Aryl Hydrocarbon Receptor Agonists Induce MicroRNA-335 Expression and Inhibit Lung Metastasis of Estrogen Receptor Negative Breast Cancer Cells

Shu Zhang1, KyoungHyun Kim1, Un Ho Jin1, Catherine Pfent2, Huojun Cao1, Brad Amendt1, Xinyi Liu1, Heather Wilson-Robles3, and Stephen Safe1,4

Abstract

The aryl hydrocarbon receptor (AHR) was initially identified as a receptor that bound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related environmental toxicants; however, there is increasing evidence that the AHR is an important new drug target for treating multiple diseases including breast cancer. Treatment of estrogen receptor (ER)-negative MDA-MB-231 and BT474 breast cancer cells with TCDD or the selective AHR modulator 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) inhibited breast cancer cell invasion in a Boyden chamber assay. These results were similar to those previously reported for the anti-metastatic microRNA-335 (miR-335). Both TCDD and MCDF induced miR-335 in MDA-MB-231 and BT474 cells and this was accompanied by downregulation of SOX4, a miR-335-regulated (inhibited) gene. The effects of TCDD and MCDF on miR-335 and SOX4 expression and interactions of miR-335 with the 3’-UTR target sequence in the SOX4 gene were all inhibited in cells transfected with an oligonucleotide (iAHR) that knocks down the AHR, thus confirming AHR-miR-335 interactions. MCDF (40 mg/kg/d) also inhibited lung metastasis of MDA-MB-231 cells in a tail vein injection model, showing that the AHR is a potential new target for treating patients with ER-negative breast cancer, a disease where treatment options and their effectiveness are limited. Mol Cancer Ther; 1–11. ©2011 AACR.

Introduction

The aryl hydrocarbon receptor (AHR) was initially identified as an intracellular protein that bound with high affinity to the environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; ref. 1). It was also shown that the AHR bound other structurally related aromatic hydrocarbons and several carcinogenic polycyclic aromatic hydrocarbons. Studies with AHR−/− mice confirmed a role for this receptor in mediating the toxic and carcinogenic effects of these compounds (2–4). Subsequent studies showed that the AHR binds structurally diverse compounds including aromatic compounds, flavonoids, and many other phytochemicals, various drugs including sulindac and omeprazole, and endogenous biochemicals such as bilirubin, arachadonic acid derivatives, and indigoids (5–12).

The identification of AHR ligands with well-described health-promoting or beneficial pharmaceutical effects has also spurred research on development of drugs that target the AHR for treatment of specific tumors, immune disorders, and inflammatory disease and also for enhanced production of hematopoietic stem cells (13–21). 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) was initially characterized as an AHR antagonist that blocked TCDD-induced toxicities in rodent models (22–25); however, MCDF did not inhibit TCDD-induced antiestrogenic activity in the rodent uterus, human breast cancer cells, or tumors (26–29). MCDF exhibited AHR agonist activity for these responses and, in carcinogen-induced female Sprague–Dawley rats, MCDF alone and in combination with tamoxifen was a potent inhibitor of mammary tumor growth but did not inhibit tamoxifen-induced bone lengthening (29). In subsequent studies, we showed that MCDF, TCDD, and related halogenated aromatics inhibited growth of several estrogen receptor (ER)-negative breast cancer cell lines (30), suggesting a potential role for selective AHR modulators (SAHRMs) such as MCDF for treatment of patients with ER-negative tumors for which the prognosis is poor and treatment options are limited primarily to cytotoxic drug therapy (31).

AHR agonists also inhibit breast cancer cell invasiveness and colony formation and promote breast cancer cell...
of differentiation (32), and we hypothesized that this may be due, in part, to AHR-microRNA (miR) interactions. Using a miR array as a screening tool, we identified microRNA-335 (miR-335) as an Ah-responsive miR induced by MCDF and TCDD in ER-negative breast cancer cells. MiR-335 is an antimetastatic miR, and overexpression of miR-335 in the MDA-MB-231 LM2 cell variant inhibits breast cancer cell invasion and metastasis in vivo (33). This study reports that AHR agonists MCDF and TCDD induce miR-335 and decrease SOX-4 expression in breast cancer cells, and MCDF inhibits lung metastasis of these cells in an in vivo tail vein injection model. These results show the potential clinical importance of the AHR as a drug target for inhibiting breast cancer metastasis and indicate a role for AHR-miR-335 interactions in mediating inhibition of tumor metastasis.

Materials and Methods

Cell lines and cell culture

The MDA-MB-231 and BT474 cell were purchased from the American Type Culture Collection (ATCC) and were authenticated as indicated below.

MDA-MB-231 from ATCC:


BT474 from ATCC:

http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-20&Template=cellBiology

Cells were initially grown and multiple aliquots were frozen and stored at −80°C for future use. Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. MDA-MB-231 and BT474 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS and 10 ml/L charcoal-stripped FBS for 16 hours and then treated with either vehicle (DMSO) or the compounds. Cellular lysates and their subsequent separation by electrophoresis was carried out as previously described using β-actin as a loading control (30). Total RNA was extracted, real-time PCR was carried out as previously described and normalized to TBP (34). The PCR profile was as follows: one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The comparative CT method was used for relative quantitation of samples. The following primers for CYP1A1, SOX4, TBP, and PTPRN2 were synthesized by Integrated DNA Technologies: CYP1A1, forward, 5’-CTT CAG ACA TCT TCT CGG-3’; reverse, 5’-GTT TGA TCT GCC ACG GTT T-3’; SOX4, forward, 5’-CAA ACC AAT GCC GAG AAC-3’; reverse, 5’-CTC TTT TTT GCC GCC GAT-3’; TBP, forward, 5’-TGG ACA GGA GCC AAG AGT GAA-3’; reverse, 5’-CAT ACC ACA GCT CCC CAC CA-3’; PTPTN2, forward, 5’-AGT GGC TGC CCA TTC TGC TGT-3’; reverse, 5’-GCA CTG TTG CTC CTC CAC-3’.

Antibodies and reagents

CYP1A1 and β-actin antibodies were purchased from Santa Cruz Biotechnology. All the short interfering RNAs were prepared by Dharmacon Research. Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega. β-Galactosidase (β-Gal) reagent was obtained from Tropix. Lipofectamine reagents were supplied by Invitrogen. Western Lightning chemiluminescence reagents were from Perkin-Elmer Life Sciences. SOX4 3’-UTR reporter construct was generously provided by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY). MCDF and TCDD were synthesized in this laboratory to more than 98% purity as determined by gas chromatographic analysis.

Scratch assay and Boyden chamber assay

Cells were seeded in 6-well plates and allowed to attach for 16 hours. The medium was then changed to DMEM/F-12 medium containing 2.5% charcoal-stripped FBS, and then treated with either vehicle (DMSO) or the compounds for 24 hours before the scratch was made. A scratch through the central axis of the plate was gently made using a sterile pipette tip. Cells were 70% confluent when the scratch was made. Cells were then washed and treated with either vehicle (DMSO) or the compounds. Migration of the cells into the scratch was observed at 9 preselected points (3 points per well) at 0, 8, and 16 hours. Results of this study were obtained at a 16-hour time point. A 48-well micro-chemotaxis chamber (Corning Incorporated) was used for Boyden chamber assay. To evaluate invasion, the 8-μm pore filter separating the 2 wells was coated with Matrigel (Becton-Dickinson Labware; 50 μg/filter). Either vehicle (DMSO) or the compounds were dissolved in culture medium and placed in both the upper and lower well, whereas 5 × 10^3 cells were added to each upper well. After 24 hours, the filter was removed and fixed with methanol. Cell invasion was evaluated by counting spread cells adhering to the lower filter surface.

Western blot analysis and quantitative real-time PCR

Cells (2 × 10^5) were plated in 6-well plates in DMEM/F-12 (Sigma-Aldrich) media containing 2.5% charcoal-stripped FBS for 16 hours and then treated with either vehicle (DMSO) or the compounds. Cellular lysates and their subsequent separation by electrophoresis was carried out as previously described using β-actin as a loading control (30). Total RNA was extracted, real-time PCR was carried out as previously described and normalized to TBP (34). The PCR profile was as follows: one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The comparative CT method was used for relative quantitation of samples. The following primers for CYP1A1, SOX4, TBP, and PTNR2 were synthesized by Integrated DNA Technologies: CYP1A1, forward, 5’-CTT CAG ACA TCT TCT CGG-3’; reverse, 5’-GTT TGA TCT GCC ACG GTT T-3’; SOX4, forward, 5’-CAA ACC AAT GCC GAG AAC-3’; reverse, 5’-CTC TTT TTT GCC GCC GAT-3’; TBP, forward, 5’-TGG ACA GGA GCC AAG AGT GAA-3’; reverse, 5’-CAT ACC ACA GCT CCC CAC CA-3’; PTNR2, forward, 5’-AGT GGC TGC CCA TTC TGC TGT-3’; reverse, 5’-GCA CTG TTG CTC CTC CAC-3’.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was done using ChIP-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif) according to manufacturer’s protocol. BT474 or MDA-MB-231 cells (5 × 10^5 cells)
were treated with DMSO, TCDD, or MCDF for 1 hour and after several steps including reversing DNA cross-links, DNA was prepared by proteinase K digestion followed by PCR amplification. The 335-DRE14 primers were 5'-CAG GAG TGG GAC TAG CCC TCC TTG G-3' (sense), and 5'-GTT TCT TCT ACC ACC CCG AAG TGG AGC-3' (antisense). The 335-DRE56 primers were 5'-AGTCA TCCGC TAGCTGGCGTCCAC-3' (sense), and 5'-TGG ACC TGG ACC ACC ACC TGC AG-3' (antisense). The 335-DRE7 primers were 5'-TTC CCT ACG ATG AAA TTC TCT TGC-3' (sense), and 5'-CGA AGG GTG GTC TTG AAT GAT GAT G-3' (antisense). The positive control primers were 5'-TCA GGG CTT CAG CCG TAG CCG TCT TCC T-3' (sense), and 5'-GCT ACA GCC TAC CAG GAC TCG GCA G-3' (antisense), and they amplified a 122-bp region of human Cyp1A1 promoter (35).

**Cell transfection and RNA interference**

Cells were seeded in 6- or 12-well plates in phenol red-free DMEM/F12 medium (Sigma-Aldrich) supplemented with 2.5% dextran/charcoal-stripped FBS. After 16 to 20 hours when cells were 50% confluent, appropriate amounts of plasmids and short interfering RNA duplexes were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the recommendations of the manufacturer and as previously described (30). After incubation, cells were collected for Western blot analysis and quantitative real-time PCR assay.

**Tail vein injection of cells for metastasis in athymic mice**

Mice were purchased from Harlan, MDA-MB-231 cancer cells (10<sup>5</sup> cells/animal) were introduced through tail-vein injection. After 6 days, mice were gavaged daily for 21 days with MCDF (40 mg/kg/d; 7 mice) or corn oil (vehicle; 7 mice), then euthanized and lungs were examined. H&E-stained slides were scanned to create a high-quality TIF image using the Nikon Super COOLSCAN 5000. All nonlung tissue was edited from the image using Imagej software (NIH; ref. 36). The image was converted into an 8-bit binary image and the number of pixels was calculated. Next, all normal lung tissue was edited from the images so that only tumor metastases remained and the process was repeated to calculate pixels. To determine the percentage of tumor to total lung, the number of pixels from the tumors was divided by the number of pixels from the total lung tissue.

**Statistical analysis**

Statistical significance was determined by ANOVA and Scheffe’s test and the levels of probability are noted. The results are expressed as means ± SEM for at least 3 separate experiments for each treatment group.

**Results**

Figure 1A shows that 10 nmol/L TCDD and 5 μmol/L MCDF inhibited MDA-MB-231 cell migration in a scratch assay, whereas no significant migration was observed in control or treated BT474 cells (data not shown). Supplementary Fig. S1 shows that MCDF (but not TCDD) inhibited proliferation of BT474 and MDA-MB-231 cells using the MTT assay, and this effect may contribute to inhibition of MDA-MB-231 cell migration by MCDF. Results in Fig. 1B and C show that 10 and 20 nmol/L TCDD and 5 and 10 μmol/L MCDF significantly decreased MDA-MB-231 cell invasion in a Boyden chamber assay, and Fig. 1D confirms that 10 and 20 nmol/L TCDD and 5 μmol/L MCDF also inhibited BT474 invasion in the Boyden chamber assay. Results illustrated in Fig. 2A show that inhibition of AHR expression by RNA interference (RNAi) significantly blocked TCDD- and MCDF-mediated inhibition of MDA-MB-231 cell invasion and similar results were observed in BT474 cells (Fig. 2B), showing a role for the AhR in mediating this response. The effectiveness of AHR knockdown is illustrated in Fig. 2C, which shows that iAHR significantly decreased AHR protein levels in both MDA-MB-231 and BT474 cells.

We hypothesized that the AHR-dependent inhibition of cell migration may be, in part, to regulation of miRs and, the effects of TCDD and MCDF were initially investigated using a cancer microRNA-PCR array (from SA Biosciences). On the basis of published results (33), we also examined the effects of these compounds on miR-335 expression. Figure 3A confirms that both 10 nmol/L TCDD and 5 μmol/L MCDF significantly induced miR-335 after treatment for 12 and 24 hours. Moreover, the induction of miR-335 in MDA-MB-231 cells treated with TCDD and MCDF for 24 hours was decreased after knockdown of the AHR by RNAi (Fig. 3A). Results in Fig. 3B also show that TCDD and MCDF also induced miR-335 expression in BT474 cells and this response was also abrogated after knockdown of AHR by RNAi confirming that induction of miR-335 by TCDD and MCDF in both cell lines was AHR-dependent. Figure 3C shows that overexpression of miR-335 in MDA-MB-231 cells inhibited cell invasion in a Boyden chamber assay and similar results were observed in BT474 cells (Fig. 3D). The effects of the miR-335 mimic were direct (AHR-independent) and were observed in cells transfected with a nonspecific iCTL oligonucleotide or iAHR. These results confirm that miR-335 directly decreases cancer cell invasion and were comparable with that observed for TCDD and MCDF.
except that the induced miR-335 and inhibition of invasion were AHR-dependent and inhibited by AHR knockdown.

Previous array studies identified SOX4, COL1A1, PLCB1, TNC, MERTK, and PTPNR2 as miR-335–regulated genes in MDA-MB-231 cells (33). Results in Fig. 4A show that treatment of MDA-MB-231 cells with 10 nmol/L TCDD or 5 μmol/L MCDF significantly decreased SOX-4 but not PTPRN2 expression. Similar results were observed for COL1A1, PLCB1, TNC, and MERTK (data not shown). RNAi studies show that repression of SOX4 in MDA-MB-231 cells treated with TCDD or MCDF was significantly inhibited after knockdown of the AHR (iAHR; Fig. 4A) and this consistent with the AHR-dependent upregulation of miR-335 (Fig. 3A). Both TCDD and MCDF also decreased SOX4 mRNA expression in BT474 cells and knockdown of the AHR by RNAi blocked TCDD-/MCDF-mediated SOX4 mRNA downregulation (Fig. 4B) and similar results were observed for SOX4 protein (Fig. 4C). As a positive control for this experiment, Fig. 4C shows that 10 nmol/L TCDD induced CYP1A1 mRNA levels in MDA-MB-231 and BT474 cells, whereas 5 μmol/L MCDF did not induce activity in the former cell line and significantly induced a small response in BT474 cells (Fig. 4D). We also investigated the role SOX4 overexpression on TCDD-/MCDF-mediated inhibition of cell migration (scratch assay) and invasion (Boydchen chamber) in MDA-MB-231 cells. SOX4 partially blocked the inhibitory effects of TCDD and MCDF (Supplementary Figs. S2 and S3).

The SOX4-UTR-luc construct contains a seed sequence that binds miR-335 (33) and, in MDA-MB-231 cells transfected with the SOX4-UTR-luc construct, treatment with TCDD or MCDF decreased luciferase activity (Fig. 5A) and the effects of TCDD and MCDF on luciferase activity were reversed after cotransfection with as-miR-335. In addition, transfection with miR-335 mimic also inhibited luciferase activity in MDA-MB-231 cells transfected with the SOX4-3′-UTR construct (Fig. 5C). Seven major dioxin response elements (DRE) are positioned in the region upstream from miR-335 and treatment of BT474 cells with DMSO, MCDF, or TCDD and analysis of AHR complex binding in a ChIP assay did not identify formation of a TCDD-induced AHR complex (Fig. 5C). In contrast, TCDD and MCDF induced formation of an AHR complex at the proximal DRE in the CYP1A1 gene promoter as previously described (35) and Western blot analysis showed that TCDD and MCDF induced CYP1A1 protein

Figure 1. TCDD and MCDF inhibit cancer cell migration and invasion. A, inhibition of cell migration. MDA-MB-231 cells were treated with 10 nmol/L TCDD or 5 μmol/L MCDF and analyzed for inhibition of cell migration in a scratch assay as described in Materials and Methods. TCDD (B) and MCDF (C) inhibit invasion in MDA-MB-231 (B and C) and BT474 (D) cells. Cells were seeded and treated with DMSO (CTL), TCDD (10 or 20 nmol/L), or MCDF (5 or 10 μmol/L) and the effects on cell invasion were determined in a Boyden chamber assay as described in Materials and Methods. The experiments described in B–D were replicated (3–5) and results are expressed as means ± SE. *, significantly (P < 0.05) decreased invasion by TCDD and MCDF.
The ChIP and Western blot assays were also carried out in MDA-MB-231 cells and interactions of the AHR complex with the DREs was not observed (Supplementary Fig. S4). The identification of more distal cis-elements that bind the AHR complex and are required for the Ah-responsiveness of miR-335 is currently being investigated.

Tail vein injection of MDA-MB-231 cells results in formation of lung tumors (33) and this model was used to compare the effects of corn oil versus MCDF (40 mg/kg/d in corn oil) on lung tumor formation in athymic nude mice. Body weights and lung volumes in untreated (control) and corn oil- and MCDF-treated animals were not significantly different, but there were significantly higher numbers of lung tumor colonies in the corn oil- versus MCDF-treated mice (Fig. 6A). Among the corn oil-treated mice, tumor cells were identified intravascularly, destroying vessel walls and invading the surrounding lung within the peripheral pulmonary interstitium (Fig. 6B). Tumor emboli and metastasis measured up to 1.5 mm in diameter and consisted of dense pleomorphic cells arranged in trabeculae packets and nests supported by minimal stroma. The cells had distinct borders with a moderate amount of cytoplasm. The nuclei were central and round displaying marked anisokaryosis, and 1 to 4 mitotic figures were observed per ×40 field of tumor cells. There was considerable heterogeneity in the expression of miR-335 in individual mice in the corn oil- versus MCDF-treated groups; however, mean values were higher in the latter treatment group but these differences did not reach the \( P < 0.05 \) level of significance (Fig. 5C). The human \( \beta_2 \)M-globulin gene was used as a measure to determine the presence of the human MDA-MB-231 cancer cell line in lung tissue of each animal and levels were high and low in the corn oil- and MCDF-treated animals, respectively, and non-detectable in lungs from untreated mice (Fig. 5C). SOX4 mRNA levels in mice were also highly variable and immunostaining for SOX4 also gave inconsistent results (data not shown).

A histologic image analysis software technique was used to analyze and quantitate breast cancer cell metastasis to the lung in corn oil- and MCDF-treated mice (3/group; Supplementary Fig. S5) and the approach used for this assay is outlined in Materials and Methods. The% tumor area/total lung area in MCDF-treated mice 1 to 3 was 0%, 0.48%, and 0%, whereas in corn oil-treated mice (1–3), the values were 6.58%, 8.44%, and 12.34%, respectively, showing the...
remarkable effects of MCDF as an inhibitor of MDA-MB-231 cell lung metastasis.

Discussion

MiRs are small (~22 nt) noncoding RNAs (ncRNA) that play a critical role in cellular homeostasis and cancer, and it has been estimated that more than 30% of protein-encoding genes are regulated by miRs (37–40). MiRs have been extensively investigated in several tumor types and cell lines and it is clear that these small ncRNAs are important prognostic factors for cancer patients and can play critical roles as tumor promoters and tumor suppressors (41, 42). Moreover, the identification of plasma miRs opens up new possibilities for identification of miRs that may predict patient survival and favorable treatment regimens and for detecting early forms of cancer or pre-cancerous lesions (43). Several miRs play a role in cancer metastasis and these have been designated as metastasis-suppressing and metastasis-promoting metastamirs (44). miR-335 has been characterized as a metastasis-suppressing metastamir that effects cancer cell migration and invasion and in a set of 20 primary breast tumor samples, increased miR-335 expression correlated with enhanced metastasis-free survival for these patients (33).

In MDA-MB-231 breast cancer cells, overexpression of miR-335 decreases invasion in a Boyden chamber assay and overexpression of this miR also decreases lung metastasis (33). Using MDA-MB-231 and BT474 cells, we showed that both TCDD and MCDF also inhibited invasion of these cells in a Boyden chamber assay (Fig. 1). These results complement previous studies showing that these AHR agonists inhibit growth of a panel of ER-negative breast cancer cell lines (30), and Hall and colleagues reported that TCDD also inhibited invasiveness and colony formation in SKBR3 and MDA-MB-231 cells (32). The similarities between the antimetastatic effects of miR-335 and AHR agonists (TCDD/MCDF) prompted us to use a miR cancer array that identified miR-335 as a TCDD-inducible miR. We confirmed that both TCDD and MCDF inhibited MDA-MB-231 and BT474 cancer cell invasion and this response was inhibited after cotransfection with iAHR to knockdown the receptor (Figs. 1 and 2). Moreover, induction of miR-335 in MDA-MB-231 and BT474 cells treated with TCDD and MCDF was inhibited by AHR knockdown (Fig. 3A and B), showing that the antinvasive activity of TCDD and MCDF was related to the induction of miR-335 and associated with AHR-miR-335 interactions.

Previous studies linked the antimetastatic activity of miR-335 to a relative small number of genes and among them, the SOX4 and PTPRN2 3′-UTR sequences interacted most avidly with miR-335 (33). SOX4 is a member of the group C of Sry-related HMG box transcription factors; SOX4 expression is highly variable in different tissues and the gene plays an important role in embryonic development (45). SOX4 is overexpressed in multiple tumor types and in breast cancer cells, and it was reported that knockdown of SOX4 in MDA-MB-231 cell variants decreased lung metastasis in vivo and decreased invasion in vitro (33). TCDD and MCDF did not affect PTPRN2 expression in MDA-MB-231 cells; however, SOX4 mRNA and protein expression were decreased by these AHR agonists and these effects were attenuated by cotransfection with iAHR to knockdown the AHR (Fig. 4B and C). TCDD and
MCDF decreased luciferase activity in MDA-MB-231 cells transfected with p0-UTR-SOX4-luc, which contained the miR-335–binding site (Ref. 33; Fig. 5A), and these results were consistent with AHR-mediated regulation of miR-335-SOX4 (UTR) interactions. Moreover, SOX4 overexpression partially blocks TCDD- and MCDF-mediated inhibition of MDA-MB-231 cell migration and invasion (Supplementary Figs. S2 and S3) and this confirms previous studies showing that SOX4 was a prometastatic factor (33).

The AHR is a ligand-activated nuclear receptor and in most cell lines including ER-negative breast cancer...
cells, TCDD induces CYP1A1 gene expression and this is preceded by recruitment of the AHR complex to cis-acting dioxin response elements (DRE) in the CYP1 promoter (35, 46). Not surprisingly, treatment of BT474 cells with TCDD and MCDF also resulted in recruitment of the AHR complex to the CYP1-DRE (Fig. 5C). MCDF is an AHR agonist/partial antagonist and typically is a weak inducer of CYP1A1 but forms a nuclear AHR complex (47). This is consistent with the results observed in BT474 cells in which suboptimal induction of CYP1A1 was observed (Figs. 4C and 5D), even though an intense AHR-DRE (CYP1A1) band was observed (Fig. 5C).

MiR-335 is located between exon 2 and exon 3 (intron 2) of the MEST gene in chromosome 7. The region surrounding miR-335 contains 7 possible DREs containing the core GCGTG pentanucleotide sequence (Fig. 5B); 6 of these DREs are located approximately 4.8 kp upstream from MEST exon 1 and the other DRE is 1.7 kb upstream. Separate primers were designed to detect AHR complex interactions with DRE1-4, DRE5-6, and DRE7 in a ChIP assay (Fig. 5C). However, the results obtained in replicate assays did not detect AHR binding in BT474 and MDA-MB-231 cells (Fig. 5C and Supplementary Fig. S4). Receptor-dependent activation of genes is dependent on both proximal and distal promoter sequences (48), and both nuclear receptors and the AHR interact with multiple proximal response elements that may or may not affect gene expression (49). These results are also consistent with a role for DNA-bound receptors or transcription factors that affect expression of distal genes (48), and identification of distal DREs required for AHR-mediated induction of miR-335 are currently being investigated.

Tail vein injection of MDA-MB-231 cells resulted in extensive formation of lung tumors in athymic nude mice that adequately represent the development of spontaneous metastatic disease (Fig. 6 and Supplementary Fig. S5). Previous studies with miR-335 knockdown or overexpression showed that miR-335 was an antimetastatic...
miR in breast cancer (33). In mice treated with MCDF (40 mg/kg/d), there was a dramatic inhibition of lung metastasis that was quantitated using histologic image analysis software (Supplementary Fig. S5), and these results are consistent with the in vitro studies showing AHR-dependent induction of miR-335 and inhibition of breast cancer cell invasion. These results suggest that MCDF-mediated induction of miR-335 contributes to the antimetastatic effects of this AHR agonist; however, this does not exclude other AHR-dependent pathways (including other miRs) as contributing factors and these are currently being investigated. A recent study reported that the drug Tranilast, which is also an AHR agonist, prevented breast cancer metastasis in a mouse model; however, the role of miR-335 or other miRs in mediating this response were not reported (50).

Inhibitory AHR-ER cross-talk has been extensively characterized and MCDF alone or in combination with tamoxifen was an indirect antiestrogen and inhibitor of ER-positive mammary tumor growth (29), we now show that the SAhRM MCDF also inhibits metastasis in an ER-negative breast cancer model. This latter response suggest that the AHR may be an important new drug target for treating ER-negative breast cancer and we are currently evaluating the efficacy of other SAhRMs as potential drugs for treatment of this devastating disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

The financial assistance of the NIH (R01-CA142697) and Texas A&M AgriLife is gratefully acknowledged.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 21, 2011; revised September 29, 2011; accepted October 14, 2011; published OnlineFirst October 27, 2011.
Zhang et al.

References


Molecular Cancer Therapeutics

Aryl Hydrocarbon Receptor Agonists Induce MicroRNA-335 Expression and Inhibit Lung Metastasis of Estrogen Receptor Negative Breast Cancer Cells

Shu Zhang, KyoungHyun Kim, Un Ho Jin, et al.

*Mol Cancer Ther* Published OnlineFirst October 27, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0548

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/10/21/1535-7163.MCT-11-0548.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.