Depletion of the Aryl Hydrocarbon Receptor in MDA-MB-231 Human Breast Cancer Cells Altered the Expression of Genes in Key Regulatory Pathways of Cancer

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Abstract

The aryl hydrocarbon receptor (AhR), a transcription factor that is best known for its role in mediating the toxic responses elicited by poly aromatic hydrocarbons as well as many other environmental factors; is also involved in breast cancer progression. We previously reported that stable knockdown of AhR decreased the tumorigenic properties of the highly metastatic MDA-MB-231 breast cancer cell line; whereas ectopic overexpression of AhR was sufficient to transform immortalized human mammary epithelial cells to exhibit malignant phenotypes. In the present study we investigated the genes that are differentially regulated by AhR and are controlling cellular processes linked to breast cancer. We used Affymetrix Human GeneChip 1.0-ST whole transcriptome arrays to analyze alterations of gene expression resulting from stable AhR knockdown in the MDA-MB-231 breast cancer cell line. The expression of 144 genes was significantly altered with a $\geq 2.0$-fold change and a multiple test corrected p-value $\leq 0.05$, as a result of AhR knockdown. We demonstrate that AhR knockdown alters the expression of several genes known to be linked to cancer. These genes include those involved in tryptophan metabolism (KYNU), cell growth (MUC1 and IL8), cell survival (BIRC3 and BCL3), cell migration and invasion (S100A4 and AB13), multi-drug resistance (ABCC3) and angiogenesis (VEGFA and CCL2). The identification of the genes and pathways affected by AhR depletion provides new insight into possible molecular events that could explain the reported phenotypic changes. In conclusion AhR knockdown alters the expression of genes known to enhance or inhibit cancer progression; tipping the balance towards a state that counteracts tumor progression.

Introduction

The aryl hydrocarbon receptor (AhR) is a ubiquitously expressed ligand-activated transcription factor that belongs to the basic-helix-loop-helix-Per-ARNT-Sim superfamily of transcription factors [1]. The AhR-mediated regulation of metabolism of polycyclic aromatic hydrocarbons (PAH) has been implicated in a variety of cancers [2,3]. PAH-induced AhR activation increases the transcription of a variety of genes encoding phase I drug metabolizing enzymes, such as CYP1A1 and CYP1B1, and phase II drug metabolizing enzymes, such as glutathione-S-transferase A1 (GSTA1) and UDP glucuronosyl transferase 1A2 (UGT1A2). In particular the CYP enzymes promote metabolic activation of PAHs increasing the levels of PAH-DNA adducts which are associated with cancer initiation. Additionally, PAH-induced AhR activation was shown to play a role in cancer promotion and progression [4,5].

Although expressed in normal tissues, elevated AhR expression has been reported in several cancer types including lung, breast, liver, stomach and pancreas [6,7]. This elevated expression is associated with constitutive activation in the absence of exogenous ligand, evident by the predominant nuclear localization of AhR and induced expression of AhR responsive gene, CYP1A1 [8,9]. These findings suggest a role for AhR in cancer independent of exogenous ligand. In support of this notion, studies designed to mimic a constitutively active AhR showed a role for AhR in tumor promotion and progression [10,11].

In breast cancer, elevated and constitutively active levels of AhR were found in advanced human breast tumors and breast cancer cell lines, with a strong correlation between expression of AhR and the degree of the tumor malignancy [12]. Interestingly, ectopic overexpression of AhR in immortalized normal mammary epithelial cells induced a malignant phenotype with increased growth and acquired invasive capabilities proportional to the level of AhR expressed [13]. Subsequently, we showed that knockdown of the inherently elevated levels of AhR in the highly metastatic MDA-MB-231 breast cancer cell line, decreased their tumorigenic properties both \textit{in vitro} and \textit{in vivo} [9]. Based on these phenotypic changes, we hypothesize that the elevated levels of AhR in advanced breast cancer are driving signaling pathways involved in cell survival, adhesion and invasiveness. In the present study we sought to identify alterations of global gene expression in MDA-MB-231 cells following stable AhR knockdown in order to determine which of the genes and signaling pathways involved...
in breast cancer progression are affected by the elevated AhR levels.

**Materials and Methods**

**Cell Culture**

The triple negative human breast carcinoma (HBC) cell line MDA-MB-231 was purchased from ATCC (Manassas, VA). Cells were cultured in the recommended medium [L-15 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin] in a humidified atmosphere at 37°C with 5% CO2. Stable knockdowns were cultured in media supplemented with 2.5 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO).

**Stable Knockdown of AhR using RNA Interference**

The stable knockdown of AhR gene expression in MBA-MD-231 human breast carcinoma cell line and the generation of AhR knockdown clone 8 and its Scrambled (Scr) control cells was previously described [9]. Briefly, the MBA-MD-231 cells were infected with shRNA-AhR or shRNA-Scr control retroviral particles produced in Phoenix cells. Stable lines were selected by culturing in the presence of puromycin (2.5 µg/ml). Limited cell dilution was performed on shRNA-AhR and shRNA-Scr control cells to obtain single cell clones. Clone 8 of shRNA-AhR cells, which exhibited the highest AhR knockdown (~80%) was chosen for subsequent analyses. Multiple clones of Scr control with equivalent AhR protein expression to the parental heterogeneous Scr control cells, were pooled, expanded and used for the subsequent studies to represent the Scr control. Both clone 8 and Scr control were used in our previous studies [9] and were analyzed here for their comparative differential gene expression.

**Isolation of RNA**

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and further purified using a Qiagen RNeasy kit following the provider's instructions (Qiagen Inc, Valencia, CA). Total RNA was treated with Qiagen RNase-Free DNase I, prior to RNA being resuspended in RNase-free water and quantified by absorbance at 260 nm using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE). The quality and quantity of RNA was verified with the Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA). Only RNA samples with high quality (a RIN of 8 or greater) were used for microarray analysis.

**Transcriptome Microarray Analysis**

Total RNA was submitted to Vanderbilt University Microarray Shared Resources (VMSR) (Nashville, TN) for gene expression profiling using the Affymetrix Human Gene 1.0-ST whole transcriptome array (Affymetrix, Santa Clara, CA). Synthesis and labeling of complementary DNA targets, hybridization and scanning of GeneChips were carried out by VMSR on a fee for service basis. We performed transcriptome microarray data analysis using Partek Genomics Suite version 6.6 (Partek Inc., St. Louis, MO). Relative expression levels of gene transcripts were based on the average of at three clone 8 and two Scr-control replicates. Affymetrix CEL files were normalized using the Robust Multi-array Average (RMA) algorithm [14]. Transcriptome level fold changes and the significance of those changes were calculated using one way ANOVA. Significantly changed transcripts in clone 8 cells were defined as having a ≥2.0 fold expression change from Scr-controls and a Benjamini-Hochberg (BH) false discovery rate corrected P-value ≤0.05.

**KEGG Pathway and Gene Ontology (GO) Enrichment Analysis**

The WEB-based Gene Set Analysis Toolkit (WEBGASTALT) was used in order to conduct KEGG pathway and gene ontology (GO) enrichment analysis on the transcriptome array dataset. Briefly, gene transcripts showing significant changes in expression from the transcriptome array were mapped to their corresponding KEGG pathways and GO biological processes and a hypergeometric test was used to determine significant enrichment. To correct for multiple testing, the threshold for significance of the enrichment scores used a BH false discovery rate corrected P-value <0.05 [15].

**Biological Interaction Network Construction**

To populate and build a biological interaction network of the transcriptome dataset, the Michigan Molecular Interactions (MiMI) database MiMIO Cytoscape plugin (version 3.2) was used. MiMIO gathers and merges data from well-known protein interaction databases including BIND, DIP, HPRD, RetSeq, SwissProt, IPI, and CCSB-HII. The Plugin also integrates other NCBI tools for literature information, document summarization, and pathway matching [16]. The differentially expressed genes were used as the initial population nodes then MiMIO was used to query for the initial nodes and their respective nearest neighbors to one degree of biological interaction. The networks were then merged for interconnections and the global interactome was visualized in Cytoscape.

**Validation Using Quantitative Reverse Transcriptionase-Polymerase Chain Reaction (qRT-PCR)**

RNA (1 µg) was reverse transcribed to complementary DNA (cDNA) using random hexamer primers and Moloney murine leukemia virus reverse transcriptase in presence of RNase inhibitor (Promega, Madison, WI). Quantitative real-time PCR was then carried out in 96-well plates in a Bio-rad CFX96 Real Time System (Bio-Rad, Hercules, CA) using QuantiFast SYBR Green (Qiagen, Valencia, CA) to monitor the PCR amplification. The real-time PCR mixtures consisted of 12.5 µL of 2X QuantiFast SYBR Green master mix, template cDNA (≤100 ng), each primer (1 µM), and ddH2O to give a final volume of 25 µL. The following two-step cycling program was used for all PCR reactions: 95°C for 10 min, 40 cycles of (95°C, 15 sec; and 60°C, 60 sec). The specificity of each amplification reaction was verified by a dissociation curve (melting curve) consisting of 10 s incubation at 95°C, 5 s incubation at 65°C, a ramp up to 95°C. All samples were amplified in triplicates and relative quantification of the expression level of each gene was calculated using the delta CT method in CFX manager software (Bio-Rad, Hercules, CA). Ribosomal 18S was used as the endogenous reference gene. Non-template controls were included for each primer pair. Gene-specific primers were designed using Applied Biosystems Primers Express software (Life Technologies, Grand Island, NY), (Table 1).

**Analysis of Knu Expression**

Clone 8 and Scr-control cells were seeded in six-well plates at a density of 3×104 wells per well and grown for 24 h. On the following day cells were treated with 1 nM TCDD, 10 µM DIM or dimethyl sulfoxide (DMSO), for 16 hrs. After treatments, cell monolayers were lysed in 1 ml of TRIzol, which allowed for simultaneous isolation of RNA and protein. Total RNA was isolated as described above. Proteins were isolated as previously described [17] and the protein pellets were suspended in RIPA buffer containing 2% SDS and sonicated briefly.
For semi-quantitative RT-PCR, 2 μg of total RNA was reverse transcribed to first strand cDNA, and equivalent of 40 ng was amplified in a 25ul reaction using Bullseye Taq DNA polymerase 2X Master Mix (MIDSCI, St. Louis, MO). Ribosomal 18s was amplified for normalization. PCR primers used were: for KYNU (5’ to 3’), CATGCCCATACGATTAAACCTG and CATGCAAGGAACAGACCAACA; for Cyp1a1 (5’ to 3’), TAGACACTGATCTGGCTGCAG and GGGAAGGCTCCATCAGCATC. Immunoblotting was performed as previously described [13]. Primary antibodies used include: anti-AhR and anti- KYNU (Santa Cruz, Dallas, TX), anti-β-Actin (Sigma-Aldrich, St. Louis, MO).

Results

Gene Expression Profiling
In order to analyze the alterations of transcriptome expression level associated with AhR knockdown, global gene expression profiling was performed on MDA-MB-231 Scr-control cells and clone 8 (C8) of MDA-MB-231 cells with stable AhR knockdown [9]. Profiling analyses identified 308 probe-set level transcripts as being significantly differentially expressed in C8 cells compared with control cells, having a greater than two-fold change in expression and a p-value ≤0.05 (Figure 1). Mapping the probe-sets to known annotated genes yielded 144 significantly changed unique gene identifications. Of the differentially expressed genes, 66 were upregulated >2-fold and 78 were downregulated >2.0-fold in C8 cells. Table 2 summarizes the top ten upregulated or downregulated genes, ordered by the average fold differences (a complete list can be found in Table S1).

Validation of Microarray Data with Quantitative RT-PCR (qRT-PCR)
Validation of microarray data was done using quantitative RT-PCR (qRT-PCR) on the same RNA samples used for the transcriptome microarray analysis. Ten genes were selected from the 144 differentially expressed genes, choosing the top five upregulated genes (CALB1, SAMSN1, PLEKHA7, MRGPRX4, NAALADL2) and the top five downregulated genes (KYNU, SPRR2A, CSF1, HLA-DPA1, C15orf48). As shown in Figure 2, the trend (upregulation or down-regulation) in expression of all ten selected genes showed consistency with result from the microarray analysis, validating accuracy of the microarray data.

Gene Ontology Term and KEGG Pathway Enrichment Analysis
The biological processes involving differential gene expressions were identified by Gene Ontology (GO) enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. In total, 18 GO terms were identified including immune response, response to hypoxia, cell migration, cell adhesion, antigen processing and presentation and angiogenesis (Table 3).

To identify well characterized molecular pathways that were significantly represented, the genes list was also subjected to pathway analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG pathway analysis identified 18 significantly enriched pathways, including those associated with cancer. The following pathways of interest were shown to be significantly enriched: bladder cancer, cytokine-cytokine receptor interaction, mTOR signaling pathway, cell adhesion molecules, toll-like receptor pathway, B cell receptor signaling pathway and...
Figure 1. Hierarchical clustering of differentially expressed genes in MDA-MB-231 cells following AhR knockdown. (A) Illustration of the procedure used to examine differential gene expression following AhR knockdown. (B) Clusters of genes altered by AhR KD. Heat map reveals clusters of genes. Green indicates genes up-regulated compared to control cells and red indicates genes down-regulated compared to control cells. doi:10.1371/journal.pone.0100103.g001
VEGF signaling pathway (Table 4). In Table 5, we identified some individual genes that may be associated with the phenotypic changes we previously observed following AhR knockdown [9].

Next, to construct a predicted protein-protein interaction network for interactions between AhR and differentially expressed genes the Michigan Molecular Interactions (MiMI) database was

| Symbol | Description | Fold Change | P-value |
|--------|-------------|-------------|---------|
| **Up** | CALB1 | Calbindin 1, 28kDa | 23.4907 | 2.48E-03 |
| | SAMSN1 | SAM domain, SH3 domain and nuclear localization signals 1 | 9.1653 | 1.70E-02 |
| | PLEKH7 | Pleckstrin homology domain containing, family A member 7 | 8.78089 | 6.15E-03 |
| | MRGPRX4 | MAS-related GPR, member X4 | 8.4079 | 2.38E-02 |
| | NAALADL2 | N-acetylated alpha-linked acidic dipeptidase-like 2 | 8.40242 | 4.37E-02 |
| | PSG2 | Pregnancy specific beta-1-glycoprotein 2 | 7.47155 | 1.24E-02 |
| | IL18 | Interleukin 18 | 7.08637 | 1.17E-02 |
| | DPP4 | Dipeptidyl-peptidase 4 | 6.84522 | 8.42E-03 |
| | MAMDC2 | MAM domain containing 2 | 6.23418 | 2.94E-02 |
| | SNACAIP | Synuclein, alpha interacting protein | 6.21389 | 4.91E-03 |
| **DOWN** | KYNU | Kynureninase | -11.1561 | 7.86E-05 |
| | SPRR2A | Small proline-rich protein 2A | -8.28175 | 2.81E-03 |
| | CSF1 | Colony stimulating factor 1 | -6.62339 | 1.64E-02 |
| | HLA-DPA1 | Major histocompatibility complex, class II, DP alpha 1 | -5.90627 | 1.09E-02 |
| | C15orf48 | Chromosome 15 open reading frame 48 | -5.20775 | 2.63E-02 |
| | SERPINB2 | Serpin peptidase inhibitor, clade B | -5.06934 | 4.74E-02 |
| | TNF3 | TNF alpha interacting protein 3 | -5.00674 | 2.29E-03 |
| | HLA-DRA | Major histocompatibility complex, class II, DR alpha | -5.00129 | 2.81E-03 |
| | IL8 | Interleukin 8 | -4.69281 | 4.22E-02 |
| | CD74 | CD74 molecule, major histocompatibility complex, class I | -4.39972 | 1.30E-02 |

doi:10.1371/journal.pone.0100103.t002

Figure 2. Validation of microarray data by qRT-PCR. Differential gene expression from the microarrays analyses was confirmed by qRT-PCR of 10 selected genes using gene specific primers shown in Table 1. Data represents mean of triplicates, normalized to GAPDH, and presented as relative fold-change (RFC) of Clone 8 to that of Scr-control cells.
doi:10.1371/journal.pone.0100103.g002
used (Figure 3). The differentially expressed genes were used as the initial population nodes then the network was extended to one degree of biological interaction by known protein interactions from the MiMI database.

Regulation of KYNU Expression by AhR

The gene for kynureninase (KYNU), which is an enzyme involved in tryptophan catabolism was notably identified as one of the genes that was considerably downregulated following AhR depletion in C8 cells. Because kynurenine (Kyn), a tryptophan catabolite was recently identified as an endogenous ligand of AhR in neuroblastoma [18], we attempted to define the relevance of the AhR regulation of KYNU, which is the enzyme downstream of the implicated AhR endogenous ligand, Kyn. We examined the effect of activation of AhR by two exogenous ligands, DIM (natural ligand) and TCDD (synthetic ligand) on KYNU expression. Activation of AhR with both TCDD and DIM didn’t affect KYNU gene or protein expression significantly in C8 or control cells (Figure 4A & B). Induction of Cyp1a1 expression was measured as a read out of AhR activation. TCDD strongly induced CYP1A1, whereas DIM induced CYP1A1 to a lesser extent in both control and C8 cells (Figure 4A & C). Consistent with the microarray analysis, both KYNU gene and protein expression were substantially lower in C8 cells compared to control cells under basal condition (Figure 4 A–C).

Discussion

Several studies have identified a role for AhR in cancer independent of exogenous ligand. We previously demonstrated that merely reducing AhR expression altered cell proliferation, anchorage independent growth, migration and apoptosis in MDA-MB-231 cells in vitro, and reduced orthotopic xenograft tumor growth and experimental pulmonary metastasis in vivo [9]. This led

### Table 3. Functional enrichment of GO biological processes following AhR knockdown in MDA-MB-231 cells (C8).

| Accession | Biological Process                     | Count* | P-value |
|-----------|----------------------------------------|--------|---------|
| GO:0006955 | Immune response                         | 24     | 0.0049  |
| GO:001666  | Response to hypoxia                     | 9      | 0.0116  |
| GO:0016477 | Cell migration                          | 14     | 0.0270  |
| GO:0007155 | Cell adhesion                           | 22     | 0.0270  |
| GO:0019882 | Antigen processing and presentation     | 5      | 0.0360  |
| GO:0001525 | Angiogenesis                            | 9      | 0.0393  |

*Counts refer to the number of genes from the input list that fit into the given pathway. Enrichment was performed using the WebGESTALT online tool. It identified those biological processes that had significant alterations following AhR knockdown.

doi:10.1371/journal.pone.0100103.t003

### Table 4. Functional enrichment of KEGG pathway analysis following AhR knockdown in MDA-MB-231 cells.

| Category          | Term                                          | Enrichment¹ | P-value² |
|-------------------|-----------------------------------------------|--------------|----------|
| KEGG_Pathway      | Allograft rejection                           | 12.03        | 0.0087   |
| KEGG_Pathway      | Antigen processing and presentation           | 7.38         | 0.0087   |
| KEGG_Pathway      | Graft-versus-host disease                     | 13.00        | 0.0087   |
| KEGG_Pathway      | Type 1 diabetes mellitus                     | 10.15        | 0.0087   |
| KEGG_Pathway      | Autoimmune thyroid disease                   | 9.28         | 0.0104   |
| KEGG_Pathway      | Bladder Cancer                               | 8.33         | 0.0126   |
| KEGG_Pathway      | Intestinal immune network for IgA production | 7.92         | 0.0133   |
| KEGG_Pathway      | Asthma                                        | 10.60        | 0.0188   |
| KEGG_Pathway      | Cytokine-cytokine receptor interaction        | 3.00         | 0.0188   |
| KEGG_Pathway      | mTOR signaling pathway                        | 6.37         | 0.0188   |
| KEGG_Pathway      | Hematopoietic cell lineage                   | 5.01         | 0.0188   |
| KEGG_Pathway      | Cell adhesion molecules (CAMs)               | 4.03         | 0.0188   |
| KEGG_Pathway      | Axon guidance                                | 3.84         | 0.0219   |
| KEGG_Pathway      | Toll-like receptor signaling pathway          | 4.41         | 0.0232   |
| KEGG_Pathway      | Viral myocarditis                            | 5.42         | 0.0232   |
| KEGG_Pathway      | NOD-like receptor signaling pathway          | 5.42         | 0.0232   |
| KEGG_Pathway      | B cell receptor signaling pathway            | 4.51         | 0.0387   |
| KEGG_Pathway      | VEGF signaling pathway                       | 4.58         | 0.0387   |

¹Ratio of enrichment refers to the number of observed genes divided by the number of expected genes from each KEGG category.

²Multiple test adjustment (BH) FDR adjusted p<0.05 was considered significant.

KEGG pathway analysis was performed using the WebGESTALT online tool. It identified those pathways that had significant alterations following AhR knockdown.

doi:10.1371/journal.pone.0100103.t004
us to conclude that AhR has the capacity to influence key cellular process associated with breast cancer aggressiveness (promotion/progression). In order to gain an insight into the mechanisms by which elevated AhR expression influences cancer progression independent of exogenous ligand, this study aimed to identify the alterations of gene expression and possible molecular mechanisms.

Table 5. List of genes related to phenotypic changes observed following AhR knockdown in MDA-MB-231 cells.

| Symbol | Description                  | Cell Process           | Fold Change | P-value |
|--------|------------------------------|------------------------|-------------|---------|
| KYNU   | Kynureninase                 | Tryptophan Catabolism  | -11.15      | 7.8E-05 |
| MUC1   | Mucin 1                      | Cell Growth            | -2.0        | 0.0412  |
| IL-8   | Interleukin 8                | Cell Growth            | -2.08       | 1.5E-04 |
| BIRC3  | Baculoviral IAP repeat- containing 3 | Cell Survival  | -3.5        | 4.1E-04 |
| BCL3   | B-cell CLL/lymphoma 3        | Cell Survival          | -2.98       | 5.6E-04 |
| ABCC3  | ATP-binding cassette, sub-family C. member 3 | Multi-drug resistance | -3.29       | 8.3E-04 |
| S100A4 | S100 calcium binding protein A4 | Cell Migration and Invasion | -2.86       | 0.01    |
| ABI3   | Abelson interactor protein 3 | Cell Migration and Invasion | +5.15       | 5.7E-04 |
| CCL2   | Chemokine (C-C motif) ligand 2 | Angiogenesis            | -2.7        | 2.3E-03 |
| VEGFA  | Vascular endothelial growth factor A | Angiogenesis       | -3.74       | 9.5E-04 |

1P-value <0.05 and fold change ≥2.0 was considered significant.
2The fold changes in transcriptome levels and significance were calculated using one-way ANOVA.
KEGG pathway analysis was performed using the WebGESTALT online tool. List identified genes which had significant alterations following AhR knockdown 1.
doi:10.1371/journal.pone.0100103.t005

AhR Knockdown and Differential Gene Expression

Figure 3. Subnetwork of functional interactions of AhR-target networks. Initial nodes were populated with differential expressed genes. Circles represent genes of AhR targets, while diamonds represent linker genes.
doi:10.1371/journal.pone.0100103.g003
by which AhR overexpression contributes to breast cancer. Utilizing Human Gene 1.0-ST array, we show that AhR knockdown alters the expression of several genes known to be linked with cancer. These genes include those involved in tryptophan metabolism (KYNU), cell growth (MUC1 and IL8), cell survival (BIRC3 and BCL3) cell migration and invasion (S100A4 and ABI3), multi-drug resistance (ABCC3) and angiogenesis (VEGFA and CCL2).

**Tryptophan Catabolism**

Tryptophan, an essential amino acid, is metabolized in the local microenvironment of tumors through the kynurenine pathway.
leading to the production of several catalobites. Recently, kynurenine (kyn), a tryptophan catalobite that is constitutively produced in glioma cells by tryptophan-2,3-dioxygenase (TDO) in amounts sufficient for AhR activation, has been identified as an endogenous ligand of AhR [18]. In our study in the MDA-MB-231 breast cancer cell line, the AhR depletion resulted in substantial down-regulation of KYNU, the enzyme which catalyzes the conversion of kyn to anthranilic acid and the conversion of 3-hydroxykynurenine (3HK) to 3-hydroxanthranilic acid; taking into account that the mammalian KYNU preferentially targets the 3HK over kyn [19]. The lack of the effect of the AhR exogenous ligands on KYNU gene expression suggests that its’ expression is modulated by AhR levels rather than AhR ligand-dependent activation. Furthermore, the lack of a putative XRE within the KYNU promoter suggests that AhR modulates KYNU gene expression either by binding to a novel non consensus XRE or through interaction with other transcription factors, possibly serving as a co-activator. Down-regulation of KYNU subsequent to AhR depletion could potentially lead to increased accumulation of 3HK, which may induce apoptosis through increased generation of ROS [18,20]. This could partially account for the increased apoptosis previously observed in vitro and in vivo following AhR knockdown [9].

**Cell Growth and Survival**

Aggressive cell growth is a well defined characteristic of cancer cells. MUC1 and IL8 expressions were downregulated by AhR knockdown in MDA-MB-231. These genes are associated with cell growth, and therefore, their downregulated expression may contribute to the previously observed reduced proliferation of these cells following AhR knockdown [9]. Mucin 1 (MUC1) is a tumor associated glycoprotein that plays a role in cancer progression. MUC1 promotes the transcription of MAP2K1 (MEK1), JUN, PDGF, CDC25A, VEGF and ITGA1 (integrin α;); genes involved in proliferation and cell survival, by affecting the recruitment of co-activators [21]. Interleukin-8 (IL-8) is a CXC-type chemokine that has multiple functions within the tumor microenvironment; promoting cell growth, invasion and metastasis of cancer cells through binding its receptors, CXCR1 and CXCR2 in an autocrine fashion [22,23]. In many cancers MUC1 and IL-8 are overexpressed and are associated with poor prognosis [21,22].

Cancer cells often gain resistance to apoptosis providing the basis for cell survival and growth by expressing anti-apoptotic proteins. AhR knockdown resulted in down-regulation of the expression of two anti-apoptotic genes, Baculoviral IAP repeat containing 3 (BIRC3) and B-cell CLL/lymphoma 3 (BCL3). Both BIRC3 and BCL3 function to inhibit apoptosis and their elevated expression has been observed in a number of cancers [24–26]. Therefore, the downregulation of these genes may also contribute to the increased apoptosis previously observed in C3 cells compared to control cells.

**Multi-Drug Resistance**

The development of multi-drug resistance (MDR) remains a major obstacle in the chemotherapy of breast cancer and can develop by increased drug efflux via ATP-binding cassette (ABC). AhR knockdown downregulated the expression of ABCC3, which is a member of the ABC gene family ABCC3 overexpression is observed in breast cancer and has been implicated in acquired MDR [27]. Considering ABCC3 has been identified as conferring resistance to paclitaxel [28], a chemotherapeutic agent frequently used in the treatment of metastatic breast cancer, the downregulation of its expression may contribute to the sensitization of MDA-MB-231 cells to paclitaxel we previously observed following AhR knockdown [9].

**Cell Migration and Invasion**

Our analysis also revealed a down-regulation in the expression of S100 calcium binding protein A4 (S100A4) and upregulation of ablson interactor protein 3 (AB13) genes. S100A4 is associated with cell migration and invasion; key steps in cancer metastasis. It functions by interacting with target proteins involved in cytoskeleton rearrangement and cell motility in a calcium-dependent manner, including F-actin, tropomyosin, and the heavy chain of non-muscle myosin II [29]. Notably, S100A4 is overexpressed in metastatic cancers and is characterized as a marker of tumor progression [30]. On the other hand, ABI3 is a component of the Abl/WAVE complex and it regulates Rac-dependent actin polymerization and formation of lamellipodia. ABI3 functions as a tumor suppressor inhibiting ectopic metastasis of tumor cells and its expression is often lost in invasive cancer cell lines [31,32]. Therefore altered expression of both these genes may contribute to the decreased metastatic potential of MDA-MB-231 cells following AhR knockdown [9].

**Angiogenesis**

As angiogenesis has a critical role in the metastatic process, which is the primary cause of mortality in cancer patients, the down-regulation of chemokine (C-C motif) ligand 2 (CCL2) and vascular endothelial growth factor A (VEGFA), subsequent to AhR knockdown was deemed of high significance. CCL2 is a chemokine that
functions as a chemoattractant, facilitating angiogenesis through the recruitment of tumor associated macrophages (TAMs). TAMs promote a microenvironment that supports tumor growth and metastasis through the production of growth and angiogenic factors, including VEGFA [33,34]. CCL2 can also recruit endothelial cells, which express a CCL2 receptor [35]. VEGFA, a cytokine that is a major regulator of angiogenesis in cancer, is secreted by cancer cells under hypoxic conditions and leads to recruitment of endothelial cells through binding to its receptor [36]. Therefore downregulated expression of CCL2 and VEGFA following AhR knockdown may decrease the blood supply necessary for tumor growth. In our previous studies [9] we have not examined closely the impact of AhR depletion on the angiogenesis process, it will be of interest to perform angiogenesis assays in vivo to verify the relationship between AhR level and development of angiogenesis.

Conclusion

As many of these genes are multifunctional and the primary cause for cellular transformation is aberrant expression of genes/proteins, this study sheds light on molecular mechanisms through which AhR overexpression may influence breast cancer progression. The fact that AhR knockdown alters various genes involved in different biological processes suggests that the role of AhR in breast cancer merits further investigation. The protein–protein interaction network (PIN) (Figure 3) provides insight on how AhR expression might influence breast cancer. The PIN suggests that AhR transcriptional activity may be modified through direct interaction with transcription factors (TBK and SPI), co-activators (EP300 and NCOA1) and co-repressors (NRP1 and NCO2). In addition, the network links AhR with proteins involved in cellular growth, cellular migration, immune response and gene regulation. Analysis examining protein levels will be crucial, as studies have shown that mRNA levels often do not correlate with protein levels. Nevertheless, the identification of these genes has provided new insight into possible molecular events affected by reducing AhR expression that could explain the phenotypic changes we previously observed in vitro and in vivo [9]. In these studies, we showed that the depletion of AhR in metastatic MDA-MB-231 remarkably attenuated their tumorigenic growth in vivo and in vitro, as well as inhibited their lung metastasis in nude mouse model. Taken together with the sets of genes we identified in this study, we can conclude that AhR knockdown alters the expression of genes enhancing or inhibiting cancer progression; tipping the balance towards a state that counteracts tumor progression (Figure 5).

Supporting Information

Table S1 Complete List of Transcriptome Microarray Analysis of AhR KD clone 8 MDA-MB-231 cells compared to scramble control MDA-MB-231 cells.

Author Contributions

Conceived and designed the experiments: SE GG SP. Performed the experiments: GG SP. Analyzed the data: SE GG SP. Contributed reagents/materials/analysis tools: SE SP. Wrote the paper: GG SP SE.

References

1. Poland A, Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol 22: 517–534.
2. Swanston HI, Bradfield CA (1993) The Ah-receptor: genetics, structure and function. Pharmacogenetics 3: 213–230.
3. Gallero MA, Loaiza-Perez AI (2011) The role of aryl hydrocarbon receptor and crosstalk with estrogen receptor in response of breast cancer cells to the novel antimumor agents benzbromarone and anilindaflox. Int J Breast Cancer 2011: 923250.
4. Eguchi H, Ituta T, Tachibana T, Yoneda Y, Kawajiri K (1997) A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator: hypoxia-inducible factor beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. J Biol Chem 272: 17640–17647.
5. Kanno M, Miyama Y, Takara Y, Nakahama T, Inouye Y (2007) Identification of intracellular localization signals and of mechanisms underlining the nucleocytoplasmic shuttling of human aryl hydrocarbon receptor repressor. Biochem Biophys Res Commun 364: 1026–1031.
6. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity. J Biol Chem 279: 23847–23856.
7. Schlezinge R, Liu D, Farago M, Seldin DC, Belgue K, et al. (2006) A role for the aryl hydrocarbon receptor in mammary gland tumorigenesis. Biomol Med 387: 1173–1187.
8. Wong PS, Li W, Vogel CF, Matsunuma F (2009) Characterization of MCF53 mammary epithelial cell overexpressing the Arylhydrocarbon receptor (AhR). BMC Cancer 9: 234.
9. Goode GD, Ballard BR, Manning HC, Freeman ML, Kang Y, et al. (2013) Knockdown of aberrantly upregulated aryl hydrocarbon receptor reduces tumor growth and metastasis of MDA-MB-231 human breast cancer cell line. Int J Cancer 133: 2769–2780.
10. Moennikes O, Loeppen S, Buchmann A, Andersson P, Ittrich C, et al. (2004) A nuclear receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol 22: 517–534.
11. Andersson P, McGuire J, Beazer-Brayard YD, Antonellis K, et al. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4: 249–264.
12. Schwarcz R, Bruno JP, Muchowski PJ, Wu HQ (2012) Kynurenines in the mammalian brain: when physiology meets pathology. Nat Rev Neurosci 13: 465–477.
13. Lee HJ, Bach JH, Chae HS, Lee SH, Joo WS, et al. (2004) Mitogen-activated protein kinase/extracellular signal-regulated kinase attenuates 3-hydroxykynurenine-induced neuronal cell death. J Neurochem 88: 647–656.
14. Dai Y, Dent P, Grant S (2003) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) promotes mitochondrial dysfunction and apoptosis induced by 7-hydroxytaurosporine and mitogen-activated protein kinase inhibitors in human leukemia cells that ectopically express Bcl-2 and Bcl-xL. Mol Pharmacol 64: 1402–1409.
15. Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS, Jr. (2000) Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene 19: 1125–1131.
16. Partain L, Staaf J, Tanner M, Tuominen VJ, Borg A, et al. (2012) Amplification and overexpression of the ABCG2 (MRP2) gene in primary breast cancer. Genes Chromosomes Cancer 51: 832–840.
28. O’Brien C, Cavet G, Pandita A, Hu X, Haydu L, et al. (2008) Functional genomics identifies ABCC3 as a mediator of taxane resistance in HER2-amplified breast cancer. Cancer Res 68: 5380–5389.
29. Li ZH, Spektor A, Varlamova O, Bresnick AR (2003) Msl1 regulates the assembly of nonmuscle myosin-IIA. Biochemistry 42: 14258–14266.
30. Ismail TM, Zhang S, Fernig DG, Gross S, Martin-Fernandez ML, et al. (2010) Self-association of calcium-binding protein S100A4 and metastasis. J Biol Chem 285: 914–922.
31. Miyazaki K, Matsuda S, Ichigotani Y, Takenouchi Y, Hayashi K, et al. (2000) Isolation and characterization of a novel human gene (NESH) which encodes a putative signaling molecule similar to e3B1 protein. Biochim Biophys Acta 1493: 217–241.
32. Cerutti JM, Delcelo R, Amadei MJ, Nakabashi C, Maciel RM, et al. (2004) A preoperative diagnostic test that distinguishes benign from malignant thyroid carcinoma based on gene expression. J Clin Invest 113: 1234–1242.
33. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, et al. (2000) Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 96: 34–40.
34. Low-Marchelli JM, Ardi VC, Vizzara EA, van Rooijen N, Quigley JP, et al. (2013) Twist1 induces CCL2 and recruits macrophages to promote angiogenesis. Cancer Res 73: 662–671.
35. Stamatovic SM, Keep RF, Mostarica-Stojkovic M, Andjelkovic AV (2006) CCL2 regulates angiogenesis via activation of Ets-1 transcription factor. J Immunol 177: 2651–2661.
36. Fondevila C, Metges JP, Fuster J, Grau JJ, Palacin A, et al. (2004) p53 and VEGF expression are independent predictors of tumour recurrence and survival following curative resection of gastric cancer. Br J Cancer 90: 206–215.