE ID	*fold change	GENE ID	*fold change	GENE ID	*fold change	GENE ID	*fold change
MYLIP	0.802096578	RND3	0.824025407	SLC39A6	1.239425522	DLC1	1.356945237
ST3GAL1	0.573180864	MYB	1.538360507	TUFT1	0.704391074	ADCY1	1.301094779
GPRC5A	1.207910304	ABCG2	0.359869277	PIK3R1	1.277219373	TNFRSF11B	0.611478095
PTPN21	1.381218888	MFSD1	0.663404044	C9orf3	0.736838039	PLEKHF1	0.414179586
NTN4	0.681822478	MSX2	0.80009415	CRIM1	1.26251853	YIF1B	0.765092431
FOSL2	0.76343792	SOCS2	1.299622085	TMEM45B	0.552147883	FAM83B	1.238570568
TP73	1.27755078	RAMP3	1.537558068	SPOCK1	0.429769601	NPR1	0.486624749
KCNK2	0.53577002	PREX1	1.368259706	ACOXL	0.705962135	CHST11	0.661659483
PITPNM2	0.612266033	CDH26	0.550789411	GPR115	0.556788075	AGR3	1.657756668
ESR1	1.242955418	VIL1	1.286171847	SEMA3D	1.460941605	TSKU	0.526450429
TIMP3	0.678358994	GAD1	0.57281971	TDH	0.702355238	ALDH1A3	0.673243346
PYGL	0.603272436	PPARG	0.620796173	TTC39B	0.511040521	DRD1	0.555398086
MET	1.292037898	DCLK1	0.769661296	SH3RF2	0.707331314	C3orf70	1.320237175
AHR	0.245846079	CCNJL	0.762501798	CLSTN2	0.48144621	ARL4C	0.732119812
MAPK10	0.582554716	BRIP1	1.315901173	IL6R	0.678256519	CLDN4	0.783196813
KAT2B	0.690073646	PRCP	1.2423756	IER5	0.807121799	EVL	1.377514622
HES1	0.820976576	STRA6	0.602652873	RNF149	0.784847051	SVIL	1.202244505
ABCC5	0.695456439	CYP1B1	0.448965655	SRGAP2	1.207060918	TXNRD1	0.795095818
EFEMP1	1.386922297	AGPAT9	0.602182209	S100P	0.643296423	PAPSS2	0.759199786
IGFBP5	1.67229658	CYP1A1	0.047383928	FOXQ1	0.20435077		

*RNA-seq fold change expression values from AHR knockdown cells compared to controls expressed as a ratio of AHR knockdown/control.

Figure 19. Genes in common between AHR knockdown RNA-Seq expression profiles and TCDD-ChIP-Seq data. Analysis of reported MCF-7 TCDD-ChIP-Seq data revealed that 80 of the 634 AHR-RNA-Seq genes were TCDD-AHR bound genes. The specific 80 ADGs that overlapped with TCDD-ChIP-Seq genes in MCF-7 cells are shown in the table.

28 ADGs that overlap with reported AHR-regulated genes in liver.	
GeneID	*fold change
ABCC3	0.431623029
ABCG2	0.359869277
ADORA1	2.033421256
AGPAT9	0.602182209
AKR1C1	0.304418760
AKR1C2	0.296001896
BLVRB	0.825905639
C19orf70	0.296001896
C9orf3	0.736838039
CCNF	1.196510021
ELOVL2	1.818790159
ESR1	1.242955418
ESRRA	0.818597062
F2R	0.564685491
HLA-B	0.666409580
LEAP2	1.597154630
ME1	0.680037420
MET	1.292037898
OGFRL1	1.508349672
PIR	0.693838416
PYGL	0.603272436
RBBP8	1.222730403
RHPN2	1.225426422
RRM2	1.366597067
SLC16A2	1.210276804
TKT	0.682068785
TTC39B	0.511040521
VAMP8	0.777080233

*RNA-seq fold expression values from AHR knockdown MCF-7 compared to controls expressed as a ratio of AHR knockdown/control.

Figure 20. Genes in common between AHR knockdown RNA-Seq expression profiles and AHR-regulated genes in mouse liver. Analysis of reported AHR gene targets in mouse liver revealed that 28 of the 634 ADGs were AHR targets in mouse liver. The specific 28 ADGs that overlapped with AHR-regulated genes in liver are shown in the table.

Boutros, 2009
AHR-Kidney   ADGs

364   15   619

15 ADGs that overlap with reported AHR-regulated genes in the kidney.	
GeneID	*fold change
AGPAT9	0.602182209
AHR	0.245846079
AKR1B10	0.250526362
F2R	0.564685491
HMGCS2	0.129756073
KCNJ8	1.446700213
LACTB	0.796970078
ME1	0.68003742
MFSD1	0.663404044
PAPSS2	0.759199786
PTGR1	0.628375867
RPS9	0.807248215
STRA6	0.602652873
TIMP3	0.678358994
TXNRD1	0.795095818
*RNA-seq fold expression values from AHR knockdown MCF-7 compared to controls expressed as a ratio of AHR knockdown/control.	

Figure 21. Genes in common between AHR knockdown RNA-Seq expression profiles and AHR-regulated genes in mouse kidney. Analysis of reported AHR gene targets in mouse kidney revealed that 15 of the 634 ADGs were AHR targets in mouse kidney. The specific 15 ADGs that overlapped with AHR-regulated genes in the kidney are shown in the table.

Microarray based expression profiles on liver and kidney from AHR null mice has been reported [278]. Twenty eight genes were shared between the mouse liver gene set and ADG set (Figure 20). A small number of mouse kidney genes (15) overlapped with ADG set (Figure 21). The specific ADGs that overlapped with AHR-liver and AHR-kidney are shown in Figure 20 and Figure 21, respectively. Differences in tissue- and species-specific expression may explain the limited overlap in these gene sets.

6.3.4. AHR modulates TNF induction of MnSOD and cytotoxicity response

Based on the finding that the ADG set is significantly associated with the TNF pathway, we sought to determine if TNF induction of SOD2 requires AHR expression. SOD2 is a nuclear gene that encodes the mitochondrial superoxide dismutase (MnSOD). We focused on MnSOD regulation because it is inducible [303] and Rico de Souza et al. [304] have reported that MnSOD levels are lower in AHR knockdown primary mouse lung fibroblasts than control cells [304]. Serum-starved control and AHR knockdown MCF-7 cells were treated with vehicle or TNF (10 ng/mL) for 12 h. As expected, AHR protein levels were lower in knockdown cells than control cells (Figure 22 (A)). While TNF stimulated MnSOD protein levels ~eight-fold in control cells, this induction was significantly abrogated in AHR knockdown cells by 60% (Figure 22 (A)). We then asked whether siRNA knockdown of AHR and NF-κB subunit RELA (also known as p65) inhibited TNF-stimulated induction of MnSOD mRNA expression. The impetus for including RELA is based on its requirement for TNF induction of the SOD2 gene [305, 306]. RELA mRNA was reduced ~90% by siRNA treatment (Figure 22 (B)). Knockdown of AHR and RELA suppressed TNF induction of MnSOD mRNA levels (Figure 22 (B)). We also asked whether TCDD would modulate TNF regulation of MnSOD. The level of MnSOD induction by TNF was not affected by TCDD (Figure 22 (B)). Collectively, these data indicate that

endogenous AHR and RELA promote TNF induction of MnSOD in MCF-7 cells through a mechanism that is independent of TCDD effects.

TNF-induced RELA stimulates SOD2 expression by binding to NF-kappa-B response elements (kappa-RE) in intron 2 [305, 306]. Physical interactions between AHR and RELA have been reported [307]. We therefore tested whether TNF signaling results in recruitment of AHR and RELA to the SOD2 kappa-RE. ChIP-qPCR experiments revealed that treatment with TNF (12 h) increased the binding of AHR and RELA on the SOD2 kappa-RE by ~2.5 fold in each case (Figure 22 (C)). AHR and RELA association with kappa-RE in vehicle treated cells was not greater than non-specific IgG (Figure 22 (C)). These results indicate that TNF signaling recruits AHR and RELA to an active kappa-RE in the SOD2 gene [304, 305].

The finding that AHR modulates TNF induction of MnSOD prompted us to investigate whether AHR is required in the response to TNF-induced cytotoxicity. To this end, MCF-7 cells were transiently transfected with non-targeting control or AHR siRNAs prior to treatment with vehicle or TNF for 12 h, followed by determination of the percentage of non-viable cells. As shown in Figure 22 (D), TNF-induced cytotoxicity was significantly higher in AHR knockdown MCF-7 cells compared with controls (Figure 22 (D)). This result suggests that AHR suppresses TNF-induced cytotoxicity.

A Immunoblot analysis

Veh TNF Veh TNF, 12hr

AHR

MNSOD

GAPDH

cRNAi   AHRi

MNSOD

normalized protein levels

Veh ■   TNF □

cRNAi   AHRi

B qRT-PCR analysis

Veh ■
TNF □

SOD2 mRNA levels

cRNAi   AHRi

SOD2 mRNA levels

cRNAi   RELAi

RELA mRNA levels

cRNAi   RELAi

SOD2 mRNA levels

NS

DMSO   TCDD

C ChIP-qPCR analysis

RELA

SOD2

1   Intron 2

% Input

P < .03

P < .03

veh TNF   veh TNF   veh TNF
  IgG   AHR   RELA

D Cell viability assay

% of dead cells

P < .05

Veh ■   TNF □

cRNAi   AHRi

116
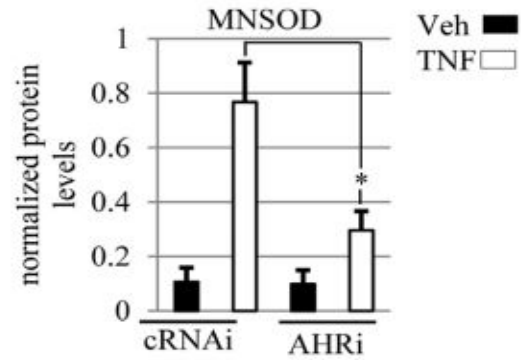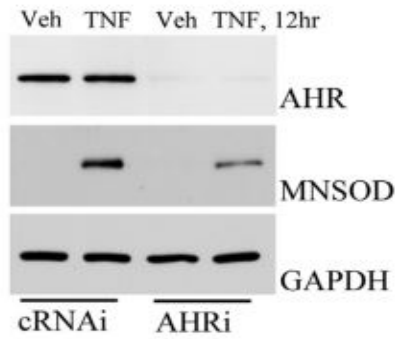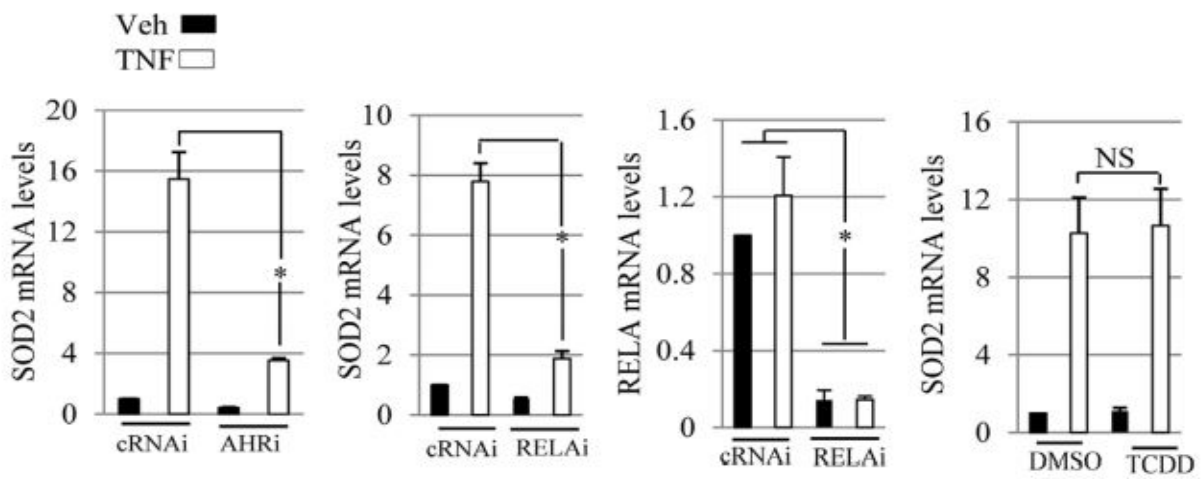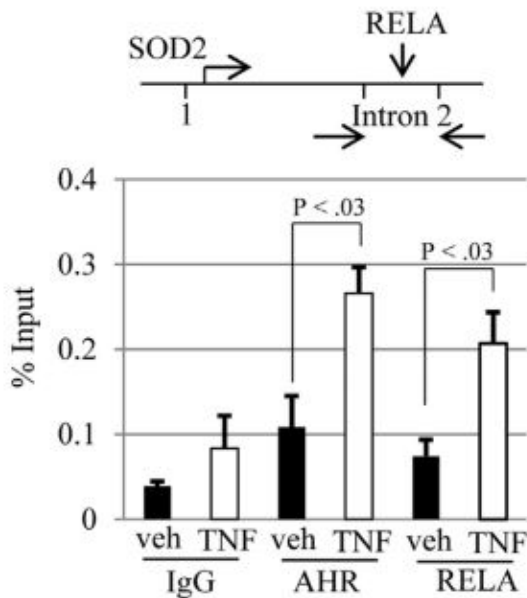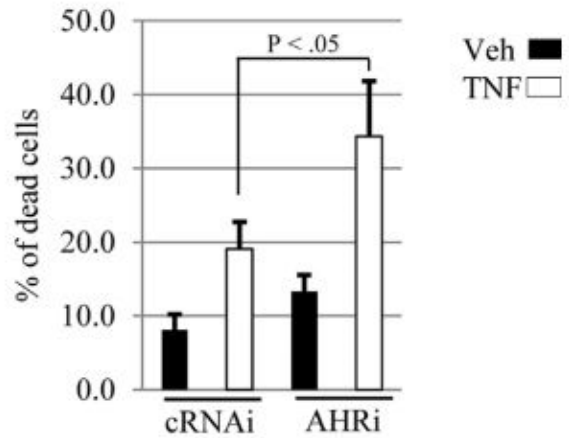
Figure 22. AHR promotes TNF induction of MnSOD. (A) MCF-7 cells were transfected with control (cRNAi) or AHR (AHRi) siRNAs (please see Section 2 for details regarding transfection) prior to treatment with vehicle (Veh) or TNF (10 ng/mL) for 12 h. Total cellular protein was then isolated and subjected to western blot analysis. The blot was then probed with the indicated antibodies. Relative level of MnSOD protein was expressed as a ratio of MnSOD/GAPDH. A significant decrease in MnSOD protein by AHRi is indicated by (* $P < 0.001$). (B) RT-qPCR analyses of SOD2 and RELA mRNA levels in MCF-7 cells transfected with control (cRNAi), AHR (AHRi) or RELA (RELAi) siRNAs prior to vehicle (Veh) or TNF (10 ng/mL) treatment for 12 h. Significant decreases in SOD2 or RELA mRNAs is indicated by (* $P < 0.001$). For TCDD stimulation, MCF-7 cells were stimulated with either dimethyl sulfoxide vehicle (DMSO-veh) or TCDD (10 nM) in the presence of vehicle or TNF (10 ng/mL) for 12 h, followed by measurement of SOD2 mRNA with RT-qPCR. No significant difference is indicated by (NS). Gene expression was normalized against GAPDH. (C) MCF-7 cells were treated with vehicle (Veh) or TNF (10 ng/mL) for 12 h and chromatin immunoprecipitation (ChIP) experiments were conducted, followed by real-time Q-PCR (ChIP-qPCR). A significant increase in AHR and RELA binding on an intronic NFKB response element in the SOD2 gene induced by TNF is indicated (* $P < 0.03$). (D) MCF-7 cells were transfected with cRNAi or AHRi prior to treatment with vehicle (Veh) or TNF (10 ng/mL) for 12 h and cell viability was determined as outlined in Section 2. Significant increases is indicated (* $P < 0.05$). ((A)–(D)) Data shown are the means -/+ S.E. of at least three independent experiments.

6.4. DISCUSSION

In this report, RNA-Seq analysis revealed that the expression of over 600 genes in MCF-7 cells is dependent on AHR based on our knockdown experiments. Pathway analysis revealed that a significant number of ADGs were present in toxicant and TNF pathways (Table 10). TNF induction of MnSOD required AHR and RELA expression, and this process involved recruitment of RELA and AHR to a TNF-responsive NF-kappa-B element in the SOD2 gene (Figure 22). Consistent with AHR/RELA recruitment to MnSOD, the cellular response to TNF was dependent on AHR expression as demonstrated in knockdown experiments (Figure 22).

There is little current evidence that demonstrates that cancer progression requires the expression of AHR; however, it is clear that AHR responds to and modulates cancer signals. From our prior report, we know that insulin like growth factor 2 (IGF-2) signaling rapidly increases AHR mRNA and protein levels in MCF-7 cells and that upregulated AHR promoted the activation of the CCND1 gene upon binding to the CCND1 gene promoter [114]. In this report we demonstrate that AHR modulates MCF-7 responsiveness to TNF. Together these findings indicate that AHR can modulate MCF-7 cancer progression by interacting with two major cancer signaling pathways, specifically IGF-2 and TNF.

Even though AHR expression has not been directly associated with cancer, AHR activity may be aberrant in cancer cells. AHRR is a putative tumor suppressor whose expression is downregulated in multiple cancers including breast tumors due to hypermethylation of its promoter [94]. AHRR inhibits AHR activity through a mechanism that could be mediated by AHRR binding with AHR [308]. Thus, AHR activity could be higher because AHRR expression is downregulated in human cancers [94].

There are several lines of evidence that AHR through interactions with RELA regulates proinflammatory genes; our data suggests this interaction is also important for regulating MnSOD, a major antioxidant enzyme. DiNatale and colleagues demonstrated that TCDD and interleukin 1 (IL-1) synergistically induce IL-6 transcription [309]. This was mediated through DREs in the IL-6 gene promoter [309]. Recently, AHR itself, in the absence of TCDD, has been reported to activate the IL-6 gene by pairing with RELA at kappa-RE in the IL-6 gene [310]. AHR interaction with NF-kappa-B is not restricted to RELA, considering that AHR binding with RELB activates the IL-8 gene [311, 312]. TCDD inhibits NF-kappa-B activity when measured with EMSA and a kappa-B-RE luciferase reporter construct [307]. We found that TNF induction of MnSOD is refractory to TCDD (Figure 22). So in some cases TCDD interactions with NF-κB therefore could be gene specific.

AHR-deficient MCF-7 cells were more sensitive to TNF-induced cytotoxicity than controls (Figure 22 (D)). TNF signaling stimulates opposing cell survival and death pathways [313]. TNF-induced NF-kappa-B protects cells from TNF-induced cell death by upregulating the expression of antioxidant and antiapoptotic genes [313]. Upregulation of MnSOD by NF-kappa-B inhibits TNF-induced ROS accumulation and cell death [228, 314]. The levels of MnSOD were lower in AHR knockdown MCF-7 cells in response to TNF compared with controls (Figure 22). Thus, AHR could in part protect MCF-7 cells from TNF-induced cytotoxicity by promoting upregulation of MnSOD (Figure 22).

Our RNA-Seq data and IPA analyses are consistent with many reports showing that AHR regulates gene expression in the absence of TCDD. There are potential mechanisms to explain AHR activity in MCF-7 cells in the absence of TCDD. Chiaro et al. discovered that the 5-lipoxygenase (5-LOX) pathway generates 5,6-dihydroxyeicosatetraenoic acid isomers (5,6-

DiHETEs) that induce expression of a DRE-promoter reporter construct, the formation of AHR-DNA binding complexes in EMSA assays, and increases in CYP1A1 mRNA in hepatocytes [315]. DiNatale and colleagues reported that the tryptophan metabolite kynurenic acid induced CYP1A1 mRNA, DRE-promoter reporter activity and the formation of an AHR-DNA complex, and competitively displaced labeled AHR ligand from AHR in hepatocytes [95]. Kynurenine has been reported to be secreted at μM levels from glioma cells and to induce DRE-promoter reporter activity, CYP1A1 mRNA levels and to competitively displace labeled AHR ligand from AHR in glioma cells [75]. 5,6-DiHETEs, kynurenic acid and kynurenine therefore may serve as endogenous AHR ligands that stimulate AHR activity and expression of AHR target genes in MCF-7 cells in the absence of TCDD. Considering our data showing that TNF-induced AHR binding at an active NFκB-RE, we postulate that AHR may be recruited to gene promoters by activated RELA (perhaps in an AHR ligand independent mechanism).

In conclusion, our RNA-Seq data suggest a role for AHR in toxicant and TNF pathways. Further, AHR and RELA are clearly required for induction of MnSOD and the cytoprotective response to TNF. In a similar vein, AHR protects lung cells from cigarette induced cytotoxicity by maintaining MNSOD expression [304]. As a whole, our findings implicate unliganded AHR expression in a new aspect of cancer progression.

6.5. COMPETING INTERESTS

The authors declare that they have no competing interests.

6.6. ACKNOWLEDGEMENTS

to Travis Salisbury. RNA-Seq and related data analyses were performed by the Marshall

University School of Medicine Genomics Core Facility.

CHAPTER VII: ARYL HYDROCARBON RECEPTOR (AHR) REGULATION OF L-TYPE AMINO ACID TRANSPORTER 1 (LAT-1) EXPRESSION IN MCF-7 AND MDA-MB-231 BREAST CANCER CELLS.

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that is regulated by environmental toxicants that function as AHR agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). L-Type Amino Acid Transporter 1 (LAT1) is a leucine transporter that is overexpressed in cancer. The regulation of LAT1 by AHR in MCF-7 and MDA-MB-231 breast cancer cells (BCCs) was investigated in this report. Ingenuity pathway analysis (IPA) revealed a significant association between TCDD-regulated genes (TRGs) and molecular transport. Overlapping the TCDD-RNA-Seq dataset obtained in this study with a published TCDD-ChIP-seq dataset identified LAT1 as a primary target of AHR-dependent TCDD induction. Short interfering RNA (siRNA)-directed knockdown of AHR confirmed that TCDD-stimulated increases in LAT1 mRNA and protein required AHR expression. TCDD-stimulated increases in LAT1 mRNA were also inhibited by the AHR antagonist CH-223191. Upregulation of LAT1 by TCDD coincided with increases in leucine uptake by MCF-7 cells in response to TCDD. Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assays revealed increases in AHR, AHR nuclear translocator (ARNT) and p300 binding and histone H3 acetylation at an AHR binding site in the LAT1 gene in response to TCDD. In MCF-7 and MDA-MB-231 cells, endogenous levels of LAT1 mRNA and protein were reduced in response to knockdown of AHR expression. Knockdown experiments demonstrated that proliferation of MCF-7 and MDA-MB-231 cells is dependent on both LAT1 and AHR. Collectively, these findings confirm the dependence of cancer cells on leucine uptake and establish a mechanism for extrinsic and intrinsic regulation of LAT1 by AHR.

Key Words: Aryl Hydrocarbon Receptor (AHR); L-Type Amino Acid Transporter 1 (LAT1), TCDD, gene expression; breast cancer

GRAPHICAL ABSTRACT
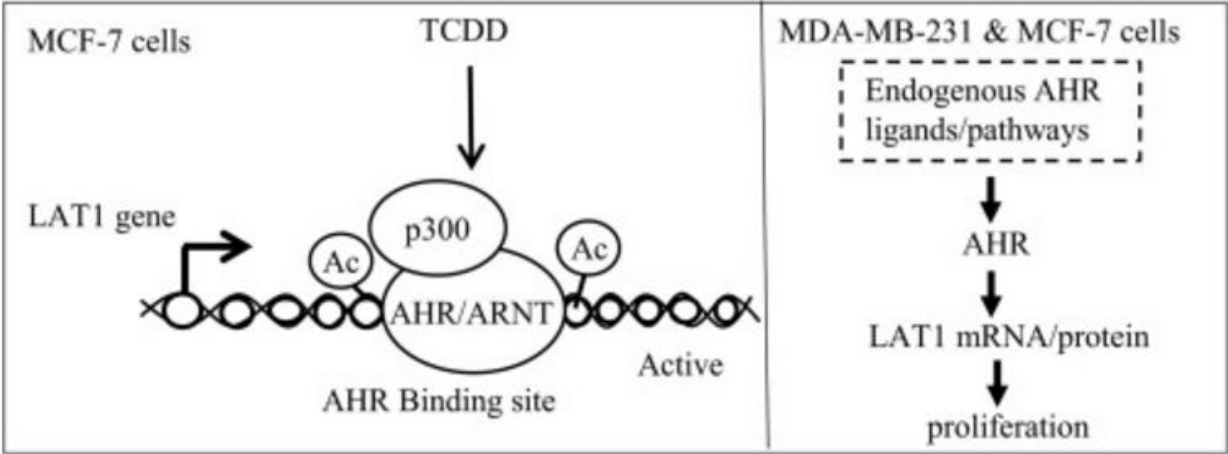
7.1. INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs) are environmental toxicants that are formed as byproducts of industry and municipal waste incineration [316, 317]. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exhibits the highest affinity for AHR compared with other HAHs [318]. In the absence of ligand, AHR is associated with chaperone proteins including heat shock protein 90 (HSP90) [319, 320], Aryl Hydrocarbon Receptor Interacting Protein (also known as XAP2) [20, 321, 322], and the co-chaperone protein p23 [21]in the cytoplasm. Upon binding to TCDD, AHR translocates from the cytoplasm into the nucleus and binds AHR nuclear translocator (ARNT) [36, 253, 323]. TCDD-induced AHR/ARNT dimers confer transcriptional activity specifically to AHR response elements (AHR-REs) that cluster near the promoter regions of TCDD target genes [36]. CYP1A1 and CYP1B1 are phase I xenobiotic metabolizing enzymes that are transcriptionally induced by TCDD via AHR [36]. The induction of CYP1A1 and CYP1B1 transcription by TCDD also requires several transcriptional coactivators including steroid receptor coactivator 2 (SRC1), steroid receptor coactivator 2 (SRC2), p300 and BRG-1 [37, 324, 325].

In addition to xenobiotic metabolism, immune responses are modulated by AHR and the outcome is dependent on the AHR ligand. For instance, T regulatory cells (Tregs) suppress excessive immune responses and their differentiation is promoted by TCDD or kynurenine (Kyn), and both of these AHR ligands are immunosuppressive [73, 74]. Th17 cells are proinflammatory T cells and their expansion and differentiation is enhanced by the endogenous AHR ligand 6-formylindolo [3,2-b] carbazole (FICZ), but suppressed by TCDD [74, 77]. Developmental and functional immunity is dependent on AHR and the dietary AHR ligands indolo [3,2-b] carbazole (ICZ) and 3,3-diidolylmethane (DIM) [326, 327]. Finally, cytokine and

chemokine gene expression in dendritic cells (DC) and macrophages is increased by AHR ligands, and the transcription of AHR is increased by nuclear factor kappa B in response to lipopolysaccharide (LPS) in innate immune cells [49, 95, 328].

TCDD-RNA-Seq analysis described herein identified 137 TCDD-regulated genes (TRGs) in MCF-7 breast cancer cells (BCCs) among which is L-Type Amino Acid Transporter 1 (LAT1). The uptake of large neutral acids including: leucine, arginine, phenylalanine, tyrosine, and tryptophan is mediated by LAT1 [230-232]. Breast, colorectal, head and neck, leukemia, lymphoma, melanoma, prostate and parathyroid cancers express higher levels of LAT1 compared with corresponding normal tissue [233]. LAT1 promotes proliferation of cancer cells by stimulating the uptake of amino acids that are important for protein synthesis [233]. In addition, its ability to promote cellular uptake of leucine would also increase the activity of mTORC1 which has been reported to be important for the growth and survival of some cancers [235]. TCDD has been reported to increase LAT1 mRNA in HEPG2 cells, which are a model of hepatocellular carcinoma [329]. These observations strongly suggest that LAT1 is critical for cancer cell growth and survival. However, the mechanism by which TCDD or AHR regulates LAT1 expression has not been determined.

The objective of this report was to investigate extrinsic regulation of LAT1 by TCDD/AHR and intrinsic (endogenous) regulation of LAT1 by AHR. Extrinsic regulation of LAT1 by TCDD/AHR was investigated in MCF-7 cells. Intrinsic regulation of LAT1 by AHR was investigated in MCF-7 and MDA-MB-231 cells because these BCC lines have been reported to exhibit endogenous AHR activity [59, 107-109]. Based on our findings, we report a new role for AHR as an extrinsic and intrinsic regulator of LAT1 expression in BCCs and show that AHR binds to LAT1 AHR-REs as part of a transcriptional activator complex.

127

7.2. METHODS

7.2.1. Materials

Dulbecco's Modified Eagle Medium/High glucose (DMEM) with l-glutamine and sodium pyruvate, phenol red-free DMEM, phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin, streptomycin, and dimethyl sulfoxide (DMSO) were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Sodium dodecyl sulfate (SDS), 30% acrylamide/bis solution, ammonium persulfate, Tween-20, 2-mercaptoethanol and polyvinylidene difluoride (PVDF) membranes were obtained from BIO-RAD (Hercules, CA). Non-targeting short interfering RNA (siRNA) (cat # D-001810-01-20), ON-TARGET plus human siRNAs against AHR (cat # J-004990-08-0010, and cat # J-004990-06-0010) and LAT1 (cat # J-004953-09-0010) were purchased from GE Dharmacon (Lafayette, CO). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotopes Laboratory (Andover, MA). The AHR antagonist CH-223191 was purchased from Sigma–Aldrich (St. Louis, MO). MCF-7 and MDA-MB-231 BCCs were purchased from ATCC (Manassas, VA) and maintained in DMEM, 10% FBS, with penicillin (100 IU/mL) and streptomycin (100 IU/mL).

7.2.2. TCDD RNA-Seq

250,000 MCF-7 cells were seeded in 35 mm plates in DMEM supplemented with 10% FBS for 24 h, followed by overnight serum-starvation in phenol red-free DMEM, and then treated with vehicle (DMSO) or 10 nM TCDD for six hr. RNA-Seq analysis was based on four biological replicates in each experimental group. Total RNA purification kits (Qiagen, Valencia, CA) were used to extract total RNA. RNA sample quality was assessed using Bioanalyzer RNA Nano chips (Agilent); all RNA samples had an RNA Integrity Number greater than or equal to eight. RNA-Seq libraries were prepared from 1 μg of total RNA using a TruSeq RNA Prep Kit

(Illumina Inc., San Diego, CA). RNA-Seq was performed using an Illumina HiSeq1000 in a $2 \times$ 100 base paired end design yielding a minimum of 50 million reads per sample. Differentially expressed genes were identified at a False Discovery Rate (FDR) of 5% as detailed in our prior report [59]. Raw reads and processed data (unnormalized and normalized read counts by gene) were deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information and are accessible via accession number GSE76608.

7.2.3. Ingenuity pathway analysis (IPA)

TCDD-regulated genes (TRGs) were expressed as a ratio of TCDD/DMSO and loaded into IPA software (Ingenuity Systems, Redwood City, CA). Of the 137 TRGs identified by RNA-Seq, 116 were mapped to known functions and pathways by IPA. The Core Analysis tool and the Fisher Exact Test in IPA were used to identify statistically significant associations between TRGs and cellular and molecular pathways. We configured the core analysis to report Benjamini–Hochberg corrected p-values.

7.2.4. Reverse transcription and real-time polymerase chain reaction (RT-qPCR)

RT-qPCR assays were carried out as described in our prior reports [59, 114]. In brief, total RNA was extracted using RNA purification columns (Qiagen) and 100–300 ng of extracted RNA was reverse transcribed to cDNA using High Capacity Reverse Transcription kits (Thermo Fisher Scientific). Real-time qPCR reactions were performed in triplicate using SYBR Green Master Mix according to the manufacturer's instructions (Thermo Fisher Scientific). Relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ formula as described by Livak and Schmittgen [239]. Glyceraldehyde-3-phosphate (GAPDH) mRNA levels served as the internal control. The sequences of the qPCR primers used to amplify GAPDH and AHR mRNA have been published [59]. LAT1 mRNA qPCR primers were: forward, 5′-ccgaggagaaggaagaggc-3′; reverse, 5′-

gaagatgcccgagccgataa-3′. The Student–Newman–Keuls (SNK) post hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA).

7.2.5. Short interfering RNA (siRNA) assays and Western blotting

The siRNA knockdowns were performed as detailed in our prior reports [59, 114]. Briefly, 200,000 cells (MCF-7 or MDA-MB-231) in 1 mL of DMEM supplemented with 10% FBS were mixed directly with 100 nM of siRNA that was non-targeting, AHR-targeting or LAT1-targeting and 3 μL of Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) and immediately plated in 35 mm tissue culture plates for 48 h. MCF-7 cells were then treated with vehicle (DMSO) or 10 nM TCDD for 16 h. Treatments were removed and cells were rinsed once with PBS. For Western blotting, total protein was extracted by scraping cells in 2× Laemmli Sample Buffer containing β-mercaptoethanol (BME). Laemmli sample buffer and BME were purchased from BIO-RAD. Standard Western blotting techniques were used to analyze ~10 μg of protein per sample (please refer to our prior reports for technical details [59, 114]). Western blot analysis of GAPDH was used to confirm equal protein loading. Blots were probed with anti-GAPDH antibody (diluted 1:10,000), anti-AHR antibody (diluted 1:5,000) or anti-LAT1 antibody (diluted 1:2,000) overnight at 4° C, followed by incubation with anti-HRP secondary antibody (1:5000) for one hr at room temperature. The blots were then rinsed with PBS + 0.1% tween 20, and then developed with enhanced chemiluminescent substrate Millipore Corp., (Billerica, MA). The anti-GAPDH antibody was purchased from Millipore (Cat #MAB374). The anti-AHR antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, Cat #H-211) and the anti-LAT1 antibody was purchased from Cell Signaling Technology (Danvers, MA, Cat #5347). Densitometry was calculated with ImageJ PC-based software

130

(National Institute of Health). The Student–Newman–Keuls (SNK) post hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA).

7.2.6. Chromatin immunoprecipitation followed by qPCR (ChIP-qPCR)

The ChIP-qPCR assays were carried out as described in our previous report [114]. In brief, nonspecific IgG, and antibodies that were specific for AHR, ARNT or p300 were obtained from Santa Cruz Biotechnology. The antibodies against acetylated lysine 9 or lysine 14 in histone H3 were purchased from Cell Signaling Technology. The magnetic protein A beads and proteinase K were purchased from Life Technologies (Carlsbad, CA). A recent TCDD-ChIP-Seq report identified an AHR binding site within a 900 bp region in LAT1 corresponding to coordinates 87,840,300–87,841,199 (human genome version 19 (Hg19)) [277]. This AHR binding site was investigated in this report by ChIP-qPCR with primers that span coordinates 87,840,403–87,840,544, which were: [forward 5′-GCACGTACCTGTAGGGGTTG-3′ and reverse 5′-ATGCTCTCTCCCCGGTGATT-3′]. The ChIP-qPCR primers used to amplify the AHR binding sites in the CYP1B1 gene have been published [330]. ChIP-qPCR data were expressed as % input in which signals obtained from the ChIP are divided by signals obtained from an input sample. Statistical differences among groups were determined by the SNK post hoc test following one-way analysis of variance (ANOVA).

7.2.7. Leucine uptake experiments

Leucine uptake experiments were performed in MCF-7 cells grown to confluence on 24 well plates. The cells were first washed twice with Na-free buffer (130 mM TMACl, 4.7 mM KCl, 1 mM MgSO4, 1.25 mM CaCl2, 20 mM HEPES; pH 7.4) and incubated with the same for ten min at room temperature. The uptake was then initiated by incubating the cells for 30 s with

131

Na-HEPES buffer (130 mM NaCl, 4.7 mM KCl, 1 mM MgSO4, 1.25 mM CaCl2, 20 mM HEPES; pH 7.4) with 10 μCi of ^{3}H-l-Leucine (PerkinElmer; Waltham, MA) and 10 μM l-Leucine (Sigma–Aldrich). The reaction was stopped with ice cold Na-HEPES buffer after which the cells were washed twice with the same ice-cold buffer. The cells were then lysed in 500 μl of 1 N NaOH followed by incubation for 20 min at 70° C. The lysed contents of each well was collected in a 7 ml scintillation tube and mixed with 5 ml Ecoscint A (National Diagnostics; Atlanta, GA). Leucine uptake experiments were conducted using chemicals obtained from Sigma–Aldrich. The vials were kept in the dark for 48 hr and the radioactivity was determined in a Beckman 6500 scintillation counter.

7.2.8. MDA-MB-231 and MCF-7 proliferation experiments

20,000 MDA-MB-231 or 10,000 MCF-7 cells in 1 mL of DMEM + 10% FBS were mixed directly with siRNA's (100 nM) that were non-targeting, AHR-targeting or LAT1-targeting and 3 μL of RNAiMax Transfection Reagent. The cells were then plated into 96 well plates at a density of 2000 MDA-MB-231 or 1000 MCF-7 cells per well. After three days, cell proliferation was assayed with the Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) in accordance with the manufacturer's protocol.

7.3. RESULTS

7.3.1. TCDD RNA-Seq

RNA-Seq analysis revealed that TCDD-regulated the expression of 137 genes in MCF-7 BCCs at an FDR <5%, with all fold changes greater than or equal to 1.3 compared with vehicle. Of these 137 TCDD-regulated genes (TRGs), 116 were mapped to known functions by IPA. Comparison of TRGs with a published TCDD/AHR-ChIP-Seq dataset [277] revealed that 47 genes were shared between the two gene sets (Figure 23). These 47 genes included known TCDD target genes such as CYP1A1 [331], CYP1B1 [331] and ALDH3A1 [299] (Figure 23). Bioinformatic analysis revealed that the 47 TCDD target genes were significantly associated with metabolic pathways including: lipid metabolism, carbohydrate metabolism, nucleic acid metabolism, vitamin and mineral metabolism and energy production (Table 11). The 47 TCDD target genes were also associated with cancer processes including: cell death and survival, cell cycle, cellular growth and proliferation, and molecular transport (Table 11).

7.3.2. TCDD/AHR regulation of LAT1 and leucine uptake

The TCDD-RNA-Seq indicated that LAT1 (also known as SLC7A5) was induced 2.33-fold by TCDD (Table 11), while TCDD-ChIP-Seq evidence suggested that AHR could directly bind to intron 2 of the LAT1 gene [277]. We decided to further investigate extrinsic regulation of LAT1 by TCDD, considering that prior reports indicate that upregulation of LAT1 could be important for breast cancer progression [117, 332, 333]. To investigate whether TCDD increases LAT1 expression through AHR, MCF-7 cells were transfected with short interfering RNA against AHR (AHRi). Control MCF-7 cells were transfected with non-targeting siRNA (cRNAi).

Lo, R 2012
AHR/ARNT ChIP-seq

10 nM TRGs

69   47   660
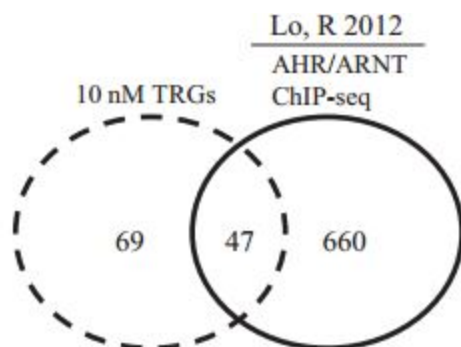
47 TRGs genes that overlap with reported TCDD-AHR/ARNT ChIP set.					
GENE ID	*fold change	GENE ID	*fold change	GENE ID	*fold change
CYP1A1	424.40	NEDD9	2.15	PYGL	1.57
CYP1B1	8.29	TMEM45B	2.03	AGPAT9	1.56
DRD1	6.08	NFE2L2	1.86	ATP9A	1.56
ALDH1A3	6.06	PPARG	1.85	SLC16A7	1.50
PITPNM2	3.77	NBPF10	1.80	SLC27A2	1.50
LMCD1	3.75	CABLES1	1.75	LRIG3	1.49
BMF	3.13	SYT12	1.79	FOSL2	1.49
GLDN	2.62	DNMBP	1.74	ELF4	1.48
ST3GAL1	2.61	SLC16A14	1.73	TMEM120B	1.47
FAM105A	2.49	STC2	1.70	ADAP1	1.43
RUNX2	2.38	TMTC2	1.68	DDIT4	1.41
SLC7A5 (LAT1)	2.33	HES1	1.64	GDF15	1.41
TNFRSF11B	2.32	AHRR	1.61	TUFT1	1.38
TTC39B	2.29	PRPS1	1.60	PSPC1	1.37
MB21D2	2.20	DLL1	1.59	C6orf132	0.70
PLEKHF1	2.18	LRP5	1.58		
*RNA-seq fold change expression values from TCDD-treated MCF7 cells compared to vehicle expressed as a ratio of TCDD/vehicle					

Figure 23. Genes common between TCDD RNA-Seq data and TCDD-ChIP-Seq data.
Analysis of reported MCF-7 TCDD-ChIP-Seq data revealed that 47 of the 116 TCDD-RNA-Seq genes were TCDD-AHR/ARNT bound genes. The specific 47 TCDD-regulated genes and their associated fold changes are shown in the table.

134

Category	*B-H p-value	Target molecules in dataset
Lipid metabolism	8.55E–03-3.52E–02	14
Small molecule biochemistry	8.55E–03-4.11E–02	26
Cell death and survival	8.55E–03-4.11E–02	30
Gene expression	8.55E–03-3.2E–02	12
Cell cycle	8.55E–03-3.91E–02	16
Cellular development	8.55E–03-3.54E–02	37
Cellular growth and proliferation	8.55E–03-3.93E–02	42
Carbohydrate metabolism	8.55E–03-3.2E–02	9
Drug metabolism	8.55E–03-3.71E–02	10
Energy production	8.55E–03-3.27E–02	8
Molecular transport	8.55E–03-3.71E–02	15
Vitamin and mineral metabolism	9.07E–03-3.2E–02	8
Nucleic acid metabolism	9.68E–03-3.2E–02	4

* p-Values are calculated by Fishers exact test and corrected for multiple testing by the Benjamini–Hochberger p-values (B–H) method (B–H p-value). Column 2 shows the range of B–H corrected p-values for the biofunctions in a given category. Target molecules in dataset are the number of RNA-Seq TRGs in a given biofunction.

Table 11. IPA cellular and molecular functions associated with the 47 TRGs that overlap with reported TCDD-AHR/ARNT ChIP-seq.

SiRNA-treated cells were then exposed to either vehicle or TCDD (10 nM) for six hr. Reductions in AHR mRNA were confirmed in MCF-7 cells transfected with AHRi compared with those transfected with cRNAi (Figure 24 (A)). As expected, TCDD-stimulated increases (three-fold) in LAT1 mRNA compared with vehicle in MCF-7 cells transfected with cRNAi (Figure 24 (B)). In contrast, AHRi significantly reduced the stimulatory effect of TCDD on LAT1 expression ($P <$ 0.01; Figure 24 (B)). These results indicate that LAT1 regulation by TCDD is mediated by AHR.

To investigate whether TCDD increases LAT1 expression by binding to AHR, MCF-7 cells were treated with CH-221391, which is an AHR antagonist that specifically inhibits the binding of TCDD to AHR [334]. TCDD induction of CYP1A1 transcription is a commonly used readout of TCDD-induced AHR activity that requires TCDD to bind AHR. To verify that CH-221391 is an AHR antagonist in MCF-7 cells, its ability to suppress induction of CYP1A1 by TCDD was measured by RT-qPCR. The findings revealed that TCDD-induced increases in CYP1A1 were indeed reduced by CH-221391, indicating that it is an effective AHR antagonist in MCF-7 cells (Figure 24 (C)). As expected, TCDD-stimulated increases ($\sim$three-fold) in LAT1 mRNA in control MCF-7 cells not treated with CH-221391 (Figure 24 (D)). In contrast, CH-221391 significantly ($P < 0.01$) suppressed the stimulatory effect of TCDD on LAT1 expression (Figure 24 (D)). These results indicate that regulation of LAT1 by TCDD requires TCDD to bind AHR.

Western blot experiments were conducted to confirm that TCDD induction of LAT1 mRNA leads to increases in LAT1 protein. Exposure to TCDD stimulated robust increases ($\sim$ten-fold) in LAT1 protein in MCF-7 cells transfected with cRNAi (Figure 25 (A) and (B)). Induction of LAT1 protein by TCDD was completely suppressed in MCF-7 cells transfected with AHRi (Figure 25 (A) and (B)). As expected, AHR protein levels in MCF-7 cells transfected with AHRi
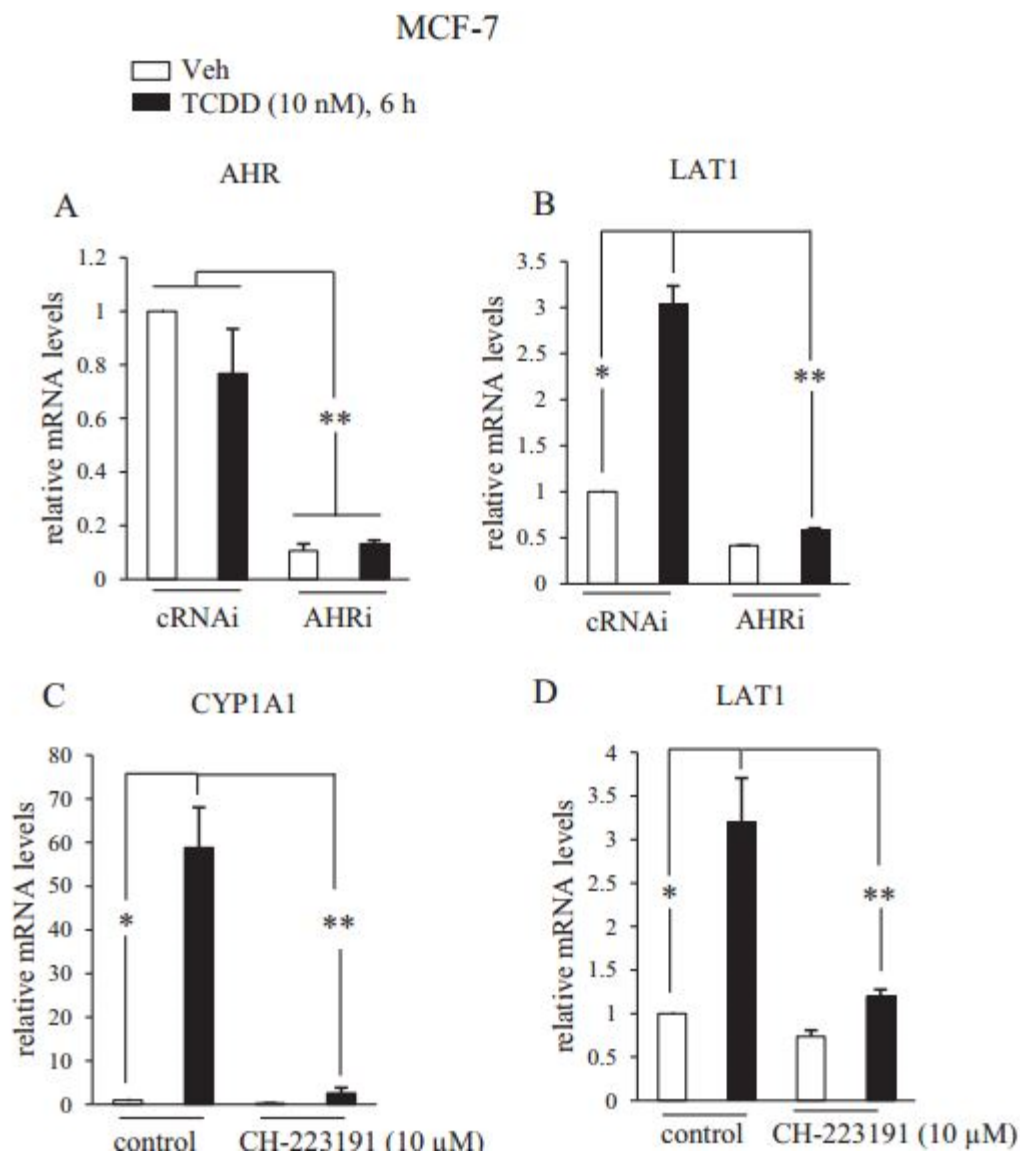
Figure 24. AHR mediates TCDD-stimulated increases in LAT1 mRNA. (A and B) MCF-7 cells were transfected with siRNAs that were either non-targeting (cRNAi) or AHR targeting (AHRi) and then treated with DMSO vehicle (Veh) or TCDD (10 nM) for 6 h. (C and D) MCF-7 cells were treated vehicle or TCDD (10 nM) in the absence (controls) or presence of CH-223191 (10 μM) for 6 h. (A–D) AHR, LAT1, CYP1A1 or GAPDH mRNA were quantified by qRT-PCR from total RNA. GAPDH mRNA levels were used to normalize samples. * P < 0.05; **P > 0.01. Data shown are the mean ± S.E. of three independent experiments.
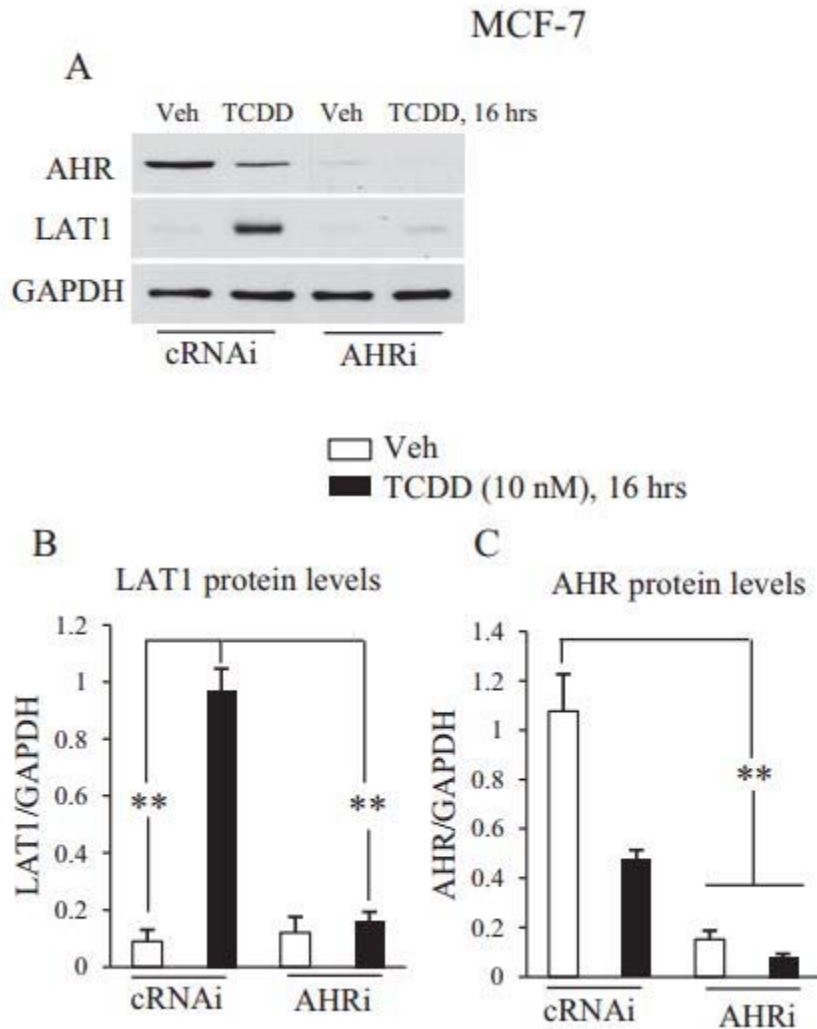
Figure 25. AHR mediates TCDD-stimulated increases in LAT1 protein. (A–C) MCF-7 cells were transfected with siRNAs that were either non-targeting (cRNAi) or AHR targeting (AHRi) and then treated with vehicle (Veh) or TCDD (10 nM) for 16 h. Total cellular protein was then isolated and subjected to Western blot analysis. The blot was then probed with the indicated antibodies. Relative levels of AHR or LAT1 protein were expressed as a ratio to GAPDH loading control. * P < 0.05; **P > 0.01. Data shown are the mean ± S.E. of three independent experiments.

138

were lower than those transfected with cRNAi (Figure 25 (A) and (C)). Leucine uptake experiments were performed to investigate if the induction of LAT1 protein by TCDD coincided with increases in leucine uptake by MCF-7 cells. Significant increases in leucine uptake by cells was observed in response to TCDD exposure compared with vehicle-treated cells (Figure 26). Taken together, these results indicate that the induction of LAT1 protein by TCDD leads to a functional increase in leucine uptake by MCF-7 cells.

7.3.3. TCDD-induced AHR/ARNT/p300 recruitment to an AHR binding site in the LAT1 gene

A prior report by Lo and Matthews (2012) identified an AHR binding site in intron 2 of the LAT1 gene by TCDD-ChIP-seq analysis in MCF-7 cells [277]. In order to characterize the mechanism of LAT1 induction, we measured AHR recruitment to the AHR response elements (AHR-REs) in LAT1 intron 2 by ChIP-qPCR analysis. The results showed a significant ($P <$ 0.05) 4-fold increase in AHR binding to the intron 2 site in response to TCDD compared with vehicle (Figure 27 (A)). In accordance with its known mechanism of action, TCDD-increased (by 37-fold) AHR binding to AHR-REs that are located upstream from the CYP1B1 transcription start site (Figure 27 (D)).

By binding to AHR, the ARNT transcription factor promotes AHR recruitment to AHR-REs in TCDD target genes [36, 253]. We assessed ARNT binding to the LAT1 intron 2 and CYP1B1 using ChIP-qPCR analysis and the findings revealed its recruitment to AHR-REs in the LAT1 and CYP1B1 genes was increased by 12- and 55-fold, respectively in response to TCDD (Figure 27 (B) and (E)). Prior reports indicate that AHR/ARNT heterodimers recruit the p300 transcription complex to TCDD target genes [37]. The AHR-REs in LAT1 and CYP1B1 genes exhibited three-fold and ten-fold increases, respectively, in p300 binding in response to TCDD

139
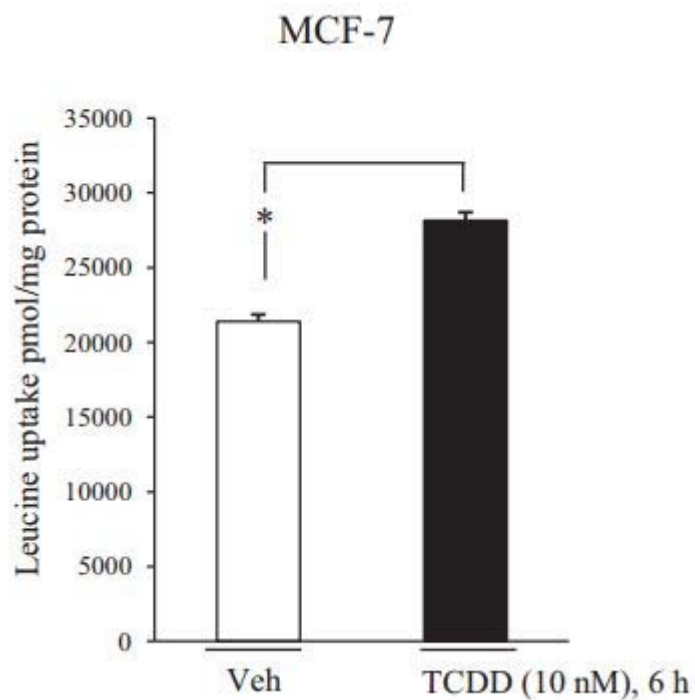
Figure 26. TCDD increases leucine uptake. MCF-7 cells were treated with vehicle or 10 nM TCDD for 6 h, followed by analysis of leucine uptake as detailed in the material and methods. * $P < 0.05$. Data shown are the mean ± S.E. of three independent experiments
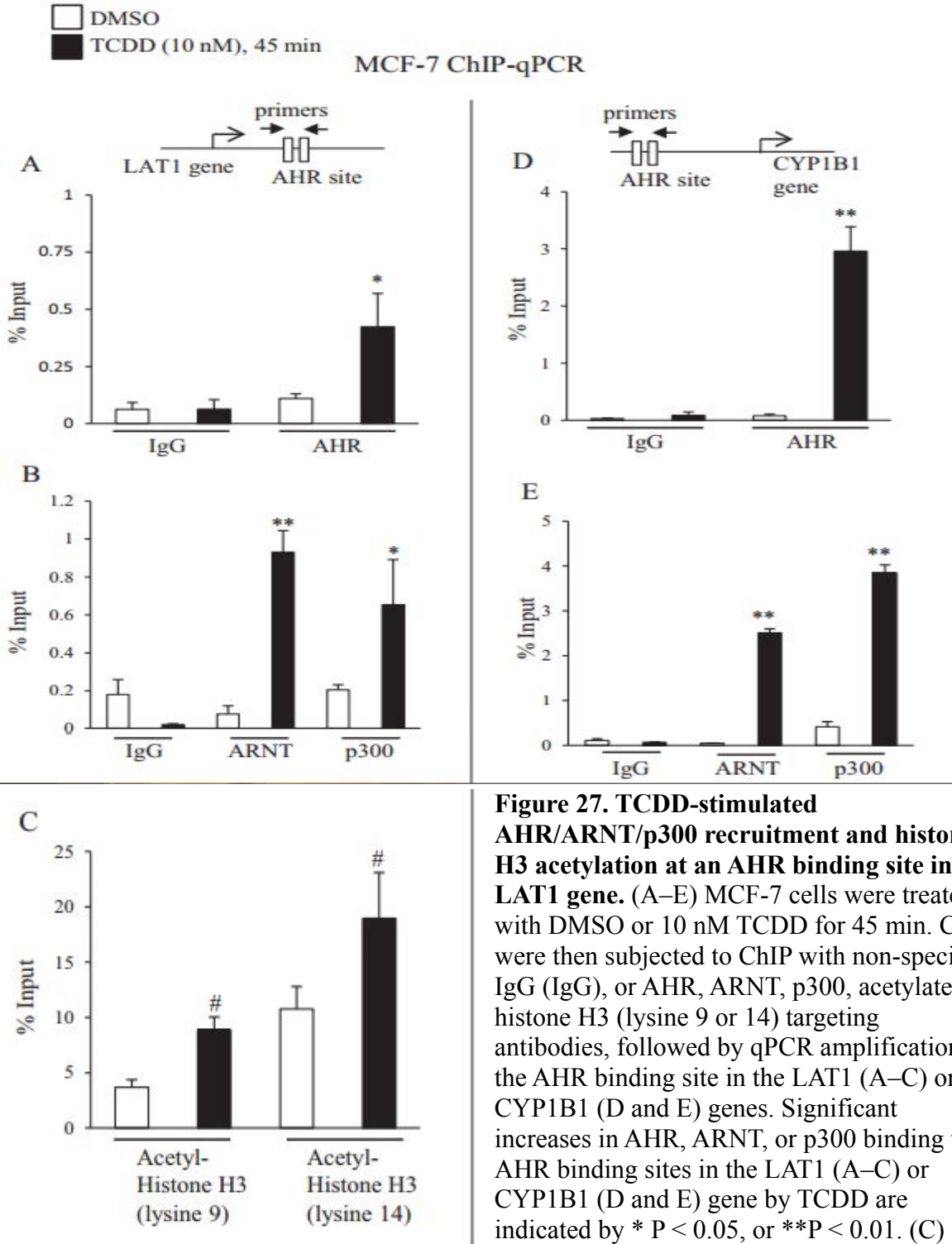
Figure 27. TCDD-stimulated AHR/ARNT/p300 recruitment and histone H3 acetylation at an AHR binding site in the LAT1 gene. (A–E) MCF-7 cells were treated with DMSO or 10 nM TCDD for 45 min. Cells were then subjected to ChIP with non-specific IgG (IgG), or AHR, ARNT, p300, acetylated histone H3 (lysine 9 or 14) targeting antibodies, followed by qPCR amplification of the AHR binding site in the LAT1 (A–C) or CYP1B1 (D and E) genes. Significant increases in AHR, ARNT, or p300 binding to AHR binding sites in the LAT1 (A–C) or CYP1B1 (D and E) gene by TCDD are indicated by * $P < 0.05$, or **$P < 0.01$. (C) Significant increases in the acetylation of lysine 9 or 14 in histone H3 at the AHR binding site in the LAT1 gene by TCDD are indicated by #$P < 0.05$. Data shown are the mean ± S.E. of three independent experiments.

compared with vehicle (Figure 27 (B) and (E)).

The p300 complex has inherent histone acetylase activity and is known to increase in histone H3 acetylation at lysine 9 and lysine 14, both of which are markers of active transcription [335]. Consistent with its physical recruitment to the LAT1 AHR-RE, we observed increases in the acetylation of histone H3 at lysine 9 and lysine 14 (by 2.4 and 1.8-fold, respectively) at the AHR-RE in response to TCDD (Figure 27 (C)). Collectively, these data indicate that extrinsic regulation of LAT1 by TCDD is mediated via the AHR binding and recruitment of p300 to the AHR-RE in the LAT1 gene.

7.3.4. Endogenous regulation of LAT1 by AHR promotes MCF-7 and MDA-MB-231 proliferation

Intrinsic regulation of LAT1 by AHR was investigated in MCF-7 and MDA-MB-231 cells because these BCC lines have been reported to exhibit endogenous AHR activity [59, 107-109]. Western blot analysis revealed basal AHR and LAT1 protein expression in MCF-7 (Figure 28 (A)) and MDA-MB-231 (Figure 28 (C)) cells transfected with cRNAi. Transfection with AHRi reduced AHR protein in MCF-7 (Figure 28 (A)) and MDA-MB-231 cells (Figure 28 (C)) to levels that were not detected by standard Western blot analysis. Reducing AHR protein expression with AHRi also suppressed the levels of LAT1 protein in MCF-7 (by ∼70%) (Figure 28 (A)) and MDA-MB-231 (by ∼60%) (Figure 28 (C)) and the levels of LAT1 mRNA in MCF-7 (by ∼50%) (Figure 28 (B)) or MDA-MB-231 (by ∼40%) (Figure 28 (D)) compared with controls. These data indicate that endogenous AHR activity regulates LAT1 expression in MCF-7 and MDA-MB-231 cells.

Since LAT1 is known to promote cancer cell proliferation by stimulating the amino acid uptake [233], we sought to determine if AHR expression and its regulation of LAT1 in MCF-7
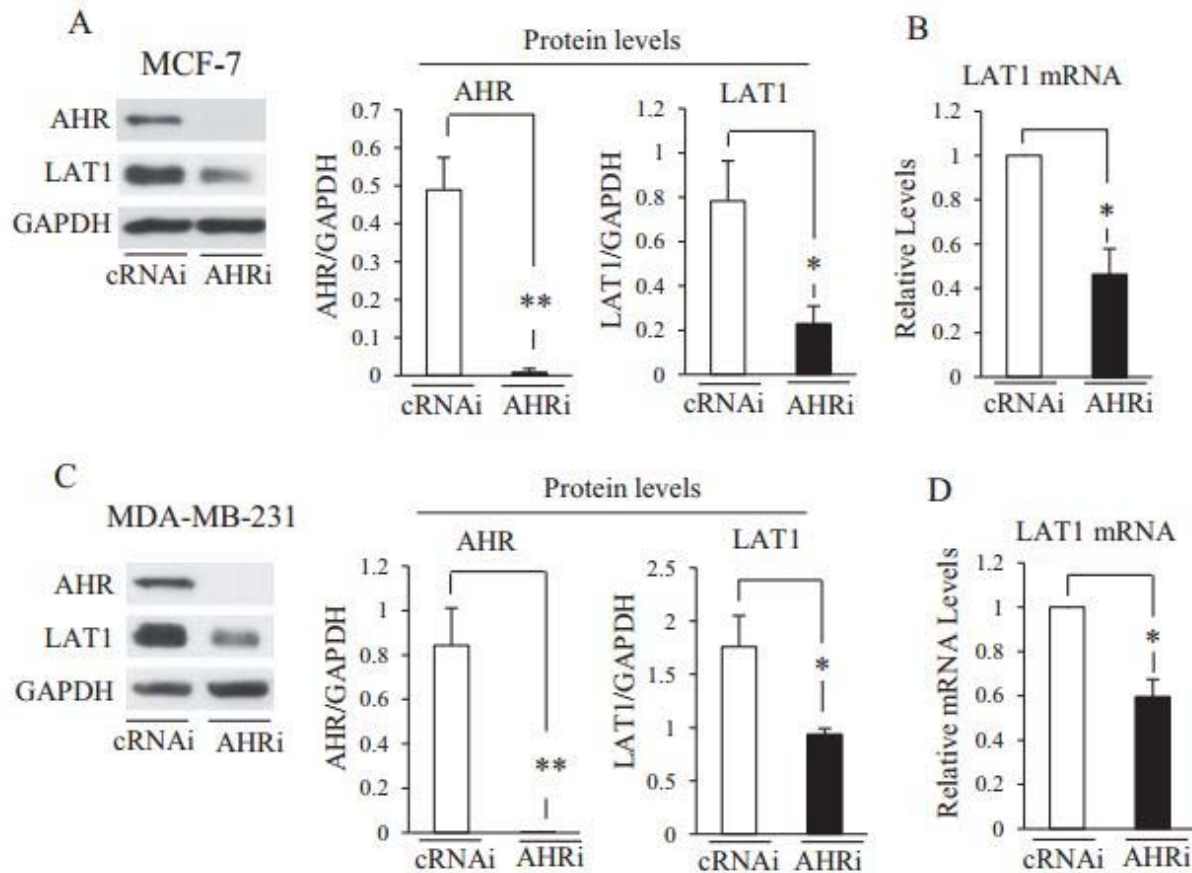
Figure 28. Endogenous regulation of LAT1 by AHR in MCF-7 and MDA-MB-231 cells. (A–D), MCF-7 (A and B) or MDA-MB-231 (C and D) cells were transfected with control (cRNAi) or AHR (AHRi) siRNA for 72 or 48 h, respectively. (A and C) Total cellular protein was then isolated and subjected to Western blot analysis. Blots were then probed with the indicated antibodies. Relative levels of AHR or LAT1 protein was expressed as a ratio of AHR/GAPDH or LAT1/GAPDH, respectively. * P < 0.05; **P > 0.01. (B and D) RTqPCR analyses of LAT1 mRNA levels in MCF-7 (B) or MDA-MB-231 (D) cells transfected with cRNAi or AHRi. GAPDH mRNA levels were used to normalize samples. * P < 0.05. (A–D) Data shown are the mean ± S.E. of three independent experiments.
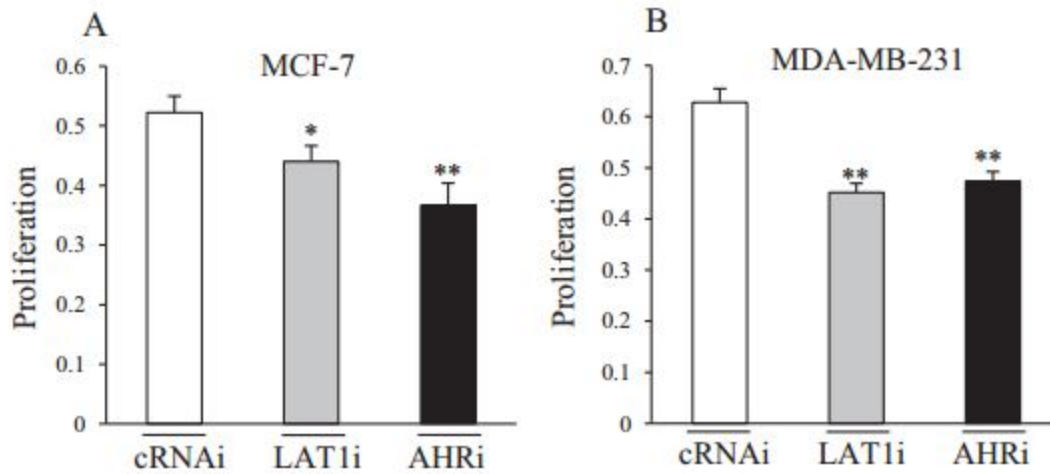
Figure 29. AHR and LAT1 promote MCF-7 and MDA-MB-231 proliferation. (A and B), MCF-7 (A) or MDA-MB-231 (B) cells were transfected with cRNAi, LAT1i or AHRi for 3 days, followed by analysis of proliferation (please see methods for details). Significant decreases in proliferation by LAT1i or AHRi are indicated by * $P < 0.05$, or **$P < 0.01$. Data shown are the mean ± S.E. of nine replicates.

144

and MDA-MB-231 are important for proliferation. To this end, MCF-7 and MDA-MD-231 cells were transfected with cRNAi, AHRi or LAT1-targeting siRNA (LAT1i). After three days, significant reductions in proliferation was observed in MCF-7 (Figure 29 (A)) and MDA-MB-231 (Figure 29 (B)) cells expressing LAT1i or AHRi compared with those transfected with cRNAi. These findings suggest that AHR regulation of LAT1 and LAT1 expression are important for the proliferation of MCF-7 and MDA-MB-231 cells.

7.4. DISCUSSION

The findings of this report provide new insight into extrinsic and intrinsic regulation of LAT1 by AHR. Reducing AHR with AHRi suppressed extrinsic regulation of LAT1 by TCDD in MCF-7 cells (Figures 24 and 25) and intrinsic regulation of LAT1 in MCF-7 and MDA-MB-231 cells (Figure 28). These findings indicate that AHR regulates LAT1 expression. ChIP-qPCR results indicate that extrinsic regulation of LAT1 by TCDD is mediated via the AHR binding site in the LAT1 gene (Figure 27). Indeed, the binding of AHR/ARNT/p300 and the acetylation of histone H3 at the AHR site in the LAT1 gene was increased by TCDD (Figure 27). Consistent with reports showing that AHR promotes MCF-7 and MDA-MB-231 cancer processes [109, 114], our proliferation assays indicate that reducing AHR suppressed their proliferation (Figure 29).

Prior reports have provided important insights into amino acid uptake by other transporters in MCF-7 and MDA-MB-231 cells. Karunakaran et al. demonstrated that SLC6A14 (also known as ATBo+) is a Na+ dependent, estrogen-induced transporter that mediates the uptake of all essential amino acids, including leucine, in MCF-7 cells [336]. The SLC6A14 inhibitor α-methyl-dl-tryptophan stimulated apoptosis of MCF-7, but not MDA-MB-231 cells, which was attributed to selective expression of SLC6A14 in MCF-7, but not MDA-MB-231 cells [336]. Shennan et al. established that MCF-7, but not MDA-MB-231 cells express LAT2, which is an isoform of LAT1 that also mediates leucine uptake [337]. Our observation that MCF-7 cells exhibit high basal leucine uptake activity in the absence of TCDD can be explained by the transporter activity of SLC6A14 and LAT2 as well as basal LAT1 activity (Figure 26). Since TCDD induction of SLC6A14 and LAT2 mRNA was not observed in our RNA-Seq data, we

conclude that the increase in leucine uptake in the presence of TCDD in MCF-7 cells is mediated via increased expression of LAT1.

CYP1A1 and CYP1B1 harbor upstream AHR-REs within 1 kb of their transcription start sites [330, 338, 339]. Reported TCDD-ChIP-seq data indicated that the LAT1 gene lacks promoter AHR-REs [277], but its expression is regulated by an AHR binding site located in intron 2 (Figure 27), which is 29 kb from the LAT1 promoter. Although long distance regulation of gene promoters by AHR-REs is relatively novel for TCDD, it is not uncommon for gene promoters to be regulated by distal enhancers [340].

AHR stimulation of transcription may rely on endogenous ligands. D'Amato et al. demonstrated that MDA-MB-231 cells synthesize kynurenine, which is a tryptophan metabolite and a known endogenous AHR ligand [109]. Production of kynurenine by MDA-MB-231 cells is mediated by tryptophan 2,3-dioxygenase (TDO2), which is the first and rate-limiting enzyme in the kynurenine synthesis pathway [109]. Thus, the observed AHR activity that is required for LAT1 expression in MDA-MB-231 cells could be attributed to kynurenine interacting with AHR. However, endogenous AHR activity could also reflect its interaction with the other tryptophan metabolites that also function as AHR agonists such as kynurenic acid or xanthurenic acid [95]. Regulation of LAT1 by unliganded AHR is also a possibility, considering that AHR can be regulated by cyclic AMP [341, 342].

Previous reports have provided important evidence that breast cancer progression may require LAT1. For instance, LAT1 expression is upregulated in human breast tumors compared with normal breast tissue [332]. Shennan et al. demonstrated that 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), which inhibits L type transporters, suppressed the proliferation of MCF-7, MDA-MB-231 and ZR-75-1 BCCs [117]. Our findings now establish that specifically

147

knocking down the expression of LAT1 with siRNA inhibits proliferation of MCF-7 and MDA-MB-231 cells (Figure 29).

In addition to breast cancer cells, normal cells that require high levels of amino acid uptake for their proliferation and differentiation have been reported to express LAT1 and AHR. In this regard, leucine uptake by activated T cells is mediated through LAT1 and LAT1 null T cells exhibit defects in proliferation and effector activity [343-345]. As noted in the introduction, AHR promotes the differentiation of Tregs and Th17 cells in response to TCDD or FICZ, respectively [73, 74, 77]. Given these prior reports, it is possible that in addition to carcinogenesis, the induction of LAT1 by AHR may promote the proliferation and differentiation of Tregs or Th17 cells depending on which AHR ligand is present.

7.5. COMPETING INTERESTS

The authors declare that they have no competing interests

7.6. ACKNOWLEDGEMENTS

CHAPTER VIII: DISCUSSION AND FUTURE DIRECTIONS

8.1. DISCUSSION.

At the end of my research project, I still find AHR signaling to be as fascinating now as it was to me when I first began studying it four years ago. This is due in part to the vastness of AHR biology, which seems to reveal itself slowly and veer into new directions with each decade. In toxicology, AHR is without a doubt the most highly studied receptor, given the breadth of compounds which work through AHR to exert their effects. While the AHR has been extensively studied in response to environmental pollutants/toxicants, there is still much to be learned about endogenous AHR cellular signaling. To date, over 400 exogenous ligands for the AHR have been found [346]. AHR is an evolutionarily conserved protein [347], and over the past decade great strides have been made in understanding endogenous AHR function as well as the characterization of endogenous AHR ligands [44, 59, 75, 95, 107-109]. While AHR research has shifted from toxicology-based to a more cancer-based emphasis, it is clear there is still much to learn regarding AHR functioning in both normal and cancerous tissues.

The objectives for our research were simple, yet with a broad spectrum: to reveal new tumor promoting roles for the AHR in breast cancer. We have done that, and continue to approach this objective from many new angles, as I will detail in the next sections. First, we revealed that adipokine-induced increases in MCF-7 BCC proliferation required AHR, considering that its knockdown suppressed MCF-7 proliferation in response to adipo-CM or IGF-2 compared with controls (Figure 16). In addition to characterizing new roles for AHR, our work also identified that IGF-2 is an important adipocyte-secreted factor that promotes MCF-7 and T-47D proliferation. Our finding that IGF-2-mediated cancer effects are mediated in part through AHR suggests that combining AHR antagonists with IGF1-R blocking antibodies [348]

may increase the efficiency of IGF1-R blocking antibody therapy in women with breast cancer. This combination could be particularly effective in obese women with breast cancer, considering that many obese women have hyperinsulinemia and the fact that adipose tissue secretes IGF-1 and IGF-2 [111-113]. Measuring gene expression with cancer profiling gene arrays revealed that TCDD, which potentially suppresses endogenous AHR signaling by downregulating AHR protein, induced decreases in the expression of CCND1 in IGF-2-treated MCF-7 BCCs (Table 7). This novel result opened up a new line of study, investigating whether IGF-2 upregulation of CCND1 required endogenous AHR. We addressed this question in my second publication as detailed in the next paragraph.

In our next study, we extended upon our first report showing that AHR responds to and mediates the effects of adipokines/IGF-2 and asked whether the adipokine/IGF target CCND1 was in fact a primary AHR gene target. We discovered that IGF-2 signaling increased both AHR mRNA and AHR protein levels, as well as the recruitment of AHR to AP-1 and TCF response elements within the CCND1 promoter (Figures 14 and 15). This recruitment was undoubtedly very important, as silencing AHR with AHR-targeting siRNA significantly blunted IGF-2-stimulated increases in CCND1 mRNA and protein when compared to control cells (Figure 16). Based on the findings from Barhoover et al. [105], we hypothesized that increases in AHR would promote its interaction with the CCND1 and CDK4 complex, and that AHR/CCND1/CDK4 complexes would in turn suppress Rb activity by phosphorylating Rb, which would promote cell cycle advancement (Figure 17 (B)). The observation that AHR-null cells are less responsive to the proliferative effects of IGF-2 supports the requirement of AHR for the proper phosphorylation of Rb in BCCs (Figure 17 (A)) [105]. Our findings in this study complemented those found by Barhoover et al [105], leading to two unique but ultimately linked mechanisms by

which endogenous AHR functions to promote cell cycle progression in response to growth factors like IGF-2.

Considering our finding that reducing AHR decreased the expression of CCND1 (Figure 16), we hypothesized that AHR regulates endogenous gene expression in MCF-7 BCCs. To address this hypothesis, we performed RNA-seq analysis to establish genome-wide changes in gene expression in response to AHR knockdown in MCF-7 BCCs. RNA-seq revealed that AHR knockdown altered the expression of over 600 genes in MCF-7 cells. Ingenuity Pathway Analysis revealed that AHR-regulated genes were significantly associated with toxicant and tumor necrosis factor (TNF) pathways (Table 10). We then treated MCF-7 BCCs with TNF and established that TNF-stimulated increases in MnSOD mRNA and protein required AHR and RELA, and that this coincided with AHR and RELA recruitment to a transcriptionally active NF-κB response element in the SOD2 gene (Figure 22). AHR-null cells were more sensitive to TNF-induced cytotoxicity than controls (Figure 22 (D)). Considering that MnSOD protects cancer cells from TNF-induced cell death by reducing ROS production [228, 314], we hypothesize that the reduced levels of MnSOD in AHR-null MCF-7 BCCs increases ROS levels in response to TNF, causing cell death. Our finding extends prior reports showing that AHR and NF-KB interact to modulate signaling induced by cytokines. For instance, IL-1 has been shown to promote the binding of AHR and RELA to the IL-6 gene promoter [309], and this stimulates the expression of IL-6 [309]. AHR also interacts with RELB to regulate IL-8 expression [311, 312]. Collectively, our work established that IGF-2 and TNF, which are major cancer pathways, regulate gene expression through AHR in MCF-7 BCCs.

To further characterize AHR regulation of gene expression, we conducted RNA-seq to identify TCDD/AHR regulated genes in MCF-7 BCCs. To this end, MCF-7 BCCs were treated

151

with either DMSO vehicle or 10 nM TCDD for six hr. We identified that TCDD regulated the

expression of 118 genes (Figure 23). TCDD-regulated mRNAs were overlapped with a reported

TCDD-ChIP-seq dataset to identify primary TCDD/AHR gene targets [277]. This analysis

identified that SLC7A5 was a primary TCDD/AHR target gene. Considering that SLC7A5 is

reportedly overexpressed in breast cancer compared with normal tissue [233], we investigated its

regulation by TCDD/AHR in further detail. ChIP analysis revealed that TCDD induced

recruitment of a transcriptional complex to intron 2 of the LAT1 gene and that this complex

consisted of AHR, ARNT, and p300, and finally that their binding correlated with increases in

histone H3 acetylation (Figure 27). Further characterization of AHR regulation of LAT1

expression established that endogenous AHR activity promoted the expression of LAT1 in MCF-

7 and MDA-MB-231 BCCs (Figure 28). We postulate that endogenous AHR ligands produced

by MCF-7 and MDA-MB-231 BCCs activate AHR and that this, in turn, stimulates LAT1

expression. Potential endogenous AHR ligands that could be involved in the regulation of LAT1

include the breakdown products of tryptophan metabolism: kynurenine, kynurenic acid, and

xanthurenic acid [75, 95]. Given prior reports from our lab and others suggesting that AHR is

crucial for BCC proliferation [107, 108, 111, 114], it was expected that reductions in AHR or

LAT1 would reduce the proliferation of MCF-7 or MDA-MB-231 BCCs. Supporting this,

siRNA-mediated knockdown of AHR or LAT1 confirmed that both genes are required for the

proliferation of MCF-7 and MDA-MB-231 BCCs (Figure 29). Even though MCF-7 also express

LAT2 and SLC6A14 [336, 337], it is important to note that these two leucine transporters cannot

compensate for the loss of LAT1, as knockdown of LAT1 reduced MCF-7 proliferation (Figure

29). Our work supports the findings of prior reports showing that the LAT system inhibitor, 2-

amino-bicyclo[2.2.1]heptane-2-carboxylic acid (BCH), reduced the proliferation of MCF-7, ZR-

75-1 and MDA-MB-231 BCC proliferation [117], providing further evidence for the importance of leucine transport in breast cancer.

In short, we have uncovered several novel roles for AHR in breast cancer. AHR is required for the proliferative effects of adipokines on breast tumor cells (Chapter IV). IGF-2, a dominant adipokine, recruits AHR to the CCND1 promoter to increase CCND1 mRNA and protein, and silencing AHR blocked this induction (Chapter V). Finally, RNA sequencing studies revealed AHR is needed for maximal induction of SOD2 by TNF in MCF-7 BCCs (Chapter VI), and that AHR regulates the expression of the important amino acid transporter LAT1 in MCF-7 and MDA-MB-231 BCCs (Chapter VII).

8.2. PRELIMINARY DATA.

In addition to investigating the mechanism how TCDD/AHR-induced increases in LAT1 expression, I also have addressed if estrogen (E2) and adipo-CM also regulate LAT1 expression. In order to reduce confounding estrogenic effects from serum, or phenol-red, experiments were conducted in serum-free, phenol-red free cell culture medium. Stimulating MCF-7 and T-47D BCCs with estrogen increased LAT1 mRNA and protein (Figure 30 A and B). We questioned whether E2 induced ERα binding to the AHR binding site in the LAT1 gene. ChIP analysis revealed that E2 increased the binding of ERα to the AHR binding site in intron 2 of the LAT1 promoter (Figure 30 C). Given our lab's focus on obesity-driven increases in breast cancer, we questioned whether adipo-CM could exert the same effect on LAT1. MCF-7 cells were treated with either unconditioned media (con-CM), fibroblast-conditioned media (fibro-CM), or adipocyte-conditioned media (adipo-CM) which was devoid of FBS and phenol-red for 24 h. Adipo-CM increased the levels of LAT1 protein relative to other treatment conditions. As described in our report [111], adipo-CM contains a cocktail of adipokines including leptin and

153
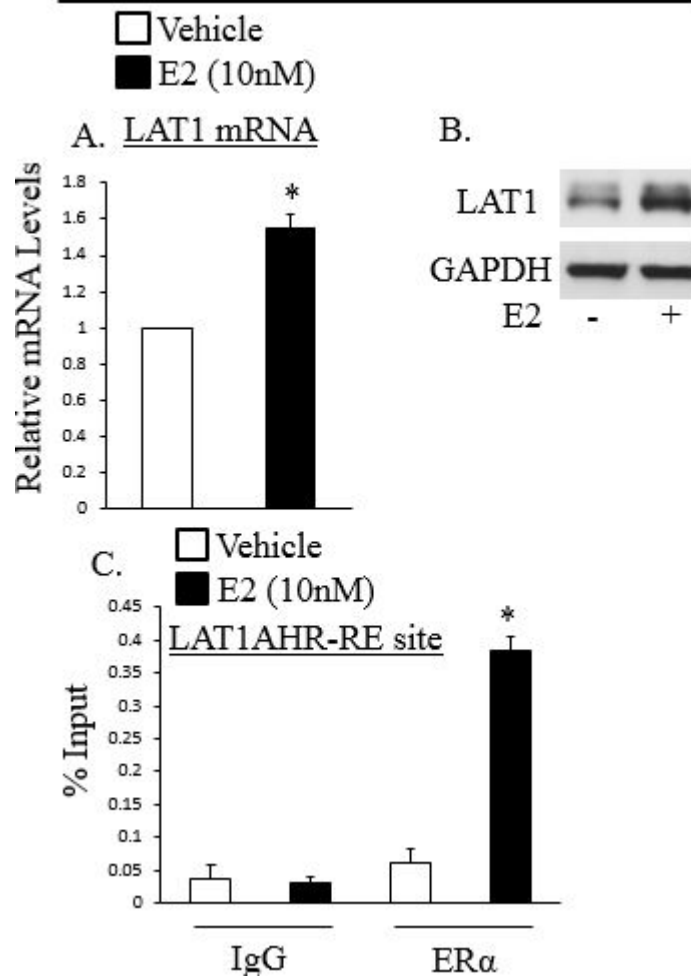
Figure 30. Estrogen treatment increases LAT1 expression and the binding of ERα to the AHR binding site in the LAT1 gene in MCF-7 cells. (A & B) MCF-7 cells maintained in E2-free conditions for 48 h were then treated with vehicle or 10 nM estradiol (E2) for 24 h. LAT1 mRNA (A) and protein (B) levels were then determined. (C) MCF-7 cells were maintained in E2-free conditions for 48 h before treatment with vehicle or E2 for 1 h. ChIP was performed with a non-specific IgG or an ERα specific antibody. Recruitment expressed as % input as determined through qPCR. "*" denotes statistical significance reached (P < 0.05) as determined by Student's T-test (A & B) or one-way ANOVA (C).

154
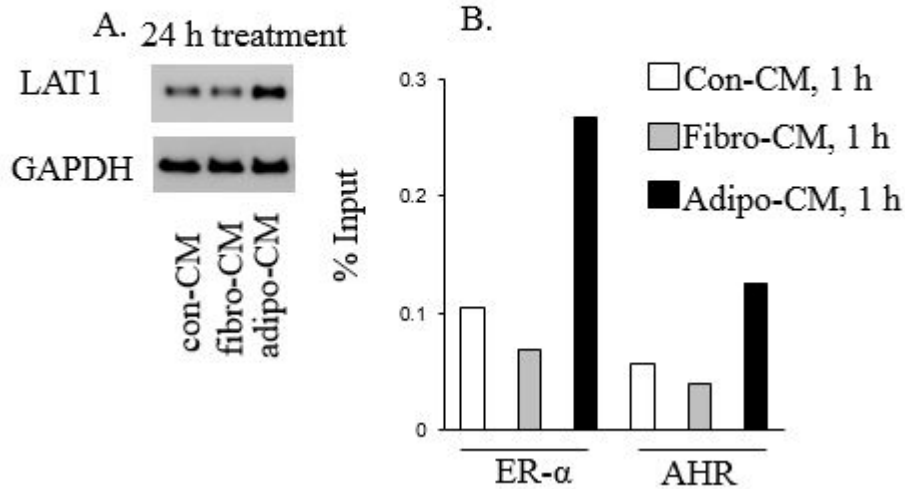
Figure 31. Adipo-CM treatment increases LAT1 expression via ERα and AHR recruitment to AHR response elements in the LAT1 promoter in MCF-7 cells. (A) MCF-7 cells were maintained in E2-free conditions for 48 h prior to treatment with con-CM, fibro-CM, or adipo-CM for 24 h. Total protein was extracted and LAT1 protein levels were determined by western blot. (B) MCF-7 cells were maintained in E2-free conditions for 48 h before treatment with con-CM, fibro-CM, or adipo-CM for 1 h. ChIP assays were conducted with anti-ERα or anti-AHR antibodies. Recruitment expressed as % input as determined through qPCR.
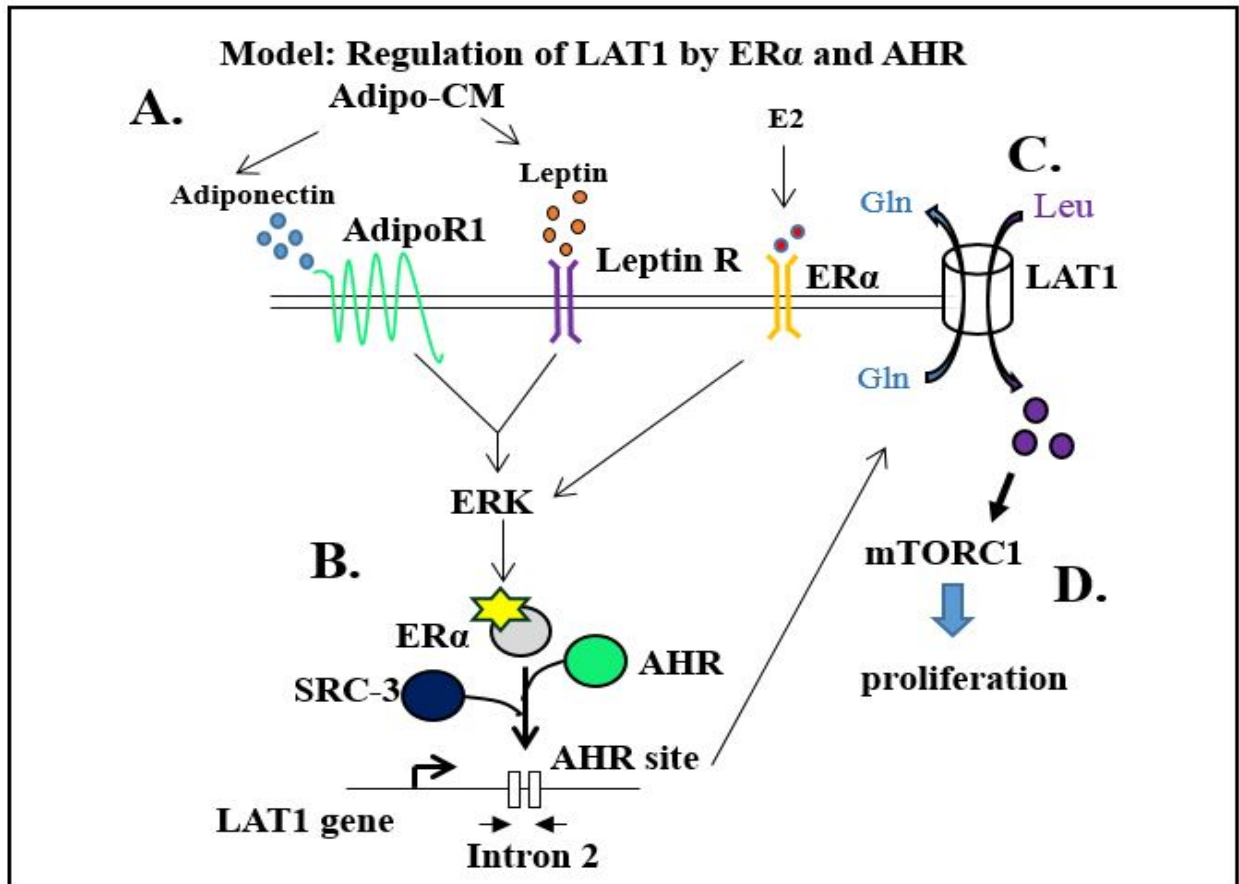
Figure 32. Proposed mechanism by which E2 and adipo-CM increase LAT1 expression. (A) E2, leptin and adiponectin upon binding their cognate receptors induce signaling that increases ERK activation. (B) Upon its activation, ERK phosphorylates and activates ERα, facilitating interactions between AHR and SRC-3 and their recruitment to an AHR binding site in the LAT1 gene. (C) Increased LAT1 transcription drives the accumulation of LAT1 and the transport of leucine. (D) Leucine activates mTOR signaling and increases proliferation. Adipo-CM = adipocyte conditioned media, E2 = 17-β-Estradiol, ERK = extracellular regulated kinase, AHR = aryl hydrocarbon receptor, ERα = estrogen receptor alpha, SRC-3 = steroid receptor coactivator 3, LAT1 = l-type amino acid transporter 1, Gln = glutamine, Leu = leucine, mTORC1 = mammalian target of rapamycin complex 1.

156

adiponectin. These results suggest the high levels of leptin and low levels of adiponectin secreted by adipocytes in obesity could promote the expression of LAT1 in breast tumors in obese patients (Figure 31 A). ChIP analysis was conducted to investigate if adipo-CM promoted the binding of AHR and ERα to the AHR binding site in the LAT1 gene, and the findings revealed increased binding of both transcription factors (Figure 31 B). Collectively, these data suggest that adipocyte-secreted factors in combination with environmental pollutants like TCDD may promote the expression of LAT1 in BCCs via AHR and ERα. Such regulation would be expected to increase BCC proliferation, considering that increases in LAT1 would promote the activity of the mTORC1 pathway (Figure 32). The following sections will outline the rationale and proposed experiments to study this new finding.

8.3. RATIONALE FOR NEW EXPERIMENTS.

As shown in our model shown in Figure 32, we hypothesize that estrogen and adipo-CM will increase the expression of LAT1 by stimulating the binding of AHR, ERα and SRC-3 to the AHR binding site of the LAT1 gene. The rational for this hypothesis stems from prior reports showing that obese women have higher levels of estrogen due to excess adipose tissue than lean women [161]. Upon estrogen binding, ERα binds to and recruits the transcriptional co-activator SRC-3 to ER target genes that stimulate cancer, including CCND1 and cMYC [349, 350]. The binding of ERα to SRC-3 is highly relevant to breast cancer, considering that transgenic overexpression of SRC-3 is sufficient to induce breast cancer in mice [351]. In human breast cancer, the SRC-3 gene is often amplified, and one report noted SRC-3 expression was elevated in over 60% of breast cancer cases [352]. As mentioned, SRC-3 is an ERα transcriptional co-activator and amplification of SRC-3 is typically seen in ER-expressing breast tumors. In this regard, one study noted SRC-3 was critical for MCF-7 BCC growth *in vitro* and *in vivo* [353].

High SRC-3 expression is linked to chemotherapeutic resistance to tamoxifen in breast cancer patients [354]. SLC7A5 (LAT1) expression has also been linked to tamoxifen resistance [237, 238]. Phosphorylation of both SRC-3 and ERα by ERK increase their transcriptional activity [355, 356], and E2 and certain adipokines have been shown to increase ERK signaling [357-359]. Our preliminary data demonstrates that E2 and adipo-CM increase the expression of LAT1, and we provide the first evidence that this could be mediated through ERα and AHR recruitment to AHR-RE in intron 2 of the LAT1 gene (Figures 28 and 29). Given the reports linking both LAT1 and SRC-3 to tamoxifen resistance, we hypothesize that E2 and adipo-CM stimulate increases in LAT1 expression through formation of a transcriptional complex including ERα, AHR, and SRC-3 at AHR binding site in the LAT1 gene leading to upregulation of LAT1 expression in breast tumors and activation of mTORC1 (Figure 32). The following sections will detail future experiments that will be done to test our hypothesis.

8.4. ERα, AHR, AND SRC-3 KNOCKDOWN TO TEST THEIR ROLE IN THE REGULATION OF LAT1

To investigate whether AHR, ERα, and SRC-3 are important for stimulus-induced LAT1 expression, non-malignant MCF-10A cells, ERα positive (MCF-7, T-47D, or ZR-75-1) or ERα negative (MDA-MB-231) BCCs would be transfected with non-targeting (control), AHR, SRC-3, ERα targeting siRNA's using methods that we have published [44, 114], and detailed in Chapter III. After 24 h, siRNA's would be removed and cells will be serum-starved overnight in phenol red-free DMEM devoid of FBS prior to treatment with vehicle, E2 (10 nM), con-CM, fibro-CM, or adipo-CM for time course studies. Western blot and real time qPCR analysis of ERα, AHR, and SRC-3 would be conducted to verify their knockdown by their respective siRNA's compared with non-targeting siRNA. Protein and mRNA levels of LAT1 would be

measured by western blot and real time qPCR, respectively, to investigate our hypothesis that its regulation is dependent on ERα, AHR, and SRC-3. I expect ERα, AHR, and SRC-3 knockdown will compromise induction of LAT1 expression upon treatment with E2 or adipo-CM, given our preliminary data showing ERα and AHR recruitment to the AHR binding site in the LAT1 gene (Figures 30 & 31), and the extensive literature showing SRC-3 to be an important ER-co-activator [349, 362-364].

8.5. CHIP ANALYSIS TO EVALUATE THE BINDING OF ERα, AHR, AND SRC-3 TO THE AHR SITE IN THE LAT1 GENE.

The binding of ERα, AHR and SRC-3 to the AHR binding site in the LAT1 gene would need to be investigated using ChIP-qPCR assays, as described in Chapter III. Non-cancerous MCF-10A cells, ERα positive BCCs (MCF-7, T-47D or ZR-75-1) or ERα negative BCCs (MDA-MB-231) would be seeded into 60mm plates for 24 h prior to overnight serum-starvation in phenol-red free DMEM devoid of FBS then stimulated with vehicle, E2 (10 nM), fibro-CM, or adipo-CM for time course studies. ChIP-qPCR analysis using methods detailed in our recent publications [44, 114] would then be performed to assess the binding of ERα, AHR, and SRC-3 at the AHR binding site in intron 2 of the LAT1 gene. The acetylation of histone H3 at lysine 9 and lysine 14 are markers of active transcription, and these histone H3 modifications in intron 2 of the LAT1 gene would also be investigated by ChIP-qPCR. Non-specific IgG would be used to control for antibody binding specificity, as we have used in our prior reports [44, 114]. The comparative CT method will be used to calculate transcription factor binding, with input ChIP DNA used to normalize samples as detailed in our prior reports [44, 114] and Chapter III. I expect these experiments would reveal ERα, AHR and SRC-3 form an active transcriptional complex at the AHR binding site of LAT1 to regulate its expression.

8.6. ANALYSIS OF TRANSCRIPTIONAL ACTIVITY OF AHR BINDING SITE IN INTRON 2 OF LAT1 GENE.

These experiments would establish the transcriptional activity of the AHR-REs in intron 2 of the LAT1 gene in response to E2 or adipo-CM stimulus, a very important question to address. The region of the LAT1 gene that harbors intron 2 and accompanying AHR-REs would be subcloned into promoter reporter luciferase constructs following methods we have previously published [272]. Non-cancerous MCF-10A cells, ERα positive BCCs (MCF-7, T-47D, ZR-75-1) or ERα negative BCCs (MDA-MB-231) would be seeded into 96 well plates at a density of 5,000 cells per well for 24 h prior to transfection with 100 ng of an "empty" luciferase reporter vector (Dual Light Reporter Gene Assay System; Life Technologies) or the luciferase reporter vector which will express the 1 KB region containing intron 2 of the LAT1 gene. After 24 h, cells would be treated with vehicle, E2 (10 nM), con-CM, fibro-CM, or adipo-CM for time course studies and reporter activity would be measured using a luminometer. To verify that increases in intron 2 of the LAT1 gene is attributed to AHR-REs, these specific sites in intron 2 of the LAT1 gene could be mutated using standard procedures (QuikChange II XL Site-Directed Mutagenesis Kits, Agilent Technologies). Given that we found there are three putative AHR-REs in the LAT1 gene, these AHR-REs may differ in activity. To address this, several different LAT1 intron 2 constructs could be made that either contain a mutation in one of the AHR-REs, two of the AHR-REs, or a construct in which all three of AHR-REs have been mutated to assess activity of the different sites. I expect that AHR is able to bind to all of these regions, and therefore, constructs containing the three mutated AHR-REs would inhibit transcriptional activity at the AHR binding site most effectively.

8.7. SILENCING AHR, ERα, OR SRC-3 WITH TAMOXIFEN TREATMENT: INCREASED TUMOR CELL DEATH?

Given reports that have linked LAT-1 and SRC-3 expression to tamoxifen resistance [237, 238, 354], and our recent report characterizing AHR regulation of LAT-1 [44], we would assay whether silencing ERα, AHR, or SRC-3 sensitizes ERα-expressing tumor cells to tamoxifen treatment. To this end, we would reverse transfect 200,000 ERα positive (MCF-7, T-47D, or ZR-75-1) BCCs in the presence of con-siRNA, ERα-siRNA, AHR-siRNA, or SRC-3-siRNA for 36 h prior to treatment with tamoxifen (0.001, 0.01, 0.1, or 1 µM). Lower concentrations which have been shown to have minimal effect or which only induce cell-cycle arrest will be used, instead of higher concentrations (above 5 µM) which have been shown to induce apoptosis in MCF-7 BCCs [360]. Cell viability would then be determined utilizing the Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI), which we used to assess cell number in our recent report [44], as well as manual cell counting using the trypan blue exclusion method to identify dead cells [240]. If viability is reduced, parallel western blot experiments would be performed looking at hallmarks of apoptosis, including cleavage of caspases as well as cleavage of poly ADP ribose polymerase (PARP) [361], upon treatment. Considering that obesity induces breast tumors in post-menopausal women [112, 113], and tamoxifen is especially indicated for use in ERα-expressing tumors in post-menopausal women [360], experiments silencing AHR, ERα, or SRC-3 to see if loss of these important transcriptional regulators confers sensitivity to tamoxifen treatment will be especially informative.

8.8. ANTICIPATED AND ALTERNATIVE OUTCOMES FOR PROPOSED EXPERIMENTS.

Given our preliminary data showing E2 and adipo-CM can stimulate a marked increase in LAT1 expression in MCF-7 BCCs (Figure 30 A and B, Figure 31 A), as well as increased recruitment of ERα and AHR to AHR-RE in intron 2 of LAT1 in response to E2 or adipo-CM stimulus (Figure 30 C, Figure 31 B), I expect that knockdown of ERα and AHR will abrogate E2 and adipo-CM regulation of LAT1 expression. I also expect SRC-3 knockdown to compromise stimulus-driven increases in LAT1, due to the extensive literature showing SRC-3 is a transcriptional co-activator of ERα signaling [349, 362-364]. Our prior report has characterized the AHR binding site in intron 2 of LAT1 to be important for TCDD-stimulated increases in LAT1 [44]. Experiments looking at transcriptional activity of intron 2 of LAT1 upon stimulus would provide additional insights as to the role of this 1 KB fragment in E2 or adipo-CM-stimulated increases in LAT1. Site-directed mutagenesis experiments would prove important in determining if one, two, or all three of the putative AHR binding sites in this fragment are important for conferring transcriptional activity upon stimulus. Finally, I predict silencing AHR, ERα, or SRC-3 will allow tamoxifen to induce apoptosis in ER-expressing BCCs at lower concentrations due to reductions in LAT1 expression, as LAT1 levels have been linked to tamoxifen resistance in breast tumors [237, 238].

Alternative outcomes exist for these experiments. An important alternative outcome is that silencing SRC-3 may prove insignificant to blunt E2 and adipo-CM-stimulated induction of LAT1. If this is the case, looking at other ERα co-activators which ERα-signaling has been shown to utilize to regulate gene expression, including other members of the p160 family like SRC-1 [365], would provide additional insight. SRC-1 has been shown to interact both with ERα and AHR [37, 365], where SRC-3 has only been shown to interact with ERα. The proposed ERα-AHR crosstalk mechanism for regulating LAT1 in response to E2 and adipo-CM may recruit

SRC-1 to AHR-RE to regulate LAT1 as part of the transcriptional complex in place of SRC-3. Similarly, p300 is a known ERα and AHR co-activator [366]. We have already shown p300 recruitment to the AHR binding site upon TCDD stimulus [44]. Thus, p300 binding at the AHR site in response to E2 and adipo-CM stimulus may be a critical player in the observed increases in LAT1 we have seen (Figures 30 & 31). Once the co-activator(s) have been determined, the alternative co-activator(s) could then be silenced using targeting siRNAs to reduce their expression and determine first, whether LAT1 expression is compromised upon their silencing, and second, whether sensitivity to tamoxifen occurs with their loss.

I do not anticipate any pitfalls in completing these experiments, as we have used all of the proposed methods (western blotting, real time qPCR, ChIP, etc) numerous times in previously published reports to generate data [44, 59, 111, 114, 272]. Overall, I expect these future experiments to reveal that E2 and mitogenic factors present in adipo-CM (i.e. leptin) increase LAT1 expression through recruitment of ERα, AHR, and SRC-3 to the characterized AHR binding site in intron 2 of the LAT1 gene. To build on our adipo-CM findings, determining which factor or cocktail of factors critical for the observed increases in LAT1 upon adipo-CM treatment could then be assessed. I feel that targeting members of this transcriptional complex, such as the use of AHR antagonists, as part of a combinatorial treatment regimen could be beneficial in treatment of breast cancer in obese patients, as well as sensitizing ERα-expressing breast tumors to tamoxifen treatment.

8.9. CONCLUSION

My thesis research over the past four years has led to a better understanding of the complex roles that AHR plays in breast cancer. By uncovering some novel tumor promoting roles for AHR, it begs the question whether more emphasis should be given to AHR modulating

drugs for use in cancer patients. While targeting the IGF-1 and PI3K/AKT/mTOR pathways have received great attention over the years, recent strides in AHR research suggests that targeting AHR warrants continued investigation. In this regard, AHR and its roles in tumor initiation, invasion, and metastasis are being explored in many cancer types in addition to breast cancer, including prostatic, ovarian, colorectal, melanoma, and gliomas. Targeting AHR as a chemotherapeutic could, therefore, have broad-spectrum effects on the treatment outcome of a variety of cancers in future patients.

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APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

MARSHALL UNIVERSITY.
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Office of Research Integrity

January 29, 2016

Justin K. Tomblin
105 Louis Ct
Huntington, WV 25705

Dear Mr. Tomblin:

This letter is in response to the submitted dissertation abstract entitled *"Uncovering New Roles for the Aryl Hydrocarbon Receptor (AHR) in Breast Cancer."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: ABBREVIATIONS

3-MC…3-methylcholanthrene

4EBP…4E binding protein

5-LOX… arachidonate 5-lipoxygenase

5,6-DiHETEs…5,6-dihydroxyeicosatetraenoic acid isomers

ABCG2…ATP-Binding Cassette, Sub-Family G (WHITE), Member 2 (Junior Blood Group)

ACPR30…adiponectin

ADG…AHR dependent gene

Adipo-CM…adipocyte conditioned media

AdipoR1…adiponectin receptor 1

AdipoR2…adiponectin receptor 2

ADORA…adenosine receptor

AHH…aryl hydrocarbon hydroxylase

AHR…aryl hydrocarbon receptor

AHR -/-…AHR-null mice

AHRi… short inferfering RNA targeting the AHR gene

AHRR…aryl hydrocarbon receptor repressor

AHR-RE… aryl hydrocarbon receptor response element

AHR-siRNA…short inferfering RNA targeting the AHR gene

AIP…AHR interacting protein

AKT…protein kinase B

ALDH1A3…Aldehyde Dehydrogenase 1 Family, Member A3

ALDH3A1… Aldehyde Dehydrogenase 3 Family, Member A1

ALOX5… Arachidonate 5-Lipoxygenase

AMPK…adenosine monophosphate dependent kinase

ANOVA…analysis of variance

AP-1…activator protein-1

AP-1-RE… activator protein-1 response element

AR…androgen receptor

ARE…androgen response element

ARNT…aryl hydrocarbon nuclear translocator

ATP… adenosine triphosphate

B(a)P… benzo(α)pyrene

BCC…breast cancer cell

BCH…2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid

bFGF…basic fibroblast growth factor

bHLH…basic helix loop helix

BME…β-mercaptoethanol

BRG-1…brahma related gene-1

bZIP… Basic Leucine Zipper Domain

CBP…CREB binding protein

CCND1…cyclin D1

CD98… 4F2 cell-surface antigen heavy chain

CDK…cyclin dependent kinase

CDK4… cyclin dependent kinase 4

CDKN1A… Cyclin-Dependent Kinase Inhibitor 1A

ChIP…chromatin immunoprecipitation

ChIP-Seq… chromatin immunoprecipitation sequencing

Co-IP…co-immunoprecipitation

Con-CM…unconditioned/control media

Con-siRNA…non-specific control short interfering RNA

CREB3L… cAMP-responsive element-binding protein 3-like protein

cRNAi… non-specific control short interfering RNA

CSC…cancer stem cell

CSP…chondroitin sulfate proteoglycan

CUL4B…cullin 4B

CYP1A1…cytochrome P450 1A1

CYP1B1…cytochrome P450 1B1

DDB1…damaged DNA binding protein-1

DIM…3,3-diidolylmethane

DMBA…7,12-dimethylbenz[a]anthracene

DMEM… Dulbecco's Modified Eagle Medium

DMSO…dimethyl sulphoxide

DRE… dioxin response element

E2… 17β-estradiol

E2F4…E2F transcription factor 4

EDTA… ethylenediaminetetraacetic acid

EGF…epidermal growth factor

eIF…eukaryotic initiation factor

EMT…epithelial to mesenchymal transition

ER…estrogen receptor

ERRBB2… Erb-B2 Receptor Tyrosine Kinase 2

ERK…extracellular regulated kinase

FBS…fetal bovine serum

FDR…false discovery rate

Fibro-CM…fibroblast conditioned media

FICZ…6-formylindolo [3,2-b] carbazole

FSH…follicle stimulating hormone

GAPDH… glyceraldehyde 3-phosphate dehydrogenase

GEO…gene expression omnibus

GPX…glutathione peroxidase

GR…glutathione reductase

GSH…reduced glutathione

GSSG…oxidized glutathione

GST…glutathione-S-transferase

H_2O_2…hydrogen peroxide

HAH…halogenated aromatic hydrocarbon

Hepa-1…mouse derived liver cell line

HER2…epidermal growth factor receptor 2

HMGCS2…3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (Mitochondrial)

HRP…horseradish peroxidase

HSP90…heat shock protein-90

I3C…indole-3-carbinol

IBMX… 3-isobutyl-1-methylxanthine

ICZ…indolo [3,2-b] carbazole

IGF-1…insulin-like growth factor 1

IGF1-R…insulin-like growth factor 1 receptor

IGF1-R/IR…hybrid IGF/IR receptor

IGF-2…insulin-like growth factor 2

IGF-2R…insulin-like growth factor 2 receptor

IgG…immunoglobulin G

IL-1…interleukin 1

IL-1β…interleukin 1-beta

IL-6…interleukin 6

IL-8…interleukin 8

INBRE… IDeA Network of Biomedical Research Excellence

IPA…ingenuity pathway analysis

IR…insulin receptor

IR-A…type A insulin receptor

JAK…janus kinase

JUND…Jun D proto-oncogene

Kyn…kynurenine

LAT1…L-type amino acid transporter-1

LEPR…leptin receptor

LEPR-b…dominant isoform of leptin receptor

LH…luteinizing hormone

LOX1…lipoxygenase 1

LPS…lipopolysaccharide

MAPK…mitogen activated protein kinase

MCL…mantle cell lymphoma

MEF…mouse embryonic fibroblast

MGP… Matrix Gla Protein

MMP…matrix metalloproteinase

MnSOD…manganese superoxide dismutase

MPE…malignant pleural effusion

MRK… dominant-negative IGF1-R mice

mRNA…messenger ribonucleic acid

mTOR…mammalian target of rapamycin

MUC1…mucin 1

MYB… Avian Myeloblastosis Viral Oncogene Homolog

NANOG…nanog homeobox

NADPH… nicotinamide adenine dinucleotide phosphate

NCBI… National Center for Biotechnology Information

NCOA-1…nuclear receptor co-activator 1

NF-κB…nuclear factor kappa-light-chain-enhancer of activated B cells

NF-κB-RE… NF-κB response element

NRF2… nuclear factor erythroid 2-related factor

NRP…neuropilin

$O_2{}^-$…superoxide anion radical

OAS1…2'-5'-Oligoadenylate Synthetase 1

OCT4…octamer-binding transcription factor 4

OxLDL…oxidized low-density lipoprotein

p70S6K…ribosomal S6 kinase

PAH… polycyclic aromatic hydrocarbon

PBS…phosphate buffered saline

PDFG…platelet derived growth factor

PGR…progesterone receptor

PhRMA… the Pharmaceutical Research and Manufacturers of America

PI3K… phosphatidylinositol-3-kinase

PKD1L1… Polycystic Kidney Disease 1 Like 1

PLA2G2… phospholipase A2 group IIA

PMA… phorbol 12-myristate 13-acetate

PPP…picropodophyllin

PVDF… polyvinylidene difluoride

PYDC1… pyrin domain containing 1

qPCR…quantitative polymerase chain reaction

Rb…retinoblastoma protein

RBX-1… ring box-1

RELA…p65 subunit of NF-κB

RELB…p50 subunit of NF-κB

RNA…ribonucleic acid

RNA-Seq…RNA sequencing

ROS…reactive oxygen species

SDS…sodium dodeacyl sulfate

SERPIN3A…serpin 3a

SERPIN5A…serpin 5a

siRNA…short-interfering ribonucleic acid

SLC7A5… solute carrier family 7 (amino acid transporter light chain, L system) member 5

SNK…Student Newman-Keuls test

SOD1… superoxide dismutase 1

SOD2…superoxide dismutase 2

SOD3… superoxide dismutase 3

SOX2…SRY (sex determining region Y)-box 2

SRC…steroid receptor co-activator

STAT…signal transducer and activator of transcription

SU5416… [3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydro-indole-2-one]

SWI/SNF… SWItch/Sucrose Non-Fermentable

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

TCF…T-cell factor

TCF/LEF…T-cell factor/lymphoid enhancing factor

TCF-RE…TCF response element

TDO2…tryptophan dioxygenase 2

TGFα…transforming growth factor alpha

Th17…T-helper 17 cells

TNBC…triple-negative breast bancer

TNF…tumor necrosis factor α

TP53…p53 tumor suppressor gene

Tregs…T regulatory cells

TRGs…TCDD regulated genes

TSS…transcriptional start site

UGTAs… UDP-glucuronosyltransferase genes

VEGF…vascular endothelial growth factor

VEGFR…VEGF receptor

XAP2… hepatitis B virus X-associated protein 2

XRE…xenobiotic response element

CURRICULUM VITAE

JUSTIN KIRK TOMBLIN

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EDUCATION

Marshall University · Huntington, WV (2011-present)
- ❖ Biomedical Sciences Graduate Program; GPA: 3.51
- ❖ Ph.D. Candidate –Toxicology and Environmental Health

Marshall University · Huntington, WV
- ❖ Bachelors of Science, Dept. of Integrated Science and Technology
- ❖ Major: Biotechnology, Minor: Chemistry

WORK EXPERIENCE

Center for Bioengineering and Biomanufacturing Commercialization · Huntington, WV
Research Assistant, August 2010 – September 2011

Graduate Researcher · Huntington, WV
Salisbury Lab, May 2012 – Present

AWARDS

2014	Best Research Performance Award for the academic year 2013-2014
	■ Biomedical Sciences Graduate Program
2016	Best Oral Presentation, Basic Science Category
	■ MUSOM Research Day 2016

SKILLS

Cell Culture	Gene silencing	Gene Overexpression
RT-qPCR	Western blots	Flow Cytometry
Chromatin	Toxicity assays	Proficient with Microsoft
Immunoprecipitation	like MTT, TUNEL, etc	Office, EndNote and Graphpad
Prism		

GRANTS FUNDED

2015	Graduate Research Fellowship Award
	■ NASA West Virginia Space Grant Consortium

PROFESSIONAL AFFILIATIONS

- Society of Toxicology
- American Chemical Society

- American Society of Pharmacology and Experimental Therapeutics

PRESENTATIONS

2016	Oral presentation at MUSOM Research Day 2016
2015	Oral presentation at Appalachian Regional Cell Conference 2016
2015	Poster presentation at Experimental Biology meeting 2015
2015	Lecture on insulin/insulin-like growth factors in cancer at Marshall University
2014	Scientific review lecture for peers at Marshall University
2014	30 min lay talk to audience at Marshall University
2012	Poster presentation at Society of Toxicology meeting 2012
2012	MUSOM Research Day Oral Presentation

PUBLICATIONS

❖ **Tomblin JK**, Arthur S, Primerano DA, Chaudhry AR, Fan J, Denvir J, Salisbury TB. 2016 Aryl hydrocarbon receptor (AHR) regulation of L-Type Amino Acid Transporter 1 (LAT-1) expression in MCF-7 and MDA-MB-231 breast cancer cells. Biochem Pharmacol. 2016 Mar 1. pii: S0006-2952(16)00134-9. doi: 10.1016/j.bcp.2016.02.020.

❖ Salisbury TB, **Tomblin JK.** 2015 Insulin/Insulin-like growth factors in cancer: new roles for the aryl hydrocarbon receptor, tumor resistance mechanisms, and new blocking strategies. Front Endocrinol (Lausanne). Feb 2;6:12. doi: 10.3389/fendo.2015.00012. eCollection 2015.

❖ Salisbury TB, **Tomblin JK,** Primerano D, Boskovic G, Fan J, Mehmi I, Fletcher J, Santanam N, Hurn E, Morris GZ, Denvir J. 2014 Endogenous aryl hydrocarbon receptor promotes basal and inducible expression of tumor necrosis factor target genes in MCF-7 cancer cells. Biochem Pharmacol. Oct 1;91(3):390-9. doi: 10.1016/j.bcp.2014.06.015.

❖ **Tomblin JK,** Salisbury TB 2014 Insulin like growth factor 2 regulation of aryl hydrocarbon receptor in MCF-7 breast cancer cells. Biochem Biophys Res Commun. 2014 Jan 17;443(3):1092-6.

❖ Salisbury TB, Morris G, **Tomblin JK**, Chaudhry A, Cook C, and Santanam N 2013 Aryl hydrocarbon receptor ligands inhibit IGF-II and adipokine stimulated breast cancer cell proliferation. ISRN Endo. Sep 23;2013:104850.

❖ Selvaraj V, **Tomblin JK,** Yeager Armistead M, Murray E 2013 Selenium (sodium selenite) causes cytotoxicity and apoptotic mediated cell death in PLHC-1 fish cell line through DNA and mitochondrial membrane potential damage. Ecotoxicol Environ Saf. Jan;87:80-8.