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The Aryl Hydrocarbon Receptor (AhR) Contributes to the Proliferation of Human Medulloblastoma Cells

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MOL #77305

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MOL #77305

ABSTRACT:
The AhR, a ligand-activated member of the basic-helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) transcription superfamily, is known to regulate the toxicity of polyaromatic halogenated hydrocarbon environmental chemicals, most notably dioxin. However, the AhR has also been implicated in multiple stages of tumorigenesis. Medulloblastoma (MB), a primary cerebellar brain tumor arising in infants and children, is thought to originate from abnormally proliferating cerebellar granule neuron precursors (GNPs). GNPs express high levels of the aryl hydrocarbon receptor (AhR) in the external germinal layer of the developing cerebellum. Moreover, our lab has previously reported that either abnormal activation or deletion of the AhR leads to dysregulation of GNP cell cycle activity and maturation. These observations led to the hypothesis that the AhR promotes the growth of MB. Therefore, this study evaluated whether AhR serves a pro-proliferative role in an immortalized MB tumor cell line (DAOY). We produced a stable AhR knockdown DAOY cell line (AhR shRNA), which exhibited a 70% reduction in AhR protein levels. Compared to wild type DAOY cells, AhR shRNA DAOY cells displayed an impaired G1 to S cell cycle transition, decreased DNA synthesis, and reduced proliferation. Furthermore, these cell cycle perturbations were correlated with decreased levels of the pro-proliferative gene Hes1 and increased levels of the cell cycle inhibitor p27kip1. Supplementation experiments with human AhR restored the proliferative activity in AhR shRNA DAOY cells. Collectively, our data show that the AhR promotes proliferation of MB cells, suggesting that this pathway should be considered as a potential therapeutic target for MB treatment.
MOL #77305

INTRODUCTION:

The aryl hydrocarbon receptor (AhR), a ligand-activated member of the bHLH-PAS transcription factor family, regulates genes associated with cell growth and differentiation (Puga et al., 2005). Polycyclic aromatic hydrocarbons (PAHs), most notably the potent environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), mediate their developmental toxicity through binding AhR, which leads to the activation of gene programs that disrupt normal cell growth. The AhR has also been associated with various stages of carcinogenesis, such as initiation, progression, and migration (Gasiewicz et al., 2008). AhR activation upregulates xenobiotic metabolizing enzymes, which have been linked to cancer development through altered metabolic clearing of carcinogenic xenobiotics that may also bioactivate procarcinogens (Gasiewicz et al., 2008). Additionally, AhR modulates the expression of growth-associated genes such as c-Myc, Hes-1, p21\textsuperscript{CIP1}, and p27\textsuperscript{KIP1} (Thomsen et al., 2004 and Yang et al., 2005).

Increasing evidence suggests an endogenous role for the AhR in controlling the cell cycle (Puga et al., 2002). For example, mouse embryonic fibroblasts from AhR\textsuperscript{-/-} mice exhibit slower growth and accumulation in the G\textsubscript{2}/M phase of the cell cycle (Elizondo et al., 2000). Additionally, stable knockdown of the AhR in human keratinocytes induces expression of p27\textsuperscript{kip1} and cell cycle arrest (Kalmes et al., 2011). Moreover, AhR expression is elevated in cycling fibroblasts, compared to non-dividing fibroblasts (Vaziri et al., 1996). The AhR-regulated signaling pathways responsible for modulating the cell cycle are, in most cases, unknown. Although these investigations provide considerable evidence that the AhR serves to promote cell growth in certain
tissues, considerable data indicate that the effects are likely to be cell and differentiation stage specific.

Several studies in tumor cells describe AhR upregulation and/or activity in the absence of exogenous ligands. For example, AhR is elevated in several rodent and human tumors, including leukemias and mammary tumor cells (Abdelrahim et al., 2003 and Hayashibara et al., 2003). Inhibition of AhR also reduced BrdU incorporation and clonogenic survival in human glioblastoma cells (Gramatzki et al., 2009). Moreover, ectopic expression of AhR in mammary epithelial cells resulted in malignant transformation (Brooks et al., 2011). These studies indicate that AhR has a role in promoting the growth and survival of tumor cells.

Medulloblastoma (MB), one of the most common pediatric malignancies, with its prevalence increasing 2-3% over the last 30 years, is a primary cerebellar tumor that occurs predominantly in children between ages 5 and 10 (Louis et al., 2007). Five-year survival rates remain less than 50% and the patients that do survive often have impaired intellectual and physical development (Zakhary et al., 1999). MB is hypothesized to arise from abnormal proliferating cerebellar granule neuron precursors (GNPs) in the external germinal layer (EGL) of the developing cerebellum (Wechsler-Reya et al., 2001). Our laboratory has published results suggesting that the AhR is highly expressed and transcriptionally active during the peak proliferative phase of GNP neurogenesis. Moreover, abnormal activation of the AhR by TCDD dysregulated GNP proliferation and maturation, suggesting the AhR has a role in the proliferation of GNPs (Williamson et al., 2005 and Collins et al., 2008).
Common genes are involved in medulloblastoma pathogenesis and GNP proliferation (Fogarty et al., 2005). For example, the Notch signaling pathway, which is upregulated in MB tissue, promotes proliferation and inhibits cell cycle exit of GNPs, through the induction of the basic-helix-loop-helix transcription factor Hes1 (Solecki et al., 2001 and Fogarty et al., 2005). Interestingly, Hes1 has been reported as an AhR target gene (Thomsen, et al., 2005). Several genes have been identified within the inner EGL that act as intrinsic promoters of GNP cell cycle exit, including p27kip1 and p21cip1, which have both been suspected in MB pathogenesis (Argenti et al., 2005). p27kip1 has also been reported as a transcriptional repression target for Hes-1 in embryonic carcinoma cells (Murata et al., 2005).

This study tested the hypothesis that AhR plays a role in MB proliferation. The human MB DAOY cell line served as a model to explore whether AhR promotes MB growth. DAOY cells were shown to express a functional AhR signaling pathway. To examine the role of AhR in absence of exogenous ligand exposure, we created a stable cell line that has reduced AhR protein levels. Our data demonstrate that reduced AhR signaling in DAOY cells attenuates proliferation, which correlated with decreases Hes1 and increases p27kip1 expression. Furthermore, supplementation of human AhR restored DAOY proliferation. Our findings suggest that abnormal AhR activity, in the absence of exogenous ligands, positively promotes MB cell proliferation through a signaling pathway that includes Hes1 and p27kip1.
MATERIALS AND METHODS:

Reagents

The human medulloblastoma cell line (DAOY) was purchased from ATCC (Manassas, VA). TCDD (Cambridge Isotopes) was solubilized in dimethyl sulfoxide (DMSO). DMSO, phenylmethanesulfonyl (PMSF), antiprotease cocktail, Triton X-100, trypsin, puromycin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Dulbecco’s modified of Eagle’s medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), EDTA and L-glutamine were purchased from Gibco (Grand Island, NY).

DAOY cell culture

The immortalized DAOY cell line was derived from biopsy tissue taken from the posterior fossa of a 4-year-old boy with MB (Jacobsen et al., 1985). DAOY cells were maintained in DMEM containing 10% FBS, 1% L-glutamine (2mM), 1% penicillin/streptomycin and kept in a humidified atmosphere of 5% CO₂ at 37°C. This media is referred to as a growth media, which was replaced every 2-3 days.

Immunoblot analysis

Cells were harvested for protein analysis in ice-cold PBS supplemented with 0.1% Triton X-100, 1.0% phenylmethanesulfonyl (PMSF), 1.0% EDTA and 1.0% antiprotease cocktail. Protein concentrations were determined by using the microBCA protein assay (Pierce). 10μg of total protein was fractionated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (BioRad). Subsequently, membranes were blocked with 5% powdered milk containing 0.2% Tween-20 and probed for AhR
(1:1000; Enzo), Cyp1A1 (1:1000; Santa Cruz), β-actin (1:5000; Sigma), p27kip1 (1:1000; Santa Cruz), Cyclin D1 (1:1000; Oncogene), p21Cip1 (1:1000; Santa Cruz), Hes-1 (1:1000; Chemicon), MYCN (1:1000; Santa Cruz), c-Myc (1:1000; Santa Cruz) or GFP (1:2000; Invitrogen) overnight at 4°C. Following overnight incubation, membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1h at room temperature. Proteins were visualized with LumiGLO chemiluminescent substrate reagent (Kirkegaard & Perry Laboratories). Semi-quantitative densitometric analysis of proteins was accomplished with ImageJ software (National Institutes of Health).

**Plasmids**

Lentiviral shRNA transfer plasmid (pLKO.1) targeting human Aryl hydrocarbon receptor (hAhR) mRNA and empty control vector (Empty) were purchased from Open Biosystems (Huntsville, AL). Addgene plasmid 12260 packaging plasmid (psPAX2) and 12259 VSV-G envelope plasmid were a kind gift from Dr. Didier Trono (Trono Labs). pcDNA3.1/CT-GFP-TOPO (Invitrogen) vector was used to tag the cDNA coding sequence of hAhR with GFP enabling our laboratory to confirm positive transfection and to identify the localization of hAhR in live cells. DRE-EGFP reporter plasmid (pGreen1.1) was a kind gift from Dr. Michael Denison (University of California, Davis).

**Transient transfections**

DAOY cells were seeded in 6-well plates (BD falcon) at a density of 1x10^5 per well and were grown to 70% confluency. 1μg of DRE-EGFP DNA plasmid was transiently transfected using 3μl Fugene 6 transfection reagent (Roche). Twenty-four hours post-transfection, cells were exposed to DMSO, or 1nM or 10nM TCDD for 24h. Following
24h exposure, protein was harvested for immunoblot assay. For hAhR rescue experiments, 1.0 μg of pcDNA3.1/hAhR-CT-GFP-TOPO was transiently transfected using 3.0 μl Fugene 6 transfection reagent. Following 48h transfection, cells were harvested for confocal, immunoblot, and FACS analyses.

**Lentivirus production**

Human embryonic kidney 293FT cells (Invitrogen) were grown to 60–80% confluency in Dulbecco's modified Eagle's media (GIBCO) supplemented with 10% fetal bovine serum in T-175 flasks. The VSVG pseudotyped HIV vector was generated by co-transfecting with 7.3μg of envelope plasmid (pMD2G), 18.2μg of packaging plasmid (psPAX2), and 18.2μg transfer vector (shRNA hAhR), using lipofectamine LTX (Invitrogen). Media was changed 6 hours post-transfection. Viral supernatants were harvested at 48, 72 and 96 hours post-transfection. Viral supernatants were spun down twice for 5 minutes at 2,000xg and filtered through a .45μm low protein-binding filter. Viruses were concentrated 1000 fold using Lenti-X concentrator (Clontech). The concentrated viruses were stored at -80°C until use.

**Stable shRNA cell line generation**

DAOY cells were seeded in 24-well plates (BD Falcon) at a density of 5x10^4 cells per well and grown overnight. Cells were transduced with the appropriate concentrated lentivirus (AhR shRNA or Empty shRNA) at a multiplicity of infection (MOI) of 20. Following 48h of transduction, puromycin (2μg/ml) was added to the growth media to select for virally integrated cells. Once cells reached confluency, they were trypsinized and transferred to a 6-well culture dish (BD falcon), and then to a T-75 culture dish (BD
Falcon). Puromycin was maintained in the media during stable knockdown experiments in order to ensure maximal transgene expression.

**Cell survival assay**

DAOY cell viability was assessed with the Live/Dead cytotoxicity Kit (Molecular Probes). Calcein-AM (green) and Ethidium Homodimer (red) were used to delineate living and dead cells, respectively. DAOY cells were seeded in 24-well plates (BD falcon) at a density at 2x10^4 cells per well and then exposed to DMSO, or 1nM or 10nM TCDD for 24h. Viability was also assessed in the shRNA stable cell lines in the absence of chemicals. Cells were rinsed in DPBS and incubated with serum-free DMEM supplemented with 2μM Calcein AM and 4μM Ethidium Homodimer at 37°C for 30 minutes. Fluorescence was visualized using a Nikon Eclipse T100 fluorescent microscope (20x magnification). Approximately 600 cells from 3 randomly selected areas were counted using Image-Pro Plus software Version 6.2 (Media Cybernetics). The percentage of live cells was determined as the ratio of green cells over the total cells stained for green and red. While serum supplemented media was used as a negative control, 70% methanol served as a positive control for cell death.

**3H-thymidine incorporation**

DAOY cells were seeded in 96-well plates (BD Falcon) at a density of 3x10^3 cells per well until they reached 60% confluency. Cells were exposed to growth media containing DMSO, 1nM TCDD or 10nM TCDD for 48h. For shRNA stable cell line studies, growth media was replaced after cells reached 60% confluency and cells were grown for 48h. Cells were labeled with 1μCi of methyl-^3^H-thymidine (Perkin Elmer) during the last 2h of incubation and harvested onto filter paper using a Skatron cell harvester. The amount
of incorporated radioactivity was quantified by liquid scintillation counting. The incorporated radioactivity was normalized to the amount of protein present in individual wells, to account for potential differences in cell density.

**Flow cytometry for cell cycle analysis and cell proliferation**

DAOY cells were seeded in 6-well plates (BD Falcon) at a density of 1x10^5 per well and were grown to 70% confluency. For TCDD exposure studies, cells were exposed to DMSO, 1nM or 10nM TCDD for 48h. Following exposures, cells were washed with PBS containing 0.5% BSA (FACS buffer), fixed with 70% ethanol and stored in -20°C until ready for analysis. For shRNA stable cell lines, cells were trypsinized and fixed according to the protocol listed above. On the day of analysis, cells were treated with 1 mg/ml RNase A (Roche Diagnostics) and DNA was stained with 50 μg/ml propidium iodide (Molecular Probes). Cells were analyzed with a FACS CantoII flow cytometer using a 488 nm argon blue laser (Becton Dickinson). 50,000 events were collected per sample. Cell cycle analysis was performed with FlowJo software (Tree Star).

DAOY cell proliferation studies were evaluated with the CellTrace CFSE Cell Proliferation Kit (Molecular Probes). 3x10^6 cells were resuspended in 1 ml of PBS containing 0.05% BSA. 5μM carboxyfluorescein diacetate succinimidyl ester (CFSE) was added to the suspension and incubated for 10 minutes at 37°C. The staining was quenched by adding five volumes of ice-cold growth media and stained cells were incubated on ice for five minutes. Cells were pelleted by ultracentrifugation and resuspended in 1 ml of pre-warmed growth media. Cells were seeded at a density of 7.5x10^4 cells per well. Following 96h incubation, cells were trypsinized, pelleted, and resuspended in 0.5mls FACS buffer. Cells were analyzed with a FACS CantoII flow
cytometer using a 488 nm argon blue laser (Becton Dickinson). 50,000 events were collected per sample. Mean fluorescence intensity (MFI) was obtained with FlowJo software (Tree Star).

**Immunocytochemistry**

Cells were seeded in 12-well culture plates (BD falcon) containing microglass coverslips (VWR) on the bottom of each well. After 48h in culture, cells were rinsed with DPBS and then fixed with 4% paraformaldehyde at room temperature for 30 min. After fixation, cells were blocked in PBS containing 10% normal goat serum and 0.3% Triton-X-100 at room temperature for 30 min. Fixed cells were then incubated with an AhR antibody (1:800; Enzo) overnight at 4°C. Following overnight incubation, cells were washed and incubated with the appropriate Alexa Fluor-conjugated secondary antibody (Molecular Probes) for 90 min at room temperature. Nuclei were stained with DAPI (1:5000; Molecular Probes) for fixed cell imaging. For live cell imaging, the nuclei were stained with DRAQ5 (1:1000; Cell Signal). Fluorescence was visualized with an Olympus FV1000 laser scanning confocal microscope (University of Rochester). Images were taken with the Olympus FV1000 software program. Co-localization was quantified using Pearson’s correlation coefficient, by an algorithm that measures the overlap between two fluorochromes in the same specimen (Manders et al., 1993).

**Quantitative real time polymerase chain reaction (Q-PCR)**

For TCDD exposure studies, DAOY cells were seeded in 6-well plates (BD Falcon) at a density of 1x10^5 cells per well. Following overnight incubation, cells were exposed to DMSO, or 1nM or 10nM TCDD for 6, 12, or 24h. For shRNA stable cell lines, cells were grown until subconfluency. In both experiments, cells were harvested and RNA
was isolated using the RNeasy Mini Kit (Qiagen). 1μg of RNA was reverse transcribed into cDNA using the Super Script III First-Strand cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was carried out utilizing Taqman probes and primers, which were specific to the gene of interest. PCR mix (20μl) consisted of 10ng cDNA, 200nM forward primer, 200nM reverse primer, 10nM probe and 1X IQ Supermix (BioRad). An initial denaturation was accomplished at 95°C for 2 min. Amplification was achieved by denaturation at 95°C for 30s, annealing at 60°C for 30s for 50 cycles. Amplified products were fluorometrically detected during the end of the 60°C annealing using the BioRad iCycler analyzer (Biorad). Relative mRNA expression was quantified using a previously published algorithm (Gilliland et al., 1990). Forward (Fwd), reverse (Rev) and probe (Prb) sequences for Q-PCR were as follows: Hes1; Fwd: 5’-AGGCGGACATTCTGGAAATG-3’, Rev: 5’- CGGTACTTCCCCAGCACACTT-3’, Prb: 5’FAM-AGTGAAGCACCTCCGGAACCTGCAG-BHQ-3’ Cyp1A1; Fwd: 5’-CACAGACAGCCTGATTGAGCA-3’, Rev: 5’-GTGACTGTGTCACCCAGCTCACAAGA-3’, Prb: 5’FAM-AAGCAGCTGGAGTAAACGCAAATG-BHQ-3’, p27 kip1; Fwd: 5’-CCGTTGGACCACGAAGAGT-3’, Rev: 5’- GCTCGCTCCTTCCATGTCCT-3’, Prb: 5’FAM-AACCCGGGACATTGGAGAAGCAGT-3’, GAPDH; Fwd: 5’-TCAAGAAGGTGGTGGTGAAGCAG-3’, Rev: 5’-CGCTGTGTAAGTCAGAGGAG-3’, Prb: 5’FAM-CCTCAAGGGCATTGGCTGAGCAGT-3’. Primers and probes were purchased from Biosearch Technologies (Novato, CA).

**Statistical analyses**
Data are expressed as means +/- standard error of the mean (SEM) from a minimum of 3 independent experiments. Sample sizes are indicated in the figure legends. Statistical analyses were performed by Student’s t-tests. P values of <0.05 were considered statistically significant.
RESULTS:

AhR is expressed and transcriptionally active in DAOY human medulloblastoma cells

The primary objective of this study was to elucidate whether the AhR signaling pathway contributes to the abnormal/unbalanced proliferation of medulloblastoma cells and begin to identify the mechanisms by which this occurs. We confirmed that the AhR protein is expressed in human DAOY MB cells (Fig. 1A). Mouse hepatoma cell lysates (Hepa) served as a positive control for AhR expression (Fig. 1A). Human AhR had a predicted molecular weight of 105 kDa, while mouse AhR had a predicted molecular weight of 95 kDa.

To establish that AhR is responsive and transcriptionally active, DAOY cells were exposed to TCDD, a high affinity exogenous agonist. Following 1 and 10nM TCDD exposure, DAOY cells were harvested for mRNA analysis of the known AhR target gene, Cyp1A1 (Kress et al., 1997). After 24h of 10nM TCDD exposure, there was a ~15-fold increase in Cyp1A1 mRNA (Fig. 1B). There was no statistical difference between 1nM and 10nM TCDD-mediated increase in Cyp1A1 mRNA levels, which may be due to a maximum response ceiling effect of AhR-induced transcription. Immunoblot analysis of Cyp1A1 further confirmed that activation of the AhR signaling pathway resulted in the translation of AhR target genes (Fig. 1C).

An AhR activity reporter construct was used to further confirm that the AhR signaling is being activated in DAOY cells. The pGreen1.1 construct contains several AhREs upstream of the EGFP coding sequence, which when in the presence of an
activated AhR complex, will result in the transcription of GFP protein molecules (Han et al., 2004). DAOY cells were transiently transfected with pGreen1.1 for 24h then exposed to TCDD for 24h and protein was harvested for immunoblot analysis of GFP (Fig. 1D). TCDD exposure induced a ~3-fold induction of GFP compared to vehicle exposed cells. Together, these results suggest that AhR is transcriptionally active in DAOY cells following exposure to an exogenous ligand.

**AhR is localized in the nucleus and transcriptionally active independent of exogenous ligand exposure in MB cells.**

We hypothesize that the AhR promotes abnormal growth of MB cells. Therefore, it would be expected that AhR would be localized in the nucleus and transcriptionally active independent of an exogenous ligand. Cellular localization of AhR in DAOY cells was evaluated by immunocytochemistry. Representative images indicate that the AhR was localized both in the nucleus and cytoplasm of DAOY cells. These two distinct populations can be seen readily from the dot plot (Fig. 2A). Utilizing Pearson’s correlation coefficient ($r_p$) to determine co-localization of two fluorophores, we found a ~69.5% association of AhR with the nuclear stain DAPI. This association is considered statistically significant based on previously publications using this algorithm (Manders et al., 1993).

To confirm that the AhR was transcriptionally active independent of exogenous ligand exposure, DAOY cells were transiently transfected with the pGreen1.1 reporter construct. Following 24h transfection, DAOY cells were harvested for immunoblot analysis of GFP. As expected, the mock transfected group was devoid of GFP expression, while DAOY cells transfected with the pGreen 1.1 reporter construct
exhibited an ~8-fold induction in GFP protein expression (Fig. 2B). These results indicate that the AhR is localized in the nucleus and transcriptionally active in DAOY human medulloblastoma cells in the absence of exogenous ligand.

**Knockdown of the AhR in DAOY cells results in G1 cell cycle arrest and reduced proliferation**

To understand if the AhR promotes the abnormal growth of medulloblastoma cells, an shRNA lentiviral approach served to down-regulate AhR expression in DAOY cells. Stable cell lines were generated by transducing DAOY cells (mock) with lentiviral particles that encoded either an shRNA vector targeting human AhR (AhR shRNA) or an empty shRNA vector (empty shRNA). Compared to the empty shRNA DAOY cell line, the AhR shRNA stable cell line exhibited a ~70% reduction in AhR protein levels (Fig. 3A). This decrease in AhR protein expression persisted between several cell passages (data not shown). Moreover, AhR shRNA DAOY cell survival did not differ compared to mock DAOY cells (Fig. 3B).

We next evaluated whether down regulation of AhR in DAOY cells impacted the MB cell cycle activity. Mock, AhR shRNA, and empty shRNA DAOY cells were grown in complete growth media until 70% confluent then harvested and analyzed for cell cycle distribution via PI staining. Down regulation of the AhR lead to a ~17% increase in the percentage of cells in the G0/G1 phase of the cell cycle, which was accompanied by a ~17% decrease in the percentage of cells in the S phase of the cell cycle (Fig. 4A). To further confirm that knockdown of the AhR resulted in fewer cells entering the S phase, we investigated ³H-thymidine incorporation into AhR shRNA cells. When compared
with empty shRNA DAOY cells, AhR shRNA DAOY cells incorporated ~50% less \(^3\)H-thymidine (Fig. 4B).

To further characterize the impact AhR knockdown on cell cycle activity, we evaluated the number of mitotic events in AhR shRNA DAOY cells. Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a membrane permeable dye that is non-toxic and is commonly used to measure the amount of cell divisions (Hodgkin et al., 1996). The label is inherited by daughter cells after mitosis, diluting the dye in half. Therefore, increased numbers of cell divisions are inversely proportional to the levels of dye that will be present in each cell (Fig. 4C). AhR shRNA DAOY cells, mock DAOY cells, and empty shRNA DAOY cells were labeled with CFSE and grown for 96h and subsequently harvested for FACS analysis to quantify intensity of CFSE dye. AhR shRNA DAOY cells exhibited a ~4 fold increase in the intensity of CFSE dye compared to empty shRNA DAOY cells (Fig. 4C). These results suggest that the AhR contributes to proliferation of MB tumor cells.

**Hes1 protein expression is decreased following AhR reduction in DAOY cells**

Hes1 promotes proliferation of GNPs and subsequently is involved in the pathogenesis of MB (Solecki et al., 2001 and Fogarty et al, 2005). Also, Hes-1 has been established as an AhR target gene (Thomsen et al., 2004). Therefore, we investigated if Hes1 expression was altered following the knockdown of AhR in DAOY cells. Q-PCR analysis revealed a 30% reduction in Hes1 mRNA expression following knockdown of AhR in DAOY cells, but the decrease in Hes1 mRNA was not statistically significant (Fig. 5A). However, immunoblot analysis demonstrated a 50% decrease in Hes1 protein
expression in AhR shRNA DAOY cells (Fig. 5B). These data suggest that AhR could regulate Hes1 expression in MB DAOY cells to promote growth.

**Loss of AhR in DAOY cells results in increased p27kip1 mRNA and protein expression.**

To further evaluate the cell cycle perturbations seen following reduction of AhR, p27kip1 was investigated because Hes1 has been shown to transcriptionally repress p27kip1 expression in order to promote proliferation (Murata *et al.*, 2005). Therefore, we hypothesized that decreasing AhR levels would positively impact p27kip1 expression through decreased Hes1 expression. There was a ~2-fold increase in p27kip1 mRNA expression, which was accompanied by a ~6-fold increase in p27kip1 protein expression (Fig. 6A and 6B). In addition, we investigated the cell cycle regulatory proteins, Cyclin D1, p21cip1, and c-Myc because they regulate GNP proliferation and subsequently are implicated in MB pathogenesis. We found no statistical differences in these proteins following densitometric quantification when AhR protein expression was reduced in DAOY cells (Fig. 6C). Together, these results demonstrate that down regulation of AhR in DAOY MB cells is correlated with an elevation in the cell cycle arrest protein p27kip1, while other cell cycle proteins investigated remained unchanged.

**Supplementation of human AhR in AhR shRNA DAOY cells increased percentage of cells in S phase of cell cycle**

Supplementation experiments were accomplished in order to evaluate whether the replenishment of hAhR into AhR shRNA DAOY cells restores the proliferative capacity of MB cells. Transient transfection of shRNA AhR DAOY cells with hAhR for 48h resulted in increased AhR expression (Fig. 7A). Furthermore, live cell confocal analysis indicated that supplemented hAhR was localized in the nucleus of AhR shRNA DAOY cells.
cells (Fig. 7B). Following 48h transfection, AhR shRNA DAOY cells were harvested for cell cycle analysis, via PI staining. Consistent with our previous observations, there was an increase population of cells in G₀/G₁ when AhR protein expression is knocked down in DAOY cells (Fig. 7C). Compared to AhR shRNA DAOY cells, AhR shRNA DAOY cells supplemented with hAhR displayed an increases percentage of cells in S-phase (Fig. 6C). These results suggest that AhR contributed to the proliferation of MB cells by controlling the G₀/G₁ to S cell cycle transition.

**Increased AhR expression in AhR shRNA DAOY cells correlates with increased Hes1 and decreased p27kip1 levels.**

Because proliferative activity was restored following the supplementation of hAhR in AhR shRNA DAOY cells, we evaluated whether Hes1 and p27kip1 expression patterns would correlate with the increased number of cells in S-phase in these cells. Densitometric quantification revealed a ~75% increase in Hes1 protein expression in AhR shRNA DAOY cells supplemented with hAhR compared to AhR shRNA (Fig. 8B). Furthermore, a ~72% decrease in p27kip1 expression was seen in AhR shRNA DAOY cells supplemented with hAhR (Fig. 8C). These results suggest that AhR positively impacted Hes1 and negatively influenced p27kip1 expression.
MOL #77305

DISCUSSION:

Our laboratory previously established that the AhR is robustly expressed and transcriptionally active during the expansion phase of granule neuron precursors (GNPs) in the developing cerebellum (Williamson et al., 2005). Moreover, abnormal activation of the AhR by dioxin interfered with GNP proliferation and differentiation, suggesting that the AhR has some involvement in the maturation of these progenitors (Williamson et al., 2005; Collins et al., 2008). Interestingly, MB tumors are thought to arise from GNPs that lose growth control (Wechsler-Reya et al., 2001). Therefore, we tested the hypothesis that AhR activity influences abnormal DAOY cell growth, an immortalized cell culture model that was derived from a patient with MB (Jacobsen et al., 1985). We determined that the AhR is highly expressed in the absence of exogenous ligand exposure in DAOY cells. AhR was also transcriptionally active in the absence and presence of exogenous ligand exposure. Moreover, the reduction of AhR protein expression in DAOY cells attenuated the proliferative capacity and DNA synthesis, possibly through a signaling mechanism involving Hes1 and/or p27kip1. These observations suggest that a signaling pathway involving AhR, Hes1, and p27kip1 plays an important, if not major, role in promoting growth of MB cancer cells.

Several studies have indicated that the AhR functions endogenously to regulate normal cell growth independent of exogenous ligand treatment (Puga et al., 2002). AhR was shown to regulate cell cycle progression in human breast cancer cells via a functional interaction with cyclin-dependent kinase 4, but treatment with TCDD disrupted this complex resulting in cell cycle arrest (Barhoover et al., 2010). However, there have been
conflicting reports regarding the function of AhR as a cell cycle regulator, which may be due to differential responses in various cell types. For example, AhR overexpression in neuroblastoma cells resulted in a neuronal phenotype due to augmented differentiation (Akahoshi et al., 2006). AhR has been reported to control the expression of genes that influence growth and differentiation, which are dysregulated in cancer, suggesting that AhR may be involved in tumor development and progression (Thomsen et al., 2004 and Yang et al., 2005).

The AhR has been reported to be overexpressed in several cancers, including human lung and gastric carcinomas (Lin et al., 2003 and Peng et al., 2009). Additionally, expression of a constitutively active AhR variant in transgenic mice causes pro-proliferative effects, such as induction of stomach tumors and the promotion of hepatocarcinogenesis (Andersson et al., 2002 and Moennikes et al., 2004). We identified that the AhR is highly expressed in human MB cells (Fig. 1). Furthermore, AhR was consistently localized in the nucleus and transcriptionally active either in the presence or absence of exposure to an exogenous ligand (Fig. 2). Accordingly, several investigators have published that the AhR is expressed at higher levels and localized predominantly in the nucleus of transformed cells, compared to non-transformed cells (Yang et al., 2005).

We also detected AhR in the cytoplasm of DAOY cells (Fig. 2). However, this would be expected because prior to activation, AhR normally resides in the cytoplasm (Hankinson et al., 1995). Upon activation by endogenous pathways, the AhR subsequently translocates to the nucleus and regulates transcription. These observations strongly suggest that AhR activation by endogenous pathways may play a role in promoting MB growth.
Following AhR knockdown, DAOY cells exhibited decreased proliferation, diminished DNA synthesis, and a higher accumulation in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (Fig. 4). However, proliferation was not completely abrogated and there was a modest yet statistically significant percentage change in G<sub>0</sub>/G<sub>1</sub> and S-phases of the cell cycle. A few possibilities could explain our observations in cell proliferation following reduction of AhR in DAOY cells. First, the AhR is unlikely to be a master regulator of proliferation in MB cells but may function in concert with other signaling pathways to promote abnormal growth of these cells. There may also be compensatory proliferative signals that result from the permanent decrease in AhR expression. Additionally, we did not observe complete knockdown (70%) of the AhR following shRNA lentiviral-mediated stable cell line generation. Nevertheless, we observed a ~4-fold reduction in DAOY cell proliferation over a 96h timeframe (Fig. 4), which could have implications for MB growth.

As mentioned above, MB is thought to arise from abnormally dividing GNPs residing in the EGL. Accordingly, common genes such as those regulated by the Notch pathway are involved in medulloblastoma pathogenesis and GNP proliferation (Wechsler-Reya et al., 2001 and Fogarty et al., 2005). The Notch signaling cascade initiates transcription of target genes, most characterized are the Hairy and Enhancer of Split homologs Hes1 and Hes5 (Solecki et al., 2001). Interestingly, Hes1 is also an AhR target gene (Thomsen et al., 2004). In our study, Hes1 protein expression was decreased when AhR expression was knocked down in DAOY cells (Fig. 5). Hes1 functions to transcriptionally activate pro-proliferative genes, such as Cyclin D1 and D2 (Fogarty et al., 2005). Based on our proliferation experiments, we would have expected Cyclin D1
to decrease following AhR down regulation. However, we did not observe differences in Cyclin D1 protein expression following downregulation of AhR in DAoy cells (Fig. 6). Cyclin proteins are mitogen-activated and their activation triggers entry into the S-phase of the cell cycle. Therefore, perhaps the activities of the cyclins were diminished, rather than protein expression. However, Hes1 also functions to repress cell cycle inhibitory molecules like Cyclin-dependent kinase inhibitor p27kip1, in order to maintain cell proliferation (Solecki et al., 2001). We identified an AhR-dependent (directly or indirectly) expression of p27kip1 in DAoy cells (Fig. 6).

Low level p27kip1 expression has been associated with poor prognosis in a variety of neuronal tumors, including astrocytoma, glioblastoma, and neuroblastoma (Chu et al., 2008). Although MB tumors have not been investigated for p27kip1 expression, a recent report indicated that an MB mouse model expressing a mutant p27kip1 allele, developed an accelerated and more severe form of MB (Ayrault et al., 2009). Therefore, we evaluated whether AhR impacted p27kip1 levels, which could ultimately modulate MB growth. Our results suggest that AhR is associated with reduced p27kip1 expression, which would be consistent with promoting MB growth, possibly through Hes1 induction (Fig. 6A and 6B). However, we cannot conclude that the observed differences in p27kip1 expression are directly related to Hes1 or AhR-mediated gene transcription. AhR could be destabilizing p27kip1 mRNA and/or protein. Alternatively, AhR could be repressing p27kip1 promoter activity or positively regulating a transcription factor that negatively regulates p27kip1, such as Hes1. Because Cyclin-dependent kinase 2 (CDK2) activity is required for entry into the S-phase, its inhibition by p27kip1 could ultimately result in G0/G1 cell cycle arrest (Kolluri et al., 1999). Regardless of the mechanisms involved, our
experiments provide compelling reasons to further investigate the role of AhR in the regulation of expression of Hes1, p27kip1, and MB cell cycle.

We investigated the GNP cell cycle regulatory molecules p21cip1 and c-Myc because they have been shown to regulate GNP proliferation, differentiation, and are also suspected to be involved in MB pathogenesis (Yang et al., 2005 and Wechsler-Reya et al., 2001). Furthermore, p21cip1 and c-Myc are suspected to be AhR target genes (Gasiewicz et al., 2008). However, immunoblot quantification revealed no significant changes in the expression of c-Myc and p21cip1 following AhR reduction in DAOY cells (Fig. 7C). These data further support the contention that the AhR and Notch signaling pathways may converge to promote MB proliferation, possibly through regulating p27kip1.

Recent studies have demonstrated a relationship between AhR activity/expression and tumorigenesis. AhR gene polymorphisms have been associated with an increased risk of lung, breast and pancreatic tumors in humans (Kim et al., 2007 and Long et al., 2006). Interestingly, substantial evidence regarding AhR activity and tumor growth has resulted in an increased effort for targeting this transcription factor for cancer therapy (Koliopanos et al., 2002). We propose that the AhR has a role in promoting the proliferation of MB cells, specifically through a mechanism that involves Hes1 and p27kip1 expression. Our observations suggest that the AhR has the potential of acting as a proto-oncogene in MB cells and warrants future mechanistic studies concerning the relationship between AhR and MB progression. Therefore, AhR represents another potential therapeutic target to consider for MB treatment.
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FOOTNOTES:

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FIGURE LEGENDS:

Fig 1. AhR is expressed and transcriptionally active in DAOY human medulloblastoma cells. A, Protein was harvested from DAOY cells and protein (10μg) was separated by SDS-PAGE then analyzed for AhR expression by immunoblot. B, DAOY cells were treated with TCDD and RNA was harvested as designated time points. Gene expression was normalized to GAPDH. C, Cells were exposed to TCDD for 24h and protein (50μg) was separated by SDS-PAGE then analyzed for Cyp1A1 and actin expression by immunoblot. D, DAOY cells were transfected with DRE-GFP reporter construct for 24 hours followed by TCDD treatment for 24 hours. Protein was harvested and processed for GFP and actin detection by immunoblot. Data represent mean +/- SEM (n=4, *p <0.05, significantly different from DMSO, Student’s t-test).

Fig 2. AhR is localized in the nucleus and transcriptionally active independent of exogenous ligand exposure. A, DAOY were grown on glass coverslips and fixed with 4% PFA. Representative confocal images at 20x (a-c). Representative confocal images at 20x with 3x zoom (d-f). Red=AhR; Blue=Dapi. Co-localization is shown in the merge panel depicting two representative DAOY cells. Graph on the right indicates overlap of the two fluorophores. B, DAOY cells were transfected with DRE-GFP reporter construct for 24 hours. Protein was harvested immunoblot analysis was accomplished for the detection of GFP and β-actin. Data represent mean +/- SEM (n=3, *p<0.001, significantly different from mock, Student’s t-test).
Fig 3. Lentiviral-mediated knockdown of AhR does not impact DAOY cell survival.  
A, AhR knockdown stable cell line was generated and protein was harvested. Protein (10μg) was separated by SDS-PAGE then analyzed for AhR expression by immunoblot. Densitometry was carried out with ImageJ software. B, neuron viability was assessed with the Live/Dead Cytotoxicity kit. Data represent mean +/- SEM (n=3).

Fig 4. Knockdown of the AhR in DAOY cells results in G_1 cell cycle arrest and reduced proliferation. A, DAOY cells were harvested into a single cell suspension, fixed and stained with propidium iodide (PI). Cells were acquired on a FACS CantoII flow cytometer and data were analyzed with FlowJo software. Histograms are presented on the left and graphed values are located on the right. B, Cells were pulsed with 1μCi of methyl-^3H-thymidine for 2 h. Values were normalized to the amount of protein in each well. C, CFSE stained DAOY Cells were grown for 96 h. Stained cells were analyzed with a FACS CantoII flow cytometer and a mean CFSE intensity was calculated with FlowJo. Graphical representation of mean CFSE staining is to the right of histogram. Data represent mean +/- SEM (n=3, *p <0.05, **p <0.005, ***p <0.001, significantly different from empty shRNA, Student’s t-test).

Fig 5. Hes1 protein expression is decreased following AhR reduction in DAOY cells.  
A, Hes1 mRNA was quantified by Q-PCR and normalized to GAPDH mRNA expression. B, Hes1 protein expression was quantified with ImageJ software and
normalized to β-actin expression. Data represent mean +/- SEM (n=3, *p <0.05, significantly different from empty shRNA, Student’s t-test).

**Fig 6. Loss of AhR in DAOY cells results in increased p27kip1 mRNA and protein expression.** **A,** p27kip1 mRNA was quantified by Q-PCR and normalized to GAPDH mRNA expression. **B,** p27kip1 protein expression was quantified with ImageJ software and normalized to β-actin expression. **C,** Cyclin D, p21Cip1, MYCN, and c-Myc protein levels were assayed via immunoblot. Statistically significant differences were not observed following quantification with ImageJ software. Data represent mean +/- SEM (n=3, *p <0.05, significantly different from empty shRNA, Student’s t-test).

**Fig 7. Supplementation of human AhR in AhR shRNA DAOY cells increased percentage of cells in S-phase of cell cycle.** **A,** Following 48h transfection of hAhR, protein was harvested and protein (10μg) was separated by SDS-PAGE then analyzed for AhR and β-actin expression by western blot. **B,** (a-c) Depicts representative live cell confocal images (20x magnification) of AhR shRNA DAOY cells transfected with hAhR-GFP. (d-f) 20x with 3x zoom image of one cell showing co-localization of hAhR within the nucleus. Green=hAhR-GFP; Red=DRAQ5 **C,** DAOY AhR shRNA cells were harvested into a single cell suspension fixed and stained with propidium iodide (PI). Cells were acquired on a FACS CantoII flow cytometer and data were analyzed using FlowJo software. Data represent mean +/- SEM (n=3, *p <0.05, ***p <0.001, significantly different from empty shRNA; ^p <0.05, ^^p <0.01, ^^^p<0.001, significantly different from AhR shRNA, Student’s t-test).
Fig. 8. Increased AhR expression in AhR shRNA DAOY cells correlates with an increase in Hes1 expression and a decrease in p27kip1 expression. A, A representative western blot showing AhR, Hes1, p27kip1, and β-actin expression. B, Hes1 protein expression was quantified with ImageJ software and normalized to β-actin expression. C, p27kip1 protein expression was quantified with ImageJ software and normalized to β-actin expression. Data represent mean +/- SEM (n=3, *p <0.05, significantly different from empty shRNA; ^p <0.05, significantly different from AhR shRNA, Student’s t-test).
Figure 3

A

Mock Empty shRNA AhR shRNA

AhR

Actin

B

% Live

100
90
80
70
60
50
40
30
20
10
0

Mock Empty shRNA AhR shRNA
Figure 5

A

![Graph showing relative Hes1 mRNA levels normalized to GAPDH.](image)

B

![Graph showing relative Hes1 protein expression normalized to b-actin.](image)
Figure 6

A

Relative p27kip1 mRNA expression (normalized to GAPDH)

Mock Empty shRNA AhR shRNA

B

Relative p27kip1 protein expression (normalized to b-actin)

Mock Empty shRNA AhR shRNA

C

Mock Empty AhR

c-Myc

Cyclin D1

p21Cip1

Actin