Ah receptor antagonism inhibits constitutive and cytokine inducible IL6 production in head and neck tumor cell lines

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Abstract

There is increasing evidence that the aryl hydrocarbon receptor (AHR) plays a role in tumor progression through numerous mechanisms. We have previously shown that, in certain cancer cell lines that are typically non-responsive to cytokine-mediated IL6 induction, activation of the AHR with the agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin derepresses the *IL6* promoter and allows for synergistic induction following IL1β treatment. The mechanism by which this occurs involves liganded AHR binding upstream from the transcription start site and dismissing HDAC-containing corepressor complexes, giving rise to a promoter structure that is more amenable to NF-κB activation. This fact, combined with observations of multiple endogenously-produced chemicals activating the AHR, led us to study its role in basal expression among high cytokine-producing cancer cell lines. The current study provides evidence that several head and neck squamous cell carcinoma cell lines have a level of constitutively bound AHR at the *IL6* promoter, allowing for higher basal and readily inducible *IL6* transcription. Treatment of these cell lines with an AHR antagonist led to dismissal of the AHR from the *IL6* promoter and recruitment of corepressor complexes, thus diminishing cytokine expression. Head and neck squamous cell carcinoma is typically a high cytokine-producing tumor type, with IL6 expression levels correlating with disease aggressiveness. For this reason, AHR antagonist treatment could represent a novel adjuvant therapy for patients, lowering pro-growth and anti-apoptotic signaling with minimal systemic side effects.

Keywords

AHR; aryl hydrocarbon receptor; IL6; cytokines; antagonist

INTRODUCTION

The aryl hydrocarbon receptor (AHR) has been historically examined as a mediator of response to xenobiotic exposure, leading to subsequent metabolism of the compounds. A ligand-activated transcription factor of the basic helix-loop-helix, Per-Arnt-Sim class of proteins, research has begun to show that the AHR plays numerous physiological roles outside of its xenobiotic niche and does so through various molecular mechanisms. The AHR-mediated signaling pathway has been documented extensively, and recent reviews highlight the array of modes through which the AHR produces its effects (1). Prior to activation, the AHR resides largely in the cytoplasm, in a core complex with a 90 kDa heat shock protein dimer (hsp90) and the X-associated protein 2 (XAP2). Following activation by agonist binding, the receptor translocates to the nucleus, where it releases its chaperone

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proteins and dimerizes with its partner protein, the aryl hydrocarbon receptor nuclear translocator (ARNT). The AHR binds a variety of xenobiotics including polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (B[a]P) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). PAHs are common environmental pollutants resulting from car exhaust, manufacturing, iron foundries, and cigarette smoke, in addition to other sources. The xenobiotic role of the AHR has typically been studied in reference to its ligand-mediated binding to dioxin response elements (DREs) in the promoters of cytochrome P4501A genes, which express enzymes that act in phase I drug metabolism. Research into the disparate endogenous activities of the AHR has shown that it plays a role in Th17 immune cell differentiation, regulation of acute phase response genes, antiestrogenic activities, and modulation of NF-κB protein activity (2–5).

Several mechanisms have been documented by which the AHR can affect gene regulation, as outlined in the review by Beischlag, et al (1). The prototypical AHR activation pathway involves ligand activation, heterodimerization with ARNT and binding to DRE sequences in the promoter of a target gene to regulate transcription. Multiple instances of protein-protein interactions have been demonstrated, including AHR interactions with ERα (6), RELB (7), glucocorticoid receptor (8), and β-catenin (9). This last interaction is due to the AHR acting as an E3 ubiquitin ligase and inducing turnover of β-catenin. Additionally, ligand binding by the AHR has been shown to affect other cellular processes through mechanisms unknown at this time, such as the ability to repress acute phase response genes in the absence of DRE binding (3).

We have previously shown that ligand-activated AHR plays a role in the synergistic induction of *IL6* following IL1β cotreatment in MCF-7 breast cancer cells (10,11). In these cells, the presence of an AHR ligand or an inflammatory signal (e.g., IL1β) alone leads to only a modest level of *IL6* induction. The mechanism by which the presence of AHR at the *IL6* promoter mediates *IL6* induction in what is typically an unresponsive cell line centers on the activated AHR/ARNT heterodimer binding to imperfect DREs upstream from the *IL6* transcription start site and displacing corepressor complexes. This in turn allows for IL1β-mediated induction of *IL6* through recruitment of NF-κB family members to the promoter. The presence of the HDAC1-containing corepressor complex at the *IL6* promoter is at least partially responsible for preventing basal expression, and perhaps plays a role in the weakly metastatic phenotype of MCF-7 cells. Comparatively, aggressive cell lines often display high constitutive cytokine expression, as well as highly invasive and metastatic phenotypes. Following elucidation of the mechanism by which the AHR mediates the de-repression of the *IL6* promoter in some cell lines, our research turned to whether the AHR plays a role in constitutive *IL6* expression in a variety of tumor cell lines.

IL6 induction is most commonly seen in acute phase response signaling. Cancer cells have been shown to express IL6 in certain situations, often accompanied by phenotypic changes. Prostate cancer cells have been shown to have increased anti-apoptotic properties, and prostate and breast cancer cells have both been shown to have increased chemo-resistance in conjunction with higher IL6 production (12,13). Likewise, breast cancer cells have been shown to have decreased adhesive properties and greater migratory ability, along with increased proliferation, following an increase in IL6 production (14–17).

Squamous cell carcinoma of the head and neck (HNSCC) is an umbrella term that covers solid tumors of the larynx, pharynx, oral cavity, tongue, and nasal passages. Squamous cell carcinoma of the head and neck (HNSCC) has been tied to high cytokine expression both *in vitro* and in human patients (18–20). Expression of IL6 in HNSCC is associated with increased disease invasiveness, as well as patient prognosis and recurrence rates (21). The results of the current study point to a level of constitutively active AHR in numerous

HNSCC cell lines, which leads to a direct effect on *IL6* mRNA and protein expression. An AHR antagonist is able to significantly attenuate IL6 expression by reducing the level of AHR occupancy at the *IL6* promoter, and thus allow for re-occupancy by the corepressor complex observed previously (11). In this manner, treatment of HNSCC tumors with an AHR antagonist could represent a well-tolerated method by which pro-growth and metastasis signaling could be reduced prior to typical chemotherapy and radiation therapy.

MATERIALS AND METHODS

Chemicals

6, 2′, 4′-trimethoxyflavone (TMF) was purchased from Indofine Chemical Company 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was kindly provided by Dr. Steve Safe, Texas A&M University

Cell culture—All head and neck squamous cell carcinoma [HNSCC] lines (HN4, HN13, HN15, HN30, HN2095) were maintained at 37°C, 5% CO_2 in a high glucose 1:1 DMEM:F12 (Sigma), supplemented with 10% fetal bovine serum (FBS; Hyclone Labs.), 1,000 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). HN4, HN15, and HN2095 cells were obtained from Susan Mallery, following their purchase from ATCC (22,23). HN13 and HN30 cells were obtained from J. Silvio Gutkind and were first developed and characterized by Yeudall, et al (24). BEAS-2B cells were purchased from ATCC and maintained in supplemented BEBM media with 1,000 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma).

Gene expression—All HNSCC cell lines were serum-starved 18 h before treatment. Treatment of cells was performed by diluting compounds to the desired working concentration in serum-free media supplemented with 5 mg/ml bovine serum albumin (BSA). Total RNA was extracted from the cells using TRI reagent (Sigma) as specified by the manufacturer. The ABI high-capacity cDNA archive kit (Applied Biosystems) was used to prepare cDNA from isolated RNA. mRNA expression for all samples was measured by quantitative real-time PCR using the Quanta SYBR Green kit on an iCycler DNA engine equipped with the MyiQ single color real-time PCR detection system (Bio-Rad). Expressed quantities of mRNA were normalized to *GAPDH* or *L13A* mRNA levels and plotted using GraphPad Prism 4.0 (GraphPad Software). Histograms are plotted as mean values of three biological replicates, error bars represent the standard deviation of replicates. Real time primers used are shown in supplemental methods. Statistical significance was calculated using the student's T test, one-way ANOVA, and two-way ANOVA, as appropriate for the number of values and comparisons made.

Immunoblotting—Whole cell extracts were prepared by lysing cells in 1× radioimmunoprecipitation assay buffer [RIPA; 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS] supplemented with 1% NP40, 300 mM NaCl, and protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 21,000×g for 30 min at 4° C, and the soluble fraction was collected as whole cell extract. Protein concentrations were determined using the detergent compatible DC protein assay kit (Bio-Rad). Protein samples were resolved by Tricine SDS-PAGE and transferred to PVDF membrane. Primary antibodies used to detect specific proteins are shown in supplemental methods and were visualized using biotin-conjugated secondary antibodies (Jackson Immunoresearch) in conjunction with ^{125}I streptavidin (Amersham). Quantification of protein levels was conducted in triplicate by removal of ^{125}I labeled protein bands and analysis of radioactivity via gamma counter.

Chromatin immunoprecipitation assays—HNSCC cell lines were grown to approximately 90% confluency in 150 cm^2 dishes and serum-starved 18 h before treatment. Cells were treated in serum-free media supplemented with 5 mg/ml BSA by diluting compounds to the desired working concentrations. Following treatment, cells were washed once with warm PBS and chromatin complexes were chemically crosslinked using a 1% formaldehyde/PBS solution (final concentration) for 10 min at room temperature. Crosslinking was stopped by addition of glycine solution to a final concentration of 0.125 M, cells were then washed twice with ice cold PBS, scraped, and collected in 2 ml of harvest buffer (100 mM Tris, pH 8.3, 10 mM dithiothreitol). Cells were centrifuged, washed in ice cold PBS, and resuspended in 600 μl of lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.1, 10 mM EDTA). Chromatin was sheared with the Bioruptor water bath sonicator (Diagenode, Sparta, NJ) to an average size of 500 bp - 1 kb. Complexes were precleared with protein A agarose (Pierce) and incubated overnight with specific antibodies, listed in supplemental methods. Immunoadsorbed complexes were captured on protein A agarose and washed once with TE8 (10 mM Tris-HCl (pH 8.0) and 0.5 M EDTA). Agarose-bound complexes were then resuspended in TE8, layered on top of a sucrose solution (1 M sucrose, 200 mM NaCl, 1% NP40) and centrifuged for 3 min. Agarose-bound complexes were then washed once with 0.5× RIPA buffer, followed by four washes with TE8. Samples were eluted off the agarose using 200 μl elution buffer (100 mM NaHCO3, 1% SDS), and crosslinks were reversed at 65° C overnight. Eluted DNA was isolated, washed, and concentrated using the ChIP DNA Clean & Concentrator Kit (ZYMO Research). Immunoadsorbed DNA was analyzed by PCR with primers listed in supplemental methods. Results shown are representative of multiple experiments.

Gene silencing—Specific protein levels were decreased using the Dharmacon small interfering RNA (siRNA) [control oligo D-001810-0X, AHR oligo J-004990-07, BRG1 (SMARCA4) oligo D-010431-01]. Electroporation/nucleofection was performed using the Amaxa nucleofection system essentially as described in manufacturer protocols. Briefly, cells were washed and suspended at a concentration of 2.0×10^6 per 100 μL of nucleofection solution. Control or targeted siRNA was added to the sample for a final concentration of 1.5 μmol/L. Samples were electroporated using manufacturer's MCF-7 high efficiency program and plated into six-well dishes in complete media.

ELISA assay—To quantify IL6 protein expression, media were collected from cell lines treated for 24 h with vehicle or 10 μM TMF and frozen at −80°C until analyzed. Briefly, 96-well optical dishes were prebound with 50 μL of 2 μg/ml anti-human IL6 monoclonal antibody (R&D Systems) in 0.1 M NaHCO3 overnight at 4°C. Plates were washed extensively with PBS plus 0.05% Tween 20 (PBST) and then blocked with 1% Block Ace diluted in nanopure water for at least 2 h. Plates were washed extensively with PBST. Samples were added (100 μL/well) in duplicate and incubated overnight at 4°C. Recombinant human IL6 (10,000-10 pg/ml; R&D Systems, rh IL6) diluted in serum-free culture media was used as a standard. Plates were washed with PBST and incubated with 100 μL of 0.2 μg/ml biotinylated goat anti-human IL6 antibody (R&D Systems) for 2 h. Plates were washed extensively with PBST and incubated with 100 μL of 1 μg/ml streptavidin (Pierce) diluted in PBS for 30 min at room temperature. Plates were washed extensively with PBST, followed by a final incubation with 100 μL of a 0.3 mg/ml 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) and 100 mM citric acid solution (pH 4.35) plus 0.03% H_2O_2 in the dark. Colorimetric assay was performed 60 min later by reading absorbance at 405 nm on a Spectracount spectrophotometer, and data were analyzed using I-Smart software (Perkin-Elmer).

RESULTS

Both benign cells of the human airway and malignant cells of the oral cavity have readily inducible *IL6*

Our previous research has explored the manner in which the activated AHR primes MCF-7 human breast cancer cells for IL1β-mediated *IL6* induction. MCF-7 cells are typically seen as unresponsive to cytokine-mediated *IL6* induction and exhibit relatively low levels of basal and induced *IL6* expression when compared to more aggressive breast cancer lines, such as MDA-MB231 cells (25). The molecular mechanism by which the AHR mediates synergistic *IL6* expression in MCF-7 cells centers on ligand-bound AHR occupying imperfect DREs in the *IL6* promoter and dismissing HDACs, which then allows for full IL1β-mediated recruitment of NF-κB family members and leads to a transcriptional increase (11). However, not all cell lines display constitutively repressed *IL6* promoters, as evidenced by the benign bronchial epithelial cell line BEAS-2B, and the human HNSCC cell line 2095 (HN2095) (Fig 1A). While BEAS-2B cells do undergo a statistical synergy with TCDD and IL1β cotreatment, the ability of IL1β alone to induce significant *IL6* expression points to a promoter structure that is more readily accessible to inflammatory signals in its native state than that observed in MCF-7 cells. *IL6* in BEAS-2B cells is only slightly more inducible following AHR activation. HN2095 cells show higher basal *IL6* expression relative to maximal induction levels, and achieve full induction following IL1β treatment alone. Interestingly, TCDD treatment alone also mediates *IL6* induction (Fig 1B). This event was examined in two other HNSCC cell lines, HN13 and HN30, both of which were also shown to have significant *IL6* induction following TCDD treatment (Supp Fig 1). Activation of the AHR following TCDD treatment leads to significant increases in *CYP1A1* transcription in both cell lines (Figs 1C, 1D), illustrating that the AHR exhibits considerable transactivation potential. We have previously demonstrated that AHR-mediated *IL6* synergy in MCF-7 cells is dependent upon activated AHR/ARNT complexes binding to imperfect DREs in the *IL6* promoter. The lack of significant basal *CYP1A1* transcription in both BEAS-2B and HN2095 cells suggests that the presence of substantial levels of endogenous AHR ligands that behave as agonists is unlikely.

AHR is required for basal and induced *IL6* in HN2095 cells

The synergistic induction of *IL6* observed in MCF-7 cells following cotreatment with IL1β and an AHR ligand was clearly found to be an AHR/ARNT mediated effect (11). However, the fact that HN2095 cells did not show constitutively high *CYP1A1* expression called into question whether a basal level of AHR was constitutively occupying the *IL6* promoter, leading to elevated basal *IL6* expression. Furthermore, it is also possible that the *IL6* promoter was accessible to IL1β-mediated induction in an AHR-independent manner. To differentiate between these possibilities, HN2095 cells were transfected with AHR-targeting siRNA oligonucleotides, which resulted in a greater than 50% decrease in AHR protein levels; a representative protein blot is shown in figure 2A. AHR knockdown was sufficient to block the low level of constitutive expression and significantly reduce TCDD-mediated *CYP1A1* activity (Fig 2B). Incomplete knockdown of AHR protein prevented full reduction of *IL6* levels, but led to significant reductions in constitutive levels, as well as cytokine and AHR ligand induced levels (Fig 2C). *COX2* is known to be an inflammatory response gene that is up-regulated by both IL1β and TCDD, though the two have not been linked to a common induction pathway. AHR knockdown in HN2095 cells resulted in loss of TCDD-induced *COX2* induction, but failed to change basal and IL1β-induced levels (Fig 2D). Thus, the AHR appears to play a direct role in *IL6* transcription, while only mediating TCDD-induced *COX2* expression.

An AHR antagonist can reduce basal and induced *IL6* mRNA and protein levels in multiple HNSCC cell lines

IL6 has been shown to lead to pro-growth signaling in various cancer cell lines, and its up-regulation has also been noted in numerous head and neck squamous cell carcinoma lines. The heterogeneity of the cell population contained under the HNSCC classification led us to investigate whether different HNSCC cell lines showed the same AHR involvement in *IL6* transcriptional regulation as observed in HN2095. This hypothesis was evaluated using TMF, an AHR antagonist that inhibits receptor activated events in the presence of agonist activity (26). Treating three HNSCC cell lines for 12 h with 10 μM TMF significantly lowered basal *IL6* gene expression in two of the lines; HN30 and HN2095. TMF pretreatment prior to 2 h IL1β treatment led to a significant decrease in induced *IL6* levels in all three HNSCC lines tested (Fig 3A). The same effect was also seen with HN4 and HN15 cell lines (Supp Fig 1A), but HN13, HN30 and HN2095 were selected for further study as they appeared to represent varying levels of basal and inducible *IL6* expression. HN13, HN30 and HN2095 cells further showed significantly lower basal IL6 protein levels following TMF treatment (Fig 3B). The variability of *IL6* expression between lines is evidenced by the fact that HN30 has two-fold higher basal *IL6* expression than HN2095 which, in turn, has greater than two-fold higher basal expression than HN13. HN30 also shows basal *CYP1A1* levels significantly higher than the other cell lines. Variable expression levels of the AHR protein among the three lines could account for some of the difference between basal expression of AHR target genes. Untreated HN30 cells have a significantly higher level of AHR protein expression as compared to the other lines (Fig 3C, 3D, Supp Fig 2B).

AHR involvement in inflammatory signaling is both gene and cell line specific

The finding that siRNA-mediated knockdown of AHR protein resulted in a loss of TCDD-induced *COX2* expression but not IL1β-induced *COX2* in HN2095 cells (Fig 2D) led to the question of whether TMF treatment would similarly show a targeted role in inflammatory mediation. Treatment of HN13, HN30 and HN2095 cells for 12 h with 10 μM TMF did not affect basal *IL8* expression levels or IL1β-mediated *IL8* induction across the three cell lines (Fig 4A). However, in examining *IL1β* transcription levels, TMF treatment leads to a significant repression in both HN13 and HN30 cell lines, but has no effect on HN2095 cells (Fig 4B). Taken together, these data argue that the TMF concentrations used in these experiments do not result in a generalized pro- or anti-inflammatory effect. However, the role played by the AHR in *IL1β* expression remains unclear, and is cell line dependent.

IL6 promoter analysis after TMF treatment reveals coactivator/corepressor switching

The mechanism by which activated AHR leads to synergistic *IL6* production following IL1β treatment in MCF-7 cells was demonstrated to involve receptor displacement of HDAC1-containing corepressor complexes, allowing for increased recruitment and full activation of NF-κB transcriptional activators (11). The question of whether constitutive AHR occupancy of the *IL6* promoter in HNSCC cell lines would lead to the same outcome was determined by ChIP assays. We have shown that HN2095 cells treated for 12 h with 10 μM TMF exhibit a significant decrease in basal AHR occupancy at the *CYP1A1* promoter (26). This same treatment leads to a significant decrease in AHR and ARNT presence upstream from the transcription start site in the *IL6* gene. This dismissal of AHR coincided with decreased p65 occupancy and increased HDAC1 occupancy, as would be expected from our previous studies (11). There was also a decrease in acetylated p65, a hallmark of optimal NF-κB activation (Fig 5A). siRNA-mediated repression of AHR protein expression in HN2095 cells was carried out (Fig 5B), and control or AHR ablated cells were then similarly analyzed by ChIP. AHR knockdown is shown to result in effects similar to TMF treatment; namely, AHR occupancy is shown to be decreased at the *CYP1A1* promoter, and protein loss from

the *IL6* promoter correlates with loss of p65 and increase in HDAC1 presence (Fig 5C). Just as HN13 and HN30 show similar AHR-mediated *IL6* expression, ChIP analysis of the *IL6* promoter in both cell lines following TMF treatment shows evidence of a loss of basal AHR presence. Interestingly, HN30 cells, having significantly higher AHR protein levels and *CYP1A1* activity, required 36 h of TMF treatment for discernible AHR dismissal from the *IL6* promoter, compared to the 12 h needed in HN13 and HN2095 cells (Fig 5D). ChIP analysis of the *IL6* promoter in HN2095 cells provided evidence that the presence of the AHR correlates with increased transcriptional potential.

AHR presence at the *IL6* promoter influences *IL6* expression through mediation of BRG1 activity

It has previously been shown that AHR ligand-induced *CYP1A1* transcription relies on the AHR/ARNT heterodimer and Brahma-related gene-1 (BRG1, SMARCA4) binding in the CYP1A1 enhancer region (27,28). BRG1 is one of two main catalytic subunits that drive the mammalian SWI/SNF complex as the ATP engine. Human SWI/SNF complexes have been shown to interact with various nuclear receptors and mediate their recruitment to gene promoters. These complexes subsequently act by changing histone methylation and influencing other post-translational modifications. Thus, BRG1 plays an integral role in regulating the chromatin structure in the promoter regions of a variety of genes (reviewed in 29). The presence of BRG1 at the *IL6* promoter was assessed within the same ChIP experiments presented in figure 5. In addition to lowering basal AHR presence at the *IL6* promoter, 12 h TMF treatment of HN2095 cells reduces BRG1 presence in the same region (Fig 6A). Concurrently, TMF treatment leads to a significant increase in dimethylated histone H3 on lysine 4 (Fig 6A). Studies examining the promoter assembly of the *CIITA pIV* gene following IFNγ stimulation have revealed that a lack of BRG1 correlates with an increase in this same histone modification and promoter repression, due to a closed chromatin conformation (30). Similarly, siRNA-mediated ablation of AHR protein expression followed by ChIP analysis revealed a parallel loss of BRG1 at the *IL6* promoter (Fig 6B). In addition, transfection of HN2095 cells with BRG1-targeted siRNA oligonucleotides resulted in a substantial reduction in BRG1 expression (Fig 6C). This decrease in BRG1 protein led to a significant reduction in basal *IL6* transcription, which was further reduced following TMF treatment, when compared to control siRNA transfections (Fig 6D).

DISCUSSION

It has been established that the AHR is involved in numerous physiological processes aside from its traditional role as a xenobiotic receptor. AHR activation has been shown to play an influential role in multiple inflammatory signaling pathways, macrophage function, malignant cellular phenotypes, and even cancer patient prognosis, with variation in effects seen between cell and tumor types (31–34). Our previous research has shown that activation of the AHR with an exogenous ligand derepresses the *IL6* promoter in MCF-7 cells, leading to synergistic IL1β-mediated *IL6* induction in what is normally an unresponsive cell line (10,11). The determination of the mechanism, in conjunction with the knowledge that endogenous ligands for the AHR can be produced in cellular environments (35–38) led us to question whether there could be instances of high basal levels of activated AHR mediating constitutive or inducible *IL6* expression.

A survey of the literature revealed that head and neck squamous cell carcinomas often exhibit elevated IL6 production. This finding led us to investigate malignant cell lines from the upper airway and related tissues. Tumors of the larynx and pharynx fall under the umbrella of squamous cell carcinoma of the head and neck (HNSCC), which also includes the oral cavity and other loco-regional tissues. Initial results showed that the commonly used

HN2095 cell line had even more readily inducible *IL6* expression, with IL1β treatment alone being enough for maximal induction. Both basal and induced *IL6* expression have some measure of AHR dependency, thereby pointing to a level of constitutive receptor activation in HNSCC, but one which is not sufficient or competent to efficiently drive target gene expression (i.e., *CYP1A1*).

HNSCC cell lines have been shown to experience an autocrine loop of pro-growth and anti-apoptotic signaling, at least partly due to STAT3 activation (39). A positive feedback loop based upon IL6 autocrine signaling would presumably add to this STAT3 activation effect. Constitutively active STAT3 has likewise been seen as a hallmark of HNSCC, and is frequently considered a potential target for therapeutic intervention. STAT3-mediating therapies have, in fact, been shown to increase apoptosis and cell cycle arrest, decrease invasion, and sensitize HNSCC cell lines to chemotherapy treatment (40–43). However, systemic treatment with a compound to fully inhibit STAT3 activation or even decrease the endogenous STAT3 levels would likely induce high grade cellular side effects in a patient. When possible, HNSCC is generally treated with a combination of surgical resection via radical neck dissection followed by adjuvant chemotherapy and/or radiation therapy (44). Any pretreatment that results in systemic side effects would likely limit later treatment options, including length and dose of radiation course, and chemotherapy regimen choice. Thus, a therapeutic option that could lower IL6 autocrine signaling with minimal toxic cellular effects would represent a potential chemo/radiation-sensitizer that could be used without affecting choices for adjuvant treatment.

Our research has shown that the compound 6,2′,4′-trimethoxyflavone (TMF) acts as an AHR antagonist without any partial agonist activity, unlike many other antagonists (26). For this reason, TMF could represent a therapeutically viable treatment for blocking AHR-mediated inflammatory effects without inducing DRE-mediated target genes, particularly those involved in Phase I xenobiotic metabolism. Although HNSCC encompasses a heterogeneous mixture of tumors, the ability of TMF treatment to reduce *IL6* mRNA and secreted protein levels in multiple cell lines reinforces the hypothesis that high constitutive *IL6* expression in these tumors is at least partly the result of a common AHR-mediated mechanism. Similarly, the variable level of endogenous AHR protein levels among three representative cell lines shows a loose correlation with basal *IL6* and *CYP1A1* transcription levels. Interestingly, nuclear levels of AHR protein have been tied to disease aggression and patient response rates in urothelial cancer (34). In this respect, the ability of 12 h, 10 μM TMF treatment to reduce both basal and induced levels of AHR-mediated *IL6* demonstrates the viability of antagonist treatment, yet highlights the further need for compounds displaying stronger affinity in order to minimize dosing even in high AHR expressing lines. The lack of a more generalized anti-inflammatory response following TMF treatment supports a targeted mechanism of action at relevant doses.

The mechanism by which activated AHR synergistically induces *IL6* in MCF-7 cells appears to be reflected in the HNSCC cell lines tested. Binding of the AHR to upstream DREs in the *IL6* promoter has been shown to prime the DNA for NFκB-mediated induction through a dismissal of an HDAC-containing repressor complex. Our research shows that a basal level of AHR is bound to the *IL6* promoter in HNSCC lines, thereby maintaining the DNA in a primed state that is readily accessible to transcriptional activators. In addition to the mechanism outlined previously, the identification of a role for the AHR in BRG1 occupancy and histone modification of the *IL6* promoter simply adds to the complexity of the role that the AHR plays in *IL6* transcriptional regulation.

On a molecular level, the AHR has been shown to have both ligand-dependent and independent effects on the transcription of cytoskeletal and migratory proteins including

NEDD9, FASCIN-1, and VAV3, all of which are differentially transcribed in malignant cells (45–47). These effects highlight the extensive role that the receptor could play in cancerous tissue with respect to non-canonical target genes. Similarly, the AHR has also been implicated in a number of phenotypic end points across various cancer models. Agonist treatment has shown that activated AHR plays a role in increased breast cancer invasion (48), decreased contact inhibition in liver cells (49), and increased MMP9 expression and invasive ability in gastric cancer (50). Conversely, use of AHR antagonists or selective AHR modulators (SAhRMs) has been shown to inhibit prostate tumor metastases (51), breast cancer cell proliferation and tumor growth (52), and various inflammatory responses (53). A SAhRM has been identified that represses AHR-mediated inflammatory reactions without inducing CYP target gene activity. While some of these mechanisms remain undetermined, the expectation is that use of a SAhRM or antagonist would block numerous effects simply by competing for an activating ligand, thus reducing nuclear AHR levels. Elucidating the mechanisms by which a SAhRM or antagonist works, together with an understanding of the molecular interactions that lead to each AHR-dependent phenotypic endpoint would allow for rational treatment approaches to individual disease settings. For this reason, TMF treatment represents a potentially superior adjuvant therapy for HNSCC, as blocking constitutive pro-growth IL6 production may be only one of its beneficial endpoints. One simple outcome of AHR antagonist-receptor binding would be to block effects mediated by exogenous agonist exposure. The experiments performed in this study represent the level of constitutive AHR activation that is observed under controlled conditions in cell culture. In contrast, within an in vivo context, there are a diverse set of environmental sources of AHR ligands that humans are exposed to including air pollution, diet, and intestinal bacterial metabolites. For example, an important secondary effect of antagonist treatment would be to help reduce *IL6* induction and other cellular effects initiated by B[*a*]P in HNSCC patients who smoke, or were previously exposed to B[*a*]P through manufacturing, car exhaust, or other sources. Patients who continue to smoke cigarettes while undergoing radiation therapy for head and neck cancer have been shown to have lower response rates and 2 year survival outcomes, as well as higher complications due to therapy (54,55). Treatment of B[*a*]P-exposed patients with an AHR antagonist prior to radiation therapy, in conjunction with smoking cessation when applicable, could help raise outcome levels by blocking AHR-mediated anti-apoptotic effects that were the result of agonist exposure. Yet another source of AHR ligands is through the production of endogenous ligands during various disease states, and this concept is underscored by the recent discovery that indole metabolites (e.g. indoxyl sulfate, kynurenic acid) are potent human AHR ligands (56,57).

The constitutively high cytokine expression found in HNSCC cell lines likely impacts the growth and anti-apoptotic effects common to these tumors when challenged with radiation and chemotherapy. Our findings that the AHR plays a key role in priming the *IL6* promoter for expression, as well as the ability of antagonist treatment to significantly reduce cytokine expression in multiple lines, point to a potential role for the receptor in disease management. Use of an AHR antagonist has the potential to inhibit pro-growth and anti-apoptotic signaling, thus sensitizing the cells to more aggressive treatments typically employed. Further, AHR antagonist treatment would likely be free of the systemic side effects wrought by full organism blockades of cytokine signaling and major pathway inhibition. Modulation of AHR-induced IL6 and its autocrine loop is likely only one mechanism by which an antagonist or SAhRM would have an effect on tumor cell phenotype. Therefore, the role of an AHR antagonist on tumor aggressiveness and as a chemo/radio-sensitizer warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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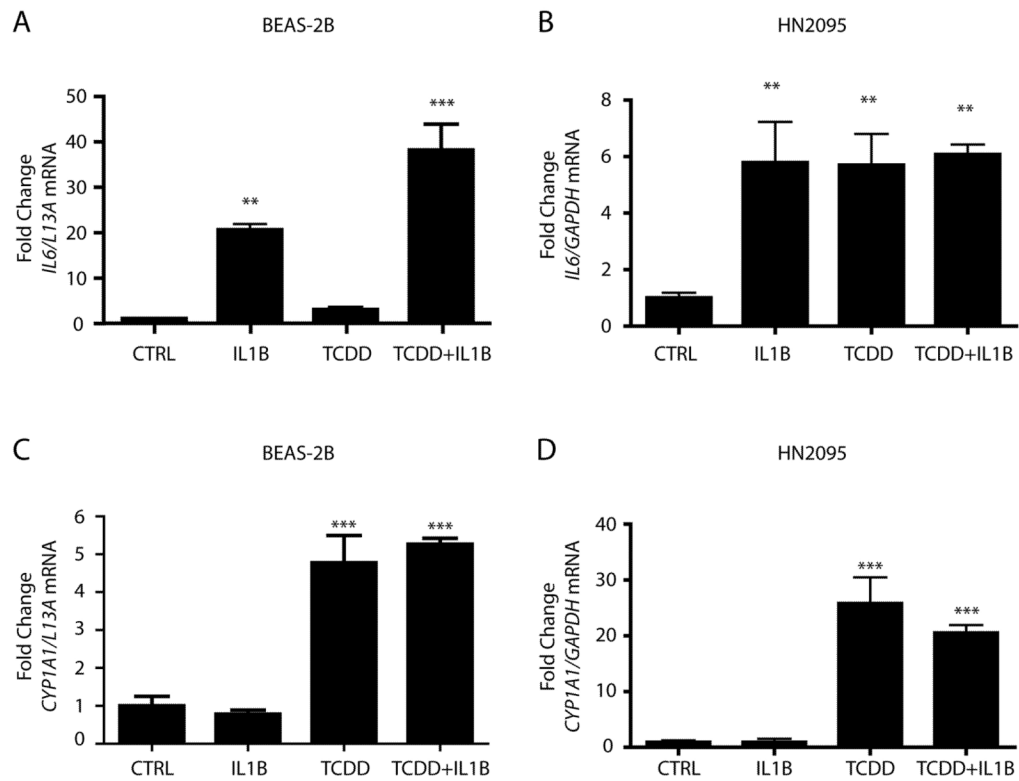
Fig 1. Both nonmalignant and malignant cells of the head and neck display readily inducible *IL6*
Serum starved BEAS-2B (A,C) and HN2095 (B,D) cells were treated for 2 h with vehicle, 10 ng/ml IL1β, 1 nM TCDD, or TCDD+IL1β. Total RNA was then isolated, cDNA prepared, and relative *IL6* (A,B) and *CYP1A1* (C,D) mRNA levels were determined by quantitative real-time PCR.
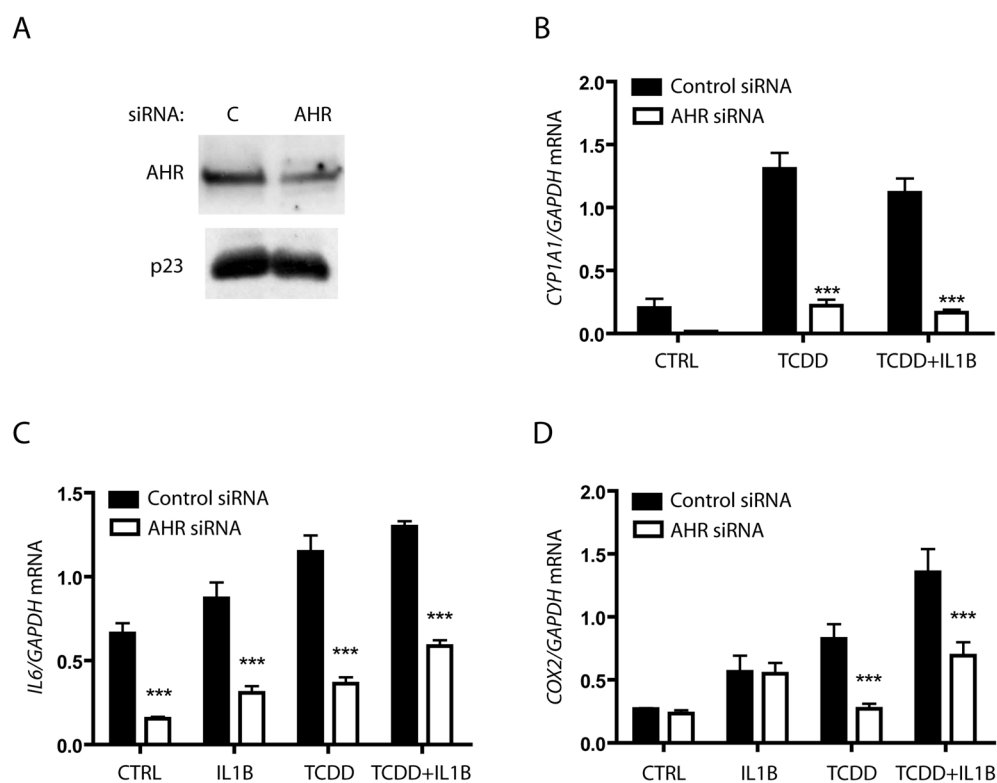
Fig 2. AHR plays a role in both basal and induced *IL6* expression in HNSCC
HN2095 cells were electroporated with control or AHR siRNA, plated for 24 h, and serum
starved for 18 h. A) Whole cell extracts were prepared and expression levels of AHR and
p23 (control) were assessed by immunoblot. B–D) Cells were electroporated as in A, plated
into 6 well dishes, then serum starved before being treated for 2 h with vehicle, 10 ng/ml
IL1β, 1 nM TCDD, or TCDD+ IL1β. Total RNA was isolated, cDNA prepared, and relative
CYP1A1, IL6, and *COX2* mRNA levels were determined by quantitative real-time PCR.
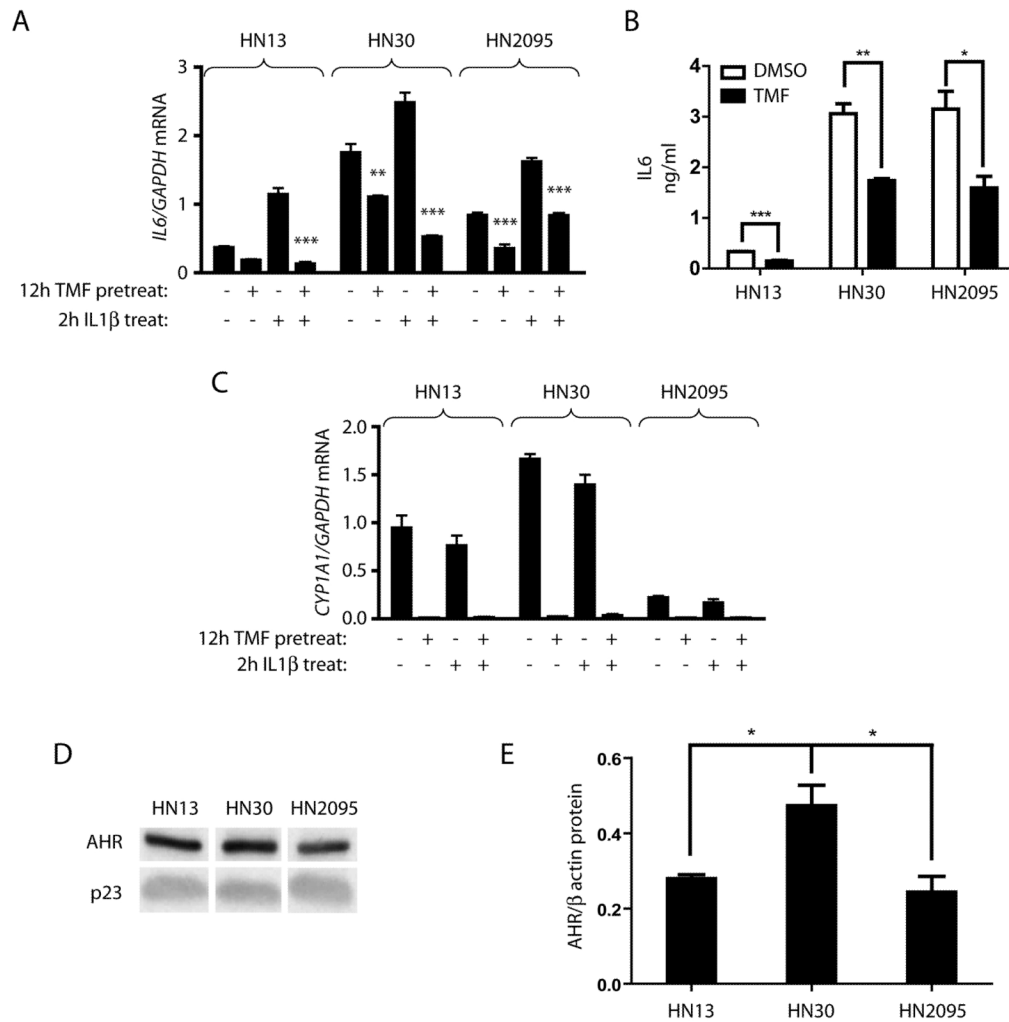
Fig 3. AHR antagonist treatment reduces *IL6* mRNA and protein in numerous AHR expressing HNSCC cell lines

Serum starved HN13, HN30, and HN2095 cells were treated for 12 h (A,C) or 24 h (B) with vehicle or 10 μM TMF. A) Following 12 h pretreatment, cells were treated with vehicle or 10 ng/ml IL1β for 2 h. Total RNA was then isolated, cDNA prepared, and relative *IL6* mRNA levels were determined by quantitative real-time PCR. Significance testing indicates that TMF pretreatment decreases basal and IL1β-induced *IL6* expression. B) Media was collected following 24 h treatment and subjected to ELISA. C) mRNA from 12 h TMF treatments in (A) were assessed for *CYP1A1* expression. D,E) HN13, HN30 and HN2095 cells were plated and serum starved, after which whole cell extracts were made. D) Representative western blot showing endogenous AHR levels. E) Endogenous AHR levels were quantified and normalized to p23 control. Samples were in triplicate.
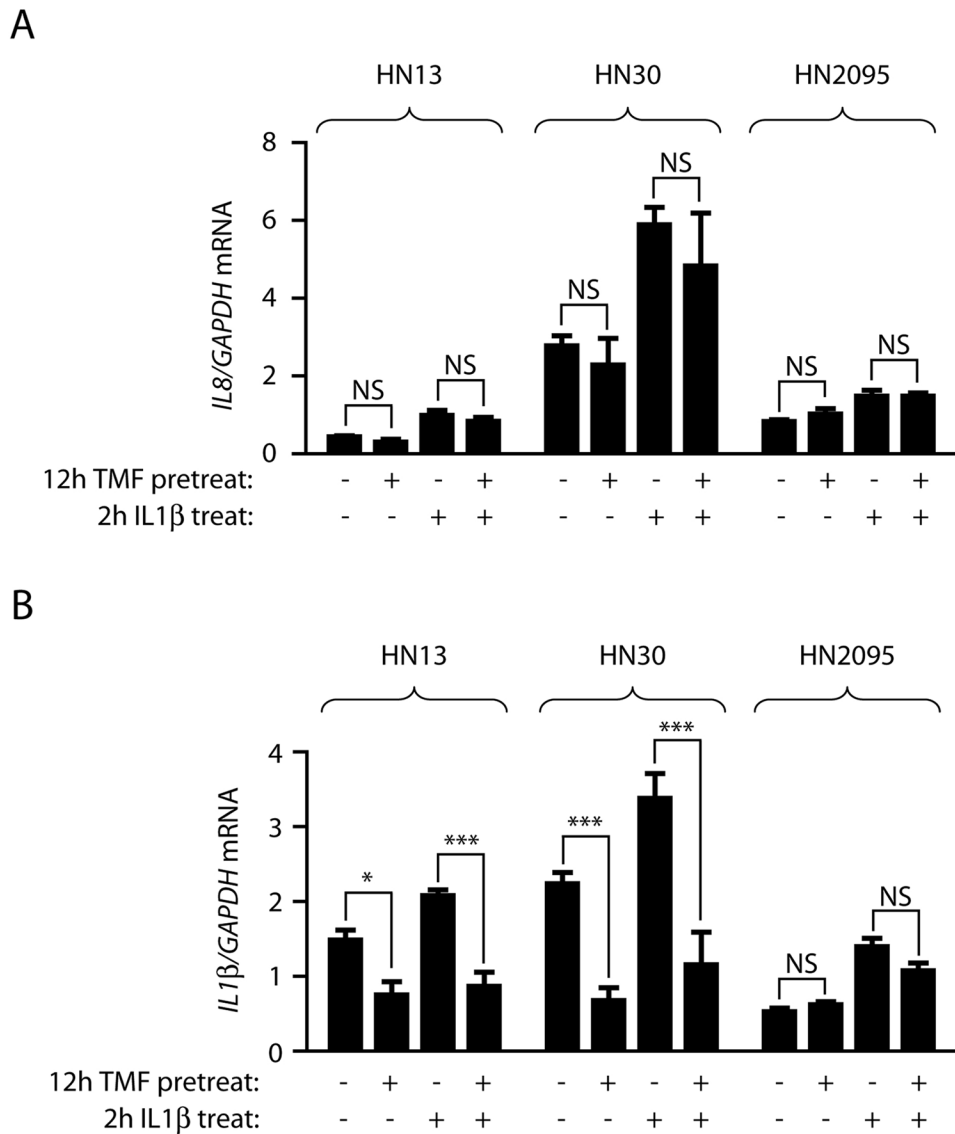
A

B

Fig 4. The role played by the AHR in inflammatory signaling is both cell line- and gene-specific
HN13, HN30 and HN2095 cells serum starved and pretreated for 12 h with vehicle or 10 μM TMF prior to treatment with vehicle or 10 ng/ml IL1β for 2 h. Total RNA was then isolated, cDNA prepared, and relative mRNA levels determined by quantitative real-time PCR.
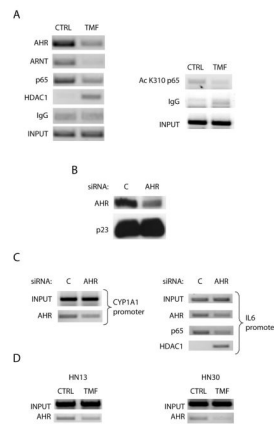
Fig 5. Reduction of AHR occupancy at the *IL6* promoter leads to a shift from coactivators to corepressors
A) ChIP analyses of the *IL6* promoter in HN2095 cells following 12 h treatment with vehicle or 10 μM TMF. B) HN2095 cells were electroporated with control or AHR targeted siRNA oligonucleotides, plated for 24 h and serum starved for 24 h. Whole cell extracts were prepared, and protein levels of AHR and p23 (control) were assessed by immunoblot. C) HN2095 cells were electroporated and serum starved for 12 h, then treated for 12 h with vehicle or 10 μM TMF. ChIP analyses of the *CYP1A1* and *IL6* promoters were then carried out for the specified proteins. D) Serum starved HN13 and HN30 cells were treated with 10 μM TMF for 12 h and 36 h, respectively, followed by ChIP analysis of the *IL6* promoter.
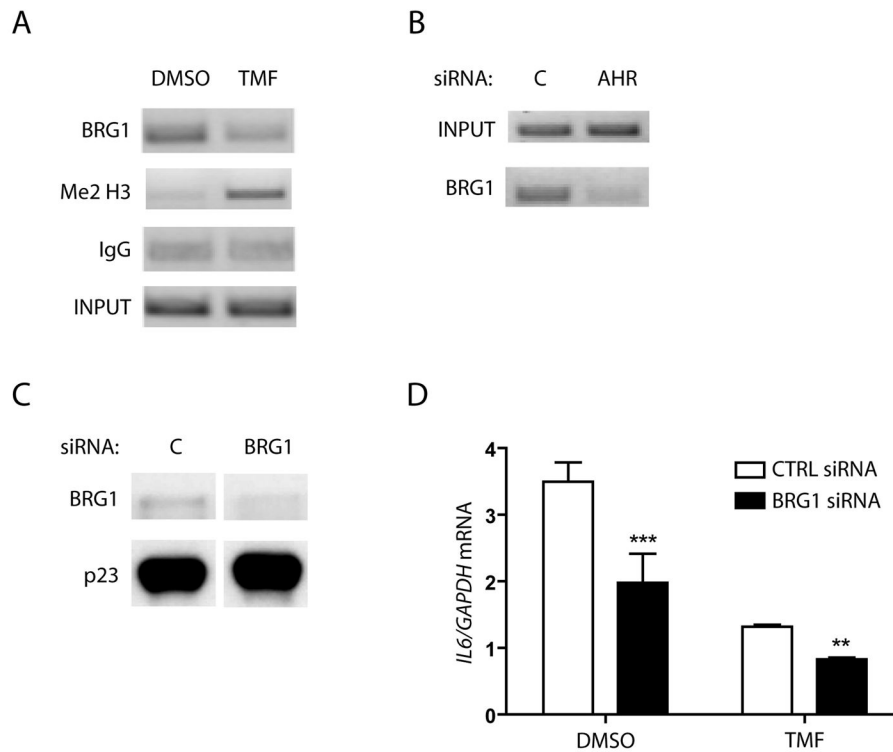
Fig 6. AHR plays a role in BRG1 occupancy and modification of the *IL6* promoter
A) ChIP analysis from figure 5A of the *IL6* promoter in HN2095 cells for BRG1 protein
following 12 h TMF treatment. B) AHR siRNA transfection and ChIP analysis of the *IL6*
promoter in HN2095 cells from figures 5B,C for BRG1 protein C) HN2095 cells were
electroporated with control or BRG1 siRNA, plated for 24 h, serum starved for 18 h, and
whole cell extracts were then prepared. Levels of BRG1 and p23 (control) expression were
assessed by immunoblot. D) HN2095 cells were electroporated, plated into 6 well dishes,
then serum starved before being treated for 12 h with vehicle or 10 μM TMF. Total RNA
was isolated, cDNA prepared, and relative *IL6* mRNA levels were determined by
quantitative real-time PCR.