A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer

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Abstract

The ability of a cancer cell to develop resistance to anoikis, a programmed cell death process triggered by substratum detachment, is a critical step in the metastatic cascade. Triple-negative breast cancers (TNBC) exhibit higher rates of metastasis after diagnosis, relative to estrogen-positive breast cancers, but while TNBC cells are relatively more resistant to anoikis, the mechanisms involved are unclear. Through gene expression and metabolomic profiling of TNBC cells in forced suspension culture, we identified a molecular pathway critical for anchorage-independent cell survival. TNBC cells in suspension upregulated multiple genes in the kynurenine pathway of tryptophan catabolism, including the enzyme tryptophan 2,3-dioxygenase (TDO2), in an NF-kB–dependent manner. Kynurenine production mediated by TDO2 in TNBC cells was sufficient to activate aryl hydrocarbon receptor (AhR), an endogenous kynurenine receptor. Notably, pharmacologic inhibition or genetic attenuation of TDO2 or AhR increased cellular sensitivity to anoikis, and also reduced proliferation, migration, and invasion of TNBC cells. In vivo, TDO2 inhibitor–treated TNBC cells inhibited colonization of the lung, suggesting that TDO2 enhanced metastatic capacity. In clinical specimens of TNBC, elevated expression of TDO2 was associated with increased disease grade, estrogen receptor–negative status, and shorter overall survival. Our results define an NF-kB–regulated signaling axis that promotes anoikis resistance, suggest functional connections with inflammatory modulation by the kynurenine pathway, and highlight TDO2 as an attractive target for treatment of this aggressive breast cancer subtype.

Introduction

The vast majority of breast cancer deaths are caused by complications from metastases (1–3). A high rate of metastasis is characteristic of triple-negative breast cancers (TNBC), which lack expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification, and thus do not respond to current endocrine therapies or HER2-targeted therapies (4). Due in part to the absence of effective targeted therapies, but likely also to inherent properties of this subtype, patients with metastatic TNBC have a poor prognosis with a median survival of 13 months (5). Thus, identification of new targeted therapies that inhibit or slow metastasis is of critical importance to improve the prognosis of women with TNBC.

Metastasis is a complex process with multiple steps, including detachment of cancer cells from the primary tumor, invasion through local tissue, intravasation into the vasculature and lymphatics, survival while in transit, extravasation, and colonization of secondary sites (6). Normal epithelial cells are programmed to undergo apoptosis if they become detached from the basement membrane, a process termed anoikis (7). Anoikis resistance is thought to facilitate survival of tumor cells that detach from the primary tumor and thereby facilitate metastasis (8, 9). Multiple mechanisms of anoikis resistance have been identified, including deregulation of integrin expression and aberrant activation of prosurvival pathways (as reviewed in ref. 9), as well as altered metabolism (10, 11). However, the present study is the first to globally profile gene expression alterations in forced suspension culture with the goal of identifying targetable pathways important for survival in suspension. This objective screening approach identified multiple components of the kynurenine pathway of tryptophan catabolism and the aryl hydrocarbon receptor (AhR), which is activated by kynurenine (12), as being upregulated by TNBC in suspension culture.

Altered tryptophan metabolism and increased secretion of tryptophan metabolites by solid tumors, including in breast cancer, have long been recognized (13, 14). The essential amino acid tryptophan is required for protein synthesis and is a precursor for the formation of multiple signaling molecules including serotonin (15). The majority of tryptophan catabolism occurs via the kynurenine pathway, leading to synthesis of NAD+ along with intermediate products, including quinolinic acid and...
kynurenine (Kyn; ref. 16). The first step of the kynurenine pathway can be catalyzed by indoleamine 2,3-dioxygenase 1 (IDO1), IDO2, or tryptophan 2,3-dioxygenase (TDO2; refs. 17–19). IDO1 is expressed in tissues throughout the body, whereas TDO2 is predominantly expressed in the liver (20). Much research on the kynurenine pathway in cancer has focused on the ability of Kyn to decrease immune surveillance (as reviewed in ref. 21), but in glioma, Kyn, acting in both a paracrine and an autocrine fashion, suppressed antitumor immune responses and promoted tumor cell survival and motility. Kyn was demonstrated to serve as an endogenous ligand to activate AhR in both immune cells and the tumor cells themselves (12).

AhR is a member of the basic-helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) superfamily of transcription factors best known for its activation by xenobiotics such as polycyclic aromatic hydrocarbons (PAH; refs. 22, 23). PAH binding leads to transcription of AhR target genes, including drug-metabolizing enzymes such as CYP1A1 and CYP1B1 (24). Increased expression of AhR and its target genes has been found in several cancer types, including lung, cervical, ovarian, and breast (16). AhR is required for normal mammary gland development (25, 26), and AhR overexpression increased migration, invasion, and proliferation of immortalized mammary epithelial cells (27). AhR knockdown in MDA-MB-231 TNBC cells decreased expression of genes involved in these same processes (28). Interestingly, increased AhR activity in mouse hepatoma cells grown in suspension was observed nearly 20 years ago (29); however, neither the mechanism of activation nor the functional significance was tested.

Using global expression analysis and metabolomic profiling of TNBC cells in forced suspension, we identify a novel TDO2-AhR signaling axis, mechanistically dependent on NF-κB, which promotes anoikis resistance, as well as migratory and invasive capacity. Indeed, we found that pharmacologic inhibition or knockdown of TDO2 or AhR decreased anchorage-independent growth and invasive capacity in vitro and TDO2 inhibition decreased lung metastasis in a TNBC preclinical model. Collectively, these data and the fact that TDO2 expression confers a shorter overall survival in breast cancer patients suggest that TDO2 inhibition may be a rational targeted therapy to reduce TNBC metastasis and resultant mortality.

Materials and Methods

Cell culture and treatments

All cell lines were authenticated by short tandem repeat analysis and tested negative for mycoplasma in July of 2014. SUM159PT cells were purchased from the University of Colorado Hospital Cancer Center Tissue Culture Core in August of 2013 and were grown in Ham’s F-12 with 5% FBS, penicillin/streptomycin, hydrocortisone, insulin, HEPES, and t-glutamine supplementation. MDA-MB-231 (MDA-231) cells were purchased from the ATCC in August of 2008 and were grown in minimum essential media with 5% FBS, penicillin/streptomycin, HEPES, t-glutamine, non-essential amino acids, and insulin supplementation. BT549 cells, purchased from the ATCC in 2008, were grown in RPMI Medium 1640 with 10% FBS, penicillin/streptomycin, and insulin. MCF7 and T47D cells were purchased from the ATCC and were grown in DMEM with 10% FBS and 2 mmol/L t-glutamine.

Forced suspension culture

Poly-2-hydroxyethyl methacrylate (poly-HEMA, from Sigma-Aldrich) was reconstituted in 95% ethanol to 12 mg/mL. Ethanol was allowed to evaporate overnight, and plates were washed with PBS prior to use.

Kynurenine high performance liquid chromatography

Experimental samples were generated by plating 500,000 cells in 2 mL culture media in a 6-well plate in triplicate. After 48 hours, media were collected and protein was precipitated using trichloroacetic acid at a final concentration of 7%, samples were centrifuged, and the supernatant was analyzed on an Agilent 1260 high performance liquid chromatography (HPLC) with a Kinetex BiPhenyl column (particle size 2.7 μm, 4 mm internal diameter by 150 mm; Phenomenex, catalog no. 00F-4622-EO). Kynurenine was eluted using a binary gradient consisting of 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B). The separation gradient was 5% to 60% B over 6 minutes at a flow rate of 0.75 mL/min, and the kynurenine peak was monitored at 360 nm. To quantify kynurenine in the samples, peak areas were compared with a serial dilution of L-kynurenine (Sigma; catalog no. K8625) in tissue culture media, which was prepared with the same precipitation method as the samples. In brief, 100 μL of standard or supernatant was injected onto the system, and peak area was calculated using Agilent ChemStation software.

Cellular assays and reagents

Cells were treated with 680C91 and CH223191 (TOCRIS Bioscience) prepared in dimethyl sulfoxide (DMSO). Alpha-naphthoflavone (Sigma-Aldrich) was diluted in methanol:ethyl acetate (3:1), and 1-D,L-methyl-tryptophan and crystalline L-kynurenine (Sigma-Aldrich) were prepared in 0.25N HCl. PS1145 (TOCRIS Bioscience) was prepared in DMSO. IL1β and TNFα were obtained from eBioscience.

Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology) as per the manufacturer’s instructions.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Plus Kit (QIAGEN) according to the manufacturer’s instructions. cDNA was synthesized with the Applied Biosystems reverse transcription Kit. qRT-PCR was performed in an ABI 7600 FAST thermal cycler using Absolute Blue qPCR SYBR Green Low ROX Mix (Thermo Scientific). All fold-change data were normalized to β-actin. All experiments were performed in biologic triplicate. For primer sequences, see Supplementary Methods.

Immunoblotting

Whole-cell protein extracts (50 μg) were denatured, separated on SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. After blocking in 3% BSA in Tris-buffered saline–Tween, membranes were probed overnight at 4°C. Primary antibodies used include AhR (#13790; 1:1,000 dilution; Cell Signaling Technology), KNYU (H00008942-B01; 1:1,000 dilution; NOVUS Biologicals), TDO2 (H00006999-B01P; 1:1,000 dilution; Abnova), TOP1 (C-21; 1:100 dilution; Santa Cruz Biotechnology, Inc.), and α-tubulin (clone B-5-1-2; 1:30,000 dilution; Sigma Aldrich). Following secondary antibody incubation, results were detected using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer). Densitometry quantification was performed using Image Studio Lite software.
Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue sections (5 µm) were heat immobilized onto glass slides and deparaffinized in a series of xylenes and graded ethanol. Antigens were heat retrieved in 10 mM Tris, 1 mM EDTA, pH 9.0 solution, and blocked for endogenous peroxidase followed by 10% normal goat serum prior to antibody incubation. Tris-buffered saline with 0.05% Tween (TBST) was used for all washes. TDO2 antibody (Abnova; # H0006999-B01P) was incubated overnight at room temperature at 1:200 in TBST, and AhR antibody (CST #13790) was incubated for 1 hour at room temperature at 1:50 in TBST. Envision polymer (Dako) was used for detection, followed by 3,3’-diaminobenzidine (Dako), and slides were counterstained with dilute hematoxylin.

Gene expression array analysis

BT549 cells were grown in either attached conditions or forced-suspension conditions on poly-HEMA-coated plates in quadruplicate for 24 hours. RNA was harvested at 24 hours using Trizol method, and hybridized onto Affymetrix Human Gene 1.0ST arrays for determination of luciferase activity. For analysis, AhR or NF-kB reporter activity was normalized to SV40 reporter activity to control for differences in transfection efficiency.

Measurement of anoikis

Soft-agar assays were performed in 0.5% bottom and 0.25% top-layer agar (Difco Agar Noble; BD Biosciences). Media with treatment were refreshed every 4 days.

Caspase-3/7 activity was measured using a Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer’s protocol. Briefly, 8 × 10^4 TNBC cells were plated in a clear bottom, white-walled 96-well plate coated with poly-HEMA. The cells were incubated for 48 hours at 37°C. Following incubation, each well was mixed with Caspase-Glo 3/7 reagent in equal volume to the culture medium. The 96-well plate was then covered in foil, shook for 30 seconds, and incubated at room temperature for 30 minutes. Luminescence was determined by luminometer, measuring luminescence after 1 second.

In vivo metastasis experiment

Tail-vein injection experiments were approved by the University of Colorado Institutional Animal Care and Use Committee [IACUC protocol—83612(10)11E]. All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. Prior to tail-vein injection, luciferase-expressing MDA-MB-231 cells were grown in forced suspension conditions for 48 hours in the presence of either vehicle control or 10 µmol/L 680C91. A total of 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD.CB17-Prkdcscid/l (NOD/SCID) mice (The Jackson Laboratory). Following injection, luminescence was monitored every 7 days for a total of 4 weeks. At the completion of this experiment, lungs of 5 mice from each group were imaged by IVIS ex vivo. Lungs from the remaining 5 mice per group were formalin-fixed and paraffin-embedded for IHC.

Proliferation, migration, and invasion

Proliferation assays were performed using the Incucyte ZOOM live-cell imaging system (Essen BioSciences). Migration scratch wound assays were performed per the manufacturer’s instructions and scanned with the Incucyte ZOOM apparatus (Essen BioSciences). Trans-well invasion assays were performed with BD BioCoat Matrigel Invasion Chambers (BD Biosciences) per the manufacturer’s protocol.

Results

Multiple enzymes of the kynurenine pathway are upregulated in detached TNBC cell lines

To model loss of attachment in vitro, we grew breast cancer cells on tissue culture plates coated with poly(2-hydroxyethyl
methylacrylate) (poly-HEMA). We previously demonstrated that TNBC cells grown in forced suspension upregulate proteins involved in anoikis resistance and motility (30, 31). To identify additional pathways responsible for resistance to anoikis in an unbiased, global fashion, expression profiling was performed on the TNBC cell line BT549 grown in standard attached conditions for 24 hours as compared with the same number of cells grown in forced suspension for 24 hours in quadruplicate. In total, we identified 367 genes that change more than 1.5-fold in a statistically significant manner (P < 0.05, log-rank test). Of these genes, 217 (59%) were upregulated and 150 (41%) were downregulated in suspended cells (Supplementary Fig. S1A). Ingenuity Pathway Analysis of these data revealed two related pathways strongly upregulated in suspension: tryptophan catabolism and AhR signaling (Supplementary Fig. S1B and S1C).

Figure 1A shows unsupervised hierarchical clustering of the top 35 genes significantly upregulated by at least 2-fold in suspended compared with attached conditions (P < 0.05, log-rank test). Among the genes most highly upregulated in suspension were two enzymes involved in the kynurenine pathway, which converts tryptophan to NAD⁺. The rate-limiting enzyme tryptophan 2,3-dioxygenase (TDO2) was the most highly upregulated gene in suspension, and the downstream enzyme kynureninase (KYNU) was also among the top fold-changing genes (Fig. 1A). To confirm our gene array data, we performed qRT-PCR for TDO2 and KYNU in multiple breast cancer cell lines, including both luminal (ER⁺) and TNBC (ER⁻) lines, after 24 hours in suspension (Fig. 1B and C). In all three TNBC lines tested, TDO2 and KYNU were significantly increased in suspension compared with attached culture. In the two ER⁺ breast cancer cell lines tested, expression of these genes trended slightly higher in suspension, but this change was not significant.

Western blot analysis of whole-cell extracts also demonstrated an increase in TDO2 and KYNU proteins in TNBC cell lines (MDA-231, BT549, and SUM159) grown in suspension for 24 hours (Fig. 1D; Supplementary Fig. S3A). The increase in TDO2 protein was confirmed by IHC in BT549 cells grown in suspension for 48 hours compared with cells grown in the attached condition (Fig. 1E).

Global metabolomic profiling of intracellular and secreted metabolites from BT549 cells grown in standard attached conditions or in forced suspension for 24 hours was also performed. Two intermediate products of the kynurenine pathway, Kyn and formylkynurenine, were the intracellular metabolites with the highest fold-change increase in suspension. Among secreted metabolites, kynurenine had the third-highest fold-change increase (Supplementary Fig. S2). Together with the gene expression data, this demonstrates that the kynurenine pathway is strongly upregulated in TNBC cells upon loss of attachment.

Using HPLC to verify the metabolomic profiling data, we found that secreted Kyn levels were more than 2-fold higher in conditioned media from BT549 cells in forced suspension for 48 hours than in media from the same number of cells in the attached condition (Fig. 1F). Furthermore, addition of the TDO2-specific inhibitor 680C91 to cells in suspension completely prevented the increase in secreted Kyn, demonstrating that increased secretion of Kyn in TNBC cells in suspension is dependent on TDO2 activity (Fig. 1F). TDO2 mRNA was expressed at a higher copy number at baseline than IDO1 in two of the three TNBC cell lines tested, and TDO2 expression increased more strongly in suspension than IDO1 (Supplementary Fig. S3B).

Aryl hydrocarbon receptor expression and activity are increased in suspension

In addition to the components of the kynurenine pathway, AhR and AhR-regulated genes were also identified among the genes most highly upregulated by TNBC cells in suspension (Fig. 1A and Supplementary Fig. S4). Increased AhR mRNA expression in suspended compared with attached TNBC cells was confirmed by qRT-PCR. Similar to TDO2 and KYNU, AhR was not significantly increased in ER⁺ cell lines in suspension (Fig. 2A). Furthermore, AhR protein was increased in all three TNBC cell lines tested following 24 hours in suspension as tested by Western blot (Fig. 1D; Supplementary Fig. S3A), and in BT549 cells grown in suspension for 48 hours as measured by IHC (Fig. 2C). In attached SUM159 cells, while AhR protein was found in both the cytoplasmic and nuclear fractions, after 48 hours in forced suspension, AhR protein was almost exclusively nuclear (Fig. 2B). Because AhR nuclear translocation and transcriptional activity are known to be ligand-mediated (32), the movement of AhR to the nucleus in suspension suggests that the receptor is ligand-bound and transcriptionally active.

We assessed AhR transcriptional activity using a luciferase reporter containing six dioxin response elements (DRE), the consensus AhR-binding site. In BT549 and MDA-231 cells, luciferase activity was upregulated more than 2-fold in suspended compared with attached cells (Fig. 2D). The AhR antagonist α-naphthoflavone inhibited the suspension-mediated increase in luciferase activity, demonstrating specificity of the reporter construct for AhR activity (Fig. 2D; Supplementary Fig. S3C).

TDO2-mediated kynurenine production activates AhR

Kyn is an endogenous ligand for AhR (12, 33–35), suggesting a potential relationship between increased kynurenine pathway enzyme expression and increased AhR activity in TNBC cells in suspension. First, we tested whether inhibition of TDO2 and consequent Kyn production could prevent AhR activation in suspended TNBC cells. Treatment with 10 μmol/L 680C91, which prevented the suspension-induced increase in Kyn, significantly decreased AhR activity in BT549 and MDA-MB-231 cells in suspension (Fig. 2E; Supplementary Fig. S3D). Furthermore, the suspension-mediated increase in AhR target genes CYP1A1 and CYP1B1 was abrogated by both the AhR inhibitor CH-223191 and the TDO2 inhibitor 680C91 (Fig. 2F), linking TDO2 activity to AhR transcriptional activity. Addition of 100 μmol/L exogenous Kyn also resulted in a significant increase in AhR luciferase reporter activity in TNBC cells in both attached and suspended culture conditions (Fig. 2G), demonstrating that Kyn is able to activate AhR in TNBC cells.

Inhibition of TDO2 and AhR decreases TNBC anoikis resistance, proliferation, migration, and invasion

Because TDO2 and AhR were upregulated by TNBC cells in suspension, we next tested their functional importance by assessing whether pharmacologic inhibition or genetic knockdown could reduce anchorage-independent growth in soft agar or increase sensitivity of TNBC cells to anoikis in forced suspension culture. BT549 and SUM159PT cells were pretreated in the attached condition with vehicle, the TDO2 inhibitor 680C91, or the AhR antagonist CH-223191 for 24 hours. Then an equal number of cells were plated in soft agar in the continued presence of treatment. Both 680C91 and CH-223191 significantly decreased anchorage-independent growth of BT549 and SUM159 cells in soft agar, demonstrating that these inhibitors further reduce TNBC cell anoikis-resistance.
To test the effect of Kyn on anoikis resistance, we treated cells in forced suspension culture for 48 hours with Kyn and found that this significantly decreased apoptosis as measured by cleaved caspase activity compared with vehicle control treatment (Fig. 3B). We then performed knockdown of TDO2 and AhR using two shRNA constructs each, and decreased protein expression was confirmed by Western blot (Fig. 3C). Knockdown of either TDO2 or AhR also significantly decreased growth of BT549 cells in soft agar (Fig. 3D). Knockdown of either TDO2 or AhR also significantly increased apoptosis in BT549 and MDA-MB-231 cells grown in suspension for 48 hours (Fig. 3E), demonstrating that TDO2 and AhR promote survival in anchorage-independent conditions.

Figure 1.
Kynurenine pathway components and activity are increased in TNBC cells in suspension and suppressed by TDO2 inhibition. A, BT549 cells were grown in either attached or forced-suspension conditions on poly-HEMA–coated plates in biologic quadruplicate for 24 hours. RNA was harvested at 24 hours and hybridized onto Affymetrix Human Gene 1.0ST array. Gene expression profile analysis identified genes significantly upregulated in suspension culture (P < 0.05). Those involved in tryptophan metabolism and AhR signaling are highlighted in red. B and C, relative TDO2 (B) and KYNU (C) mRNA levels were determined by qRT-PCR in luminal and TNBC cell lines grown in attached or suspended culture for 24 hours. D, Western blot for TDO2, KYNU, and AhR protein in three TNBC cell lines following 24 hours of attached or suspended culture. E, TDO2 protein detected by IHC in BT549 cell pellets grown in attached or suspended culture for 48 hours. Bar, 50 μm. F, secreted kynurenine levels, as measured by HPLC, from BT549 cells grown for 48 hours in the attached versus suspended conditions or suspended plus 10 μmol/L 680C91 (a specific TDO2 inhibitor). *, P < 0.05; **, P < 0.001; ***, P < 0.0001 by ANOVA with Bonferroni multiple comparison test.
Figure 2. AhR expression and localization are affected by suspension culture, and kynurenine activates AhR in TNBC cells in suspension. Breast cancer cell lines were plated on uncoated (attached) or poly-HEMA-coated (suspended) tissue culture plates for 24 hours. A, relative AhR levels measured by qRT-PCR in ER+ or TNBC cells in attached or suspended culture for 24 hours. B, Western blot for AhR levels in nuclear and cytoplasmic fractions of SUM-159 cells in attached or suspended culture for 24 hours. C, AhR protein as detected by IHC in BT549 cell pellets grown in attached or suspended culture for 48 hours. Bar, 50 μm. D and E, AhR reporter activity in BT549 cells in attached or suspended culture for 24 hours treated with vehicle [0.1% MeOH:EA (3:1); D] or 10 mmol/L α-Naphthoflavone (AhR antagonist) or vehicle (0.1% DMSO; E) or 10 mmol/L 680C91 (TDOi). F, relative expression of AhR target genes by qRT-PCR in BT549 cells in attached or suspended culture treated with vehicle, 680C91, or CH-223191 (AhR antagonist) for 24 hours. G, AhR reporter activity in BT549 cells in attached or suspended culture for 24 hours treated with vehicle (2.5 × 10⁻⁶ M HCl) or crystalline kynurenine (100 μmol/L). *, P < 0.05; **, P < 0.01; ****, P < 0.0001 by ANOVA with Bonferroni multiple comparison test.
To determine if TDO2 and AhR also mediate growth of TNBC cells, MDA-MB-231 and BT549 cells were treated with 680C91 or CH-223191, and cell number was measured over time. Both inhibitors significantly decreased the number of cells when grown in traditional attached conditions, and the combination was more effective than either drug alone (Fig. 4A). MDA-MB-231, BT549, and SUM159 cells were also treated with a range of 680C91 concentrations, and GI50 values were calculated as 20 μmol/L, 61 μmol/L, and 102 μmol/L, respectively (Supplementary Fig. S5). Knockdown of TDO2 or AhR similarly resulted in decreased growth of MDA-231 cells (Supplementary Fig. S6), demonstrating a role for TDO2 and AhR signaling in baseline growth of TNBC cells in vitro even in attached culture.

Figure 3.
Targeting the kynurenine pathway or AhR increases cell death of TNBC cells in suspension. A, anchorage-independent growth of SUM-159 or BT549 cells pretreated with vehicle (0.01% DMSO), 10 μmol/L 680C91 (TDOi), or 10 μmol/L CH-223191 (AhR antagonist) for 24 hours, then plated in 0.25% soft agar where respective treatments were maintained for 18 to 21 days. B, caspase 3/7 activity in TNBC cells (BT549 and SUM159) treated with either vehicle control or 100 μmol/L kynurenine while cultured in suspension for 48 hours. C, Western blot of TDO2 or AhR in attached BT549 or MDA-MB-231 cells following transduction with nontargeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2. D, anchorage-independent soft-agar growth of BT549 cells with knockdown of TDO2 or AhR grown in soft agar for 14 days. E, caspase 3/7 activity in BT549 or MDA-MB-231 cells transduced with nontargeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2, and plated in poly-HEMA–coated plates for 48 hours. **, P < 0.01; ****, P < 0.0001 by ANOVA with Bonferroni multiple comparison test.

To determine if TDO2 and AhR also mediate growth of TNBC cells, MDA-MB-231 and BT549 cells were treated with 680C91 or CH-223191, and cell number was measured over time. Both inhibitors significantly decreased the number of cells when grown in traditional attached conditions, and the combination was more effective than either drug alone (Fig. 4A). MDA-MB-231, BT549, and SUM159 cells were also treated with a range of 680C91 concentrations, and GI50 values were calculated as 20 μmol/L, 61 μmol/L, and 102 μmol/L, respectively (Supplementary Fig. S5). Knockdown of TDO2 or AhR similarly resulted in decreased growth of MDA-231 cells (Supplementary Fig. S6), demonstrating a role for TDO2 and AhR signaling in baseline growth of TNBC cells in vitro even in attached culture.
In addition, pharmacologic inhibition of TDO2 and AhR each significantly reduced migration of MDA-MB-231 and BT549 cells in a scratch wound assay, and again the combination was more effective than either inhibitor alone (Fig. 4B). Knockdown of either TDO2 or AhR recapitulated this effect, significantly diminishing migration in the scratch wound assay in both BT549 and MDA-231 cells (Fig. 4C and D).

Figure 4.
TDO2 and AhR inhibition decreases cell number, migration, and invasion of TNBC cells. BT549 and MDA-MB-231 cells (TNBC) were treated with either vehicle control (0.01% DMSO), 10 μmol/L 680C91 (TDOi), 10 μmol/L CH-223191 (AhR antagonist), or in combination. A, crystal violet assay of BT549 and MDA-MB-231 cells treated for 8 days. B, scratch wound assay of BT549 and MDA-MB-231 cells treated in media containing 0.5% FBS. C and D, scratch wound assay of BT549 and MDA-MB-231 cells transduced with a nontargeting control (shNeg) or shRNAs targeting AhR (shAhR-85, shAhR-86; C) or TDO2 (shTDO2-82, shTDO2-98; D). E, Boyden chamber invasion assay of SUM-159 cells pretreated with either vehicle control (0.01% DMSO), 10 μmol/L 680C91 (TDOi), or 10 μmol/L CH-223191 (AhR antagonist) in the attached or suspended conditions for 24 hours, then seeded into the upper chamber and allowed to invade through a Matrigel-coated membrane. *, P < 0.05; **, P < 0.01; ****, P < 0.0001 by ANOVA with Bonferroni multiple comparison test.
To test whether the increased TDO2 and AhR expression observed in cells grown in suspension affects invasive capacity, SJM159PT cells were grown for 48 hours either in the attached condition, or in forced suspension culture with or without addition of 10 μmol/L of the TDO2 inhibitor 680C91 or the AhR inhibitor CH-223191. After 24 hours, 25,000 viable cells were plated on Matrigel-coated transwell invasion chamber with continuous treatment. Cells grown in suspension for 24 hours were significantly more invasive than control cells grown in the attached condition (Fig. 4E). The addition of the TDO2 inhibitor 680C91 during suspension culture greatly decreased the invasive capacity of viable cells. Although the cells treated with the AhR inhibitor showed decreased invasion, this effect was not statistically significant (Fig. 4E).

NF-κB regulates expression of kynurenine pathway genes and AhR in TNBC cells

We previously demonstrated that NF-κB activity is substantially increased in TNBC cells in suspension, but not in ER+ cells (31). Here, we again demonstrate increased NF-κB activity in TNBC cells in suspension as measured by an NF-κB luciferase reporter, and this increase is not observed using a reporter containing a mutated binding site (Fig. 5A). Interestingly, a recent global profiling study of ovarian cancer in vivo found that knockdown of Ilcx-epsilon, a positive regulator of NF-κB activity, decreased expression of TDO2 and KNYU, suggesting NF-κB regulation of these genes (36); however, the regulatory mechanism was not directly tested.

To investigate the link between increased NF-κB activity, TDO2, and KNYU, we treated BT549 cells with a cocktail of TNPt and IL1β to activate NF-κB and found that expression of TDO2, KNYU, and AhR (Fig. 5B), as well as the NF-κB target genes IL6 and IL8 (data not shown), was significantly increased at 24 hours. Next, we tested whether inhibition of NF-κB in suspension could reduce upregulation of Kyn pathway genes and AhR in TNBC cells grown in suspension. Upstream inhibition of NF-κB with the Ikκ inhibitor PS1145 significantly reduced the expression of TDO2, KNYU, and AhR (Fig. 5C), as well as the NF-κB target gene IL6 (not shown), demonstrating that NF-κB activity mediates the increased TDO2, KNYU, and AhR expression in TNBC cells in suspension. Lastly, a constitutively active form of Ikκ, a negative regulator of NF-κB, was expressed in BT549 cells, and this ablated the expression-induced increase in TDO2, KNYU, and AhR (Fig. 5D). These data demonstrate that the increased transcription of TDO2, KNYU, and AhR in suspension is specifically induced by and dependent on NF-κB in TNBC.

TDO2 inhibition decreases metastatic colonization in vivo

To test the potential contribution of TDO2 activity to the metastatic capacity of TNBC cells in vivo, we grew luciferase-expressing MDA-MB-231 cells in forced suspension conditions for 48 hours in the presence of either vehicle control or the TDO2 inhibitor 680C91. A total of 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD/SCID mice, and luminescence was monitored over time. Seven days after injection, mice that received vehicle-treated cells had significantly higher luminescence compared with mice that received cells treated with the TDO2 inhibitor (Fig. 6C). A significant decrease in the number of metastatic nodules in the lungs from mice receiving cells treated with 680C91 was also observed by hematoxylin and eosin (H&E; Fig. 6D). Together, these data demonstrate that TDO2 inhibition decreases the ability of TNBC cells to successfully metastasize following tail vein injection in vivo.

TDO2 is more highly expressed in ER− than ER+ breast cancer and correlates with poor prognosis

To determine the clinical relevance of our findings, we examined TDO2 expression in primary TNBC samples by IHC. As shown in Fig. 7A, primary TNBC were strongly positive for TDO2 expression. We evaluated TDO2 gene expression in patient samples using publicly available gene expression microarray datasets from Oncomine. The Curtis and colleagues dataset (37) had the largest patient population (n = 1,998) and was therefore selected for analysis. Notably, TDO2 was the sixth most highly overexpressed gene in breast carcinoma compared with normal breast tissue (top 1% of overexpressed genes, P < 0.0001; Fig. 7B). TDO2 gene expression was significantly higher in ER-negative breast tumors compared with ER-positive tumors (P < 0.001; Fig. 7C). High TDO2 expression was also associated with increasing grade (P < 0.001; Fig. 7D), supporting the hypothesis that tumors with high TDO2 expression may have increased metastatic potential. Similarly, IDO1 was overexpressed in breast carcinoma compared with normal breast tissue, although it was not as highly overexpressed as TDO2 (Supplementary Fig. S7A). IDO1 was also more highly expressed in ER− than ER+ breast tumors in both the Curtis and TCGA datasets (Supplementary Fig. S7B and S7C).

Finally, patients whose tumors had above-median TDO2 expression had approximately 3 years shorter overall survival when compared with those with below-median TDO2 expression (median overall survival 10.62 years vs. 13.31 years, respectively; P = 0.0002, log-rank test; Fig. 7E), suggesting that TDO2 expression may contribute to tumor progression and poor prognosis. Similarly, patients with above-median IDO1 expression had a shorter survival than those with lower IDO1, but the difference in survival was not as large (Supplementary Fig. S7D). This further supports the importance of targeting TDO2 in breast cancer patients, possibly in combination with IDO1, rather than targeting IDO1 alone as in current clinical trials.

Discussion

TNBC has no effective targeted therapies and the worst prognosis of the breast cancer subtypes, due in part to its propensity for rapid metastasis (3). Indeed, patients with metastatic TNBC have a median survival of only 13 months (5). However, few studies have examined pathways that support anoikis resistance in TNBC. In this study, global gene expression profiling revealed the novel finding that TNBC cells in forced suspension upregulate two related pathways: AhR signaling and the kynurenine pathway of tryptophan catabolism. Indeed, global metabolic profiling identified formylkynurenine and Kyn as the most highly upregulated intracellular metabolites, and Kyn as the third most-highly
increased secreted metabolite in conditioned media from cells in forced suspension.

Based on our data demonstrating that TNBC cells in suspension upregulate AhR and TDO2, increase secretion of Kyn, and rely on TDO2 and AhR for anchorage-independent growth and invasive potential, we hypothesized that suspended TNBC cells utilize an autocrine signaling loop in which Kyn activates AhR to support anoikis resistance and anchorage-independent growth, as well as migration and invasion (Fig. 7F). Central to this model is upregulation of TDO2, a key enzyme in the Kyn pathway. Our observation that pharmacologic inhibition of TDO2 decreased the ability of MDA-MB-231 cells to form lung...
metastases in vivo suggests that TDO2 inhibition represents a novel opportunity for targeted therapy to inhibit TNBC metastasis. Further, we show that the Kyn pathway components TDO2 and KYNU, as well as AhR, are regulated by the transcription factor NF-kB in suspension. We previously identified a different
NF-kB–regulated autocrine signaling loop involving the neurotrophic tyrosine kinase, type 2 (NTRK2 or TrkB), and the TrkB ligand neurotrophin 3 (NTF3) that promotes survival of TNBC cells in suspension (31), suggesting that increased NF-kB signaling in suspended cells supports multiple prometastatic attributes of TNBC.

In addition to its autocrine role affecting growth, migration, and invasion of tumor cells that we describe here, the Kyn pathway may also facilitate metastasis through paracrine signaling mechanisms involving suppression of immune surveillance. Specifically, TDO2-mediated Kyn production prevented T-cell–mediated immune rejection of the immunogenic P815 mouse tumor cell line in immunized mice (38). In dendritic cells, IDO1 activity can lead to the AhR-dependent generation of regulatory T cells via a coordinated increase in Kyn and decrease in tryptophan (33). Inhibition of IDO1 also sensitizes cancer cells to chemotherapy in a T-cell–dependent manner (39).

Figure 7. TDO2 expression in breast cancer clinical samples. A, TDO2 staining in primary TNBC patient samples; bar, 50 μm. B–E, using the Curtis breast cohort, TDO2 expression was compared between normal breast and invasive ductal carcinoma (B). TDO2 gene expression in breast tumor samples divided by ER+ versus ER− (C) or by tumor grade (D). E, patients were then split into TDO2–high or –low groups based on median TDO2 gene expression, and overall survival was plotted. F, a model of TDO2-AhR signaling in TNBC cells. NF-kB activity increases in suspension, increasing expression of TDO2, which leads to increased Kyn (both intracellular and secreted), which subsequently activates AhR and promotes anoikis resistance, migration, and invasion.

P < 0.001 by log-rank test.
Multiple clinical trials testing the efficacy of Indoximod (1-methyl-D-tryptophan), an IDO1/2 inhibitor, are currently under way, including one in metastatic breast cancer in combination with conventional chemotherapies or immunotherapy (NCT01792050). These trials are based on the ability of IDO1/2 inhibition to reverse immune suppression and enhance the antitumor immune response. However, 1-MT does not target TDO2 (40). Importantly, our data combined with data mined from publicly available breast cancer clinical cohorts suggest that TDO2 may be the more relevant target in TNBC. Interestingly, the membrane protein responsible for tryptophan import into cells, LAT1, is also more highly expressed in TNBC than in other breast cancer subtypes (41). Although a recently published analysis of serum metabolite concentrations in breast cancer patients found no difference in tryptophan levels between ER+ and ER- breast cancer patients, median levels of Kyn were significantly higher in patients with ER+ tumors (42). Although this finding was attributed to IDO1 activity, we find that that TDO2 is higher in ER+ tumors than ER- in two publicly available datasets, and that TDO2 expression is higher than IDO1 at baseline in two of three TNBC cell lines tested and TDO2 increases more strongly in suspension in all cell lines. Lastly, and perhaps the strongest indicator of the relative role of TDO2 versus IDO1, blocking TDO2 activity completely prevented the detachment-induced increase in Kyn secretion observed in TNBC cells in suspension (Fig. 1F).

Collectively, our results suggest that in TNBC, TDO2 is an important contributor to Kyn production, and thus should be considered as a therapeutic target. High TDO2 in primary breast tumors from publicly available datasets correlates with shorter overall survival. TDO2 was significantly upregulated in metastases of leiomyosarcoma patients compared with primary tumors (43), and increased KYNU expression is associated with metastasis of breast cancer cells to the lung and brain (44, 45). In addition to abrogating the autocrine protumorigenic effects of Kyn, TDO2 inhibition may also inhibit Kyn activation of AhR in immune cells and thereby reduce TNBC immune evasion, but this remains to be tested.

Although we demonstrate a significant increase in Kyn levels in TNBC cells in suspension, it is possible that the effects of TDO2 could be mediated in part through production of other intermediate molecules or NAD+ production. However, AhR nuclear localization and transcriptional activity are clearly increased in TNBC in suspension culture, and other recent studies find that AhR affects growth and migration of breast cancer cells (27, 28) and castrate-resistant prostate cancer cells (46). Studies examining AhR expression in breast cancer primary tumors found that higher AhR expression correlates with good prognosis (47). However, our data suggest that AhR expression, nuclear localization, and transcriptional activity may be low until cells begin the metastatic process by detaching from the primary tumor. Thus, AhR in the primary tumor may not reflect its role in supporting the metastatic cascade.

The independent tumor-promoting roles of the Kyn pathway (particularly via IDO1) and AhR signaling have been recognized separately for many years. Recently, the connection of these two pathways via Kyn serving as an AhR ligand was discovered (12, 33–35). Our study demonstrates that the TDO2-AhR signaling axis may be of particular importance in TNBC because it is strongly activated in this highly metastatic breast cancer subtype in response to detachment, and supports anchorage-independent survival and invasive capacity. The combination of protumorigenic autocrine effects and potential immunosuppressive paracrine signaling effects makes this pathway an attractive target for therapeutic intervention. Our findings that inhibition of the Kyn or AhR pathways decreased in vitro characteristics associated with multiple steps in the metastatic cascade, and that pharmacologic inhibition of TDO2 decreased TNBC metastasis in vivo, suggest that inhibitors of TDO2 may represent an exciting opportunity for targeted therapy for TNBC to decrease mortality from this aggressive breast cancer subtype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer

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