The aryl hydrocarbon receptor (AhR) plays an important role in maintaining cellular homeostasis and also in pathophysiology. For example, the interplay between the gut microbiome and microbiobially derived AhR ligands protects against inflammation along the gut–brain axis. The AhR and its ligands also inhibit colon carcinogenesis, but it has been reported that the AhR and its ligand kynurenine enhance glioblastoma (GBM). In this study, using both established and patient-derived GBM cells, we re-examined the role of kynurenine and the AhR in GBM, observing that kynurenine does not modulate AhR-mediated gene expression and does not affect invasion of GBM cells. Therefore, using an array of approaches, including ChIP, quantitative real-time PCR, and cell migration assays, we primarily focused on investigating the role of the AhR in GBM at the functional molecular and genomic levels. The results of transient and stable CRISPR/Cas9-mediated AhR knockdown in GBM cells indicated that loss of AhR enhances GBM tumor growth in a mouse xenograft model, increases GBM cell invasion, and up-regulates expression of pro-invasion/pro-migration genes, as determined by ingenuity pathway analysis of RNA-Seq data. We conclude that the AhR is a tumor suppressor–like gene in GBM; future studies are required to investigate whether the AhR could be a potential drug target for treating patients with GBM who express this receptor.

The aryl hydrocarbon receptor (AhR) is a ligand-activated nuclear transcription factor that forms a heterodimer with the AhR nuclear translocator to activate gene expression (1). The AhR was initially discovered as the intracellular protein that binds 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and is related toxic halogenated aromatic compounds (2), and the bound AhR complex is required for mediating the toxic effects of these compounds (3). Subsequent and ongoing studies have identified structurally diverse endogenous compounds, other industrial products, pharmaceuticals, microbiota-derived metabolites, and health-promoting phytochemicals that also bind the AhR (reviewed in Refs. 4–6). Moreover, studies with AhR knockout mouse models demonstrate that the AhR plays an important role in maintaining cellular homeostasis and pathophysiology (7–10) and is a druggable target (11, 12). AhR ligands and their effects are highly tissue- and response-specific because of several factors, including ligand structure–dependent induction of various receptor conformations that differentially interact with critical nuclear cofactors, and this is commonly observed with nuclear receptors that also bind small molecules (13, 14). This concept is supported by mutational analysis of the AhR, which shows that different structural classes of AhR ligands interact with different sets of amino acids in the AhR binding pocket (15–18).

Gut microbiotas metabolize dietary tryptophan to multiple AhR-active compounds that exhibit AhR-dependent anti-inflammatory activity, and both the AhR and AhR ligands enhance gut health (19–21). A tryptophan-enriched diet that increases AhR-active metabolite formation also protects against neuroinflammation in an animal model of multiple sclerosis (22, 23), demonstrating comparable AhR-dependent protective responses along the gut–brain axis. The AhR and its ligands also protect against intestinal cancer (24, 25), but this is in contrast to the reported tumor-promoting activity of the AhR and the AhR-active tryptophan metabolite kynurenine in glioblastoma (GBM) (26). The contrasting roles of the AhR in the intestine and brain for cancer endpoints clearly differed from the parallel anti-inflammatory effects observed for non-cancer endpoints. Thus, we reinvestigated the role of the AhR and kynurenine in established and patient-derived glioma (PDG) cells. In contrast with previous studies, our data show that kynurenine exhibits minimal effects on GBM cell invasion, and results of functional, genomic, and molecular studies confirm that the AhR protects against GBM cell invasion and growth.

**Results**

Based on the reported differences in the role of the AhR and tryptophan metabolites, including kynurenine in colorectal and brain cancers, we initially compared the Ah responsiveness of
kynurenine and its metabolites in Caco2 colon and U87 GBM cells using induction of CYP1A1 and CYP1B1 as endpoints (Figs. S1 and S2). The overall pattern of Ah responsiveness was similar in both cell lines with kynurenic acid and xanthurenic acid, which exhibits AhR activity in at least one of the cell lines, whereas kynurenine was inactive. The lack of AhR activity of kynurenine was somewhat surprising because of positive results in many cell lines (27–35); however, kynurenine was also inactive in hepatoma cells (36). In some studies, AhR activity was determined using the AhR-responsive pgudLuc 6.1 plasmid reporter gene assay, and the results (Fig. S1D) show that kynurenine induced luciferase activity in U87 and Caco2 cells transfected with a DRE-luc construct. These results suggest that the AhR responsiveness of this compound is response- and cell context–dependent. The pro-invasion effects of kynurenine were observed previously in U87 GBM cells (26), and we used this cell line and three patient-derived GBM cell lines to further investigate this response. Fig. 1A confirms expression of AhR mRNA and protein in RNA extracts (by real-time PCR) and whole-cell lysates (by western blotting) and effects of transfection were determined. B, with two different siAhR oligonucleotides or a control (siCT), nonspecific oligonucleotide on AhR expression was determined by western blotting. C, effects of AhR silencing on invasion of these cells was determined in a Boyden chamber assay as outlined under “Experimental procedures.” D and E, U87 and PDG 14–104s cells (D) and PDG 14–1015s and PDG 15–037 cells (E) were transfected with siAhR or a control and treated with DMSO or 100 μM kynurenine (Kyn) or kynurenic acid (KA). F, cell invasion was determined in a Boyden chamber assay, and the results were quantitated. Results are expressed as means ± S.D. for at least three replicates for each treatment group, and significant induction of invasion is indicated (*, p < 0.05). The diameter of the Boyden chamber (C–E) was 6.5 mm.
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14-104s, and PDG 15-037 cells but did not affect invasion of Ah-nonresponsive PDG 14-015s cells in a Boyden chamber assay (Fig. 1C). The effect of kynurenine or kynurenic acid (in the presence or absence of the AhR) on GBM cell invasion was also investigated (Fig. 1, D and E), and the results werequantitated (Fig. 1F). Kynurenine and kynurenic acid at concentrations as high as 100 μM did not affect invasion of PDG 14-104s, PDG 15-037, or PDG 14-015s cells in the presence or absence of the AhR, whereas a small but significant increase was observed in U87 cells treated with kynurenine. Knockdown of the AhR increased invasion of U87, PDG 14-104s, and PDG 15-037 cells, with no change to PDG 14-015s cells, confirming that the endogenous AhR inhibits GBM cell invasion, in contrast to a previous report (26). Therefore, this study focused on further investigation of the role of the AhR in invasion of GBM cells.

We used CRISPR/Cas9 to stably knock out AhR expression in the highly invasive PDG 15-037 cells (Fig. S3A), and loss of the AhR was confirmed by Western blot (Fig. 2A). PCR analysis of basal and TCDD-induced CYP1A1 mRNA levels, respectively, in WT, mock-transfected, and 15-037-AhRKO cells...
showed that induction was not observed in the absence of AhR expression (Fig. 2B). Incubation of equal numbers of WT, mock-transfected PDG 15-037, and PDG 15-037-AhRKO cells for 1 or 2 days showed that loss of the AhR in 15-037-AhRKO cells resulted in a small but significant increase in cell proliferation after 24 h (Fig. 2C), and this was lower after 48 h. Loss of the AhR resulted in a >4-fold increase in cell invasion in a Boyden chamber assay (Fig. 2D), and there was a more than 3.5-fold increase in invasion using a 3D tumor spheroid invasion assay compared with WT parent and mock transfected cells (Fig. 2F) (37). Moreover, knockdown of the AhR in 15-037 cells by RNAi or CRISPR/Cas9 also enhanced migration in a scratch assay (Fig. 2F), and loss of the AhR in 15-037 cells enhanced α5-integrin and vimentin expression, consistent with an increase in epithelial–mesenchymal transition of these cells (Fig. S3B). We also observed similar results for a second AhR-nonresponsive 15-037-AhRKO cell line generated by CRISPR/Cas9 (Fig. 3, C–E). Collectively, our results indicate that the AhR exhibits tumor suppressor–like activity in GBM cells and functions as an inhibitor of GBM cell invasion. Previous reports used the AhR antagonist CH223191 to investigate the functions of the AhR in GBM (38, 39), and we used a similar approach. The results illustrated in Fig. 3A show that the AhR antagonist CH223191 slightly inhibited growth of WT PDG 15-037 cells; however, similar results were observed in AhR-deficient PDG 15-037 cells, suggesting an AhR-independent response. Moreover, a comparison of the effects of CH223191 on WT/AhRKO cell migration (Fig. 3B) (scratch assay) and invasion (Fig. 3C) showed that the inhibitory effects of CH223191 were observed in both AhR-expressing and AhR-silent cell lines, indicating that the effects of the AhR antagonist were AhR-independent. The AhR-independent action of CH223191 was also investigated in PDG 15-037 cells after knockdown by RNAi, and similar results were observed (Fig. 3C). Thus, the results of previous studies showing that CH22319 inhibits growth and invasion of glioblastoma cells (38, 39) may also be AhR-independent responses.

Thus, functional studies suggest that the AhR exhibits tumor suppressor–like activity in GBM cells, and this was further investigated at the genomic level. We examined the differences in gene expression in mock-transfected (control) PDG-15-037 versus AhR-deficient PDG 15-037 cells (transfected with siAhR) by RNA-Seq and observed that loss of the AhR altered the expression of 749 genes, with 268 decreased and 481 increased (Fig. 4A). Ingenuity pathway analysis (IPA) demonstrated changes in the expression of genes associated with multiple pathways (Fig. 4B), including cell movement (e.g. invasion and migration), cell growth, and proliferation. Causal IPA analysis of the RNA-Seq results demonstrated that the p values and activation z-scores for the differentially expressed genes associated with movement of cancer cells were 3.09E−08 and −2.159, respectively, and for cancer cell invasion, the p value and activation z-scores were 6.55E−06 and −2.043, respectively. These data are consistent with the role of the AhR as an inhibitor of cell movement/invasion in GBM cells, and this complements the functional studies on the AhR. Fig. 4C illustrates the pathway analysis and possible interconnections of cell movement/invasion genes increased by AhR knockdown, and Fig. 4D summarizes changes in expression growth, invasion, and AhR axis gene expression after loss of the AhR in 15-037 GBM cells. Thus, the differences in the transcriptome and associated pathway in PDG 15-037 cells after AhR knockdown are consistent with the functional tumor suppressor–like activity of the AhR in GBM.

The effects of AhR knockdown by RNAi and CRISPR/Cas9 on expression of CYP1A1 and three pro-invasion genes that were detected in the RNA-Seq experiment are illustrated in Fig. 5, A–D, and show parallel effects on expression of CYP1A1 mRNA (decreased) and MMP-9, CXCL12, and CXCR4 mRNA (induced) after loss of the AhR. Moreover, AhR knockdown also increased MMP-9 but not MMP-2, as determined by zymography assay (Fig. 5E). Previous studies have demonstrated that CXCL12 (ligand)–CXCR4 (cognate receptor)
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interactions induce the pro-invasion gene MMP-9 (40, 41), and knockdown of the AhR, which enhanced MMP-9 expression, was inhibited by AMD3100, which antagonizes CXCR4 signaling (Fig. 5F). The role of induced MMP-9 in enhanced invasion of AhR-deficient 15-037 cells (RNAi knockdown) was further investigated, and we observed that inhibition of CXCR4 signaling by AMD3100 or by direct MMP-9 inhibition significantly decreased invasion of 15-037 cells using the tumor spheroid invasion assay (Fig. 5G). These results indicate that activation of the CXCL12–CXCR4–MMP-9 pathway after loss of the AhR contributes to the enhanced invasion of 15-037 GBM cells in which the AhR is silenced.

We also investigated contributions of the CXCL12–CXCR4–MMP-9 axis to the enhanced invasion of 15-037 AhRKO cells and found that the CXCR4 antagonist AMD3100 abrogated the increase of MMP-9 mRNA levels in the AhR-deficient cell lines (Fig. 6A). Moreover, both AMD3100 and the MMP-9 inhibitor significantly decreased invasion in 15-037 AhRKO cells (Fig. 6B), complementing results observed in 15-037 cells after AhR knockdown by RNAi (Fig. 5G). ChIP analysis of the proximal regions of the CYP1A1, CXCL12, MMP-9, CXCR4, and ITGA5 promoters in 15-037 WT and AhRKO cells revealed that loss of the AhR decreased AhR–promoter binding, decreased CYP1A1 gene promoter–
associated pol II, and increased promoter-associated pol II on the CXCL12, MMP-9, CXCR4, and ITGA5 gene promoters (Fig. 6C). These results, showing that loss of AhR affects pol II binding, are consistent with gene/protein expression data (Fig. 5).

We also investigated tumor development in a mouse (Foxn1nu) xenograft model, and after 6 weeks, we observed palpable tumors in four and five mice injected with WT and AhRKO 15-037 cells, respectively (n = 7/cell line), and tumors derived from AhRKO cells were larger than those derived from WT cells (Fig. 6D). Histopathology also showed that loss of the AhR increased proliferating cell nuclear antigen and ITGA5 staining, which is consistent with enhanced growth and epithelial-to-mesenchymal transition. Therefore, our results suggest
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(A) MMP-9

(B) 0.1% DMSO

(C) 5 μM AMD3100

(D) 2 μM MMP-9 inhibitor

(E) CXCL12

CYP1A1, CXCL12, MMP-9, CXCR4, ITGA5, MMP-9, etc
that the AhR suppresses pro-invasion/migration pathways, as illustrated in Fig. 6E, and demonstrate that the AhR exhibits tumor suppressor–like activity at the gene and functional levels in GBM.

Discussion

Gut microbiome- and microbiota-derived metabolites contribute significantly to the gut/microbiome axis with other organs to influence their development and maintenance of homeostasis (reviewed in Ref. 34). A subset of microbial metabolites derived from metabolism of tryptophan exhibits AhR activity, and these metabolites play an important role in maintaining gut resilience through their regulation of immune responses in intestinal cells (19–21, 42, 43). Dietary or microbially mediated enrichment of AhR ligands protects against inflammatory diseases not only in the colon but also in rodent models of experimental autoimmune encephalomyelitis (7–10, 19–23, 32, 33, 35), and the AhR also plays a tumor suppressor-like role in the colon (24, 25). In contrast, a parallel gut-brain axis with respect to cancer is not apparent because the published studies are contradictory. On one hand, it has been reported that the tumor promoter activity of the AhR is linked to transforming growth factor β/integrin signaling and also to the tryptophan catabolite kynurenine, which promotes AhR-dependent tumor survival and motility and suppresses immune surveillance (26, 38). In contrast, there is evidence that the AhR and TCDD induce apoptosis and/or growth inhibition in human neuroblastoma SK-N-SH and their derivative SH-SY5Y cells and rat pheochromocytoma PC12 cells (44–47).

We first investigated the AhR activity of kynurenine and its metabolites in representative colon (Caco2) and GBM (U87) cells, and the results indicated that kynurenic and xanthurenic acids weakly activated expression of Ah-responsive genes in Caco2 and U87 cell lines, whereas kynurenine was inactive; similar results were confirmed in Ah-responsive patient-derived GBM cells (14–104s and 15-037 cells) (Figs. S1 and S2). However, kynurenine exhibited AhR activity in Caco2 and U87 cells transfected with a DRE-luc construct (Fig. S1D). Kynurenine had minimal effects on invasion of U87 cells, although it has been reported previously (26) that kynurenine is a more potent inducer of U87 cell invasion. In contrast to results of a previous study in U87 cells (26), AhR silencing in U87 and patient-derived GBM cells enhanced invasion, suggesting that the AhR acts as an inhibitor of GBM cell invasion. The role of the AhR in cancer is complex and tumor type–dependent (12). For example, the AhR exhibits tumor suppressor–like activity in colon cancer (24) and tumor promoter–like effects in head and neck cancer (48, 49). Therefore, because of the differences observed in the function of the AhR in this study and in a previous report (26), we carried out additional functional and genomic studies to further probe the role of the AhR in GBM.

Two complementary approaches were used to investigate the phenotype resulting from AhR loss in the patient-derived 15-037 cell line. The 15-037 AhRKO cell line generated by CRISPR/Cas9 exhibited only slightly increased growth and decreased expression and inducibility of CYP1A1 compared with WT cells in culture, and in a xenograft model, tumors from AhRKO cells exhibited increased growth. Further confirmation that the AhR was a tumor suppressor–like gene was obtained in AhR-deficient 15-037 cells generated by CRISPR/Cas9 or by knockdown of the AhR by RNAi (Figs. 4 and 6). Compared with WT 15-037 cells, both AhR-deficient cell lines exhibited increased invasion in the Boyden chamber and spheroid cell invasion assays and expressed higher levels of CXCL12, CXCR4, and MMP-9 mRNA, and invasion of both cell lines was inhibited by AMD3100 (a CXCR4 inhibitor) and an MMP-9 inhibitor. Loss of AhR by RNAi also resulted in activation of pro-migration/invasion genes, including CXCL12 and MMP-9.

Thus, we observed parallel effects of AhR silencing by RNAi and CRISPR/Cas9 on both functional and genomic responses in glioblastoma cells. Moreover, causal IPA analysis of RNA-Seq results showed that genes associated with these pathways after loss of AhR were consistent with the tumor suppressor-like activity of the AhR observed in functional studies.

Thus, in contrast to previous reports (26, 38), the results of this study indicate that the AhR exhibits tumor suppressor–like activity in GBM cells. Kynurenine does not affect cell invasion, and its AhR activity was response-dependent (no induction of CYP1A1 but induction of luciferase activity in cells transfected with DRE-luc). Kynurenine activity as an AhR agonist is cell context– dependent (26–36), and a recent study showed that, under storage conditions, the AhR activity of kynurenine can be increased by up to a 1000-fold because of formation of potent polycyclic condensation products (35). This enhanced activity was not observed in Caco2 or GBM cells (Figs. S1 and S2); however, this does not exclude a role of kynurenine in avoiding immune surveillance (26). Given the devastating natural history of GBM (50), more effective therapeutic strategies are critically needed to counter the tumor proliferation and invasion observed in glioblastoma patients (43). Current studies are focused on identifying AhR ligands that may be effective for treatment of GBM patients that express this receptor.

Experimental procedures

Cell lines, antibodies, reagents, and western blots

U87-MG human malignant glioma cell line was obtained from the American Type Culture Collection (Manassas, VA).
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The PDG cell lines 14-015s (GBM), 14-104s (grade IV gliosarcoma), and 15-037 (GBM) were generated from fresh tumor specimens collected from newly diagnosed patients with no prior chemo- or radiotherapy treatment. All cell lines were confirmed by sequencing within the last 6 months; the 14-015s cell line also exhibited an 83% identity to U-251 MG cells. All glioma cells were maintained in DMEM/Ham’s F-12 50:50 mixture supplemented with 1-glutamine, 10% FBS, 1X minimum Eagle’s medium nonessential amino acids, and 10 μg/ml gentamycin (Gibco). Cells were maintained at 37 °C in the presence of 5% CO₂, and the solvent (DMSO) used in the experiments was 0.2% or less. The AhR antibody (sc-5579) and AMD3100 (Santa Cruz, CA), and the GAPDH antibody was purchased from Cell Signaling Technology (Danvers, MA). MMP-9 inhibitor I (15942) was purchased from Cayman (Ann Arbor, MI). Negative control siRNA (1027281) was obtained from Qiagen (Hilden, Germany), and siAhR-1 (SASI_Hs01_00140198), siAhR-2 (SASI_Hs02_00332181), L-kynurenine (K8625), quinaldic acid (160660), anthranilic acid (A89855), kynurenic acid (K3375), 3-hydroxy-DL-kynurenine (H1771), and xanthurenic acid (D120804) were purchased from Millipore-Sigma. L-Kynurenine used in previous studies in glioblastoma cells was purchased from Sigma-Aldrich. Western blot analysis of whole-cell lysates was carried out as described previously (51).

Cell counting and proliferation (XTT) assays

Cells were counted using a hemocytometer after suspending cells with trypsin. The cytotoxic effect of compounds on cells was investigated using the XTT Cell Viability Kit (Cell Signaling Biotechnology) according to the manufacturer’s instructions. Briefly, the cells were plated in 96-well culture plates at a density of 1 × 10⁴ cells/well in 100 μl of complete DMEM for 16 h and then treated with 10 μM CH223191 in DMEM containing 2.5% FBS. After 24 h of culture, 25 μl of XTT reaction solution (sodium 3-[1-phenylamino-carbonyl]-3,4-tetrazoli- um]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the each well. The optical density was read at 490 nm wavelength in an ELISA plate reader after 6 h of incubation. All determinations were replicated in at least three separate experiments.

Generation of AhR-deficient glioblastoma cell lines

A human AhR CRISPR/Cas9 guide RNA (AGACCGACTT-AATACAGAGT) in a pSpCas9 BB-2A-GFP PX458 vector was purchased from GenScript (Piscataway, NJ). Three days after transfection, flow cytometry was used to collect high-GFP-expressing cells (single cells), which were seeded in 96-well plates (1 cell/well). After 2 to 3 weeks, cells from 50 individual wells were transferred into 6-well plates, and after 7 days, DNA was extracted and exon 2 was sequenced for expression of the single guide RNA target sequence. Thirty-six clones expressed WT AhR-exon 2 sequences, and other mutations were observed in some clones, but all of these cells expressed WT AhR protein. Two cell lines exhibited the expected frameshift deletion in exon 2 of the AhR; these cell lines were GFP-negative and were used in the experiments as CRISPR/Cas9 AhR knockout cells (15-037 AhRKO). Clonal cells were grown into larger cultures and screened for knockout of AhR proteins and its function as described previously (51, 52).

ChIP assay

The ChIP assay was performed using the ChIP-IT Express Magnetic Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. The primers for detecting the CXCL12 promoter (−741 to −562 bp) were 5’-GGT GGG CCT GTG ATG TC-3’ and 5’-TGA GAC CCG TCT TTG CAG TC-3’. The primers for detecting the MMP-9 promoter (−640 to −357 bp) were 5’-AGA TTC AGC CTG CGG AAG AGC AC-3’ and 5’-TGG TCA GCC AAG GGA AAG TG-3’. The primers for detecting the CXCR4 promoter (−949 to −779 bp) were 5’-ATC CCT GCC ATT TCA TCT CTC C-3’ and 5’-ACA ACA CCG TGT GGG TTG TAC C-3’. The primers for detecting the ITGAX promoter (−318 to −82 bp) were 5’-ATT CTC CGC TCT CTT TTC CG-3’ and 5’-CTG GGT CCT CCC TGG AAC TCT GA-3’. The primers for detecting the CYP1A1 promoter (−440 to −336 bp) were 5’-CTG GGG TCG CGC TTC TCT T-3’ and 5’-CTA CAG CCT ACC AGG ACT CG-3’. PCR products were resolved on a 2.5% agarose gel in the presence of ethidium bromide.

Quantitative real-time PCR

RNA was isolated using the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA). Quantification of mRNA was performed using the iTaq Universal SYBR Green 1-Step Kit using the manufacturer’s protocol with the CFX384 real-time PCR System (Bio-Rad). The comparative cycle threshold method was used for relative quantitation of samples. Values for each gene were normalized to expression levels of TATA-binding protein. The sequences of the primers used for real-time PCR included the following: CYP1A1, 5’-GAC CAC ACC CAC CAC CAA GAA C-3’ (sense) and 5’-AGC GAA GAA TAG GGA TGA AG-3’ (antisense); CYP1B1, 5’TAT CAC GGA CAT CTT CGG CG-3’ (sense) and 5’-ACC TGA TCC AAT TCT GCT GC-3’ (antisense); CXCL12, 5’-TGG GCT CCT ACT GTA AGG GTT-3’ (sense) and 5’-TTG ACC CCG CAA AGC TAA AGT GG-3’ (antisense); CXCR4, 5’-TTT TCT TCA CGG AAG CAA CGG-3’ (sense) and 5’-GGT ACC ATG GAG GGG ATC AG-3’ (antisense); MMP-9, 5’-TTG GTC CAC CTG CTT CCA CT-3’ (sense) and 5’-ACG ACG TCT TCC AGT ACC GA-3’ (antisense); and ITGA5- binding protein, 5’-GAT CAG AAC ACC AGT CC-3’ (sense) and 5’-TTC TGA ATA GGC TGT GGG GT-3’ (antisense).

siRNA and CRISPR/Cas9

After testing of siAhR-1 (SASI_Hs01_00140198) and siAhR-2 (SASI_Hs02_00332181), siAhR-2 was selected for further AhR knockdown experiments in this study. CRISPR/Cas9 silencing of the AhR and treatment of the cells was carried out as described previously (52, 53).

RNA-Seq analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and the RNA Mini Prep Kit (Qiagen) according to

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the manufacturers’ protocols, and any contaminating chromosomal DNA was removed by treatment with DNase I. Sequencing and analysis were carried out by the Michigan State University Genomics Core Facility (East Lansing, MI). Paired-end Illumina raw reads were processed with Trimomatic version 0.36 (54) to trim adapter sequences and low-quality bases. Transcript expression abundances were directly quantified with the clean reads using Salmon version 0.9.1 (55) with the hg38 assembly and Ensembl annotation. The transcript level expression abundances were aggregated into gene-level expression abundances with the length-corrected transcript per million using tximport version 1.4.0. Genes with at least 10 transcripts per million in three of six samples were used for subsequent differential gene expression analysis. All differential expression analyses were performed with voom from R Package Limma version 3.32.10 (56), and genes with a log2(3)-fold change and false discovery rate adjusted p value of less than 0.01 were retained as differentially expressed genes. The functional category and pathway analysis of differentially expressed genes in control and AhR knockdown cells were analyzed using the IPA (Qiagen Bioinformatics) Knowledge Base.

**Scratch and invasion assays**

The BD-Matrigel Invasion Chamber (24-Transwell with an 8-μm pore size polycarbonate membrane) was used in a modified Boyden chamber assay essentially as described previously (57). The scratch assays used 80% confluent cells in 6-well plates, and cell migration into the scratch was determined after 18 h.

**3D tumor spheroid invasion assay**

Spheroids were generated using the Matrigel technique as described previously (37). The cells were suspended in complete medium (2 × 10⁴ cells/ml). Spheroids were produced by seeding 200 μl of the cell suspension into a well of a 96-well round-bottomed ultra-low attachment culture plate. After incubation at 37 °C in a 5% CO₂ incubator for 24 h, 100 μl/well growth medium from the spheroid plates was removed, and 100 μl/well Matrigel (Corning, 356234) was added on the bottom of each well. The plate was transferred to the incubator for 1 h, and 100 μl of complete medium containing three times the desired final concentration of compounds was supplemented and then incubated for 3–5 days, followed by fixation in 4% formaldehyde. Spheroid invasion was determined by measuring the cross-sectional areas of the spheroid center and the rim of invaded cells using Image J software (34).

**MMP-9 zymography**

To measure gelatinase activity, GBM cells were plated and incubated in serum-free medium for 24 h. The gelatin zymography protocol from Abcam was used to detect the activity of gelatinase enzymes, MMP-9, and MMP-2, as previously described (58).

**Xenograft studies in athymic mice**

Female athymic nude mice (Foxn1nu, age, 7 weeks) were purchased from Envigo (Huntingdon, UK) and subcutaneously implanted with 1 × 10⁶ cells suspended in Matrigel (Corning) in the flank. Animals were examined every other day for tumor burden (approximated by external caliper measurements, where (width² × length)/2), animal weight, and overall well-being. At the end of the study, animals were humanely sacrificed, and tumors were harvested for analysis. All animal studies were carried out according to procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

**Statistics**

All data were expressed as the means (S.D.). Statistical significance was analyzed using either unpaired Student’s t test (two-tailed) or analysis of variance. A p value of less than 0.05 was considered statistically significant.

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