Stem Cells International

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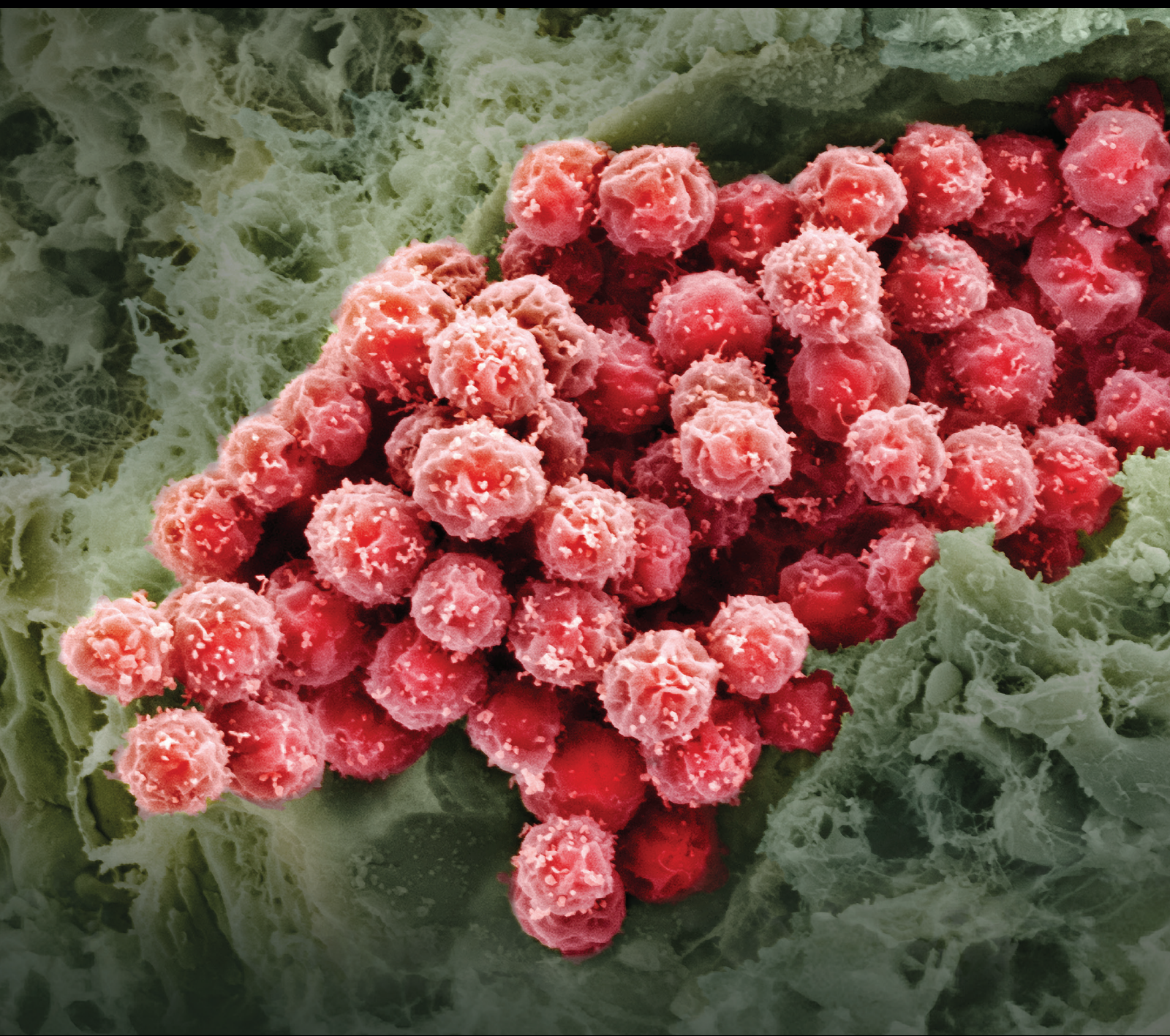
Guest Editors: Thomas A. Gasiewicz, Kameshwar P. Singh, and Fanny L. Casado



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# Editorial Board

# Contents

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*Editorial*

# Aryl Hydrocarbon Receptor and Stem Cells

**Thomas A. Gasiewicz,[1] Kameshwar P. Singh,[1] and Fanny L. Casado[2]**

[1]*Department of Environmental Medicine, University of Rochester Medical Center, Rochester, NY 14642, USA*
[2]*Instituto de Ciencias Omicas y Biotecnología Aplicada, Pontificia Universidad Católica del Perú, Avenida Universitaria 1801, San Miguel, Lima 12, Peru*

Correspondence should be addressed to Thomas A. Gasiewicz; tom_gasiewicz@urmc.rochester.edu

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From the biochemical point of view, the aryl hydrocarbon receptor (AHR) is a highly conserved, developmentally regulated, and ligand-activated member of the bHLH/PAS family of transcription factors. The AHR was originally discovered because of the human and animal toxicity of its most potent and persistent ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin, usually just referred to as "dioxin." The canonical understanding of the AHR indicates that it is present in the cytosol as one of the components of a multiprotein complex containing the immunophilin-like protein XAP2/AIP/ARA9, the 23 kDa cochaperone protein p23, and two molecules of HSP90. After ligand binding, it translocates to the nucleus forming a heterodimer with HIF-1beta a.k.a. ARNT and together they can activate gene transcription.

Later studies demonstrated that dioxins also act as endocrine disruptors [1] and as carcinogens with tumor-promoting properties and indirect roles in chemically induced carcinogenesis during initiation, progression, and metastasis. Epidemiological studies linked human exposure to environmental AHR ligands to increased incidence of diverse cancers [2], as well as diabetes and obesity [3]. While more recent research has focused on the physiologically relevant AHR ligands which might be expressed in diseased tissues or active innate and adaptive immune, the physiological relevance of the AHR without ligand binding was not fully appreciated until studies showed a link with stem cells of various tissues of origin [4]. Furthermore, AHR completely left the specialized realm of toxicological research when Boitano et al. [5] showed the pharmacological potential of the receptor by promoting expansion of hematopoietic stem cells in vitro.

In this special issue, original reports as well as thorough reviews are presented addressing the different contexts provided by the tissues and making AHR in stem cells prone to respond to different stimuli such as ligand binding and oxidative stress. Also, it is evident that current biotechnological approaches are providing a more comprehensive understanding of AHR behavior in multiple cell types which is now possible thanks to genome editing tools. With worldwide banning of polyhalogenated pesticides usage and decreases in cigarette-smoke exposures, AHR research leads the efforts to a more sophisticated understanding about the meanings of toxicity in an era of stem cell manipulation.

*Thomas A. Gasiewicz*
*Kameshwar P. Singh*
*Fanny L. Casado*

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*Research Article*

# PAHs Target Hematopoietic Linages in Bone Marrow through Cyp1b1 Primarily in Mesenchymal Stromal Cells but Not AhR: A Reconstituted *In Vitro* Model

**Catherine M. Rondelli,[1] Michele Campaigne Larsen,[2] Alhaji N'jai,[3] Charles J. Czuprynski,[1,3] and Colin R. Jefcoate[1,2]**

[1]*Molecular and Environmental Toxicology Program, University of Wisconsin, Madison, WI, USA*
[2]*Department of Cell and Regenerative Biology, University of Wisconsin Medical School, Madison, WI 53705, USA*
[3]*Department of Pathobiological Sciences, University of Wisconsin, Madison, WI 53706, USA*

Correspondence should be addressed to Colin R. Jefcoate; jefcoate@wisc.edu

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7,12-Dimethylbenz(a)anthracene (DMBA) rapidly suppresses hematopoietic progenitors, measured as colony forming units (CFU), in mouse bone marrow (BM) leading to mature cell losses as replenishment fails. These losses are mediated by Cyp1b1, independent of the AhR, despite induction of Cyp1b1. BM mesenchymal progenitor cells (MPC) may mediate these responses since basal Cyp1b1 is minimally induced. PreB colony forming unit activity (PreB CFU) is lost within 24 hours in isolated BM cells (BMC) unless cocultured with cells derived from primary MPC (BMS2 line). The mouse embryonic OP9 line, which provides more efficient coculture support, shares similar induction-resistant Cyp1b1 characteristics. This OP9 support is suppressed by DMBA, which is then prevented by Cyp1b1 inhibitors. OP9-enriched medium partially sustains CFU activities but loses DMBA-mediated suppression, consistent with mediation by OP9 Cyp1b1. PreB CFU activity in BMC from Cyp1b1-ko mice has enhanced sensitivity to DMBA. BMC gene expression profiles identified cytokines and developmental factors that are substantially changed in Cyp1b1-ko mice. DMBA had few effects in WT mice but systematically modified many clustered responses in Cyp1b1-ko mice. Typical BMC AhR-responsive genes were insensitive to Cyp1b1 deletion. TCDD replicated Cyp1b1 interventions, suggesting alternative AhR mediation. Cyp1b1 also diminishes oxidative stress, a key cause of stem cell instability.

## 1. Introduction

People are chronically exposed to polycyclic aromatic hydrocarbons (PAHs) in multiple ways ranging from cigarette smoke to diesel fumes and coal tars [1]. Many PAHs are converted by metabolism at P450 cytochromes (Cyps) to highly reactive and mutagenic dihydrodiol epoxide metabolites [2]. The highest level of such metabolism is provided in the liver by cytochrome P4501A1 (Cyp1a1), which, however, also has high levels of enzymes, notably, glutathione transferases that provide protection against this toxicity. In the bone marrow (BM), there is similarly active cytochrome P450 1b1 (Cyp1b1), in close proximity to the hematopoietic stem cell niche [3]. Numerous studies in mice have shown that repeated daily administration of PAHs causes immunosuppression. Many effects of TCDD on the immune system are produced by direct activation of the aryl hydrocarbon receptor (AhR) [4]. This intervention applies to T cells, at both the level of thymus progenitors and Treg/T17 cells. PAHs, like TCDD, activate the AhR to induce Cyp1a1 and Cyp1b1 [5, 6]. Thus, PAHs can function both through activation of AhR and through their conversion to reactive metabolites. Here, we describe new approaches to resolving the impact of PAH metabolites on hematopoietic stem cells and the lineage progenitors *in vivo* and in a cell culture model.

In order to better understand the mechanism of this immunosuppression, we have used single intraperitoneal or oral doses. This approach provides better definition of the time course and individual steps that suppress immune cells. Colony forming unit (CFU) assays show that moderate

single doses of two PAHs, 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (BP), suppress the proliferative activity of lymphoid, myeloid, and erythroid progenitors within 6 hours. Mature BM cells are unaffected as shown by minimal gene expression responses to DMBA [5, 6]. These shared progenitor suppression responses suggest that stem cell differentiation to the respective lines is blocked by DMBA metabolites. These suppressions are removed in Cyp1b1-ko mice but are surprisingly independent of the AhR, which mediates induction of Cyp1b1 by DMBA in most cell types.

BM lymphoid and myeloid cells become depleted between 24 and 48 hours after DMBA administration. During this period, lymphocytes are similarly depleted from the thymus (T cells) and spleen (B cells). Different doses and routes of DMBA administration produce 48-hour depletions of mature lymphocytes in BM, thymus, and spleen that each correlate with 6-hour suppression of BM PreB CFU expansion activity [6]. We concluded that the initial impact of DMBA on the lymphocyte populations of each tissue was caused by suppression of the BM common lymphoid progenitors (CLP) and their progression from the stem cells. This conclusion was supported by flow analyses of the CLP and other progenitor populations. This results in a failure to replace cells that are exported from these three sources, particularly to sites of injury, such as the lung.

BM mesenchymal progenitors, however, are exceptional for robust basal Cyp1b1 expression that is relatively insensitive to AhR induction. We hypothesize, therefore, that the suppression of BM progenitor activity is mediated via mesenchymal progenitors that are in close proximity to the stem cell niche. BP is distinguished from DMBA by recovery of hematopoietic activities over 24 hours, through an AhR-dependent process. This recovery is associated with large AhR-mediated increases in multiple cytokines that we attribute to hepatic metabolism of the BP following the extensive induction of Cyp1a1. This metabolism forms BP quinones, which can activate the NF-$\kappa$B pathway, a well-characterized route to cytokine stimulation. Among the stimulated cytokines, Il6 is known to protect hematopoietic progenitors. Cyp1 family members also intervene in the generation of inflammatory regulatory molecules (protectins, resolvins) [7].

We originally identified Cyp1b1 (then P450EF) as the AhR-induced cytochrome P450 in multipotential mouse embryo fibroblasts (MEFs) [8]. Subsequent gene cloning, first from human keratinocytes [9], and parallel efforts in MEFs [10] and rat adrenals [11] established that the same Cyp1b1 gene is involved and suggested a developmental function. The linkage of mutations to glaucoma [12] established a human physiological role. This has been further emphasized by links to cellular morphology and vascular development in endothelia and pericytes [13], to murine female hypertension [14], and to obesity and NASH in male and female mice [15]. Cyp1b1 has also been shown to be highly expressed in many tumors [16]. This role was further emphasized by the endogenous activation of Wnt-induced intestinal polyps in Cyp1b1-ko mice [17].

In order to dissect the role of Cyp1b1 metabolism of PAHs in BM hematopoiesis, we have established an *in vitro* coculture model in which primary mouse BM cells, isolated by collagenase treatment, are cocultured with mesenchymal progenitor cell (MPC) lines. The OP9 cell line, derived from the aorta-gonadal mesonephros (AGM) region of the embryo [18], is routinely used as a stromal support for stem cell cultures. Here, we demonstrate that OP9 cells effectively sustain PreB progenitor proliferative activity for 24 hours, as measured in the PreB colony forming unit assay (PreB CFU). We use this coculture model to show that DMBA and BP effectively suppress this activity. Other MPC lines function in the same way but are less efficient in sustaining CFU activity. In this work, we show that Cyp1b1 activity in the OP9 cells, rather than in the BMC, mediates the PAH suppression of CFU activity. We show that OP9-enriched media support the CFU activity but also that this coculture is no longer sensitive to PAH suppression. The use of Cyp1b1-ko BMC confirms this model and also shows that Cyp1b1 contributes to *in vitro* stability of the HSC activity and, surprisingly, enhances the sensitivity of PreB progenitors to DMBA. To address this anomaly, we examined the gene expression profiles of BM cells from WT and Cyp1b1-ko BMC, as well as the *in vivo* responses to DMBA. A set of Cyp1b1-responsive cytokine and developmental markers is identified.

## 2. Materials and Methods

*2.1. Animals.* C57BL/6J (wild type, WT) mice and $AhR^d$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Cyp1b1-null (Cyp1b1-ko) mice were bred in our animal care facility at the Biotron animal care facility [19]. All mice were housed in the AAALAC certified University of Wisconsin Madison School of Veterinary Medicine and Biotron Animal Care Units and used in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

*2.2. PAH Treatment In Vivo.* Animals were treated with PAH, TCDD, or vehicle control (olive oil) via a single IP injection (DMBA, 50 mg/kg; BP, 50 mg/kg; TCDD, 0.03 mg/kg (Accustandard; New Haven, CT)) prior to isolation of the BMC for analysis [1, 2]. All TCDD exposures were completed in 12 hours, while PAHs were tested for up to 48 hours.

*2.3. Bone Marrow Isolation.* Mice were euthanized under $CO_2$. Femurs were immediately extracted and crushed in extraction media (RMPI + 2% FBS). The bone marrow was exposed and cells were released from the matrix using collagenase type 1 (1600 U) (Worthington, Lakewood, NJ) digestion for 15 min at 37°C, with shaking at 110 rpm [6]. BMC were washed and collected in extraction media using centrifugation.

*2.4. Cells/Cell Culture.* BMS2 cells were a gift from Dr. Paul Kincade [20], while C3H10T1/2 [21] and OP9 [18] cells were purchased from ATCC (Manassas, VA). Cells were cultured under standard conditions (37°C at 5% $CO_2$ in saturated atmospheric humidity), in FBS (Atlanta Biologicals, Flowery Branch, GA) supplemented media (RPMI 1640, BMS2; DMEM, C3H10T1/2; $\alpha$MEM, OP9) (Fisher Scientific,

Waltham, MA) as per recommendations, with passages at 80% confluence. Feeder stromal cultures were grown to approximately 100% confluence and treated with 10 $\mu$g/mL mitomycin C (Sigma, St. Louis, MO) for 24 hours to inhibit further cellular division. Mitomycin C (2 mg/5 mL) stocks were maintained in PBS and kept frozen in liquid nitrogen. Treated feeder layers were maintained in culture for up to 6 weeks. Cocultures were comprised of freshly isolated primary BMC on a feeder layer. Conditioned medium was defined as the media recovered from feeder layer cultures alone after 24 hours. All PAH and TCDD exposures were tested after 24-hour incubation, unless otherwise noted.

*2.5. Microarray.* Microarray analyses were completed with triplicate WT and Cyp1b1-ko mice treated with either DMBA or TCDD as per above. Vehicle control animals were treated with olive oil. RNA was isolated using Qiagen's RNeasy mini-kit (Hilden, Germany) and quantified using a Nanodrop (Thermo Fisher Scientific; Waltham, MA) and integrity was assessed using formaldehyde agarose gel electrophoresis. Cy3 and Cy5 labeling was completed using Agilent Technologies' Dual Color Gene Expression kit and analysis was completed on the Whole Mouse Genome Microarray 4 × 44 slides, using the DNA Microarray Scanner and feature extraction software (Santa Clara, CA). Analysis was completed using EDGE3 software package [6, 22].

*2.6. Treatment In Vitro.* Freshly isolated primary BMC were plated at a density of $1.05 \times 10^6$ cells/cm$^2$ in fresh culture media ($\alpha$MEM + 20% FBS). Coculture experiments were completed using feeder cells, while monocultures were completed with primary bone marrow cells on tissue culture-treated plastics. PAH, TCDD, or DMSO control treatments were added to the cell suspensions at plating. Cells were treated for 24 hours prior to analysis.

*2.7. CFU.* Colony Forming Unit (CFU) assays were completed as per manufacturer's instructions. In brief, BMC were isolated by centrifugation either directly from femur extraction/bone marrow isolation or after 24-hour treatment. The BMC were resuspended in fresh culture media at $5 \times 10^5$ cells/mL for PreB progenitors. 1/10 volume of cells was mixed with the appropriate methocult media (Stem Cell Technologies, Vancouver, BC, Canada) and incubated at standard culture conditions (37°C at 5% $CO_2$ in saturated atmospheric humidity) for 7 days. Colonies were counted by visual inspection via light microscopy. Statistical significance was determined by ANOVA with a Tukey *post hoc* test for multiple comparisons, $p < 0.05$ (GraphPad Prism, San Diego, CA).

*2.8. qPCR.* Induction of *Cyp1b1* was determined using qPCR analysis. Total RNA was isolated as described above. Reverse transcription was completed using random oligos and GoTaq, and specific expression was determined using SYBR qPCR master mix, as per manufacturer's instructions (Promega, Corp; Madison, WI). Signal was detected and integrated using the BioRad CFX Real Time PCR Detection System (Hercules, CA). Primers were obtained from IDT (Coralville, IA): *Cyp1b1* (F: CCACTATTACGGACATCTTCGG, R: CACAACCTGGTCCAACTCAG) and *actin* (F: CAACGAGCGGTTCCATG, R: GCCACAGGATTCCATACCCA). Analysis was completed using GraphPad Prism (La Jolla, CA) software. Ct values were normalized to *actin* expression, fold change was calculated for induction by DMBA treatment, and interval range was calculated for graphical presentation. Fold change data were log transformed and analyzed for statistical significance by ANOVA with Tukey *post hoc* tests.

*2.9. Residual PAH Extraction and HPLC Analysis.* OP9 cells were cultured for 24 hours with media supplemented with 3 $\mu$M DMBA. The media were collected and treated with $\beta$-glucuronidase (Sigma) at 2000 U/mL to optimize recovery of the lipid-soluble residual PAHs. Samples were spiked with an extraction control standard (dibenzo[a,l]pyrene) (NCI Chemical Carcinogen Reference Standard Repositories, Midwest Research Institute, Kansas City, MO) prior to extraction using a 2 : 1 ethyl acetate : acetone (Sigma) organic phase, with repeated extraction centrifugation. The organic phase was dried under liquid nitrogen and the extracted compounds were stored at −80°C until analysis. Dried samples were resuspended in 100% methanol immediately prior to injection onto the HPLC column. Individual PAHs were separated using a Waters (Milford, MA) 2695 HPLC instrument with a $C_{18}$ column, employing both a UV detector (wavelength 254 nm) and a Waters 470 scanning florescence detector. Data was collected with Waters Empower 3 software. Peaks were separated under a 50–100% methanol gradient over a period of 55 minutes [23].

## 3. Results

*3.1. In Vivo Effects of DMBA and BP on PreB Lymphoid Progenitors In Vivo.* We have previously used an acute dosing animal model to study the effects of PAHs on bone marrow hematopoietic lineages [5, 6, 24]. Here, we have used PreB CFU assays to measure the suppression of lymphoid progenitors by IP administration of two well-characterized PAHs (BP and DMBA, 50 mg/kg). We examine the effects after 6 and 48 hours, which correspond to, respectively, the primary hepatic clearance and the midpoint of slow release from peritoneal fat (Figure 1(a)) [5, 6]. The primary clearance is enhanced by the induction of Cyp1a1 in the liver. The clearance kinetics are not significantly different between DMBA and BP [24, 25]. After 6 hours, DMBA and BP lower PreB CFU activity by 50–70 percent. After 48 hours, these activities change in opposite directions. DMBA treatment further declines to 90 percent suppression, while BP treatment shows a complete recovery (Figure 1(b)). This difference is observed in the mature cell populations after 48 hours, wherein there is 50 percent suppression of total BMC by DMBA, but there is no suppression by BP. The progenitor changes depend on Cyp1b1 expression and extend to B and T lymphocytes in the spleen and thymus, which derive from these BM progenitors [6, 26].

In AhRd mice, where DMBA and BP fail to activate the AhR, both PAHs reach the same peak suppression levels within 6 hours (Figure 1(b)). BP, in contrast to DMBA, exhibits an AhR-mediated recovery effect on BMC progenitor

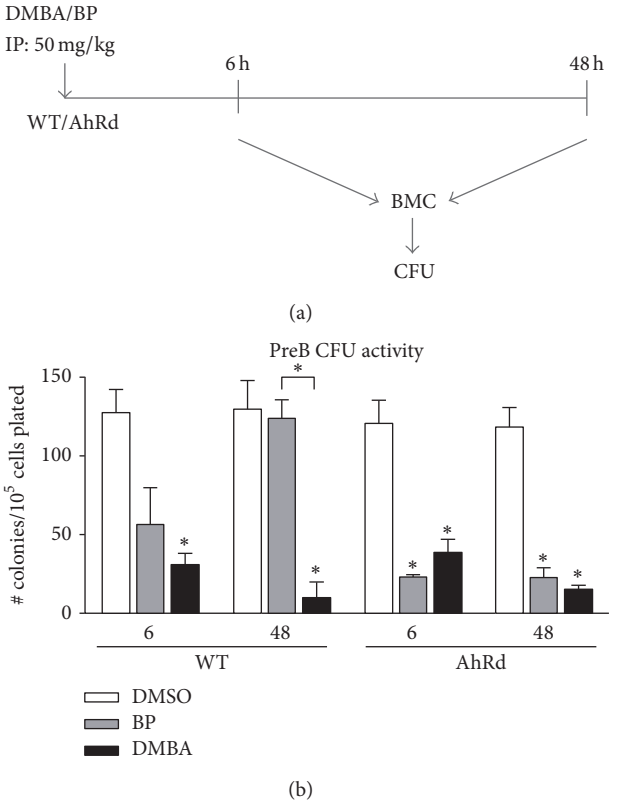


Figure 1: Comparison of progenitor activity after *in vivo* PAH treatment in mice with normal (WT) and diminished ($AhR^d$) AhR activity. (a) Diagram of *in vivo* treatments and isolation points for colony forming unit (CFU) analyses. Mice were treated with DMBA or BP (50 mg/kg) or an equivalent volume of solvent control (DMSO) via a single IP injection. Bone marrow cells (BMC) were isolated 6 and 48 hours after treatment and analyzed for PreB CFU activity. (b) Comparison of PreB CFU activity in WT and $AhR^d$ BMC 6 and 48 hours after PAH administration (50 mg/kg). Significance ($^*p < 0.05$) indicates PAH suppression from untreated control or between PAH treatments at the same time point within a genotype, as indicated.

activity, likely due to the ready formation of BP quinones [5]. Elsewhere, we have shown that these effects of DMBA are dependent on Cyp1b1 [5, 6, 24]. Cyp1a1 deletion also prevents this recovery [25, 27].

*3.2. Reconstitution of an In Vitro Model.* To better assess the mechanism of PAH effects on BM stem and progenitor cells, we have established an *in vitro* model. The first step was to sustain the BMC activity for a sufficient exposure period to see PAH effects (24 hours). BMC isolated by collagenase treatment provide extensive CFU activity when assayed directly in the methylcellulose support media of the progenitor clonal expansion assay. However, 24-hour culture in media alone, prior to transfer to the CFU assay media, resulted in a near complete loss of each CFU activity (representative example, Figure 2(a)). To sustain progenitor activities for 24 hours, we tested the capability of coculture with mesenchymal multipotential stromal lines. BMS2 cells, which were derived from continuous culture of adherent mouse BM progenitor cells [20], retained 40 percent of the activity. OP9 cells, derived from the AGM region of mouse embryos [18], have been extensively used to sustain stem cell activity [28, 29]. OP9 cells fully sustained progenitor activity for 24 hours (Figure 2(a)). C3H10T1/2 cells, which were derived from mouse embryo fibroblasts [21], also appreciably sustained PreB CFU progenitor cell activity (data not shown). Culture medium enriched by OP9 cells (OP9-EM) for 24 hours prior to culture was comparably effective as BMS2 cells in maintaining BMC progenitor activity in absence of the OP9 support (Figure 2(b)).

We also used the CFU assays designed for granulocyte-monocyte (GM) and erythrocyte (E) progenitor populations (Figure 2(c)). The erythrocyte progenitor CFU activity was sustained almost as well as PreB CFU activity. In contrast, the GM progenitor cells lost most of their activity. This may be explained by the fact that OP9 cells do not express macrophage colony stimulating factor (mCSF) [30]. Addition of this factor, however, causes a loss of the other hematopoietic lineages [31].

This OP9/BMC coculture model was then used to test the effects of PAHs on BMC progenitor activity (Figure 3(a)). BMC were either cultured with OP9 cells and serum-containing media or with OP9-EM media. The OP9/BMC coculture model was equally sensitive to both PAHs (BP and DMBA, each 1 $\mu$M) and TCDD (10 nM) treatment, resulting in significant loss of PreB progenitor activity (Figures 3(b) and 3(c)). However, when OP9-EM was used with BMC instead of OP9 cells themselves, the response to DMBA was low (Figure 3(b)). The response to DMBA, therefore, derives, mostly from the OP9 cells, and the metabolism of DMBA by the AhR-inducible Cyp1b1 in hematopoietic BMC is ineffective. However, this experiment does not rule out effects mediated by AhR induction of inhibitory factors in OP9 cells.

A likely possibility is that DMBA is metabolized to active metabolites in OP9 cells. Since BMS2 cells express basal Cyp1b1 that is resistant to AhR induction, we tested OP9 cells for similar expression. Figure 4(a) shows that Cyp1b1 mRNA is present in basal OP9 cells at higher levels than in BMS2 cells. C3H 10T1/2 cells had lower than normal basal levels, but were induced tenfold by DMBA. BMS2 and OP9 shared the same resistance to AhR induction (Figure 4(b)). This basal Cyp1b1 expression, with low AhR induction, is typical of the BM primary counterpart [3]. DMBA products seen here in the HPLC profile are unresolved 10,11- (major) and 8,9- (minor) dihydrodiols (peak A) and mixed phenols (peak B), which are typically seen for Cyp1b1 after a 3-hour incubation with DMBA [32] (Figure 4(c)). This activity in the OP9 cells strongly suggests that metabolism of DMBA in these cells contributes to their requirement for DMBA suppression of CFU activity.

*3.3. The Role of Cyp1b1 in Maintaining PreB CFU in BMC.* To test the contribution of Cyp1b1 to the PreB CFU activity, we cocultured Cyp1b1-ko BMC with OP9 cells for 24 hours (Figure 5(a)). The CFU activity of Cyp1b1-ko BMC, directly following the collagenase treatment, showed a 25 percent

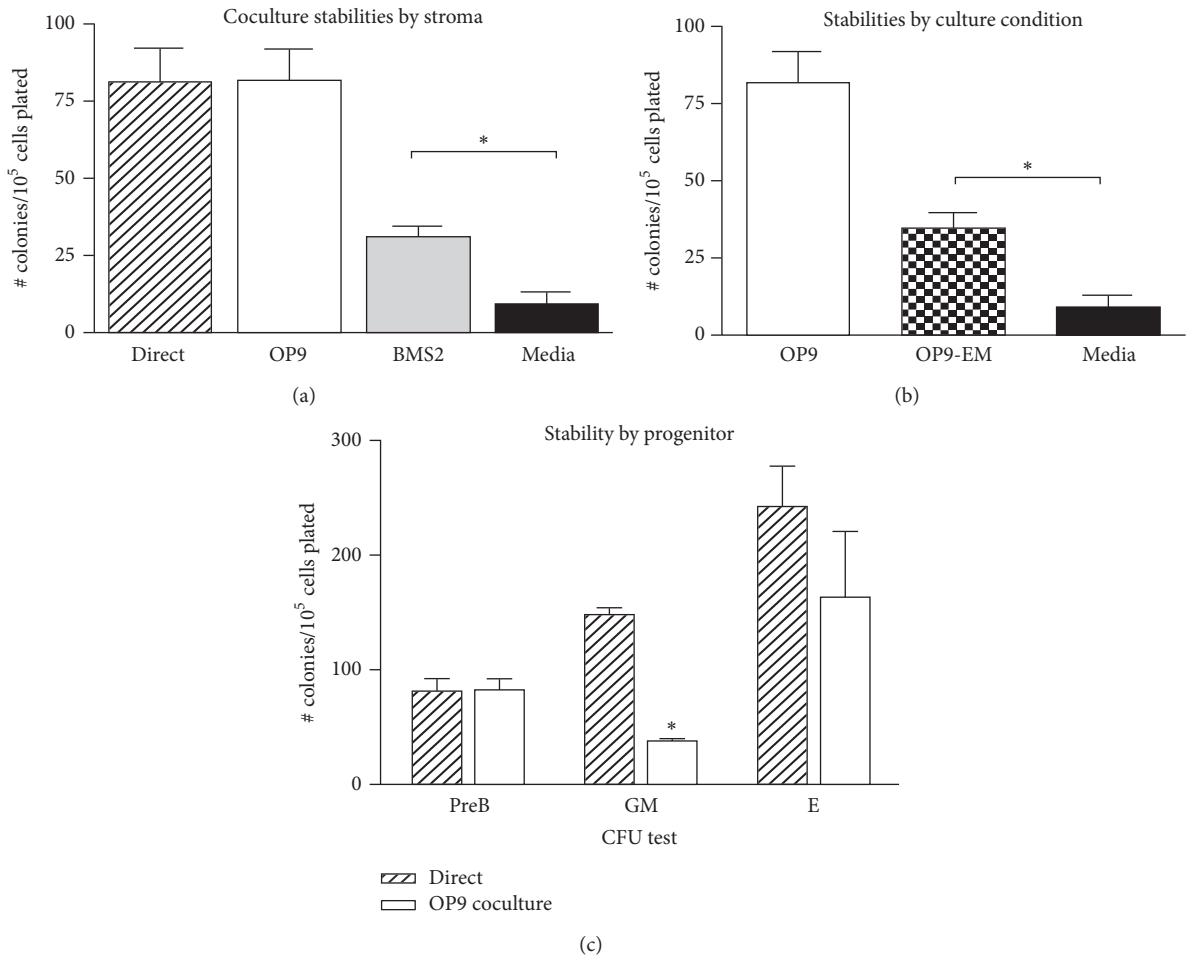


Figure 2: Conditions that sustain progenitor colony forming activities in collagenase-released bone marrow cells (BMC). (a) Comparison of PreB CFU activity in BMC maintained alone in culture media for 24 hours after isolation (media) or sustained by being cocultured for this period with an adherent monolayer of either OP9 cells or BMS2 cells. Direct cultures were placed in the methocult media immediately upon BMC isolation. $^{*}p < 0.05$. (b) BMC PreB CFU activity following 24-hour culture in media alone, in coculture with OP9 cells, or with media enriched by 24-hour preculture with OP9 cells (OP9-EM). $^{*}$Statistical significance; $p$ value < 0.05. (c) CFU progenitor cell proliferative activity (PreB, granulocyte-monocyte (GM), and erythroid (E)) in BMC, when directly isolated or after 24 hours in OP9 coculture. $^{*}p < 0.05$ for comparison between direct BMC culture and OP9 coculture.

decline after the 24-hour coculture when compared to WT BMC ($p = 0.07$). Treatment of the coculture with 1 $\mu$M DMBA indicated that the progenitor activity in Cyp1b1-ko was far more sensitive to DMBA than in WT BMC (62% versus >90% inhibition) (Figure 5(b)). This reinforces the concept that DMBA sensitivity of BMC in this coculture model primarily derives from OP9 cells, likely from conversion by Cyp1b1 reactive dihydrodiol epoxide metabolites. Accordingly, the OP9-enriched media (OP9-EM) were partially effective in supporting PreB CFU in WT and Cyp1b1-ko BMC (Figure 5(c)). In each case, there was no effect of DMBA in absence of the OP9 cells.

The much enhanced sensitivity of Cyp1b1-ko BMC suggests that Cyp1b1 provides stability by regulating constitutive progenitor/stem cell support processes of the hematopoietic cells themselves. We have previously shown that Cyp1b1 prevents oxidative stress, which is highly toxic to these hematopoietic cells [13].

*3.4. Role of Cyp1b1 in OP9 Cells.* The activity of TCDD in this coculture system leaves open the possibility that DMBA may act directly on BMC progenitors via AhR activation in OP-9 cells. Tetramethoxystilbene (TMS) and $\alpha$-NF each potently inhibit Cyp1b1. $\alpha$-NF is also an antagonist for the AhR. We have used these inhibitors to test the participation of Cyp1b1 in DMBA-mediated PreB CFU suppression, which we have now shown can only arise from OP9 cells. One problem is that DMBA binds to Cyp1b1 very potently and is, therefore, difficult to competitively inhibit. In work to be described elsewhere, we have shown that the introduction of a deficiency in the DNA repair gene, XPC (XPC−/−) [33], into the BMC greatly enhances the potency of the PAH suppression (Rondelli, unpublished observation), which is now effective at 20 nM DMBA. This increase in potency allows complete inhibition by either a-NF or TMS (1 $\mu$M each). OP9 cells, therefore, are functioning through Cyp1b1 (Figure 6).

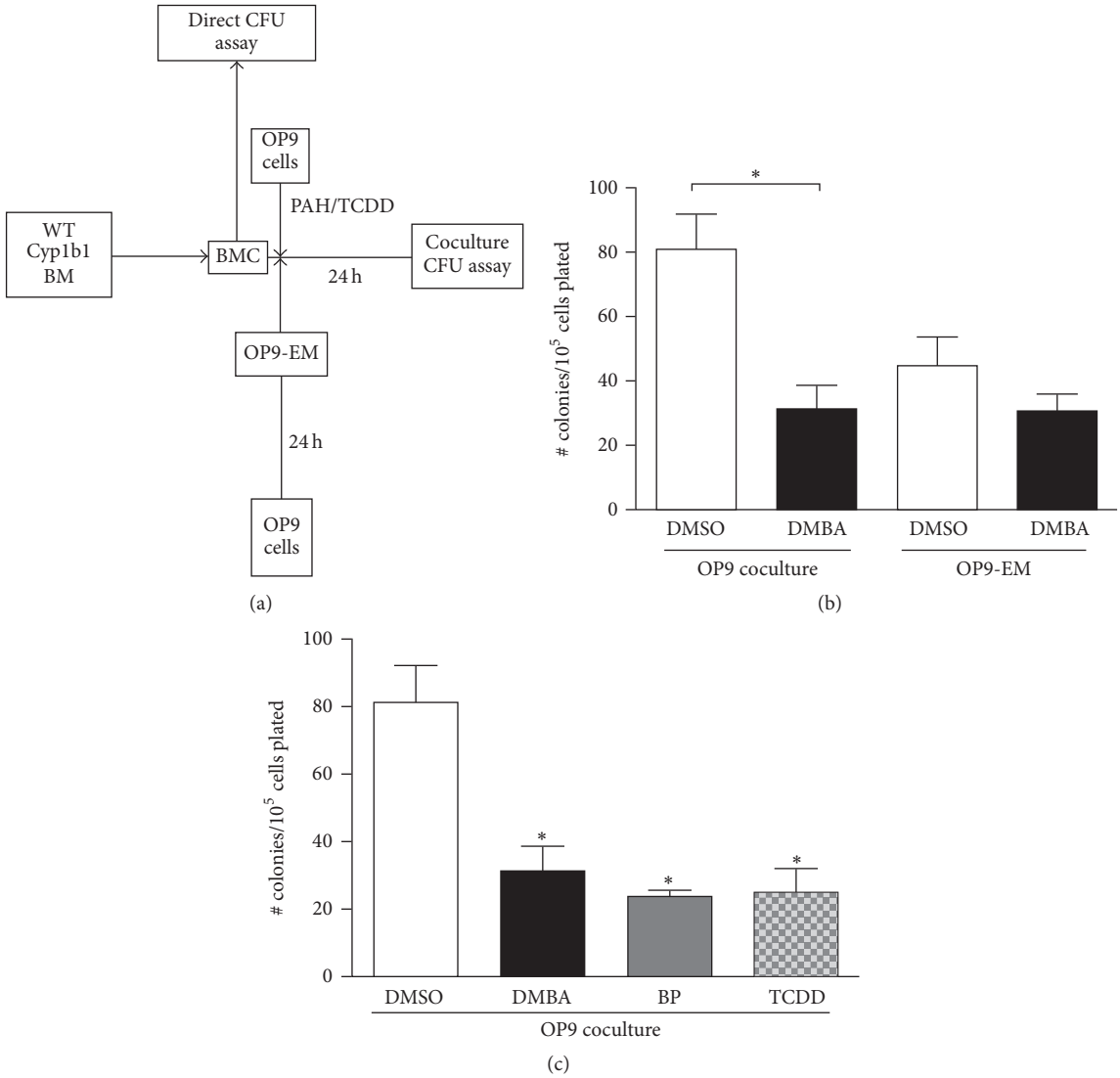


Figure 3: Effect of PAH treatment on WT progenitor activity in OP9 coculture from the fresh BMC preparation without *in vitro* culture. (a) Experimental design for assessing effects of PAHs on progenitor proliferation (PreB CFU activity) of cultured BMC. Cells isolated from the BM of mice of different genotypes are cocultured with serum media for 24 hours alone, on a monolayer stromal support (OP9 or BMS2 cells) or media enriched by 24-hour culture with OP9 cells (OP9-EM). PAH (1 $\mu$M), TCDD (0.01 $\mu$M), or an equivalent volume of DMSO solvent is added at the beginning of the 24-hour period. BMC ($5 \times 10^5$ cells) are added to the CFU methyl cellulose suspension assay at the end of 24 hours. Direct addition of BMC to the CFU assay provides a 100 percent reference activity. (b) Effect of DMBA (1 $\mu$M) in OP9 coculture compared with effect in enriched medium (OP9-EM). *Statistical significance; $p$ value < 0.05. (c) OP9 coculture experiments with DMBA (1 $\mu$M), BP (1 $\mu$M), and TCDD (0.01 $\mu$M) treatments showed significant ($^*p < 0.05$) loss of activity after 24-hour treatment, as compared with the DMSO solvent control.

*3.5. Effects of Cyp1b1-ko on Constitutive and DMBA-Induced BM Gene Expression.* Examination of gene array changes in BM, *in vivo*, after 12 and 24 hours indicated very few responses to DMBA, despite the stimulation of canonical AhR targets equal to that by either BP or TCDD and the extensive stimulation of chemokines and cytokines by BP [5, 6]. We interpreted the low response of DMBA to specific targeting of the small population of progenitor cells rather than the predominant mature cells. To understand the anomalous effects of Cyp1b1 and DMBA in the coculture model, we identified gene expression differences between WT and Cyp1b1-ko BMC (lower level of local metabolism). We also compared the *in vivo* effect of TCDD to determine whether deletion of Cyp1b1 can activate the AhR in the BM. This 12-hour treatment period corresponds to near maximal PreB CFU suppression.

Table 1 shows examples of gene expression levels in mRNA from BM cells of Cyp1b1-ko mice compared to WT mice. We show that genes, which fail to respond to DMBA in WT mice, now respond in Cyp1b1-ko mice, either up- or

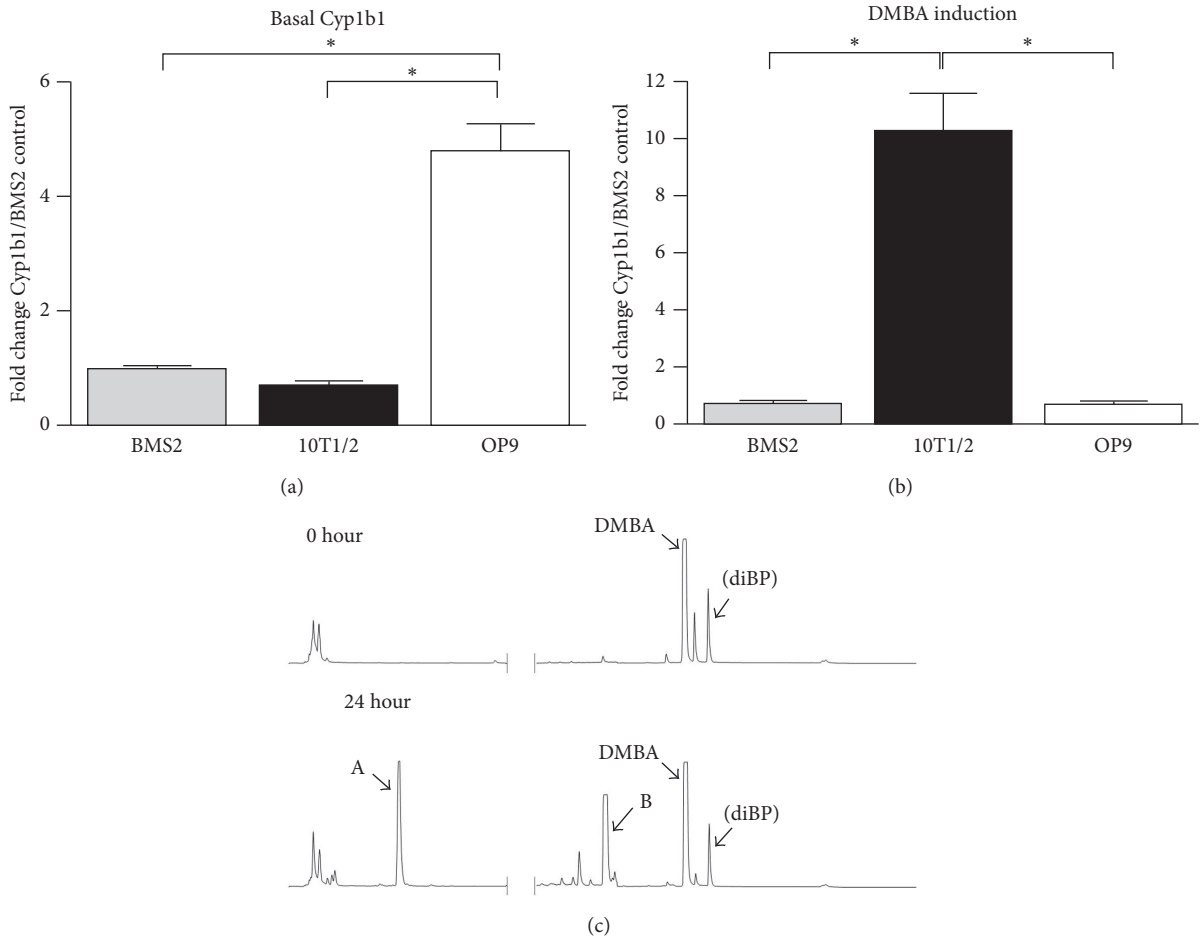


Figure 4: Cyp1b1 induction and DMBA metabolism in OP9 cells compared to other mouse multipotential progenitor lines. (a) Comparison of basal Cyp1b1 mRNA expression in OP9, C3H10T1/2 (10T1/2) cells, and BMS2 cells (set to 1.0). $^{*}p < 0.05$. (b) DMBA (24 hours, 1 $\mu$M) induction of Cyp1b1 mRNA in 10T1/2 cells but not BMS2 and OP9 cells. $^{*}p < 0.05$. (c) HPLC separation of DMBA metabolites after zero- and 24-hour incubation of 3 $\mu$M DMBA with OP9 cells (A: unresolved 8,9- and 10,11-dihydrodiols; B: unresolved 7- and 12-hydroxylmethyl). DiBP is added as an extraction standard after termination of the incubation.

downregulated. All changes shown here are to some degree shared by TCDD. Four groups (A–D) represent stimulation; one group (E) represents suppression.

Group A genes, typified by Cyp1a1, Ahrr and Cyp1b1, are classical AhR responders, with DRE elements that show similar peak upregulation by each PAH at 12 hours [5]. DMBA strongly induces expression in Cyp1b1-ko mice.

Group B comprises examples of genes involved in inflammatory stress, which are stimulated in WT mice by TCDD, but which show larger increases with BP (within 6 hours) [5]. In WT mice, Group B genes show no stimulation with DMBA, even after 12 hours, thus distinguishing these genes from the classic Group A responders. Surprisingly, the expression of each gene in Cyp1b1-ko mice is appreciably enhanced by DMBA. This extra response compared to WT mice may arise because of increased DMBA concentration or a liver metabolite, when the BM lacks metabolism by Cyp1b1.

Like genes in Group B, genes in Groups C and D also respond to TCDD in WT mice, but not to DMBA. Each shows increased expression in Cyp1b1-ko mice. The inflammatory responses in Group B are enhanced by treatment of Cyp1b1-ko mice by DMBA and Group C responses are suppressed by DMBA treatment in Cyp1b1-ko mice, while Group D increases are unaffected by DMBA treatment. Group E genes exhibit decreased expression in WT mice treated with TCDD and in Cyp1b1-ko mice in the absence of treatment, which is also unaffected by DMBA. Essentially, Group E is the suppression counterpart of Group D. Each response to Cyp1b1-ko in Groups D and E is matched by TCDD in WT mice, but not by DMBA. Many of these responses are matched by similar genes in the same family, which are listed in Table 1 legend. The majority of genes in Groups C–E that are characterized by Cyp1b1-ko mice regulate developmental responses, including hematopoiesis.

## 4. Discussion

Recent *in vivo* studies of PAH effects on hematopoietic differentiation in the BM show that single doses rapidly suppress lymphoid, myeloid, and erythroid progenitor activities, when measured with the corresponding CFU assay.

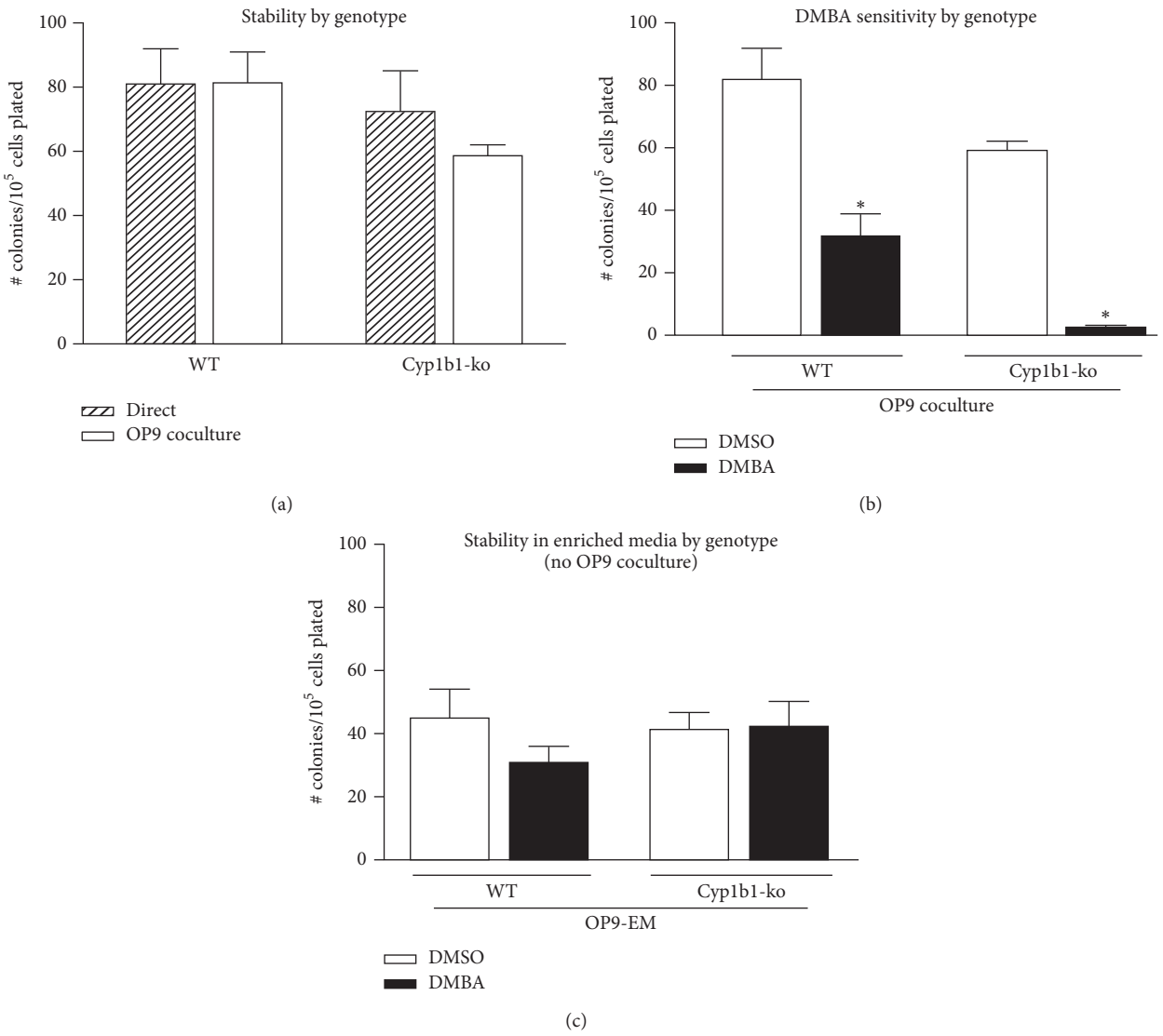


Figure 5: Comparison of WT and Cyp1b1-ko BMC with respect to PreB CFU progenitor cell activity. (a) Comparison of PreB CFU activity from BMC immediately after isolation (direct) and after 24-hour OP9 coculture. (b) Comparison of the PreB CFU activities for BMC after 24-hour solvent control (DMSO) and DMBA (1 $\mu$M) treatments, using OP9 coculture conditions. $^{*}p < 0.05$. (c) Comparison of the PreB CFU progenitor cell activity for WT and Cyp1b1-ko BMC after 24-hour solvent control (DMSO) and DMBA (1 $\mu$M) treatments in OP9 enriched medium (OP9-EM).

The shared suppression process and direct measurements of early progenitor and stem cell populations suggest that hematopoietic stem cell activity is targeted. The relationship of suppression effects to mature cell losses in BM, spleen, and thymus indicates that each of these cellularity losses arises, initially, from a PAH-induced failure to replenish mature cells from the BM hematopoietic stem cells [6]. Each of these tissues delivers mature B and T lymphocytes to the vascular and lymphatic circulation. The progenitor suppression is mediated by Cyp1b1, independent of AhR, despite appreciable induction of Cyp1b1 expression in the BM. We hypothesized that this anomaly arises because the key Cyp1b1 activity is provided by a BM mesenchymal progenitor subpopulation, wherein Cyp1b1 is not inducible.

In the present studies, we describe a coculture model that reproduces *in vivo* PAH suppression of BM progenitor cells *in vitro*. This model establishes the potential for involvement of BM mesenchymal Cyp1b1 in this suppression. Notably, we show that the PAH suppression can arise from Cyp1b1 in the supporting OP9 mesenchymal cells rather than from Cyp1b1 expressed in the BMC. OP9 cells share multipotential mesenchymal progenitor activity with the BM-derived, BMS2 cell line [29, 34–39]. Other mesenchymal lines that are derived from the AGM region of the embryo that yields the OP9 cells exhibit considerable selectivity with respect to this stem cell support capacity [35]. OP9 and BMS2 cells also share appreciable basal Cyp1b1 expression that, like the equivalent primary BM cells, scarcely responds to AhR

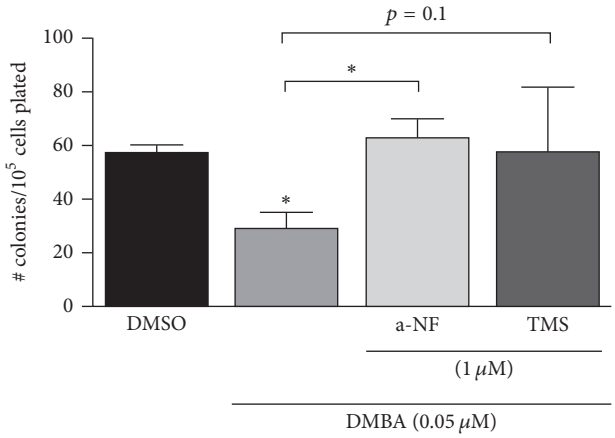


Figure 6: Reversal of DMBA suppression of PreB CFU activity from BMC by Cyp1b1 inhibitors, a-naphthoflavone (a-NF) and tetramethoxystilbene (TMS). To enhance sensitivity to DMBA, BMC from XPC−/− mice (low DNA repair) are used for the OP9 coculture. DMBA concentration is then decreased to 0.05 $\mu$M, which is more sensitive to inhibitors. Inhibitors (1 $\mu$M) were incubated with the cells for 24 hours prior to PreB CFU analyses. $^{*}p < 0.05$.

induction (Figure 4). C3H10T1/2 cells, another multipotential mesenchymal line derived from mouse embryo fibroblasts, also supported the PreB CFU progenitor activity. However, they show high AhR-mediated induction of Cyp1b1.

The origin of the Cyp1b1 activity is most clearly demonstrated by the finding that OP9-enriched media alone sustain PreB progenitor activity in BMC but are largely resistant to DMBA, despite the expression of Cyp1b1 in the BMC. TCDD also suppresses PreB CFU in the BMC/OP9 coculture model (Figure 3(c)), thus indicating a contribution from direct activation of the AhR, which may extend to DMBA. TCDD is not metabolized and may, therefore, transfer into the CFU assay. The effective reversal of DMBA activity by Cyp1b1 inhibitors, a-NF and TMS (Figure 6), indicates that metabolism by Cyp1b1 is much more important than AhR activation.

Surprisingly, coculture of Cyp1b1-ko BMC with OP9 cells greatly increases the sensitivity to PAHs. This enhanced sensitivity probably arises because Cyp1b1 also protects against oxygen-induced oxidative stress (OxS) in endothelia and pericytes, which are each present in BM vascular cells [13]. Increased OxS in Cyp1b1-ko BMC may reach the progenitors and increase their susceptibility to the PAH metabolites. Preliminary studies show that decreasing DNA repair in the BMC population, through loss of XPC activity, similarly increases lymphoid progenitor susceptibility to these metabolites (Figure 6) [33].

This DMBA suppression of the progenitor cells most likely derives from metabolites transferred from the OP9 cells. It remains to be determined why Cyp1b1 in BMC is ineffective in DMBA activation compared to the Cyp1b1 in OP9 cells. The first approach to resolving this problem will be to compare the metabolism of DMBA in these fractions. DMBA metabolites generated by Cyp1b1 in the OP9 cells may also remove progenitor support factors secreted by these

Table 1: Genes targeted by Cyp1b1 endogenous substrates and AhR. Separation by DMBA responses.

| Gene | WT (FC) | | Cyp1b1-ko (FC) | | Set |
|---|---|---|---|---|---|
| | TCDD | DMBA | Control | DMBA | |
| *Stimulated* | | | | | |
| Cyp1b1 | 3.5 | 2.5 | n/a | n/a | |
| Cyp1a1 | 9.7 | 15.0 | nd | [11.0]$^{g}$ | A |
| Ahrr | 3.8 | 4.1 | nd | [10.0]$^{g}$ | |
| IL6 | 3.0 | ns | 2.5 | 4.5 | |
| Tnfa | 2.0 | ns | 1.5 | 3.0 | B |
| Cxcl10$^{a4}$ | 2.0 | ns | 1.5 | 4.0 | |
| Egr2$^{a1}$ | 2.9 | ns | 2.5 | 4.4 | |
| Hbb-b1$^{a2}$ | 2.4 | 1.3 | 2.2 | ns | |
| Fau | 2.0 | 1.5 | 2.2 | 1.3 | |
| Alas2$^{b1}$ | 1.7 | ns | 1.6 | ns | |
| Creb3l3 | 2.9 | ns | 7.6 | 1.9 | C |
| Hoxb6$^{a5}$ | 2.6 | 1.3 | 2.7 | ns | |
| Sox19$^{a6}$ | 1.5 | ns | 5.0 | 2.2 | |
| Polr2a | 5.0 | ns | 6.2 | 2.3 | |
| Klf4 | 3.6 | 1.3 | 2.1 | 1.7 | |
| Nr4a1 | 3.6 | ns | 2.0 | 2.2 | |
| Hspa-la$^{a3}$ | 8.5 | ns | 2.6 | 3.0 | D |
| Clqc$^{a7}$ | 1.8 | 1.4 | 2.8 | 2.4 | |
| Socs3 | 2.0 | 1.5 | 2.0 | 2.8 | |
| Rag1 | −1.8 | ns | −3.0$^{*}$ | −3.0 | |
| Erdr1 | 2.5 | ns | −2.4 | −2.4 | |
| Col5a3 | −1.5 | ns | −3.0$^{*}$ | −2.0 | E |
| Faim3 | −1.5 | 1.3 | −2.9 | −1.5 | |
| Spp1 | −3.0 | 1.6 | −2.2 | −1.9 | |

Fold-changes (FC) are all significant; $p < 0.05$.
ns: not significant.
n/a: not available.
nd: not detectable.
[FC]$^{g}$: relative to Cyp1b1-ko control genotype effect on PAH.
$^{a}$Comparable responses from family members (a1, Egr1; a2, Hbb-a1/a2; a3, Hspa-lb; a4, Cxcl9; a5, Hoxb9; a6, Sox15; a7, Gimap1).
$^{b}$No response from family member, Alas1.
$^{*}$No significant difference from BP.

cells. DMBA addition, during the enrichment pretreatment, was relatively ineffective in diminishing the activity of the enriched media (EM factors) (not shown). Such factors appear to replace about half of the progenitor support activity in OP9 cells (Figure 3(b)). The remaining support comes from labile factors, which are not retained in the OP9-EM.

*In vivo*, BP-mediated suppression of progenitor activity is restored after approximately 6 hours. We have shown that this reversal derives from metabolic activation generated by AhR-mediated induction of Cyp1a1 [5]. Most likely, BP quinones produced in the liver upregulate cytokines and chemokines in BM through NF-kB activation. Cytokines, like IL6, or the antiapoptotic NF-kB effects can selectively reverse the BP toxicity on progenitor cells. This reversal of CFU suppression is not seen with BP treatment in the OP9 coculture model, where BP has comparable effects to DMBA. This parallel

is seen *in vivo*, in AhRd mice, which are resistant to PAH induction (Figure 1).

Gene expression analyses show that Cyp1b1 deletion in the BMC does much more than remove PAH metabolism. *In vivo*, effects arising from endogenous or dietary substrates are evident following the BMC deletion of Cyp1b1. Additionally, deletion of Cyp1b1 introduces *in vivo* effects of DMBA that are absent in WT mice. Such effects arise either from increased DMBA in the BM or new alternative metabolites, which can each result from removal of the Cyp1b1 metabolism. The set of chemokines and cytokines that were increased by BP and, to a lesser extent, by TCDD are elevated constitutively in Cyp1b1-ko BM. This activity is augmented by cotreatment with DMBA, which has no such effect in WT mice. The Cyp1b1 deletion alone did not increase the canonical AhR/ARNT-directed transcription. The AhR-type activity in Cyp1b1-ko BM, therefore, does not derive from AhR/ARNT complexes. We have previously concluded that the acute cytokine/chemokine responses to BP, which are only partially reproduced by TCDD, actually arise from a partnership between AhR and NF-kB transcription factors. A stimulatory partnership between AhR and Rel B increases cytokine expression [40].

The gene expression changes caused by Cyp1b1-deletion in Table 1 arise from a loss of the effects of endogenous and dietary compounds that affect BM gene expression and from changes in the ratios of the BM hematopoietic cell populations. Stem cells and hematopoietic lineage progenitors are present in low proportions and, therefore, will contribute directly to gene expression. Physiological intervention by Cyp1b1 deletion occurs through removal of estradiol and generation of hydroxyl products, which variously target nuclear (ER$\alpha$, ER$\beta$) and membrane associated receptors (ER$\alpha$, GPER), and affects an increasing number of processes, including blood pressure and cancer [41] and tryptophan dimerization products [42]. We detected many effects of Cyp1b1 deletion on developmental factors. Even though expressed at low levels, these genes can only be detected if appreciably expressed in more abundant cell types among the many present isolated from BM (perhaps >5 percent of total). By far the predominant response pattern observed is an increase or decrease in Cyp1b1-ko mice with a correspondingly diminished response in Cyp1b1-ko mice treated with DMBA. Creb3l3, Hoxb6, Hoxb9, Sox15, and Sox19 are each appreciably increased in Cyp1b1-ko mice through a mechanism that is shared by TCDD and BP in WT mice but is blocked by DMBA in Cyp1b1-ko mice. Some Cyp1b1-ko responses are unaffected by DMBA, including stimulations of Socs3 and Nr4a1, which are similarly increased by TCDD, and decreases in Rag1 and Spp1/osteopontin. These gene changes point to a novel crosstalk between the effects of endogenous Cyp1b1-ko signaling and the AhR that is selectively affected by DMBA.

## Additional Points

*Highlights*. The selective suppression of hematopoietic progenitor proliferation (CFU activity) by DMBA is directed by metabolism in BM stromal cells, which exhibit robust basal expression of Cyp1b1 but minimal induction. OP9 mouse embryo mesenchymal cells secrete factors that support proliferation of BM lymphoid and erythroid progenitors for at least 24 hours. This support is substantially replicated by OP9-enriched media and by BMS2 and C3H10T1/2 cells. Cyp1b1 is expressed at sufficient levels in OP9 cells to generate reactive DMBA metabolites that inhibit progenitor cell expansion. Cyp1b1 levels are much lower in isolated BMC and insufficient to generate inhibitory metabolites. Cyp1b1 provides basal protection to progenitors in BM, either through removal of oxidative stress or through the observed suppression of the AhR/NF-kB stimulation of chemokines and cytokines. These secreted proteins can protect progenitor cells or the activity of precursor hematopoietic stem cells.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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*Review Article*

# Antioxidant Functions of the Aryl Hydrocarbon Receptor

**Cornelia Dietrich**

*Institute of Toxicology, University Medical Center of the Johannes Gutenberg University Mainz, Obere Zahlbacherstr. 67, 55131 Mainz, Germany*

Correspondence should be addressed to Cornelia Dietrich; cdietric@uni-mainz.de

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The aryl hydrocarbon receptor (AhR) is a transcription factor belonging to the basic helix-loop-helix/PER-ARNT-SIM family. It is activated by a variety of ligands, such as environmental contaminants like polycyclic aromatic hydrocarbons or dioxins, but also by naturally occurring compounds and endogenous ligands. Binding of the ligand leads to dimerization of the AhR with aryl hydrocarbon receptor nuclear translocator (ARNT) and transcriptional activation of several xenobiotic phase I and phase II metabolizing enzymes. It is generally accepted that the toxic responses of polycyclic aromatic hydrocarbons, dioxins, and structurally related compounds are mediated by activation of the AhR. A multitude of studies indicate that the AhR operates beyond xenobiotic metabolism and exerts pleiotropic functions. Increasing evidence points to a protective role of the AhR against carcinogenesis and oxidative stress. Herein, I will highlight data demonstrating a causal role of the AhR in the antioxidant response and present novel findings on potential AhR-mediated antioxidative mechanisms.

## 1. Introduction

The AhR is a transcription factor belonging to the basic helix-loop-helix/PER-ARNT-SIM family [1]. Among this group of proteins, the AhR is the only one that is activated by a ligand. The unliganded receptor is predominantly localized in the cytosol and is associated with two heat shock proteins 90, the immunophilin homologous AhR interacting protein (AIP, also known as ARA9 or XAP2), and the cochaperone p23. After ligand binding, the complex is disrupted which leads to nuclear translocation of the AhR. After heterodimerization with aryl hydrocarbon receptor nuclear translocator (ARNT), the AhR/ARNT heterodimer binds to specific enhancer sequences, known as xenobiotic responsive elements (XREs) or dioxin responsive elements (DREs). Consequently, transactivation of several genes is induced. These genes encode phase I and II xenobiotic metabolizing enzymes, such as *cytochrome P450 monooxygenases (CYP1A1*, *CYP1A2*, and *CYP1B1)* and *glutathione-S-transferases (GSTs)*, *NADPH/quinone oxidoreductase (NQO1)*, and *aldehyde dehydrogenase 3*, respectively (for reviews, see [2, 3]). This AhR-triggered pathway is referred to as the canonical pathway and mediates xenobiotic metabolism.

The AhR was originally discovered due to its stimulation by a variety of planar aromatic hydrocarbons, such as benzo[*a*]pyrene (B[*a*]P), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs). The above-described canonical AhR signaling pathway at least partially explains the carcinogenicity of polycyclic aromatic hydrocarbons which are not only detoxified, but also metabolized at the same time to genotoxic compounds. However, it does not help to clarify the molecular mechanisms of toxic effects induced by nongenotoxic AhR ligands, such as TCDD, which is not metabolized. *In vivo* studies in two genetically different rat strains indicate that AhR-driven CYP1A1 induction and tumor promotion can be uncoupled from each other supporting the idea of additional AhR-triggered pathways [4]. To date, several novel noncanonical AhR-driven pathways have been identified and studies in $AhR^{-/-}$ mice provide strong evidence for AhR functions beyond xenobiotic metabolism [4–6]. Alternative binding regions for the AhR or the AhR/ARNT heterodimer have been identified [7–12]. It was also found that the transcription factor NF-$\kappa$B modulates AhR signaling [13]. In addition to its well-known function as a transcription factor, the AhR has been shown to possess E3 ubiquitin ligase activity [14].

Interestingly, also naturally occurring compounds, such as indoles and several flavonoids (e.g., quercetin), which are present in food, may act as AhR agonists. In search for potential endogenous AhR ligands, diverse compounds such as tryptophan derivatives, arachidonic acid metabolites, equilenin, heme metabolites, and indigoids have been characterized [15]. Also, pharmaceutical drugs may act as AhR ligands, for example, omeprazole or ketoconazole [16, 17]. Furthermore, the AhR is activated by UV photoproducts of tryptophan and regulated by nonligand signals such as cAMP [18, 19]. However, the physiological or toxicological consequences of AhR activation by these ligands are mostly unclear.

Strong evidence indicates that activation of the AhR leads to oxidative stress. This may happen due to metabolism of the ligand and by induction of CYP1 enzymes. It is known that the B[*a*]P-metabolite B[*a*]P-7,8-dihydrodiol is metabolized by aldo-keto reductases forming B[*a*]P-7,8-diol. The catechol groups are sequentially oxidized which results in the formation of a semiquinone radical and B[*a*]P-7,8-dione. Further reduction by NADPH-mediated mechanisms causes again formation of B[*a*]P-7,8-diol. This redox cycling of the B[*a*]P metabolite B[*a*]P-7,8-diol leads to the release of superoxide anions and $H_2O_2$ thereby rapidly inducing oxidative DNA damage [20]. *In vitro*, production of reactive oxygen species (ROS) can also be explained, among other mechanisms, by the induction of CYP1A1 (and CYP1B1), uncoupling of electron transfer, and hence superoxide release (for review, see [21]). However, there is increasing evidence that the AhR also displays protective functions against oxidative stress. The AhR target genes *GST* and *NQO1* are well-known enzymes playing important roles in the cellular defense against ROS. CYP1A2 protects against ROS formation by scavenging free electrons [22]. Protective functions of the AhR have also been observed *in vivo*. The AhR reduces colon carcinogenesis in the $APC^{Min/+}$ mouse [14]. $AhR^{-/-}$ mice show higher inflammation in the colon in several experimental models [23, 24], and activation of the AhR attenuates skin inflammation induced by imiquimod [25]. Here, I will highlight data suggesting a causal role of the AhR in the antioxidant response. An overview of the potential mechanisms will be presented and future directions will be proposed. Anti-inflammatory mechanisms of the AhR which also lead to reduction of oxidative stress will not be addressed in detail. Here, the reader is referred to recent excellent reviews [26–28].

## 2. The AhR-Nrf2 Pathway

One of the best studied antioxidant responses mediated by the AhR is activation of nuclear factor erythroid 2 p45-related factor 2 (Nrf2). Nrf2 is a transcription factor which is the key to protection against oxidative stress. It regulates not only a variety of antioxidant enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1), $\gamma$-glutamylcysteine synthetase, thioredoxin, or heme oxygenase-1, but also several phase I and phase II drug metabolizing enzymes, for example, UDP-glucuronosyltransferase 1A6 (UGT1A6) and glutathione S-transferase (GSTA1/2) as well as multidrug resistance-associated protein transporters (for reviews, see [29, 30]). Activity

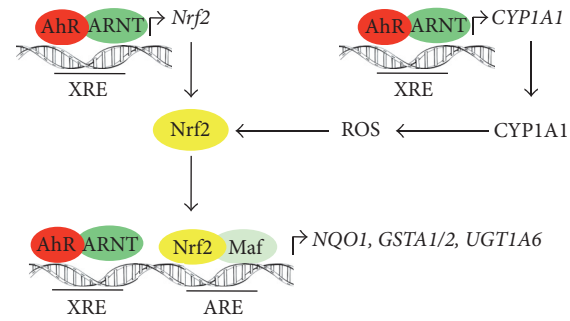


Figure 1: The proposed model for coordinate induction of Nrf2-dependent genes by AhR and Nrf2. Activation of the AhR leads to dimerization with ARNT and transcriptional activation of both Nrf2 and CYP1A1. CYP1A1 increases intracellular ROS thereby stabilizing Nrf2 protein. Nrf2, in association with Maf, binds to AREs, and the AhR/ARNT complex binds to XREs in the promoter regions of *NQO1*, *GSTA1/2*, or *UGT1A6*.

of Nrf2 is regulated by various mechanisms. Under physiological conditions, in the cytosol, Nrf2 is bound to Kelch-like ECH-associated protein-1 (Keap1), an adaptor protein for the Cullin 3-based ubiquitin E3 ligase complex (Cul3) which mediates ubiquitination of Nrf2, thereby leading to its proteasomal degradation. Hence, the basal levels of Nrf2 are kept constantly low under normoxic conditions. An increase in oxidative/electrophilic stress leads to oxidation of the cysteine residues in Keap1 and, at the same time, inhibition of the activity of the E3 ubiquitin ligase. As a result, Nrf2 protein accumulates, dissociates from Keap1, and is translocated to the nucleus where it dimerizes with Maf or c-Jun. The heterodimer then binds to the so-called antioxidant elements (AREs), also sometimes referred to as electrophile response elements (EpREs), in the promoter region of responsive genes [30, 31]. Activity of Nrf2 can further be regulated by phosphorylation, for example, by protein kinase C (PKC) or the mitogen-activated protein kinases ERK1/2 [32–34]. In addition, expression of Nrf2 is regulated epigenetically by methylation of CpG islands in the promoter and acetylation of histones and microRNA (for review, see [35]) and at the transcriptional level, for instance, in response to oncogenic activation of Ras via a TPA-responsive element (TRE) (for review, see [36]).

Interestingly, there is an overlap between Nrf2 and AhR target genes, that is, *NQO1*, *GSTA2*, and *UGT1A6*. The promoters of these genes contain functional XREs and AREs and, as a consequence, induction of these genes requires activation of AhR and Nrf2 [37] (for review, see [29]).

Two different mechanisms probably account for activation of Nrf2 by AhR, that is, (i) direct transcriptional activation of Nrf2 and (ii) generation of ROS by induction of CYP1A1 (Figure 1). Miao and coworkers were the first to show that transcription of Nrf2 is directly regulated by AhR [38]. Exposure of hepatoma cells to TCDD led to induction of Nrf2 mRNA and protein, which was abolished by siRNA, targeted against AhR. They further identified three potential XREs in the murine Nrf2 promoter which appeared to be functional as detected by site-directed mutagenesis and electrophoretic

mobility shift assays. Direct binding of the AhR to these XREs was finally shown by chromatin immunoprecipitation assay. It is worth noting that 5 XRE-like elements are found in the human Nrf2 promoter (for review, see [39]). The results of the *in vitro* studies were confirmed *in vivo*. Yeager and coworkers demonstrated that TCCD mediates induction of Nrf2 and its nuclear translocation and transactivation of the Nrf2 target genes *NQO1*, *UGTs*, and *GSTs* in mouse liver. Upregulation of Nrf2 was completely blocked in the liver of $AhR^{-/-}$ mice. Induction of NQO1 was absent in both $AhR^{-/-}$ and $Nrf2^{-/-}$ mice. Furthermore, TCDD-mediated increase in UGT1A6 and several GST isoforms was abolished in $Nrf2^{-/-}$ mice [40, 41]. Similar results concerning NQO1 upregulation have been obtained in $Nrf2^{-/-}$ mice after exposure to the AhR ligand 3-methylcholanthrene (3-MC) [42]. This implies that both AhR and Nrf2 are indispensable for TCDD-mediated NQO1 induction as well as for upregulation of UGT1A6 and GSTA1.

The second explanation for AhR-mediated activation of Nrf2 is based on the observation that activation of the AhR may increase intracellular ROS levels (for review, see [21, 43]). As stated above, production of ROS can be explained *in vitro* by AhR-mediated induction of CYP1A1 (for review, see [21]). An increase in intracellular ROS should lead to both oxidation of Keap1 and release of Nrf2 from the complex.

To date, it has not been finally proven which of the two mechanisms of Nrf2 activation in response to AhR stimulation predominates. Data exist supporting both pathways. Possibly, differences between rodents and humans help explain the divergent results. In mice, TCDD-triggered upregulation of the Nrf2 target gene *NQO1* is similar in wild-type (wt) and $Cyp1a1^{-/-}$ mouse liver [44]. In line with this observation, TCDD increases expression of Nqo1, Gsta1, and Ugt1a6 mRNA in the livers of Cyp1a1/Cyp1a2/Cyp1b1 triple-null mice [45]. In contrast, CYP1A1 is required for NQO1 induction in human hepatoma cells [46]. TCDD-triggered induction of NQO1 mRNA expression is abolished after transfection of an inactive CYP1A1 mutant and in the presence of the antioxidant N-acetyl cysteine. Oppositely, overexpression of CYP1A1 leads to a similar increase in hydrogen peroxide formation and subsequent NQO1 mRNA compared to TCDD treatment. Hence, the data speak against a pivotal role of CYP1A1 in Nrf2 activation at least in mouse and favour direct transcriptional activation of Nrf2 by the AhR whereas CYP1A1 activation seems to play a role in Nrf2 activation in human liver cells.

In addition to species or cell type specificity, which are well-known characteristics of AhR function, ligand-specific effects have been described recently in human keratinocytes. Ketoconazole, a widely used antifungal compound, leads to AhR/ARNT-dependent induction of Nrf2 protein and its nuclear translocation and induction of NQO1 protein [17]. Importantly, this results in profound inhibition of intracellular ROS generation induced by tumor necrosis factor $\alpha$ (TNF$\alpha$) or B[*a*]P. (Interestingly, B[*a*]P leads to an increase in Nrf2 protein, but not to its nuclear translocation.) Ketoconazole itself does not lead to ROS production. It is worthy of note that ketoconazole has only a weak effect on the induction of CYP1A1, thereby supporting the hypothesis that AhR-dependent pathways might be separated from each other in a ligand-dependent way. Similar results were obtained by Takei and coworkers using cynaropicrin, a phytochemical derived from artichoke [47]. Cynaropicrin results in AhR-dependent NRF2 induction, followed by an increased expression of NQO1, and thereby inhibits ROS production mediated by B[*a*]P or UVB, while CYP1A1 mRNA showed a weak increase. Extracts of *Opuntia ficus-indica* also lead to an albeit weak but nonetheless significant increase in Nrf2 activity and NQO1 expression AhR-dependently and reduction of B[*a*]P- or TNF$\alpha$-mediated ROS generation [48].

In summary, these studies show prominent antioxidant functions of the AhR by inducing the Nrf2 response with subsequent upregulation of NQO1, GSTA1/2, and/or UGT1A6. NQO1 has an important function in the reduction of quinones to quinols by a one-step $2e^-$-reduction process, thereby bypassing the semiquinone step and avoiding the generation of ROS. It also maintains endogenous antioxidants in their reduced form, such as coenzyme Q (ubiquinone) and $\alpha$-tocopherol-quinone, reduces lipid peroxidation, and quenches superoxide (for review, see [39, 49]). GSTs are required for detoxification of electrophilic compounds by reaction with glutathione [50]. UGTs also contribute to the antioxidant response by catalyzing conjugation of glucuronic acid, for instance, with quinols, thereby facilitating their excretion [51]. Interestingly, some ligands are able to differentially activate the AhR/Nrf2/NQO1 pathway while the AhR/CYP1A1 axis is only weakly induced.

## 3. Expression of Superoxide Dismutase

Superoxide dismutase (SOD) is a key enzyme in the protection of cells against the harmful superoxide anion radical which constitutively derives from leakage of the mitochondrial respiratory chain. SOD functions by dismutating the superoxide anion radical to molecular oxygen and hydrogen peroxide, the latter being detoxified by catalase. Three isoforms exist in humans: SOD1 (CuZn-SOD), a cytosolic enzyme containing copper and zinc ions in the active site, SOD2 (Mn-SOD), a mitochondrial enzyme bearing a manganese ion, and SOD3, a secreted isoform expressed only by a few cell types (for reviews, see [52, 53]). Whereas to date no XRE has been found in the *SOD2* promoter, functional XREs have been identified in the promoters of human and rat *SOD1* gene. They were originally identified by deletion/mutation analysis of promoter constructs of rat or human SOD1. Electrophoretic mobility shift assays revealed binding of a TCDD-inducible receptor complex to the XRE. Finally, endogenous SOD1 expression could be stimulated by TCDD in a human hepatoma cell line [54, 55]. The authors later showed that the promoter of *SOD1* also contains a functional ARE and that TCDD-dependent activation of *SOD1* requires both regulatory elements, that is, XRE and ARE [56]. Interestingly, basal expression of SOD1 (and SOD2) was diminished in primary lung fibroblasts derived from $AhR^{-/-}$-mice, but expression of SOD1 could not be increased by cigarette smoke extract in wt-fibroblasts [57, 58]. Very recently, it was demonstrated that

fetal pulmonary cells derived from $AhR^{-/-}$-mice displayed reduced SOD1 induction in response to hyperoxia [59]. *In vivo*, data concerning induction of SOD1 by AhR ligands are likewise inconsistent. Acute exposure of 3-MC induced upregulation of SOD1 mRNA in mouse liver [60], but not in extrahepatic tissues, such as lung, kidney, or heart tissue [61]. In contrast, no increased expression of SOD1 mRNA could be detected in mouse liver after TCDD treatment [41].

## 4. Nrf2-Independent Antioxidant Functions

Some *Brassica*-derived phytochemicals exert antioxidant functions. A prominent example is sulforaphane, a potent inducer of Nrf2. *Brassica* vegetables are rich in glucosinolates which are hydrolyzed during digestion to various products including isothiocyanates, thiocyanates, and indoles (for review, see [62]). The isothiocyanate sulforaphane is the hydrolysis product of the glucosinolate glucoraphanin, while indole-3-carbinol (I3C) is a major autolysis product derived from glucobrassicin. Indole-3-carbinol is further converted to various condensation products at acidic pH *in vivo* and *in vitro*, such as 3,3′-diindolylmethane (DIM) and indolo[3,2-*b*]carbazole (ICZ) [63, 64] (for review, see [15]). Interestingly, both ICZ and DIM are potent ligands of the AhR [63] (for review, see [15]). First evidence for a protective function of ICZ against oxidative DNA damage was provided by Bonnesen and coworkers. In the colon carcinoma cell line LS-174, pretreatment with sulforaphane together with ICZ was shown to reduce the level of DNA single-strand breaks in response to B[*a*]P or hydrogen peroxide [65]. Since both B[*a*]P and hydrogen peroxide lead to an increase in intracellular ROS formation [20, 66] (and our own unpublished data), the data suggest an antioxidant function of ICZ. We deeply investigated a potential antioxidant effect of ICZ and revealed that ICZ protects against oxidative DNA damage in various cell lines, including the colon carcinoma cell line Caco-2 [67]. ICZ decreased DNA single-strand breaks (SSB) and 8-oxo-2′-deoxyguanosine (8-oxo-dG) formation induced by hydrogen peroxide, tertiary-butyl-hydroperoxide (t-BOOH), or B[*a*]P when preincubated for 24 h. We found that intracellular ROS levels were attenuated following t-BOOH exposure. Simultaneous addition of ICZ did not protect against t-BOOH-induced SSB formation, nor could we detect a direct radical scavenging effect of ICZ as confirmed by an *in vitro* DPPH assay. Functional inhibition of the AhR and AhR/ARNT defective cell lines demonstrated that the AhR/ARNT pathway is mandatory for the observed ROS defense caused by ICZ, suggesting that AhR-mediated regulation of defense genes is involved. Protection was also detected in response to TCDD. The effect of additional AhR ligands has not been investigated yet. The downstream target(s) of the AhR/ARNT pathway mediating the protection against oxidative stress is not known yet. The observations that (i) the protective effect could not be reversed by trigonelline, an inhibitor of Nrf2, and that (ii) we could detect neither upregulation nor nuclear accumulation of Nrf2 protein speak against involvement of Nrf2. Furthermore, we did not find any increase in SOD1 protein expression after ICZ exposure (unpublished observation). Experiments to unravel the molecular mechanism of AhR-mediated protection against oxidative stress are in progress.

## 5. Induction of Paraoxonases (PONs)

The family of paraoxonases (PONs) comprises three enzymes, that is, PON1, PON2, and PON3. PON1 is predominantly found extracellularly in the blood stream where it is associated with HDL (high-density lipoprotein). PON2 and PON3 are intracellular proteins (for review, see [68]). PON1 and PON3 are synthesized in the liver, and PON2 is ubiquitously expressed in many tissues. Although the precise mechanisms of function are largely unknown, all PONs exert antioxidant functions. PON1, originally identified as a plasma hydrolase metabolizing paraoxon, has important antioxidant properties which, at least partially, account for the protective functions of HDL. For instance, PON1 decreases lipid peroxidation and generation of malondialdehyde. Malondialdehyde is known to trigger intracellular pathways which inhibit endothelial NO-synthase (eNOS) signaling and eNOS-dependent NO production. Hence, proper PON1 function is crucial for NO formation. In addition, PON1 inhibits myeloperoxidase activity in HDLs under inflammatory conditions (for review, see [68]). PON2 and PON3 also attenuate lipid peroxidation by lowering intracellular ROS, especially by maintaining proper mitochondrial function. For instance, PON2 is localized in the inner mitochondrial membrane where it is essential for correct function of the electron transport chain. As a result, PON2 decreases the release of mitochondria-derived superoxide (for reviews, see [68, 69]).

More than ten years ago, Barouki's lab revealed that activation of the AhR leads to induction of PON1 in human hepatoma cells as well as *in vivo* in mouse liver. Interestingly, 3-MC and the phytocompounds quercetin and flavone were strong inducers of PON1 whereas TCDD elicited only a marginal effect on PON1 expression [70]. The fact that induction of CYP1A1 was intense after TCDD, despite being weak after quercetin treatment, indicates again that separate stimulation of AhR pathways is feasible and probably dependent on the ligand. Functional inhibition of the AhR, either pharmacologically or by siRNA, decreased quercetin-triggered PON1 induction, whereas overexpression of the AhR enhanced it. These observations strongly indicate that quercetin-mediated PON1 activation requires AhR. However, PON1 gene expression was not mediated by a classical XRE, but rather by a noncanonical XRE (identified core sequence GCGGG) in the PON1 promoter [70]. Interestingly, resveratrol, which was originally described as an AhR antagonist [71], also led to PON1 expression in an AhR-dependent manner [72]. However, the functional consequence of AhR-mediated induction of PON1 *in vivo* has not been studied yet. Recently, Shen and coworkers demonstrated that the dioxin-like PCB126 leads not only to an increase in PON1 mRNA and activity in rat liver, but also to elevation of PON2 and PON3 [73, 74]. Importantly, elevation of PON1 activity could also be detected in the serum. No induction of any PON enzyme could be seen in the lungs of the animals after PCB126 treatment. In contrast, 3-MC upregulated PON3

expression in liver and lung, while TCDD only induced PON3 mRNA in the lung. The underlying mechanism for these ligand-specific effects on PON expression has not been clarified so far. Although the authors did not investigate a causal role of the AhR in PCB126-mediated induction of PON1 (or PON2/3) in the rat, involvement of the AhR is very likely due to the well-known action of planar PCBs on the AhR [75]. In line, non-dioxin-like PCBs did not lead to an increase, but rather to a decrease in serum PON1 activity. Unfortunately, it was not possible to analyze a potential antioxidant effect of PCB126-mediated PON induction, since the PCBs themselves generated oxidative stress [73, 74].

## 6. Upregulation of Sulfiredoxin

One important function of sulfiredoxin (Srxn) is the regeneration of oxidized peroxiredoxins. Peroxiredoxins are known to reduce peroxides which results in the formation of the hyperoxidized, cysteine-sulfinic acid form of peroxiredoxin (Prx-SH→Prx-$SO_2$H). Due to its sulfinic acid reductase activity, Srxn reverses hyperoxidation of peroxiredoxin in an ATP-dependent manner (for review, see [76]). Although it is known that transcriptional induction of murine Srxn requires Nrf2 and activator protein-1 (AP-1), Sarill and coworkers recently found that cigarette smoke extract upregulates Srxn mRNA and this depends partially on AhR function. Cigarette smoke extract-mediated Srxn induction was significantly reduced in $AhR^{-/-}$ fibroblasts. Interestingly, cigarette smoke extract induced similar induction of Srxn mRNA in fibroblasts derived from $AhR^{DBD/DBD}$-mice, which carry an AhR mutant unable to bind to XREs [77], compared to wt-mice [58]. This indicates that Srxn upregulation, in response to cigarette smoke extract, does not involve the classical AhR-XRE pathway but rather is mediated by a noncanonical AhR-dependent mechanism. One possible explanation is heterodimerization with the NF-$\kappa$B protein RelB and binding of the AhR/RelB complex to a promoter region different from the XRE [78]. However, other possible target genes have to be considered since several alternative binding regions for the AhR or the AhR/ARNT heterodimer have been identified [7–12]. Transcriptional activation could also be mediated indirectly by AhR-triggered upregulation of components of the AP-1 family of transcription factors, such as c-Jun or JunD [79–81]. Furthermore, it has been demonstrated that the AhR regulates endogenous levels of miRNAs which could account for increased stabilization of Srxn mRNA [82].

## 7. Protective Function of AhR in Hyperoxic Lung Injury

Although prooxidant functions of the AhR in hyperoxic lung injury have been described [83], other reports demonstrate a protective function of the AhR. Hyperoxia (>95% $O_2$) leads to induction of CYP1A1, NQO1, and GSTs *in vitro* and *in vivo* which is considered to require the AhR [59, 84, 85]. In contrast, hyperoxia-induced upregulation of CYP1A2 does not involve AhR signaling [84]. In line with these observations, disruption of AhR function increases ROS generation in fetal primary lung cells in response to hyperoxia and results in higher susceptibility to hyperoxic lung injury in adult and newborn mice [59, 84, 85]. Jiang and coworkers demonstrated an increase in pulmonary edema and neutrophil recruitment after hyperoxic treatment in $AhR^{-/-}$ mice [84]. Although induction of NQO1 and GSTs may partly contribute to the protective function of the AhR, several studies suggest a protective role of CYP1A enzymes. About thirty years ago, Mansour and coworkers revealed that pretreatment of rats or mice with either $\beta$-naphthoflavone ($\beta$-NF) or 3-MC decreased hyperoxia-mediated lung toxicity as assessed by pulmonary edema, lipid peroxidation, and lethality [86, 87]. It was later shown that the increase in CYP1A1 activity is correlated with the protection against hyperoxic lung injury in rats which was detected by the amount of pleural effusions [88]. Oppositely, inhibition of CYP1A isoforms by 1-aminobenzotriazole led to increased susceptibility to hyperoxic lung injury and lethality in rats [89] suggesting a protective function of CYP1A enzyme(s). The data were confirmed by studies in $Cyp1a1^{-/-}$ mice. $Cyp1a1^{-/-}$ mice were more sensitive towards hyperoxia-mediated pulmonary injury; they showed increased neutrophil infiltration and higher amounts of lipid peroxidation [90]. Noteworthy were the higher levels of $F_2$-isoprostanes (and isofurans) in the lungs of $Cyp1a1^{-/-}$ compared to wt-mice and this provides an explanation for the protective function of CYP1A1. $F_2$-isoprostanes derive from nonenzymatic peroxidation of fatty acids, predominantly arachidonic acid, thereby forming prostaglandin $F_2$-like products. $F_2$-isoprostanes are prominent markers for oxidative stress *in vivo*. Also, circulating $F_2$-isoprostanes are considered to play a role in inflammatory lung diseases by various receptor-triggered pathways (for review, see [91]). In a recent work by Wang and coworkers [92], it was shown that knocking out Cyp1a2, primarily expressed in the liver, also increased susceptibility for hyperoxic lung injury. This was assessed by the ratio $weight_{lung}/weight_{body}$ and histology, pulmonary neutrophil infiltration, cytokine expression, lipid peroxidation, and $F_2$-isoprostane levels in liver and lung [92]. The authors finally provide evidence for CYP1A2-mediated metabolism of $PGF_2$-$\alpha$ *in vitro* supporting the idea that CYP1A1 and CYP1A2 protect against oxidative stress by decreasing the amount of lung- or liver-derived circulating $F_2$-isoprostanes. Hence, these data suggest a protective function of CYP1A1 and CYP1A2 against hyperoxic lung injury, maybe due to decreased lipid peroxidation.

Finally, AhR-mediated protection might be due to its interaction with the NF-$\kappa$B protein RelB. In an *in vitro* approach, Zhang and coworkers used AhR-deficient human fetal pulmonary microvascular endothelial cells (HPMEC) to unravel mechanisms underlying the protective function of the AhR [59]. Downregulation of AhR expression by RNA interference led to increased ROS formation and augmentation of hyperoxia-mediated toxicity. The authors uncovered attenuation of CYP1A1 and NQO1 (and SOD1) expression in AhR-deficient cells and additionally a decrease in nuclear RelB expression. Indeed, several studies suggest that the AhR reduces lung inflammation by upregulating

RelB expression [57, 93]. RelB is supposed to be a negative regulator of the proinflammatory NF-$\kappa$B pathway, possibly by interaction with p50, thereby diminishing the amount of p50 to form active dimers with the p65 protein (p50 : p65), the classical NF-$\kappa$B complex. In summary, there is evidence for a protective role of the AhR in hyperoxic lung injury which is probably mediated by regulating the expression of antioxidant enzymes, such as NQO1 and CYP1A1/2. Additional mechanisms might also contribute to protection, such as increased expression of RelB leading to inhibition of the proinflammatory NF-$\kappa$B pathway.

## 8. Concluding Remarks

The presented data clearly indicate that the AhR plays a role in the antioxidant defense. Protection might be mediated by different mechanisms, such as AhR-dependent activation of Nrf2, PONs, SOD1, or CYP1A1/2 or by additional mechanisms which remain to be clarified. Also, noncanonical pathways seem to be involved, such as upregulation of sulfiredoxin which is independent of a classical XRE.

For some, but not for all of the antioxidant mechanisms, the *in vivo* relevance has been demonstrated in animal models. Nrf2$^{-/-}$ mice are prone to increased oxidative stress, inflammation, neurodegeneration, and carcinogenesis (for review, see [94]). AhR$^{-/-}$ mice are more susceptible to colon carcinogenesis, inflammation, and hyperoxic lung injury [84] (for review, see [95]). Lung fibroblasts gained from patients suffering from chronic obstructive pulmonary disease (COPD) express less AhR protein than patients without COPD and show decreased upregulation of NQO1 and Srxn in response to cigarette smoke extract [58]. Low expression of the AhR is also found in inflammatory bowel disease [96]. Oppositely, targeting the Nrf2 pathway by Nrf2-activating compounds, such as sulforaphane, protects against oxidative stress-mediated diseases like carcinogenesis, neurodegeneration, and cardiovascular illnesses in different animal models (for review, see [97]). Hence, it is conceivable that activation of the AhR-Nrf2 signaling pathway by AhR ligands should also exert chemopreventive effects. However, a direct link between AhR activation, Nrf2 induction, inhibition of ROS, and chemoprevention has not been shown *in vivo* yet. It also remains unproven whether AhR-mediated activation of PONs will lead to protection against atherosclerosis and whether ICZ is chemopreventive in animal models. Finally, the mechanism(s) of possible antioxidant functions of CYP1A1/2 remain to be elucidated.

The described findings are in contrast to the well-known increase in oxidative stress in response to AhR activation, for instance, induced by TCDD (for reviews, see [21, 43]). It has to be emphasized that the observed effects on DNA damage *in vitro* seem to depend on the cell type tested and are generally quite small [98–100]. Oxidative stress results from the net balance of oxidative and antioxidative mechanisms. Moreover, activation of the AhR will lead to induction of more than one signaling pathway. It is therefore reasonable to hypothesize that oxidant and antioxidant responses are triggered by the AhR in parallel, very likely with different kinetics (Figure 2). Protective mechanisms keeping the level

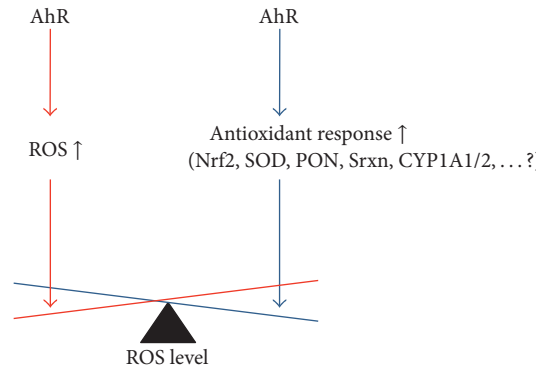


Figure 2: Hypothesis of AhR-mediated regulation of intracellular ROS. Different pathways of the AhR are activated at the same time, one leading to an increase in cellular ROS and the other(s) resulting in an antioxidant response. Possible antioxidant mechanisms are AhR-triggered activation of Nrf2, SOD, PONs, Srxn, CYP1A1/2, or, very likely, other enzymes which remain to be identified. Depending on the cell type, organ, ligand, or additional factors, either the prooxidant or the antioxidant AhR pathway predominates.

of oxidatively damaged DNA low, despite the generation of oxidative stress, would also explain the lack of TCDD-mediated mutagenicity in rats [101]. In view of the well-known cell type and organ specificity of AhR function, it is plausible to assume that, depending on the cell type or organ, oxidative or antioxidative AhR pathways predominate [67].

From a mechanistic and therapeutical view, it would be worth searching for more nontoxic ligands which selectively activate protective AhR-dependent pathways. As outlined above, discrimination could be observed by using ketoconazole, cynaropicrin, or quercetin. Different effects on AhR signaling were also detected when comparing TCDD and DIM [102]. The reasons for such ligand-specific effects remain unclear. One possible explanation is the recruitment of different cofactors [103]. Identification of selective, ideally nontoxic ligands not only would contribute to specifically triggering protective AhR signaling pathways, but also would probably help to gain a better insight into the mechanisms underlying AhR function.

## Competing Interests

The author declares that she has no competing interests regarding the publication of this paper.

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*Review Article*

# The Aryl Hydrocarbon Receptor Relays Metabolic Signals to Promote Cellular Regeneration

**Fanny L. Casado**

*Laboratorio de Investigación en Ciencias Ómicas y Biotecnología Aplicada, Pontificia Universidad Católica del Perú, Avenida Universitaria 1801, San Miguel, Lima 12, Peru*

Correspondence should be addressed to Fanny L. Casado; fanny.casado@pucp.edu.pe

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While sensing the cell environment, the aryl hydrocarbon receptor (AHR) interacts with different pathways involved in cellular homeostasis. This review summarizes evidence suggesting that cellular regeneration in the context of aging and diseases can be modulated by AHR signaling on stem cells. New insights connect orphaned observations into AHR interactions with critical signaling pathways such as WNT to propose a role of this ligand-activated transcription factor in the modulation of cellular regeneration by altering pathways that nurture cellular expansion such as changes in the metabolic efficiency rather than by directly altering cell cycling, proliferation, or cell death. Targeting the AHR to promote regeneration might prove to be a useful strategy to avoid unbalanced disruptions of homeostasis that may promote disease and also provide biological rationale for potential regenerative medicine approaches.

## 1. Understanding Self-Renewal Is Critical to Study Tissue Regeneration

Cellular regeneration involves the sum of events that renew cells, restore their function, and sustain their growth. The signals that trigger cellular regeneration can be physiological, such as cellular turnover or aging, or pathological, such as trauma or disease. Cellular regeneration is not a new field of scientific inquiry, but during the past decades it has been enriched with new insights and tools brought by stem cell research leading to the development of regenerative medicine. Regenerative medicine aims to replace, engineer, or regenerate human cells, tissues, or organs with the final goal of restoring or establishing normal function [1]. A vital connection between stem cell function and cellular regeneration is the concept of self-renewal in adult and postnatal stem cells. Self-renewal allows maintenance of the hierarchical structures of tissues when the potential of the parental cell to proliferate and differentiate is retained in one or both progenies. Thus, self-renewal is an asymmetrical type of cell division where all or some of the progeny maintains the proliferation and differentiation status of the parental cell [2].

Nowadays, genomic and proteomic approaches can be used to evaluate whether self-renewal pathways are involved in the development of disease, aging, or exposure to chemicals. However, the most robust approach available to test self-renewal functionally is the limiting dilution assay [3]. These assays have been exquisitely established in mouse models but their application in tissues other than blood and mammary gland may suffer from some limitations because the quantitation requires working with a large range of number of donor cells that may not be realistic. This assay is used to calculate the number of cells that have the ability to regenerate recipients of transplantations as a proportion of donor cells. At the beginning of the study, at least three different numbers of donor cells are tested. At the end of the experiment, the number of positively or negatively regenerated recipients is scored. The results are analyzed assuming that (1) only self-renewing cells regenerate the recipients, (2) one rare and randomly dispersed donor cell with self-renewing ability is enough to regenerate the recipient [4], and (3) the probability of regeneration follows a single-hit model of the Poisson distribution [5]. In addition, there are different in vitro serial dilution assays but since they may not recapitulate the entire tissue hierarchy, caution

needs to be used when drawing conclusions about self-renewal from them. Thus, when weighting the evidence about the involvement of certain pathways in self-renewal and maintenance of tissue homeostasis, we need to take into consideration the resolution and power of the techniques used to generate the data.

For an asymmetrical cell division to occur, some cues are governed intrinsically by organismal programs [6]. Alternatively, there can be extrinsic cues contributing to self-renewal that have been abundantly demonstrated and account for a significant flexibility in the regulation of self-renewal [7]. For instance, in the absence of cell contact, conditioned media from stromal cells in combination with the steel factor and interleukin 11 can support self-renewal of hematopoietic stem cells (HSC) [8]. This flexibility opens up opportunities in regenerative medicine to promote tissue regeneration by tapping into the signaling responsible for extrinsic self-renewal control.

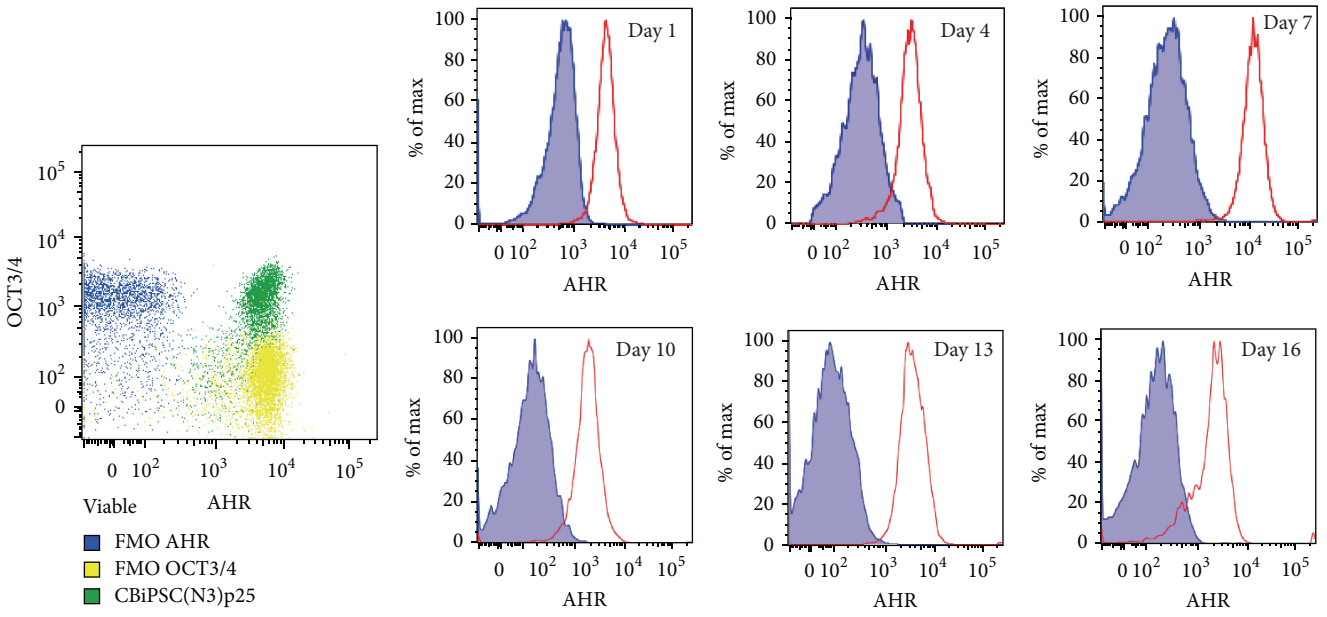
## 2. The Role of AHR in Self-Renewal of Stem Cells Is Cell Context Specific and It Is Mediated by Well-Established Self-Renewal Signaling

Evidence accumulated for the past 30 years supports that the aryl hydrocarbon receptor (AHR) is not only a xenobiotic sensor but that it also promotes changes in homeostasis of tissues whose functions rely on the maintenance of heterogeneously differentiated populations derived from self-renewing stem cells.

Historically, the AHR was discovered as the mediator of the toxic responses of halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons, including its most potent known exogenous ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [9]. Canonical ligand binding to AHR occurs in the cytosol leading to a sequence of conformational changes that ultimately transform the AHR into its high-affinity DNA binding form in the nucleus to function as a transcription factor together with its heterodimeric partner HIF1$\beta$ [10]. A TCDD-binding fingerprint of the conserved residues threonine 283, histidine 285, phenylalanine 289, tyrosine 316, isoleucine 319, phenylalanine 345, and alanine 375 within the ligand binding domain of the AHR forms a cavity conserved in mammalian AHRs necessary for optimal high-affinity TCDD binding [11]. Canonical AHR signaling induces drug-metabolizing enzymes (CYP1A1, CYP1A2, and CYP1B1), the glutathione-S-transferase, NQO1, and ALDH3 [12]. Functional studies in animal models of acute and developmental exposure to the AHR agonist TCDD [13, 14] show a direct detrimental effect in the self-renewing capacity of murine HSC. Changes in hematopoietic progenitor cell numbers have been observed in vitro when treatment with the AHR agonist benzo(a)pyrene led to decreases in the numbers of phenotypically defined human hematopoietic progenitors ($CD34^+$) [15] and increases in $CD34^+$ when exposed to the AHR antagonists StemRegenin-1 (SR1) [16] and GNF351 [17]. In addition, AHR antagonism with SR1 has been shown to be an efficient strategy to increase the numbers of $CD34^+$ by increasing self-renewal in human cells even in the context of pathological conditions such as leukemia and genetic instability [18]. Furthermore, the role of AHR signaling in self-renewal has been shown due to AHR's interaction with the RNA-binding protein MSI2 [19]. The effect of xenobiotic AHR antagonists to increase stem cells populations was an inflection point in our understanding about AHR and self-renewal because, after these findings, research on AHR went beyond the toxicology field to revitalize efforts in regenerative medicine dealing with novel ways to increase the quantity while maintaining the quality of stem cells for cellular therapy applications [20].

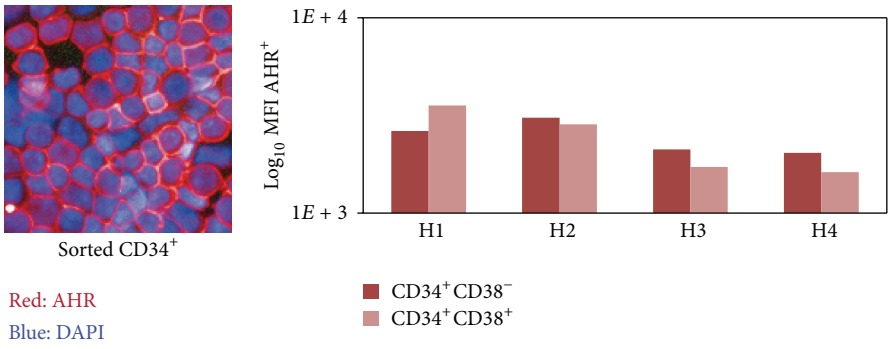
While there is a general consensus that AHR expression is ubiquitous in prenatal and adult tissues, it was not clear whether AHR levels in vitro and throughout loss of pluripotency may change. To evaluate AHR expression on an in vitro developmental model, cord blood cells were reprogrammed into induced pluripotent stem cells (iPSC) and stained for the marker of pluripotency OCT3/4 and AHR. Next, embryoid bodies were formed from iPSC and differentiated into hematopoietic lineages as previously described [21]. Flow cytometry was used to analyze AHR expression using the commercially available AHR antibody (clone RPT9) conjugated in house to allophycocyanin. As expected, AHR is expressed in undifferentiated iPSC (Figure 1(a)), and the expression is maintained even after embryoid body-mediated hematopoietic differentiation in vitro (Figure 1(b)). Furthermore, AHR is expressed in human hematopoietic stem/progenitor cells and localized mostly within the nucleus in mobilized peripheral blood with healthy self-renewal activity (Figure 1(c)) and in peripheral blood from patients with hematopoietic disease that altered their functional self-renewing activity (Figure 1(d)). For these analyses, mobilized peripheral blood and peripheral blood from patients with clinical signs of leukemia were obtained and processed as previously described [18]. Sorted $CD34^+$ were deposited in glass by high-speed centrifugation and stained for AHR (clone RPT9) and the nuclear stain DAPI. When possible, a further distinction between long-term ($CD34^+CD38^-$) and short-term ($CD34^+CD38^+$) self-renewing HSC [22] was done using flow cytometry. Intracellular expression of AHR (clone RPT9) was measured as Mean Fluorescent Intensity (MFI) using flow cytometry. Self-renewing capacity of the human hematopoietic cells was measured using xenotransplantation into mice as previously described [23]. Altogether, Figure 1 shows that AHR expression is always present in multiple cell types independent of self-renewal activity. These observations clearly demonstrate that for AHR it is differential signaling and not expression which may drive the regulation of self-renewing activity.

The mammary gland is another tissue where self-renewing stem cells are responsible for maintenance of homeostasis [24]. The evidence supporting AHR involvement in self-renewing activity of breast cells shows that when cultured breast cells were enriched in self-renewing cells, a pathological state was accompanied by changes in AHR responsive genes [25]. The same study showed that SR1 and a related AHR antagonist SR2 increase self-renewal capacity of breast cancer stem cells by upregulating LY6E

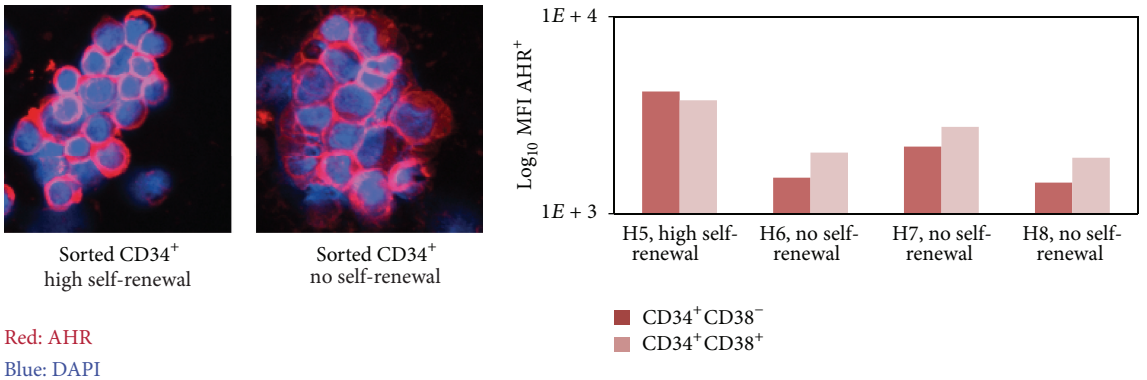



(a) Human induced pluripotent stem cells

(b) Hematopoietic differentiation from iPSC




(c) Human mobilized peripheral blood




(d) Human peripheral blood from leukemic patients

Figure 1: Ubiquitous AHR expression in iPSC and HSC regardless of potency and self-renewal activity. (a-b) AHR is expressed in (a) iPSC (OCT3/4+) and (b) cells from iPSC-derived embryoid bodies differentiating into hematopoietic lineages. Blue dots/areas correspond to fluorescence minus one (FMO) gating controls. (c-d) AHR is expressed in mobilized peripheral blood which is known to be enriched for (c) HSC and (d) leukemic cells with abnormal self-renewing activity.

which is a gene not directly involved in self-renewal but that can alter cell cycling and responses to growth factors. Surprisingly, healthy breast tissue seems to be protected of AHR-mediated carcinogenicity [26] by inhibition of small molecule metabolism-related genes whose action has been proposed to be independent of AHR. Altogether, these results show some interaction of AHR signaling with self-renewing capacity in breast cells but further studies using molecular tools may provide information about the specific players involved.

It has been recently demonstrated that persistent ligand-activation with TCDD in skin stem cells present in the sebaceous glands is responsible for the development of chloracne, a disease characterized by a dysregulation of the terminal differentiation of keratinocytes and metaplasia of sebaceous glands [27]. A complex interaction between transcription factors such as cMyc, Blimp1, and $\beta$-catenin/TCF (a prominent member of the WNT signaling) stimulate escape from quiescence to a more proliferating status but with a block in terminal sebaceous gland differentiation in favor of proliferation of interfollicular epidermal cells which results in the clinical manifestations of chloracne.

In the mouse, liver cells with multipotent capacity known as oval cells express a functional AHR and its activation has a serious impact on the numbers of these cells [28]. Increased ratios of G0 to G1 in oval cells exposed to the AHR agonist TCDD have been attributed to decreased expression of cyclin D1 and cyclin A and p27 increased expression leading to a block in the phosphorylation of the retinoblastoma protein responsible for the G0 to G1 transition. Whether these observations affect self-renewal capacity directly or just the symmetric divisions, it is not clear but the study provides further support for the flexibility of AHR responses in multiple cell contexts.

A comparison of fetal and adult murine prostate stem cells showed that AHR signaling was enriched together with WNT and HEDGEHOG pathways [29]. This study sorted adult and fetal prostate cells based on expression or lack of Sca-1. As expected, the transcriptional profiles of phenotypically defined stem cells (Sca-1$^+$) correlated with in vivo self-renewing capacity of prostate cells. In addition, AHR expression was found to be necessary for the regulation of self-renewal in Sca1$^+$ from fetal and adult prostate cells. No further evidence of AHR-HEDGEHOG interaction in other tissues has been described to date. There is some evidence for AHR-NOTCH interactions [30, 31] in the context of the development of the immune system; however, those observations indicate that these interactions affect potency and differentiation capacity rather than self-renewal.

In summary, the disruption of AHR by loss-of-function or ligand binding has repercussions on the self-renewal capacity of stem cells in multiple tissues but it is unclear whether the AHR is a self-renewing pathway to the same extent as the established self-renewal pathways WNT, NOTCH, and HEDGEHOG. Furthermore, the pleiotropic effects of AHR signaling and its ubiquitous tissue expression suggest that AHR's role in self-renewing is being an intermediary of the signaling necessary to support changes in self-renewal.

## 3. The Crosstalk between the AHR and the WNT Self-Renewal Pathway Might Be Directed by Responses to Glucose Availability

Recent studies using murine embryonic stem cells show that TCDD alters the WNT signaling pathway [32] with devastating effects on cardiomyocyte differentiation. Seminal studies in AHR KO mice, motivated by the cardiac teratogenicity of dioxins, concluded that the embryonic cardiac enlargement observed in AHR KO mice was not associated with an evident cardiac condition but to a subtle dysregulation of insulin that becomes overt with aging [33]. A clear AHR-WNT connection has also been evident when aged HSC from AHR KO mice were studied [34]. These results were consistent with epidemiological data suggesting an increased risk for diabetes in people exposed to potent xenobiotic AHR ligands [35] and a predictive behavior of background levels of xenobiotic AHR ligands other than TCDD for diabetes in the elderly [36]. In addition, loss of HIF1$\beta$, the nuclear heterodimeric partner of AHR after xenobiotic binding and HIF1$\alpha$ in hypoxic environments, mediates human type-2 diabetes [37]. Independent reports suggest that interaction of insulin growth factors (IGF) with their receptors in solid tumors favors self-renewing behavior. Also, TCDD disrupts IGF signaling in breast cancer cells via association of AHR with the CCND1 gene promoter [38]. Interestingly, functional inactivation of IGF and its receptor has been linked to diabetic onset in muscle cells [39]. Altogether, these reports suggested the involvement of AHR signaling in cellular events responsible for glucose homeostasis. A working mechanistic hypothesis that glucose may act as an endogenous ligand of AHR in endothelial cells via binding to responsive elements in the promoter of genes other than the one regulating canonical AHR signaling [40] also shed light on a little known AHR gene target: Thrombospondin-1 (THBS1). It is important to note that when the −5 kb to +2 kb region of the human THBS1 gene promoter was compared to well-known AHR responsive element promoters, there were at least two putative sequences with matching scores close to the training set [41] (Table 1). THBS1 encodes a matrix-related glycoprotein characterized by a TSP1 domain shared by a number of R-spondins [42] that gained interest because of their putative role as adult stem cell growth factors by potentiating WNT signaling [43, 44]. As expected, AHR-dependent dysregulation of WNT signaling via R-spondins has been shown to disrupt tissue regeneration in the zebrafish model [45]. A link between WNT signaling and glucose homeostasis has been extensively addressed in the literature. Broadly speaking, activation of canonical WNT signaling increases lactate production via glucose and glutamine use as carbon sources during proliferating cellular metabolism [46]. In particular, glucose availability within the physiological range can activate WNT signaling when the hexosamine pathway utilizes glucose to produce N-linked glycosylated WNT proteins [47]. Furthermore, WNT dependent stabilization of $\beta$-catenin in the nucleus of tumor-derived cell lines relies heavily on glucose [48]. Altogether, there is significant evidence that WNT-AHR

Table 1: Putative AHR responsive elements (AHRE) are present in the −5 kb to +2 kb gene promoter sequence of THBS1 and COX7B. CYP1B1 is shown as a sample gene from the training set, summarized from [41]. *Matching score with respect to a training set of known AHRE.

| Gene symbol | mRNA Refseq ID | Number of putative AHRE | Average matching score* |
|---|---|---|---|
| THBS1 | NM_003246 | 12 | 0.7939 |
| COX7B | NM_001866 | 13 | 0.7866 |
| CYP1B1 | NM_000104 | 19 | 0.8203 |

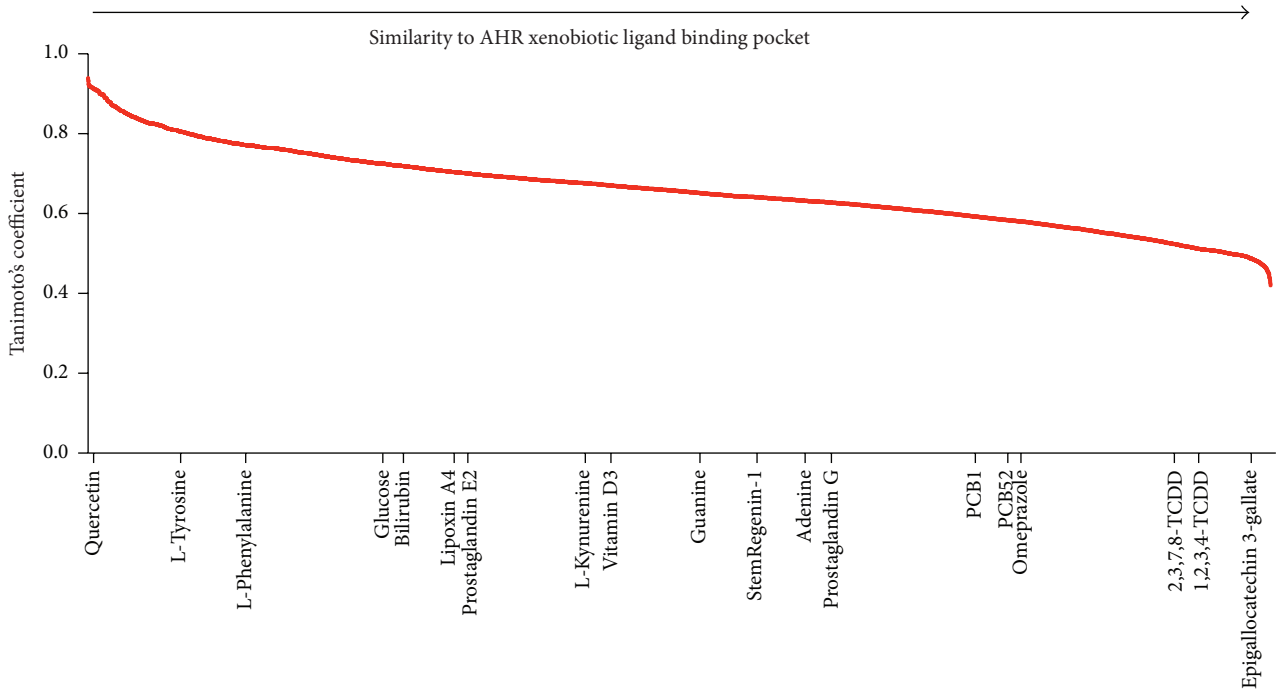



Figure 2: The AHR ligand binding pocket occupied during exposure to toxic xenobiotics such as dioxins (TCDDs) and polychlorinated biphenyls (PCBs) is not likely to accommodate ligands such as SR1, L-kynurenine, or glucose.

signaling mediates glucose metabolism [49] that regulates regenerative capacity of stem cells.

Up to date, there are not established endogenous ligands of AHR but there is certain agreement into that different cell types respond differentially to endogenous AHR ligands [50]. A possible rationale for the differences may reside in the use of multiple binding pockets that become available in a cell-specific manner. While there is not enough information to make this type of analysis, a virtual ligand screen was performed to analyze the likelihood of glucose being a ligand for AHR. The web-based software FINDSITEcomb [51] was used to generate a holographic template of a predicted ligand binding pocket for AHR by using information from the CHEMBL database which is a manually curated chemical database of bioactive molecules with drug-like properties maintained by the European Bioinformatics Institute. Since AHR (4M4X) possesses PER-ARNT-SIM ligand binding domains, the software used experimental data for ligands and ligand binding domains from PER-ARNT-SIM-containing templates obtained from Protein Data Bank files that contained the textual files for the three-dimensional structures of EPAS1/ARNT (4GHI), EPAS1/ARNT (3F1N), EPAS1/ARNT (3H7W), Sensory box histidine kinase/response regulator (3MR0), sensor protein (3BWL), and 8-oxoguanine-DNA-glycosylase (3F10). These information allowed obtaining a predicted AHR ligand binding pocket which was then used to perform a virtual ligand screen against 12 271 chemical compounds of the KEGG library including the AHR antagonist SR1. A rank of the compounds based on their similarity to the predicted AHR binding pocket was generated using their Tanimoto's coefficient. Accordingly, the 3D structures of compounds with smaller coefficients are more similar to the binding pocket. Figure 2 shows that the binding pocket is highly accommodating of xenobiotic compounds; however, glucose together with other proposed endogenous ligands and the antagonist SR1 are unlikely to interact with the same binding pocket. For instance, vitamin D receptor is a good example of a transcription factor with pleiotropic effects that uses more than one ligand binding pocket to initiate signaling [52]. Further studies need to consider whether stem cells may be regulated by AHR ligands via alternative binding pockets which may in turn lead to binding to alternative AHR responsive elements and regulation of different gene promoters.

## 4. AHR Regulates Cellular Energetics

Should glucose act as a ligand of AHR, it would be necessary to identify putative target genes that are regulated by AHR and that can directly intervene in cellular energetics. Spermatocytes in culture exposed to cigarette smoke condensates, known to contain a large number of toxic proinflammatory AHR ligands [53], have a higher glucose uptake but a decreased ATP production [54]. Also, high levels of AHR agonists in human blood serum correlate with a decreased cellular ATP production in myoblasts [55]. Patient-derived AML cells have been shown to have higher oxygen consumption rates [56] which might be consistent with an inefficient oxidative phosphorylation also known as aerobic glycolysis reported in solid tumors where glucose, the main source of energy, is converted to lactate and carbon dioxide due to the hypoxic environment rather than mitochondrial dysfunction [57, 58]. Altogether, the literature suggests that AHR might directly intervene in modulation of cellular energetic usage.

The increased self-renewal by the purine-derivative SR1, an AHR antagonist, measured as numbers of $CD34^+$ phenotypically defined HSC, has also been observed in HSC with disrupted self-renewing capacity favoring oxidative phosphorylation of the human bone marrow and peripheral blood-derived cells. While some of the patient samples showed downregulation of CYP1B1, the most consistent observation in these patient samples was the upregulation of the gene COX7B accompanied by changes in cellular metabolism [18]. CYP1B1 is considered the most relevant hematopoietic effector gene of the transcription factor AHR [16] encoding a CYP450 monooxygenase that plays important roles in the metabolism of small molecules involved in cell maintenance and survival, such as steroids [59], but its contribution to changes in cellular energetics is not clear. On the other hand, COX7B is a nuclear encoded gene for one of the subunits of cytochrome c oxidase (COX), the terminal component of the mitochondrial respiratory chain. Interestingly, changes in expression of both COX7B and THBS1 were observed in whole-blood of patients with early onset of acute coronary syndrome [60]. COX7B as well as THBS1 have putative non-canonical AHR responsive elements (Table 1). Interestingly, an alternative AHR responsive element has been described for AHR in breast cancer cells that do not respond to expected AHR ligands but to hypoxic stimuli such as low oxygen tension in culture and cobalt [61]. This study strengthens the argument for the possibility of the extrinsic cues being the most relevant to determine AHR responses since low oxygen tension is a characteristic of stem cells niches. Broadly speaking, self-renewing stem cells depend more on glycolysis than oxidative phosphorylation for ATP production [62] which is considered to be an adaptation to the low oxygen tension of the niches. The metabolic regulation of stem cells has been clearly demonstrated in studies looking at the developmental differences between the highly proliferative fetal liver HSC and adult bone marrow [63]. Similar to what was observed functionally and transcriptionally with SR1 in human HSC irrespective of their intrinsic self-renewing capacity [18], increased self-renewal potential is accompanied by increased expression of genes involved in oxidative phosphorylation for the highly proliferative stem cells. The evidence shown above about an interaction between AHR and WNT signaling in stem cells further supports the involvement of the niches because of the recognized relatively short-range action of WNT proteins in tissue self-renewal [44].

## 5. Future Directions

Further mechanistic molecular studies are needed to understand the role of AHR regulating self-renewal responses of tissue-resident stem cells niches or whether AHR participates in self-renewal by promoting release of factors by the stem cells themselves. It is expected that the results from those inquiries will place AHR at the center of studies about stem cell metabolism and its impact on aging, regeneration, and cancer stem cells. These studies might have an immediate impact on clinical applications. For instance, they may provide a rationale for the reported clinical improvement of patients with chronic heart failure with low ejection fractions that received bypass grafting plus transplantation of bone marrow-derived stem cells [64, 65] where the presence of stem cells from a different tissue and the absence of its native niche seem to favor regeneration of cardiac tissue.

## Competing Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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*Research Article*

# Aryl Hydrocarbon Receptor Deficiency in an Exon 3 Deletion Mouse Model Promotes Hematopoietic Stem Cell Proliferation and Impacts Endosteal Niche Cells

**Zeenath Unnisa, Kameshwar P. Singh, Ellen C. Henry, Catherine L. Donegan, John A. Bennett, and Thomas A. Gasiewicz**

*Department of Environmental Medicine, University of Rochester Medical Center, Rochester, NY 14642, USA*

Correspondence should be addressed to Thomas A. Gasiewicz; tom_gasiewicz@urmc.rochester.edu

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The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor belonging to the Per-Arnt-Sim (PAS) family of proteins. The AHR is involved in hematopoietic stem cell (HSC) functions including self-renewal, proliferation, quiescence, and differentiation. We hypothesize that AHR impacts HSC functions by influencing genes that have roles in HSC maintenance and function and that this may occur through regulation of bone marrow (BM) niche cells. We examined BM and niche cells harvested from 8-week-old AHR null-allele (KO) mice in which exon 3 was deleted in the *Ahr* gene and compared these data to cells from B6 control mice; young and old (10 months) animals were also compared. We report changes in HSCs and peripheral blood cells in mice lacking AHR. Serial transplantation assays revealed a significant increase in long term HSCs. There was a significant increase in mesenchymal stem cells constituting the endosteal BM niche. Gene expression analyses of HSCs revealed an increase in expression of genes involved in proliferation and maintenance of quiescence. Our studies infer that loss of AHR results in increased proliferation and self-renewal of long term HSCs, in part, by influencing the microenvironment in the niche regulating the balance between quiescence and proliferation in HSCs.

## 1. Introduction

All hematopoietic lineages arise from a small population of multipotent cells, the long term hematopoietic stem cells (LTHSCs) that are capable of self-renewal and differentiation. Through the process of multilineage differentiation, these HSCs develop into progenitor populations and lineage committed cells, the latter of which constitute the mature phenotype of blood and the immune system [1]. Hematopoiesis is, in part, regulated by interactions among the different cell populations constituting the bone marrow (BM) niche that balances the quiescence, proliferation, and differentiation of HSCs [2]. However, abnormal niche function can contribute to hematopoietic disease [3]. Several transcription factors mediate differentiation signals elicited by various inter- and intracellular factors and direct HSC lineage commitment. One such factor proposed to be involved in maintenance of self-renewal and proliferation of HSCs is the aryl hydrocarbon receptor (AHR) [4].

The AHR is a basic helix loop helix transcription factor belonging to the PAS (Per-Arnt-Sim) superfamily of proteins. These PAS domain proteins have an important role in circadian rhythms, organ development, neurogenesis, oxidation-reduction status, and response to hypoxia [5]. The PAS domain of AHR mediates ligand binding, eliciting translocation to the nucleus and dimerization with the AHR nuclear translocation protein (Arnt) to modulate gene transcription [6].

The AHR has been well studied for its role in mediating toxic responses to environmental xenobiotics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs). In order to understand the physiological function of AHR independently of xenobiotic exposure, several labs

generated *Ahr* null-allele (knockout, KO) mice using different strategies [7–9]. All these mice have shown phenotypic alterations in hepatic development, reproductive health, immunology, and vascular biology compared to wild type (WT) mice. However, some differences in the degree of phenotypic change and age-dependence of these phenotypes between KO models have been observed, possibly due, at least in part, to differences in genetic background [10]. One consistent feature among these models is altered immune system function and phenotype, although, again, the specific type and degree of immune alteration may differ [11].

In previous investigations, it was observed that lack of AHR in the "Bradfield" KO mice (B6.129-*Ahr*$^{tm1Bra/J}$), in which there is a deletion of exon 2 in the *Ahr* gene [7], alters the gene expression profile of the most primitive progenitors belonging to LTHSCs [12] and also leads to altered expression of genes associated with myeloproliferative disorders in aging mice as well as shorter lifespans [13]. So far, the specific role of AHR in regulating hematopoiesis is still not completely established and is actively being investigated.

In studies described here, we examined the role of AHR in regulating hematopoiesis using recently generated AHR-KO mice that have an *Ahr* gene exon 3 deletion. Breeding strategies have determined that these mice can be generated by mating homozygote pairs, resulting in a better birth and survival rate compared to other models. Using this model we analyzed functions of HSC and compared them with previous existing models. If results in these two different strains are found to be comparable, it would strengthen the conclusion that AHR is critical in HSC function and that earlier findings are not just peculiar to the one mutant mouse (exon 2 deleted model) and also provides another strain of AHR null-allele mouse to use for studying AHR functions that might avoid the breeding problems with the exon 2 KO mice.

Based on previous investigations, we hypothesize that AHR is responsible for functional and mechanistic regulation of HSC quiescence and proliferation. We sought to determine whether immune alterations would be observed in the exon 3 deletion model and whether these would be similar to the other KO models. If so, the use of the exon 3 KO model would greatly expedite further work to understand mechanisms involved. It is known that hematopoiesis is regulated by the interactions between the different cell populations constituting the BM niche [2]. Based on this, we further hypothesized that alterations in these niche populations might be observed in KO mice. Data reported here indicate that the AHR plays an important role in HSC quiescence, proliferation, and *in vivo* HSCs BM reconstitution ability. Furthermore, deletion of a functional AHR results in gene expression changes associated with hyperproliferation, leukemia, and accelerated aging. We also report that AHR may modulate, directly or indirectly, cell populations within the BM niche.

## 2. Materials and Methods

*2.1. Mice.* All mice used for these studies were females, purchased at 6–8 weeks of age from Taconic Farms (Germantown, NY). C57BL/6-*Ahr*$^{tm1.2Arte}$ mice carry a deletion in exon 3 of the *Ahr* gene, resulting in an out of frame splicing of exons 2 to exon 4. Results were compared to *Ahr* WT mice (C57BL/6N Tac). For adoptive transfer studies, the B6.SJL-*Ptprca*/BoyAiTac (CD45.1$^+$) and C57BL/6N Tac (CD45.2$^+$) strains were used to permit donor and recipient hematopoietic cells to be distinguished. For aging studies, 10-month-old female mice were used. Animal handling and experimental procedures were carried out in accordance with Institutional Animal Care and Use Committee at the University of Rochester.

*2.2. Organ Weights and Hematological Profile.* Animals were euthanized in a $CO_2$ chamber and organs (liver, thymus, and spleen) were harvested for wet weights. Blood was collected from the retroorbital venous plexus using capillary tubes and drained into microtainer tubes containing EDTA (Becton, Dickinson [BD] and Company, NJ). The complete blood count and other hematological parameters were analyzed using a HESKA Hematology Analyzer (HESKA Corporation, Colorado). The relative organ weights (g/100 g body weight) were calculated for the mice.

*2.3. Bone Marrow and Spleen Cell Isolation.* Bone marrow cells were harvested from femurs and tibiae by crushing the bones with a mortar and pestle. The cell suspension was collected after filtering through 40 $\mu$ membrane filter (BD). These cells were used for surface staining after red blood cell (RBC) lysis treatment with Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma). Spleens were crushed between two glass slides and isolated cells were used for surface staining with respective antibodies after RBC lysis. Surface staining was done with B220 (RA3-6132, BD), CD3 (145-2C11, BD), Gr-1 (RB6-8C5, BD), Mac1 (M170, BD), CD45.1 (A20, BD), and CD45.2 (104, BD). After surface staining with respective fluorescent antibodies, polychromatic flow acquisition was performed using a LSR II instrument (BD) at the University of Rochester Flow Cytometry Core. Flow data was analyzed using FlowJo software (version 10.0.7 Treestar, Ashland, OR).

*2.4. Histology.* Tissues were harvested and fixed in 10% neutral buffered formalin and processed for histology. Fixed tissues were embedded in paraffin, and 5-$\mu$m sections were prepared. Sections were stained with hematoxylin and eosin for histopathological evaluation.

*2.5. Cell Cycle Analysis.* Bone marrow cells were analyzed for cell cycle phases after lineage depletion and surface staining, followed by fixation and permeabilization and staining with DAPI (4-6-diamidino-2-phenylindole, dihydrochloride, Molecular Probes). Lin$^-$ cells were obtained by blocking the Fc$\gamma$III/II receptor using antibody clone 2.4 G2 and then incubated with biotinylated antibodies against Mac-1/CD11b (clone M1/70), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD3$\varepsilon$ (500A2), and Ter-119 (Ter-119) followed by streptavidin microbeads (Miltenyi Biotec). The cells were passed through magnetic columns (Miltenyi Biotec) to collect the lineage-depleted cells, and surface staining was done with fluorescently labeled stem cell antigen (Sca1) (clone D7, Biolegend)

and stem cell factor (ckit) (288, BD) antibodies. The cells were cytofixed and permeabilized (BD cytofix/cytoperm) and then stained with 1 mg/mL DAPI at room temperature for thirty minutes. Cells were centrifuged and resuspended in PBS before acquisition on LSR II. Doublets were eliminated and single cells were considered for LSK ($Lin^-Sca1^+cKit^+$) population that was analyzed for cell cycle phases based on differential staining ability.

*2.6. In Vivo Cell Proliferation Assay.* Mice were injected with 5-bromo-2′-deoxyuridine (BrdU) (0.1 mg/kg body weight) and BM cells were harvested 16 hours after injection and processed for lineage depletion. The $Lin^-$ cells were surface stained with fluorochrome labeled antibodies for Sca-1 and ckit for 20 min on ice. The cells were washed, and the pellet was resuspended in DNAse solution and incubated for one hour at 37°C. After washing in PBS, the cells were resuspended in BD cytofix/cytoperm solution for 20 min on ice followed by intracellular staining with FITC-labeled anti-BrdU (BD Biosciences, Mountain View, CA). After a final wash with PBS, the cells were subjected to LSR II flow cytometry and the data analyzed with FlowJo software.

*2.7. Differential Staining of BM Endosteal Niche Cells.* Femurs and tibiae were harvested and crushed lightly as described above to remove hematopoietic cells. The bone fragments were washed with Hank's balanced salt solution (HBSS, Invitrogen) and 2% fetal bovine serum (FBS). The bones were crushed again in the above solution to detach the adherent cells, and after centrifugation, these were resuspended in Collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) solution made in HBSS at a concentration of 3 mg/mL [15]. Cells were incubated at 37°C for 1 hour in a shaking water bath at 100 rpm. The cell suspension was filtered through 70-$\mu$ filter and washed with HBSS. The cells were counted and lineage depletion was performed as described above. After lineage depletion, the cells were stained with Sca1 (V450 clone D7, BD Pharmingen), CD31 (FITC clone 390, BD Pharmingen), CD45 (PerCP clone 30-F11, BD Pharmingen), and CD51 (PE clone RMV-7, BD Pharmingen) surface markers. Single cells were gated and analyzed for endothelial cells (EC, $Lin^-CD45^-CD31^+$), osteoblast cells (OBC, $Lin^-CD45^-CD31^-Sca1^-CD51^+$), and mesenchymal stem cells (MSC, $Lin^-CD45^-CD31^-Sca1^+CD51^+$).

*2.8. Serial BM Transplantation Assay.* Bone marrow cells ($2 \times 10^6$) from recipient $CD45.1^+$ mice were combined with cells from donor CD45.2 wild type (WT) or KO Taconic mice at a ratio of 1 : 1 ($2 \times 10^6$ BM cells) and were injected intravenously in recipient CD45.1 irradiated (550 + 550 rads, 4 h apart) mice (8 mice/group). After 16 weeks, BM cells ($1 \times 10^6$) harvested from primary recipients were injected into irradiated $CD45.1^+$ secondary recipient mice [16]. This procedure was repeated for a tertiary transplant. Bone marrow cells were harvested 16 weeks after transplantation and analyzed for the donor derived $CD45.2^+$ cells in different lineages. This was repeated for secondary and tertiary transplant recipient animals. The donor BM was also analyzed for differential HSC populations after lineage depletion as described above and further staining with Sca1, cKit, and CD48 (clone HM48-1, BD), CD150 (clone TC15-12F12.2, Biolegend), and CD135 (clone A2F10.1, BD) to differentiate the multipotent progenitors populations MPP1 [LSK, $CD135^-$, $CD48^+$, $CD150^-$], MPP2 [LSK, $CD135^-$, $CD48^+$, $CD150^+$], short term HSCs (STHSC [LSK, $CD135^-$, $CD48^+$, $CD150^-$]), and long term HSCs [LSK, $CD135^-$, $CD48^-$, $CD150^+$] [17].

*2.9. Reciprocal BM Transplantation.* Chimeric mice were generated to determine the effect of AHR-dependent extrinsic factors on BM repopulation [18]. Briefly, the WT ($CD45.1^+$), WT ($CD45.2^+$), and AHR-KO ($CD45.2^+$) recipient mice were lethally irradiated with 11 Gy (two equal divided doses of 5.5 Gy, 4 hours apart) and then injected intravenously with 2 million BM cells/mouse from donor WT ($CD45.1^+$), WT ($CD45.2^+$), or KO ($CD45.2^+$) mice in the following experimental donor → recipient combinations: WT ($CD45.1^+$) → WT ($CD45.2^+$), WT ($CD45.2^+$) → WT ($CD45.1^+$), KO ($CD45.2^+$) → WT ($CD45.1^+$), and WT ($CD45.1^+$) → KO ($CD45.2^+$) (6–8 mice/group). The level of engraftment was measured in BM of recipient mice after 16 weeks of transplantation by analyzing the $CD45.1^+$ and $CD45.2^+$ cells by flow cytometry.

*2.10. Colony-Forming Assays.* Bone marrow cells were harvested from the femurs and tibiae. Following RBC lysis, these cells were seeded onto methylcellulose media M3434 (Stemcell Technologies, BC, Canada) according to the manufacturer's instructions. These colonies were scored after 10 days for their clonogenic ability to form differential colonies.

*2.11. Real Time Quantitative PCR.* Bone marrow $Lin^-Sca1^+cKit^+$ (LSK) cells were sorted and collected in buffer RLT plus (Qiagen). These samples were then processed for RNA isolation and cDNA preparation at the University of Rochester Functional Genomics Core. Total RNA was isolated from sorted LSK cells using an RNeasy Mini Kit (Qiagen). RNA was preamplified and cDNA was produced using a WT-Ovation PicoSL kit (Nugen). For analysis, 10 ng of cDNA was used in qPCR reactions via a Bio-Rad CFX96 Real Time PCR instrument. The cDNA was then subjected to real time qPCR with standard TaqMan PCR primers and MasterMix (Applied Biosystems, Foster City, CA) for different genes. Expression of mRNA for each gene was normalized using the expression of 18S rRNA as a control endogenous gene. KO data were compared with WT data using the $2^{-\Delta\Delta Ct}$ approximation method.

*2.12. Statistical Analysis.* The results were analyzed and plotted using Graphpad Prism (Graphpad Inc., La Jolla, California). Statistical significance was determined using two-tailed Student's *t*-test. *P* values less than 0.05 were considered statistically significant ($^*P$ value < 0.05, $^{**}P$ value < 0.01, and $^{***}P$ value < 0.001 and ns = not significant).

## 3. Results

*3.1. AHR Deletion Affects the Differential White Blood Cell Count and Organ Weights.* The analyses of peripheral blood for CBC differential indicated no statistical difference in the number and types of peripheral blood cells between young WT (Taconic *Ahr* wild type) and KO (Taconic *Ahr*-KO) animals (Figure 1(a)). However, there was a significant increase in the granulocytes and monocytes of aged KO animals (10 months) compared to WT age matched controls (Figure 1(b)). There was also a lower platelet count in KO animals compared to WT (Figure 1(c)), although this was statistically different only in young animals. There was a trend for decreased relative liver weights and increased thymus and spleen weights in KO mice, although this was consistently significantly different only for spleen weights in young and aged animals (Figures 1(d) and 1(e)).

*3.2. AHR Influences HSC Proliferation and Quiescence.* Changes in the peripheral blood may arise from alterations in the BM progenitor and/or stem populations. The BM was lineage-depleted and enumerated for the expression of Sca-1 and cKit, referred to as the progenitor- and stem cell-enriched LSK ($Lin^{-}Sca\text{-}1^{+}cKit^{+}$) cells. There was an increase in the number of LSK cells in AHR-depleted animals when compared to WT (Figure 2(a)). The LSK population was further subjected to analysis for the phenotypically defined long term repopulating cells ($CD135^{-}CD48^{-}CD150^{+}$) with a potential to self-renew. There was a statistically significant reduction in the phenotypic LTHSC (Figure 2(b)) in KO BM cells. But when considering the total number of LTHSC relative to LSK population it may not differ between the two groups.

Since the increased number of LSK cells could be due to increased proliferation, we determined BrdU incorporation into these cells. At 16 hours after injection, there was a significant increase in the BrdU-labeled LSK subset of cells in AHR-KO animals compared to WT (Figure 2(c)). Possible changes in LSK cell cycle were also examined. The KO animals showed a slight but significant decline in the GO/G1 phase of the cell cycle with a concomitant statistically significant increase in the G2-M phases (Figure 2(d)). These data suggest that AHR-depleted progenitor/stem cells are less quiescent and are more prone to proliferation. We also compared the clonogenic potential of whole BM cells in both groups (Figure 2(e)). In the colony-forming assay, we found no difference in the most primitive undifferentiated CFU-GEMM (granulocyte/erythrocyte/macrophage/megakaryocyte) colonies and BFU (Burst-Forming Unit-Erythroid) colonies. However, there was a significant increase in the KO group in their ability to form differentiated granulocyte/macrophage (CFU-GM/M) colonies. This suggests that the progenitor cells in KO animals have an increased ability to differentiate towards macrophages and granulocytes.

*3.3. Functional Alteration in the AHR-KO LTHSC Population Assessed by Serial Transplantation.* The above results indicate increased proliferation and cell cycle changes in the LSK subset. To examine the self-renewal capabilities of LTHSCs to generate more differentiated progeny, the most stringent functional test for HSCs, a serial stem cell transplantation assay was performed. The results of the primary, secondary, and tertiary transplantations analyzed 16 weeks after transplantation for donor derived cells indicate increased cell counts in the BM of secondary recipients (Figure 3(a)) and decreased spleen cell counts of the primary recipients' spleens (Figure 3(b)) of KO animals compared to WT. The cell number in tertiary transplant recipients indicated no significant difference in the cell number in both the spleen and the BM. Quantification of lineage differentiation indicated no specific lineage defects except for the slightly reduced significant B220 lineage in BM of primary KO recipients (Supplementary Figure 1D in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4536187). But this difference was not observed following the secondary transplantation (Supplementary Figure 1E). There were no differences observed in the lineages of the spleen following primary transplantation or secondary transplantation, except in Mac1, which showed significant increase after secondary transplantation of KO BM cells (Supplementary Figures 1A and 1B). In tertiary transplant recipients, the differentiation towards B220 lineage was significantly reduced but there was an increased potential to differentiate into CD3 lineage (Supplementary Figure 1C). The lineage differentiation of BM cells in the transplant recipient mice of the primary and secondary transplant groups (Supplementary Figures 1D and 1E) showed a similar pattern as spleen, but the primary BM recipient had reduced B220 cells. However, tertiary recipient had reduced B220 lineage and increased Gr-1 positive cells along with a significant increase in the Mac1 lineage cells (Supplementary Figure 1F). In the BM of the primary transplant group (Figure 3(c)), we observed an increase in the phenotypic LTHSC population and a reduction in the early multipotent progenitor population (MPP2) of the secondary and tertiary transplant recipients. This suggests increased self-renewal potential of the AHR-depleted animals; this effect was more prominent in the secondary and tertiary transplant groups (Figures 3(d) and 3(e)). Together, these data suggest that absence of a functional AHR creates a hematopoietic stress-like condition and may simulate cues responsible for generating progenitor cells to reconstitute hematopoiesis.

*3.4. Reciprocal Transplantation.* The above data suggest that, consistent with previous investigations [14], deletion of AHR results in increased HSC proliferation. As such, we examined the possible involvement of cell extrinsic factors affecting HSC function in the absence of AHR by creating chimeric animals with and without AHR in hematopoietic and non-hematopoietic (including BM niche) cells. We transplanted WT cells into KO animals [18] and KO cells into WT animals as controls to analyze the BM repopulation. It is known that BM stromal cells contribute to the vascular niche in bone and are responsible for regulating the balance between self-renewal and differentiation of HSCs [19–21]. These stromal cells produce cytokines or extrinsic factors that influence hematopoietic self-renewal and differentiation. Following 16

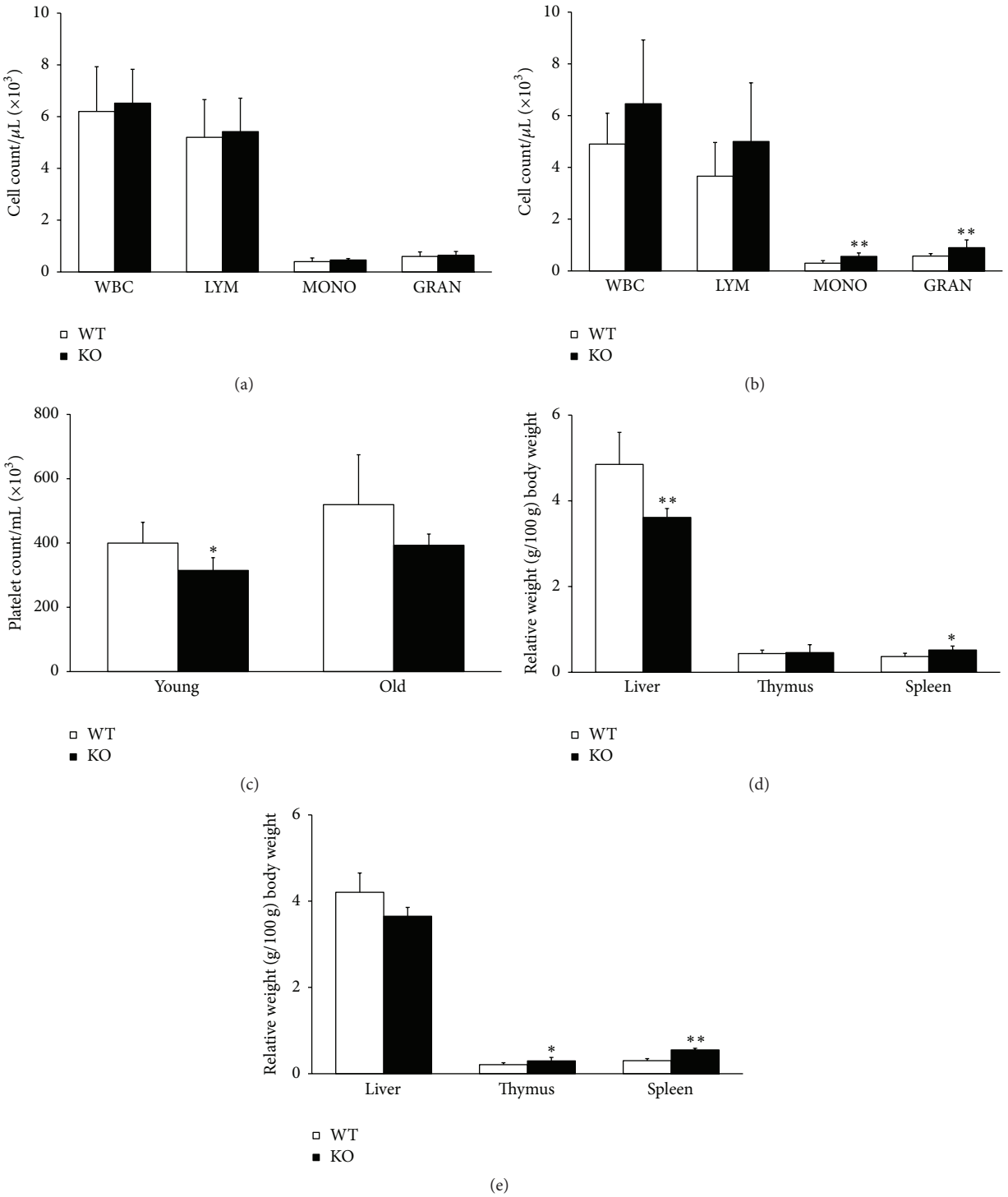


Figure 1: Loss of AHR expression influences the cellular content of peripheral blood and organ weights. Peripheral blood was analyzed by retroorbital bleeding and CBC differential was performed. (a) indicates cell count of young animals and (b) indicates old animals. (c) indicates differences in platelet counts. (d) represents relative weights of the hematopoietic organs and thymus in young animals. (e) represents relative weights of the hematopoietic organs and thymus in old animals. Data presented as mean ± SD, $n = 5$ mice/group. *Values significantly different from WT control ($P < 0.05$).

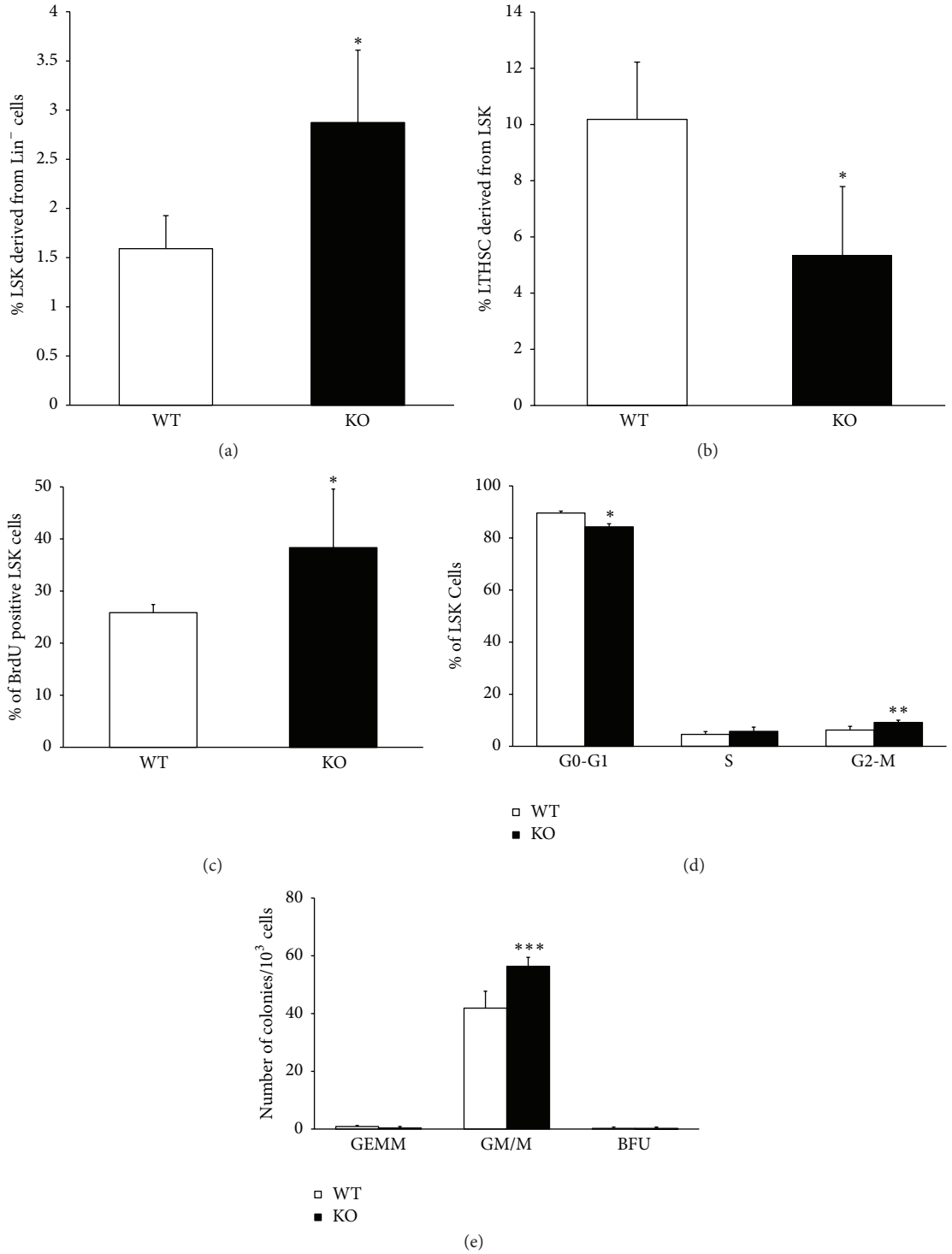


Figure 2: Bone marrow hematopoietic stem and progenitor cell populations, BrdU incorporation, cell cycle status, and colony-forming ability are altered by lack of AHR expression. (a) Bone marrow cells were processed for $Lin^-$ cell separation, stained for specific markers, and analyzed by flow cytometry for (a) LSK and (b) LT-HSC. Bone marrow $Lin^-$ cells were isolated from BrdU-treated mice, stained for LSK cells and anti-BrdU antibody, and analyzed for $BrdU^+$ LSK cells (c). For cell cycle analysis, BM $Lin^-$ cells were stained with DAPI and analyzed for LSK cells in G0-G1, S, and G2/M cell cycle stage (d). Colony-forming ability of primitive progenitors was assessed in BM cells (e). Data are mean ± SD, $n = 5$ mice/group. *Values significantly different from WT control ($P < 0.05$).

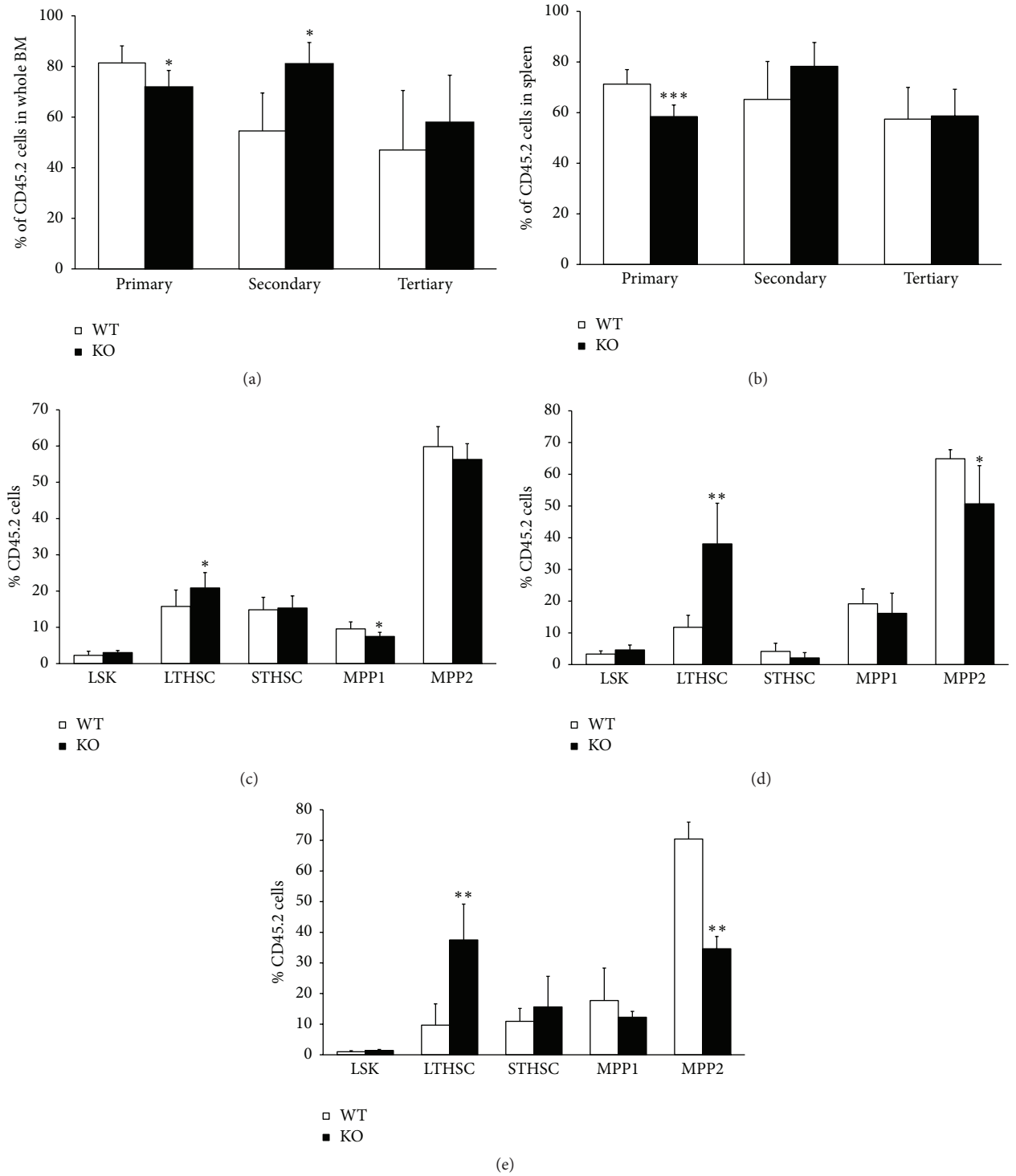


Figure 3: AHR-deficient BM cells have changes in long term competitive transplantation ability in serial transplantation. Bone marrow cells from donor AHR-KO ($CD45.2^+$) or WT ($CD45.2^+$) and recipient competitor $CD45.1^+$ mice were mixed together at a ratio of 1 : 1 ($1 \times 10^6$ cells each) and injected into irradiated $CD45.1^+$ recipient mice (8 each for donor AHR-KO and WT) for primary BM transplantation. Bone marrow cells were isolated from primary recipients after 16 weeks and serially transplanted in recipient mice as described in Section 2. (a) Bone marrow and (b) spleen cells were analyzed for $CD45.2^+$ (donor) and $CD45.1^+$ (recipient) origin after 16 weeks at each stage of transplantation. An analysis of BM cells was done for primitive hematopoietic and progenitor cells: (c) primary, (d) secondary bone, and (e) tertiary transplantation (LTHSC = LSK, $CD135^-$, $CD48^-$, $CD150^+$; STHSC = LSK $CD135^-$ $CD48^-$ $CD150^-$; MPP1 = LSK $CD135^-$ $CD48^+$ $CD150^-$; MPP2 = LSK $CD135^-$ $CD48^+$ $CD150^+$). Data shown are the mean ± SD. *Values significantly different from WT control ($P < 0.05$) ($N = 8$).

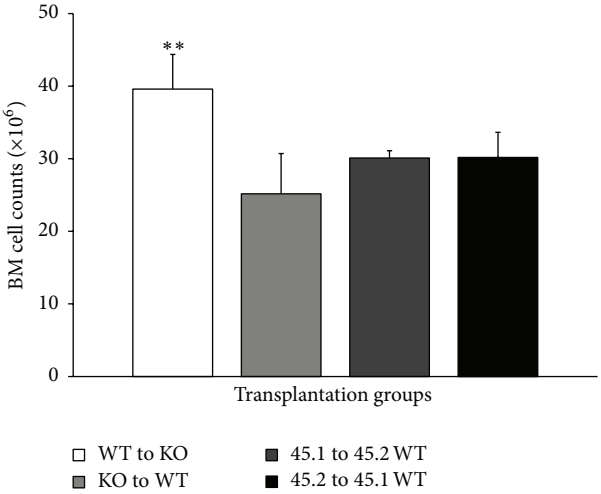


Figure 4: Lack of AHR expression in BM niche environment increases BM reconstitution ability of AHR expressing donor hematopoietic cells. Bone marrow chimera mice were prepared by lethal irradiation of WT (CD45.2$^+$), WT (CD45.1$^+$), and AHR-KO (CD45.2$^+$) mice, as described in Section 2. Irradiated WT (CD45.1$^+$) mice reconstituted with WT (CD45.2$^+$) cells [WT (CD45.2$^+$) → WT (CD45.1$^+$)], irradiated WT (CD45.2$^+$) mice reconstituted with WT (CD45.1$^+$) cells [WT (CD45.1$^+$) → WT (CD45.2$^+$)], irradiated WT (CD45.1$^+$) mice reconstituted with AHR-KO (CD45.2$^+$) cells [AHR-KO (CD45.2$^+$) → WT (CD45.1$^+$)], and irradiated AHR-KO (CD45.2$^+$) mice reconstituted with WT (CD45.1$^+$) cells [WT (CD45.1$^+$) → AHR-KO (CD45.2$^+$)] are presented. Data shown are mean ± SD, $n$ = 6–8 mice/group. $^*$Values significantly different from WT control ($P < 0.05$).

weeks after transplant, there was a significant increase in BM cell counts only in the group that had donor WT (CD45.1) cells injected into recipient KO (CD45.2) animals (WT to KO group) (Figure 4). We did not observe any significant differences in the relative percentage of donor derived lineage specific populations (data not shown). These results indicate that the donor WT cells were able to repopulate more efficiently when the AHR is absent in the niche compared to other groups containing AHR in the niche. This suggests that BM niche factors provided by the KO microenvironment may be promoting the proliferation of HSCs.

*3.5. Lack of AHR Alters the Cellular Composition of BM Stroma.* The present and previous [14] data suggest that AHR regulates hematopoiesis by balancing, directly or indirectly, the quiescence and proliferation of HSCs. The fate of HSCs is influenced, in part, by signals emanating from endosteal niche cells [22]. We examined the relative presence of endosteal niche cells in both AHR-KO and WT animals. We gated cells from the lineage negative population, further selected for endothelial cells, and the remaining cells were gated for mesenchymal stromal cells and osteoblast cells (Figure 5(a)). There was a 3-fold increase ($P = 0.003$) in the number of mesenchymal stem cells in KO as compared to WT animals (Figure 5(b)). The endothelial and osteoblastic cells also showed a slight increase in numbers, but these differences were not statistically significant.

*3.6. AHR Regulates the Expression of Genes Involved in HSC Maintenance.* The LSK population from BM of WT and AHR-KO animals was sorted and the cDNA was subjected to gene expression analysis. This analysis focused on genes (*Srpk2*, *Hes1, Mtor, Pdp1, Meis1, Gfi1, Foxo3, Stra13, Hif1α, and Cebpε*) involved in HSC proliferation; maintenance of quiescence, growth, and stem cell exhaustion; and development of myeloproliferative disorder in young and geriatric mice. We also analyzed the expression of genes (*Cxcr4* and *Angpt1*) involved in regulating niche interactions. Notably, there was an increase in the expression of *Hes1* and *Cebpε* in both young and aged (Figures 6(a) and 6(b)) KO animals. The expression of genes (*Cxcr4* and *Angpt1*) involved in niche maintenance was significantly downregulated in young and old KO animals. The higher level of *Hes1* in both KO groups may reflect the preservation of long term reconstituting ability [16]. The level of *Meis1* was also downregulated in these groups which may be related to altered *HIf1α* and reduction in *Stra13* gene expression involved in balancing ROS production [23]. There was a significant increase in the expression of *Cebpε* in both the KO groups, suggesting involvement with mature granulocyte production [24].

## 4. Discussion

In this study, we assessed the role of AHR in hematopoiesis and initiated experiments to examine the possible effect of the BM niche microenvironment in regulating hematopoiesis in absence of AHR protein. Previous reports indicating a role of AHR in regulating immune system pathways, and hematopoiesis in particular, used mouse models in which exon 2 of the *Ahr* gene was deleted [25]. In the present report, we present data from AHR-KO mice generated by deletion of exon 3, resulting in an out of frame splicing of exons 2 to exon 4. In terms of the effects of AHR absence on hematopoiesis, this exon 3 deletion mouse model appears to be similar to the "Bradfield" AHR-KO mice evaluated in previous investigations (Table 1). Combined, these data indicate that AHR signaling has a significant role in hematopoiesis by mediating and/or balancing the proliferation and maintenance of HSCs and progenitor populations in BM. Here, we also present preliminary data implicating a role of AHR in regulating the niche population that influences the proliferation and differentiation of HSCs.

Previously, it was reported that AHR-KO mice have enlarged spleens and increased number of lineage positive spleen cells and altered number of white blood cells and red blood cells, as well as increased numbers and altered functions of BM progenitors/HSCs [14]. The BM HSCs from the KO animals were highly proliferative. HSCs from AHR-KO mice have increased expression of several chemokines, cytokines, and their receptor genes. HSCs from young AHR-KO have overexpression of *Srpk2, Creb1, Hes1, Mtor*, and *Pdp1*. These genes have been associated with oxidative stress, acute myelogenous leukemia, aging and heat shock response, and

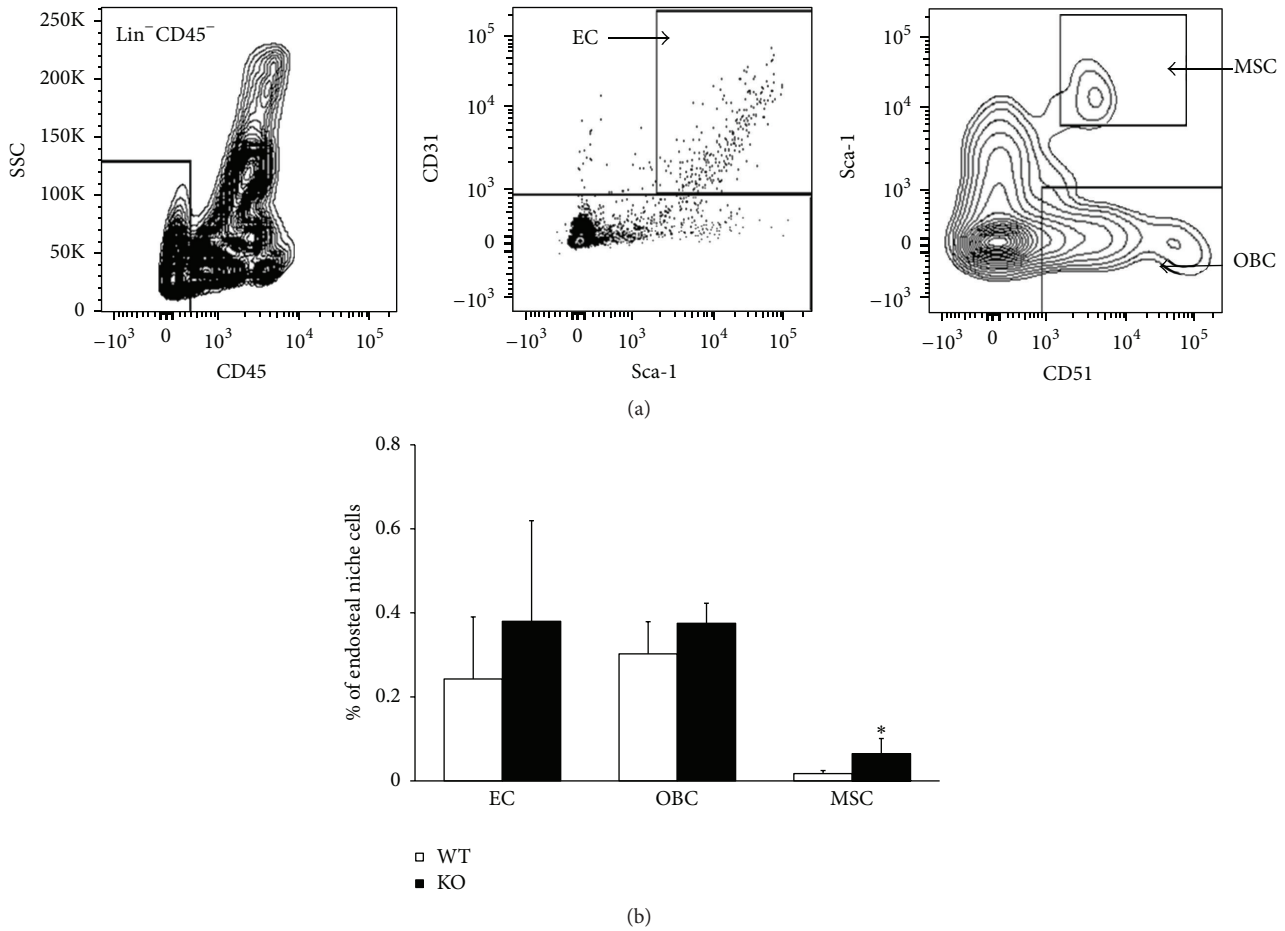


Figure 5: Lack of AHR expression alters the niche population. (a) Gating of nonhematopoietic endosteal niche cells. Endosteal niche cells are separated as $Lin^-CD45^-$, endothelial cells (EC) were gated as $Lin^-CD45^-CD31^+$ cells, and MSC (mesenchymal stem cells) and OB (osteoblast) cells were gated from the $Lin^-CD45^-CD31^-$ population based on Sca-1 and CD51 markers. The MSC are $Lin^-CD45^-CD31^-Sca\text{-}1^+CD51^+$ and OB cells are $Lin^-CD45^-CD31^-Sca\text{-}1^-CD51^+$. (b) represents the percentage of the endosteal niche cells from total endosteal BM stromal cells. Data are mean ± SD, $n = 5$ mice/group. *Values significantly different from WT control ($P < 0.05$).

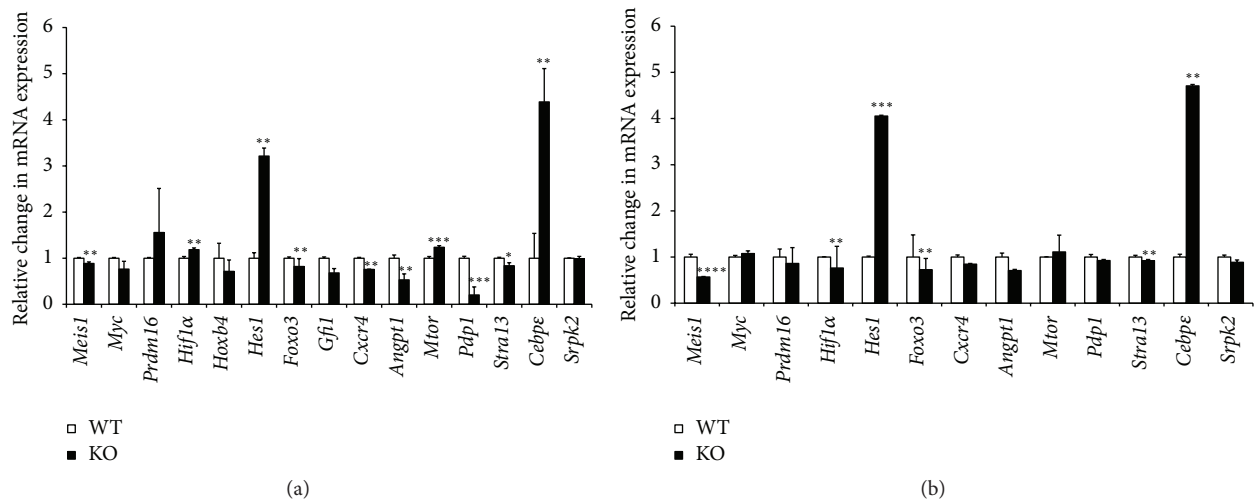


Figure 6: AHR-deficient LSK cells have alterations in gene expression related to hematopoiesis and hematopoietic disease. Quantitative real time PCR was used to analyze mRNA expression of genes associated with hematopoiesis and hematopoietic disease in LSK cells. Differential gene expression analysis of the LSK population in (a) young and (b) old mice. Data are mean ± SD, $n = 5$ mice/group. *Values significantly different from WT control ($P < 0.05$).

Table 1: AHR influences HSC characteristics.

| Hematopoietic characteristics | AHR exon 2 KO mice (Bradfield) [14] | AHR exon 3 KO mice (Taconic Biosciences) |
|---|---|---|
| Platelet counts | NR | Increased |
| WBC count | Increased | Higher but NS |
| Granulocyte and monocyte population | Reduced | Increased but NS |
| RBC number | Reduced | No change |
| Liver weight | Reduced | Reduced |
| Spleen weight and cell number | Increased | Increased |
| Functional primitive progenitor colonies | Increased numbers | Increased numbers |
| LSK cells | Increased | Increased |
| LSK cell proliferation | Higher | Higher |
| Cell cycle status of LSK cells | Reduced G0/G1 phase<br>Increased G2/M phase | Reduced G0/G1 phase<br>Increased G2/M phase |
| LTHSC in BM | NR | Higher |
| Increased self-renewal of LTHSC (serial transplantation) | ND | Higher |
| Mesenchymal stem cells in endosteal niche | ND | Higher |
| Survival rate | Reduced | Reduced |
| Gene expression changes | *Mtor* (↑) *Cebpε* (↑) *Gfi1* (↓) *Hes1* (↑) *Stra13* (↓) | *Mtor* (↑) *Cebpε* (↑) *Gfi1* (↓) *Hes1* (↑) *Stra13* (↓) |

The table depicts the difference and similarities between the two different strains of AHR-KO mice models (NR = not recorded, ND = not done, NS = not significant, (↑) = upregulation, and (↓) = downregulation).

alterations in the $\beta$-catenin/Wnt pathways. Earlier reports from AHR-KO mice indicated changes in multiple signaling pathways that promote premature HSC exhaustion and development of myeloproliferative disorder. Aging AHR-KO mice have changes in several aging associated genes that may result in shorter lifespan [12, 13].

Our results from the exon 3 deletion model show increased weights of spleen and decreased weights of liver in AHR-KO mice, as well as increased proliferation of HSCs (Figures 1(d) and 1(e)). The aging mice showed significant increase in monocyte and granulocyte numbers in peripheral blood (Figures 1(a) and 1(b)). This change may reflect skewing of lineage towards myeloid differentiation as opposed to lymphoid, reflecting early sign of aging as observed in earlier reports [13].

Consistent with previous studies [14], we found that AHR-KO mice have significant increase in numbers of LSK cells. The BrdU incorporation data and the cell cycle data showing reduced cell numbers in G0 phase indicated hyperproliferation in the AHR-KO BM progenitor and/or stem cells. Similar results were observed following the treatment of human hematopoietic progenitor/stem cells with AHR antagonists [26]. Further studies are needed to delineate the mechanisms by which AHR appears to influence the balance between quiescence and proliferation.

The repopulation potential of HSCs depends upon self-renewal, differentiation, and homing abilities. To better understand these HSC functions, we performed long term serial bone marrow transplantation using BM cells from AHR-KO and WT mice. These studies support a significant role of AHR in the maintenance of HSC self-renewal and quiescence. These results also indicate that a most primitive LTHSC population needs AHR for its functional stability and in the absence of AHR a condition resembling hematopoietic stress is created triggering the cells to mobilize and proliferate. The increase in LTHSC in KO transplant recipient animals correlates with the reduction in MPP2 populations and this could be due to a differentiation block in these LTHSC populations. This might account for the increased proliferation and exhaustion of LSK cells in KO animals leading to poor survival compared to WT animals. We also found a reduced survival rate (66%) in the 10-month-old KO animals compared to WT littermates (data not shown), supporting an earlier observation [13]. There are also reports suggesting increased HSC proliferation rates in AHR-KO mice that are also prone to early senescence and decreased lifespan [11].

The increased proliferation of cells in KO animals might indicate that altered signals emanating from the BM niche might influence cell proliferation and maintenance of quiescence in LTHSC. It is known that the BM microenvironment (the niche) regulates the quiescence, proliferation, and differentiation of HSCs [3]. Consistent with this, our preliminary studies show that loss of AHR has an impact on the niche population as indicated by the changes in the heterogeneous niche cells. The osteoblast is known to regulate the niche [15]. Endothelial cells support hematopoiesis through expression of several molecules including Angiopoietin 1 (Angpt1). This is also expressed by osteoblasts and promotes the maintenance of quiescent HSCs in osteoblastic niche [27]. It has been recently reported that *Angpt1* is highly expressed by HSCs, facilitating regeneration of BM niche by secreting

Angpt1. Notably, we observed a reduction in the *Angpt1* gene expression in LSK cells in KO animals (Figure 6). Thus, AHR absence in niche cells may be affecting the secretion of these factors supporting hematopoiesis. It has been reported that change in HSCs may induce secondary changes in BM niche cells function [28]. The observation of higher BM cell counts in chimeric mice having WT hematopoietic cells transplanted into AHR-KO hosts (Figure 4) is further evidence that lack of AHR within the BM stroma may also have a role in the HSC phenotype of the AHR-KO mice.

We performed chimeric experiments using AHR positive (WT) and negative (AHR-KO) transplantation recipient mice to further support a likely role of AHR in BM niche cells in hematopoiesis. These results provided additional evidence for the role of AHR in hematopoiesis through microenvironment signals operating through osteoblast and mesenchymal stem cells. Presently, mechanisms responsible for the delicate balance of BM cells to differentiate and proliferate rapidly are uncertain. However, the role of secretory products and direct contact signaling from the niche cells may be partly responsible for these changes. Future studies are needed to delineate the role of secretory products in the niche environment and the signaling mediated by AHR in the internal milieu of the niche cells contributing to hematopoiesis at steady state and under stress conditions.

Additional gene expression analysis of LSK population indicates changes in *Mtor*, *Hes1, PdP-1,* and *Stra13* related to various aspects of HSC maintenance. Upregulation of *mTOR* expression is associated with accelerated aging processes and aging associated diseases [29]. We observed changes in *Pdp-1* expression which acts as negative modulator of insulin/IGF-1 pathway (IIS). The IIS pathway is a regulator of longevity, development, and metabolism. It has been reported that Pdp1 may mediate this function in part by negatively regulating TGF-$\beta$ signaling to repress expression of several insulins that feed into the IIS pathway. Dysregulation of TGF-$\beta$ signaling and the IIS axis have been implicated in the onset of age-associated disease such as type 2 diabetes and cancer [30]. There was a significant increase in Hes1 in KO animals, suggesting its role in preserving the quiescence of LTHSCs [16]. It has been proposed that Hes1 is a positive regulator for the expansion of HSCs without exhausting their stem cell activity [31]. Stra13 is involved in regulating the oxidative stress in skeletal muscles [23] and we have seen increased ROS production in these animals in our previous publications.

Our studies also indicated a significant upregulation of *Cebpε* expression that may be related to altered granulocyte production in the KO animals [24]. We also observed an alteration in *Hif1α* mRNA levels in KO animals that is likely to influence the proliferation of HSCs [32]. We have also noted downregulation of *Meis1* expression involved in regulating the oxidative stress [33]. AHR mice have shown signs of early aging and myeloproliferative disease [13]. Aging AHR-KO mice have impaired glucose and lipid metabolism [34]. The most significant upregulation was observed in *Hes1* and *Cebpε* genes in both young and aging AHR-KO mice; these alterations may be associated with HSCs quiescence and granulocyte production [16, 24].

## 5. Conclusion

In conclusion, here we present data from AHR-KO mice generated by deletion of exon 3 in the AHR gene. In terms of hematopoietic effects, this mouse model of AHR-KO appears to be similar to "Bradfield" AHR-KO mice as evaluated by the parameters reported here. Taken together, our results indicate that AHR has a definitive role in the regulation of HSCs functions. Altered AHR signaling appears to produce significant effects on hematopoiesis by modulating the proliferation and maintenance of HSCs and progenitor populations in the blood and BM. The observed changes in functions of AHR-deficient HSCs may be due, in part, to changes in the AHR-KO niche cells from where critical regulatory signals emanate.

## Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

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*Review Article*

# Environmental Ligands of the Aryl Hydrocarbon Receptor and Their Effects in Models of Adult Liver Progenitor Cells

**Jan Vondráček[1] and Miroslav Machala[2]**

[1]*Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, 61265 Brno, Czech Republic*
[2]*Department of Chemistry and Toxicology, Veterinary Research Institute, 62100 Brno, Czech Republic*

Correspondence should be addressed to Jan Vondráček; vondracek@ibp.cz

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The toxicity of environmental and dietary ligands of the aryl hydrocarbon receptor (AhR) in mature liver parenchymal cells is well appreciated, while considerably less attention has been paid to their impact on cell populations exhibiting phenotypic features of liver progenitor cells. Here, we discuss the results suggesting that the consequences of the AhR activation in the cellular models derived from bipotent liver progenitors could markedly differ from those in hepatocytes. In contact-inhibited liver progenitor cells, the AhR agonists induce a range of effects potentially linked with tumor promotion. They can stimulate cell cycle progression/proliferation and deregulate cell-to-cell communication, which is associated with downregulation of proteins forming gap junctions, adherens junctions, and desmosomes (such as connexin 43, E-cadherin, $\beta$-catenin, and plakoglobin), as well as with reduced cell adhesion and inhibition of intercellular communication. At the same time, toxic AhR ligands may affect the activity of the signaling pathways contributing to regulation of liver progenitor cell activation and/or differentiation, such as downregulation of Wnt/$\beta$-catenin and TGF-$\beta$ signaling, or upregulation of transcriptional targets of YAP/TAZ, the effectors of Hippo signaling pathway. These data illustrate the need to better understand the potential role of liver progenitors in the AhR-mediated liver carcinogenesis and tumor promotion.

## 1. Introduction

The liver, a central organ responsible for maintaining the homeostasis in organism, plays an essential role in metabolism, both synthesizing a number of important molecules and metabolizing nutrients, xenobiotics, or various endogenous substrates [1]. It is primarily involved in glycogen storage, drug detoxification, bile production and secretion, as well as in production of serum proteins, and so forth. The metabolic and synthetic functions of the liver are performed primarily by hepatocytes, which make approximately 80% of the total liver mass [1]. Disruption of the liver capacity to detoxify, failure to secrete bile, or aberrant synthesis of plasma proteins leads to development of liver diseases, such as cirrhosis, which may ultimately result in the liver failure [2].

The liver is also an organ with a remarkable regeneration capacity that is capable of recovering both mass and function after an injury. Although hepatocytes have a very low turnover rate and under normal conditions almost all of them are quiescent cells (which reside in $G_0$ phase of cell cycle), following liver injury, they reenter cell cycle in order to allow restoration of the original cell mass [2, 3]. Hepatocyte proliferation represents a major mechanism responsible for the liver regeneration and homeostasis [4], and, under normal conditions, the liver regeneration is thought to be primarily mediated by self-duplication of mature hepatocytes (and biliary epithelial cells) [2, 5, 6]. Nevertheless, during strong hepatocyte depletion or when hepatocyte proliferation is inhibited, the population of liver progenitor cells may serve as a second line of defense against liver injury/failure [5, 7]. The adult liver progenitor cells can give rise both to hepatocytes and to biliary epithelial cells [8, 9], although their origin, as well as their exact contribution to liver regeneration, is a matter of an ongoing debate (for recent reviews, see [2, 5–7]).

Given the importance of the liver in detoxification of xenobiotics, it is not surprising that liver cells also constitute a major target for a number of toxicants and/or their reactive

intermediates. The toxic ligands of the aryl hydrocarbon receptor (AhR), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related dioxin-like compounds (DLCs), are well-known liver toxicants, which induce multiple forms of liver damage and contribute to hepatocarcinogenesis [10]. A brief description of the AhR functions and mechanisms of the AhR-dependent signaling is also provided as a part of this review. A significant majority of currently available studies evaluating the mechanisms underlying the toxicity of the AhR agonists in the liver have so far focused on hepatocytes as a principal target of DLCs. Therefore, the primary goal of this review is to provide an overview of experimental studies evaluating the impact of toxic AhR ligands on cellular models either derived from liver progenitor cells or exhibiting phenotypic features of liver progenitors.

## 2. Adult Liver Progenitor Cells

The progenitor cells in adult liver have been considered to represent cells that may enable the liver to regenerate upon severe or chronic injury linked with impairment of proliferative capacity of hepatocytes [8]. The activation/accumulation of cells exhibiting progenitor or mixed hepatobiliary phenotypes has been observed in a number of human liver disease conditions, including submassive liver necrosis and chronic viral hepatitis, or during both alcoholic and nonalcoholic fatty liver disease [11, 12]. These cells have been proposed to represent the human equivalent of rodent oval cells (facultative liver stem cells), which are activated during a number of experimental conditions blocking the restoration of the liver mass by hepatocytes [8, 13]. The activation of oval cells in experimental animals has been documented to occur in response to a wide range of toxic insults to the liver, including (i) application of toxins/carcinogens (such as 2-acetylaminofluorene and ethionine) in combination with partial hepatectomy; (ii) use of diets containing carbon tetrachloride or 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), or (iii) using experimental choline-deficient diet supplemented with ethionine (CDE) [14–17]. These treatments lead to emergence of oval cells with bipotential ability to differentiate into both hepatocytes and biliary epithelial cells [18, 19].

The origin of rodent oval cells is still not fully clear; however, they have been hypothesized to originate from the cells that are located within canals of Hering [20]. This structure, located between hepatocytes and biliary epithelial cells, may serve as a niche for these bipotential progenitor cells; however, as will be discussed later, other origins of liver progenitor cells have also been proposed, including hepatocytes [21, 22], hepatic stellate cells [23, 24], or cholangiocytes [5]. Oval cells express markers of both the hepatocyte and bile duct lineages, including $\alpha$-fetoprotein (AFP), delta-like 1 homolog (Dlk1), cytokeratin 19 (CK19), SRY- (sex determining region Y-) box 9 (Sox9), epithelial cell adhesion molecule (EpCAM), CD133, or MIC1-1C3 antigen [7, 8, 25]. The expression of these markers seems to be both species- and injury type-specific [25]. Therefore, additional markers of oval cells are being sought in order to improve both identification and quantification of liver progenitor cells.

The studies evaluating transdifferentiation between hepatic cell types have recently reported a number of controversial findings [2, 5–7]. Transplanted liver progenitor cells may contribute significantly to restoration of liver parenchyma, regenerating both hepatocytes and biliary epithelium [26], and the rodent liver cells expressing various markers of liver progenitors can be successfully induced to give rise to hepatocytes and/or biliary epithelial cells [27–30]. The adult bile duct derived Lgr5-positive progenitor cells have been derived and expanded from human liver, which can then be differentiated into functional hepatocytes *in vitro* or *in vivo* [31]. However, the exact contribution of adult liver progenitor cells to liver regeneration upon liver injury *in vivo* remains controversial, especially when considering the results of recent studies using genetic lineage tracing experiments. Whereas one of the first such studies has indicated that cells of biliary origin could be a major source of hepatocytes [32], others have, on the contrary, reported that adult liver progenitor cells provide only a minor fraction of cells contributing to liver regeneration, which is primarily mediated by hepatocytes under normal conditions [4, 33, 34]. Several recent studies have argued that hepatocytes arise from preexisting hepatocytes during liver regeneration or that hepatocytes within injured liver are a source of bipotential adult liver progenitors, which then contribute to restoration of hepatocyte mass through transdifferentiation [22, 35, 36]. Two recent studies have also indicated that specific progenitor/stem-like cell populations may exist in the adult liver. Recently, a preexisting population of hybrid periportal hepatocytes, expressing low levels of biliary markers, has been proposed to possess a high regenerative capacity and to contribute to restoration of liver mass after chronic hepatocyte-depleting injuries [37]. Another study has identified a population of proliferating and self-renewing Axin2-positive cells located close to the central vein within a niche established by the Wnt (wingless/integrated-1) producing endothelial cells. This population of stem cells, which is present in uninjured steady state liver, has been proposed to contribute to homeostatic liver cell renewal, similar to other organs [38].

Thus, a number of controversies currently surround both the identification of adult liver progenitor cells and their potential role(s) in homeostatic liver, during liver regeneration or in hepatocarcinogenesis. A recent study has suggested that ductular reactions may not give rise to hepatocellular carcinoma (HCC) [39], while others have proposed that dysregulated self-renewal of liver progenitor cells serves as an early event in hepatocarcinogenesis [40]. Nevertheless, regardless of the above issues concerning their origin or their role in liver regeneration, adult liver progenitor cells (which possess a significant self-renewal capacity) appear to give rise to certain types of liver cancer [41]. A significant percentage of HCC cases simultaneously exhibits both hepatocytic and biliary features [42]. A notable example is the combined hepatocholangiocarcinomas, an aggressive and heterogeneous group of liver tumors exhibiting intermediate features between hepatocytes and cholangiocytes, which have been suggested to arise from liver stem/progenitor cells [43]. This indicates that some liver cancer subtypes contain cells with phenotypic and/or functional features of liver progenitor

cells, possibly originating from adult liver progenitor cell populations. Therefore, these cell populations might also constitute an important target for liver carcinogens, including the toxic environmental AhR ligands.

## 3. Toxic Ligands of the AhR and Their Hepatotoxic and Carcinogenic Effects

The AhR is a ligand-activated transcription factor, a member of the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family of transcriptional regulators [44], which regulates the expression and activity of a number of genes participating in the regulation of liver cell function or hepatocarcinogenesis [10]. The AhR is in inactive state localized within the cytosolic protein complex containing chaperone protein hsp90 (heat shock protein 90), cochaperon p23, and immunophilin XAP2 (ARA9; AIP) protein. Following the binding of its cognate ligands, the AhR translocates to the nucleus and forms a dimer with the AhR nuclear translocator (Arnt). This dimer recognizes so-called xenobiotic/dioxin response elements (XRE/DRE) located within the regulatory regions of various AhR target genes. These include phase I xenobiotic metabolizing enzymes, such as cytochrome P450 family 1 (CYP1) enzymes and several phase II conjugation enzymes [45]. However, the AhR also regulates a number of genes contributing to the regulation of cell proliferation, differentiation, senescence, or programmed cell death [46–50]. This suggests that the AhR could play a major role in cell fate decisions; therefore, the aberrant long-term activation of the AhR by persistent toxic AhR ligands may contribute to important biological processes involved in hepatocarcinogenesis [51].

The AhR-null mice exhibit a number of liver defects, including reduced liver size, smaller hepatocytes, development of mild to severe liver fibrosis, accumulation of lipids, inflammation, or remodeling of the liver vascular architecture [52–54]. In some AhR knockout mice models, mild oval cells hyperplasia has been observed [52]. The ligand-dependent activation of AhR mediates toxicity of a variety of environmental pollutants, including polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (PCBs), or polycyclic aromatic hydrocarbons (PAHs) [44]. The exposure to TCDD leads to tumor promotion, teratogenic effects, epithelial hyperplasia, thymic involution, porphyria, and (at high doses) a severe wasting syndrome followed by death of experimental animals [55]. In the rodent liver, TCDD induces a range of effects leading to hepatocellular hypertrophy, bile duct hyperplasia, formation of multinucleate hepatocytes (in some rodents), steatosis and inflammatory cell infiltration, transient liver swelling, and, at the cellular level, plasma membrane abnormalities and proliferation of endoplasmic reticulum [56]. Most of the acute toxic effects of TCDD in rodent liver are mediated by activation of the AhR in hepatocytes [54].

The AhR activation is the major and common mode of action of DLCs [10]. This allowed establishing the "toxic equivalency factor" (TEF) approach for risk assessment of mixtures containing DLCs, which is based on the concept of determination of the potencies of DLCs to activate various AhR-dependent endpoints allowing establishment of consensus TEF values (relative to TCDD) for individual DLCs [57]. Dose-additive carcinogenicity of mixtures of DLCs has been experimentally confirmed and it supports the use of the TEF approach [58].

Importantly, apart from their acute toxicity, persistent AhR ligands have been shown to act as powerful liver tumor promoters and this effect is AhR-dependent [59, 60]. TCDD and other dioxin-like compounds, such as 2,3,4,7,8-pentachlorodibenzofuran or 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), have been listed by the International Agency for Research on Cancer as carcinogenic to humans (group 1 human carcinogens) [61]. These compounds have been shown to induce multiple cancer types in experimental animals [10, 62]. The chronic toxicity and carcinogenicity of both TCDD and PCB 126 have been evaluated in two-year bioassays in female rats [63, 64]. Increased incidence of non-neoplastic liver lesions (including hepatocyte hypertrophy, altered hepatocellular foci, inflammation, oval cell and bile duct hyperplasia, cholangiofibrosis, and nodular hyperplasia) and increased incidences of hepatocellular adenomas and cholangiocarcinomas were found after exposure to DLCs. Importantly, some of these lesions also contained a prominent component of biliary epithelium and/or oval cells [65]. This suggests that liver progenitor cells might contribute to development of cancer in experimental animals exposed to DLCs.

During recent years, the AhR has been also implicated in regulation of physiological functions of stem cells of various tissue origins, in particular in hematopoietic stem cells [66–72], or in cancer stem cells [73, 74]. Importantly, a recent study has suggested that the AhR activation can have a major impact on expansion of rodent hepatic stem cells while simultaneously reducing the viability of hepatoblasts [75]. Thus, DLCs may apparently differentially affect liver cells at less differentiated stages (including adult liver progenitor cells), and their impact on liver progenitors could markedly differ from their effects in mature hepatocytes. In this review, we summarize the results of experimental studies indicating that the AhR activation may alter various functions of adult liver progenitor cells, which include deregulation of cell cycle progression/proliferation and cell-to-cell communication, as well as modulation of activities of signaling pathways regulating liver progenitor cell activation and/or differentiation.

## 4. The Role of AhR in Regulation of Cell Cycle and Proliferation in Cellular Models Derived from Hepatocytes

The mechanisms underlying the role of the AhR in carcinogenesis have recently been reviewed by several authors and it has been proposed that the AhR can play both oncogenic and tumor suppressive roles in various cancer types, in a tissue-dependent manner [76–78]. In the liver, the AhR appears to function as tumor suppressor gene in the absence of its toxic ligands [79], whereas its aberrant long-term activation induces liver carcinogenesis [10]. This is supported also by the observation that the constitutively

active AhR mutant promotes carcinogenesis in mouse liver [80]. A number of mechanisms have been suggested to contribute to carcinogenic effects of DLCs in the liver, including altered proliferation of preneoplastic cells or inhibition of apoptosis leading to clonal expansion of altered hepatic foci [10, 47]. Disruption of cell proliferation control and loss of responsiveness to growth suppression belong among the hallmarks of cancer [81], which have been also suggested to contribute to carcinogenic effects of environmental chemicals [82].

The presence or absence of the AhR in cells may significantly modulate their proliferative behavior [83]. In rodent hepatoma cell models, TCDD treatment leads to AhR-dependent inhibition of $G_0/G_1$ to S-phase progression and accumulation of cells in $G_0/G_1$ phase of cell cycle [84]. This effect has been suggested to be mediated via various mechanisms including induction of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ [85], inhibition of E2F1-dependent gene expression, which is mediated by interactions between the AhR and retinoblastoma protein [86–88], displacement of E2F from the E2F-responsive promoters, or additional mechanisms dependent on AhR transcriptional partner, Arnt [89, 90]. TCDD and related compounds block cell cycle progression and cell proliferation in a majority of liver cell models used in toxicology [91, 92]. TCDD also suppresses liver regeneration following partial hepatectomy via the induction of $p21^{Cip1/Waf1}$ activity, which is mediated by the AhR acting together with the tumor suppressor Kruppel-like factor 6, which functions as a noncanonical AhR binding partner [93, 94]. Contrary to these observations, TCDD pretreatment increases proliferative response of hepatocytes to hepatomitogen 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) in regenerating liver, which suggests that the role of the AhR in cell cycle regulation of liver cells could be more complex than simply inhibiting cell cycle progression into S-phase [95].

## 5. The Impact of AhR Agonists on Cell Cycle and Proliferation in Models of Adult Liver Progenitor Cells

Unlike in hepatocytes or hepatoma cells, various types of AhR ligands have been found to promote proliferation of rat liver progenitor cells *in vitro*. The WB-F344 cell line, isolated from the liver of adult male F344 rat, exhibits phenotypic properties of rat oval cells [96]. Upon transplantation into the liver of syngeneic rats, these cells differentiate into hepatocytes and they retain the capacity to differentiate into both biliary and hepatic lineages [97]. When WB-F344 cells are cultivated at high cell densities, under conditions of contact inhibition of cell proliferation, TCDD stimulates their proliferation [98, 99]. The contact inhibition of cell proliferation is a tightly regulated process, which restricts the cell division of confluent nontransformed cells [81]. Since tumor promotion is characterized by unbalanced proliferation either due to increased proliferation or due to decreased level of apoptosis, it has been proposed that the loss of contact inhibition is a toxic event, which may contribute to liver tumor promotion [48]. The proliferative effect of TCDD in contact-inhibited rat liver progenitor cells can be replicated also with other classes of the AhR ligands, including PCBs, polycyclic aromatic hydrocarbons, flavonoids, or endogenous AhR ligands [100–103]. The mechanism responsible for this induction of cell proliferation is strictly AhR-dependent; however, it does not depend on presence of Arnt, a transcriptional partner of the AhR [104, 105]. The proliferative effects of TCDD or other DLCs are not limited to this model of rat liver progenitor cells; similar observations have been made using some other epithelial cell models [48, 105]. In contrast, recently, the AhR activation has been found to block cell cycle progression in isolated mouse oval cells [106]. Interestingly, we have recently observed that TCDD stimulates cell proliferation in undifferentiated human liver HepaRG cells [107]. These cells, isolated from adult human liver [108], show phenotypical features of undifferentiated bipotent liver cells when cultured at low densities, and they are capable of *in vitro* differentiation towards both hepatocyte-like and biliary-like cells [109]. Together, the present data seem to indicate that, in undifferentiated liver cells exhibiting progenitor or mixed hepatobiliary phenotypes, toxic AhR ligands can induce cell proliferation, while simultaneously suppressing proliferation of hepatocytes or hepatocyte-like cells.

In the contact-inhibited rat liver progenitor cells, activation of the AhR leads to induction of JunD expression, followed by induction of cyclin A, which in turn leads to an increased activity of cyclin A/cyclin-dependent kinase 2 complex, which drives cell proliferation [105]. The disruption of contact inhibition in this liver progenitor cell model has been found to be linked also with alterations of cell-to-cell communication and modulation of signaling pathways involved both in liver regeneration and in hepatocarcinogenesis [48, 98, 107, 110–112]. TCDD has been observed to stimulate membrane translocation of c-Src kinase in WB-F344 cells [98]. This nonreceptor tyrosine kinase has been proposed to form a part of the cytoplasmic AhR complex and it significantly modulates cell behavior, including stimulation of migration and invasion of tumor cells [113–115].

Induction of cell proliferation in contact-inhibited WB-F344 cells is also associated with decreased levels of connexin 43, a major protein forming gap junctions in epithelial cells, which corresponds with reduction of gap junction plaques at cell membranes and inhibition of gap junctional intercellular communication [110]. The AhR activation in rat liver progenitor cells reduces the expression of plakoglobin ($\gamma$-catenin), an important constituent of desmosomes and adherens junctions; this type of regulation of plakoglobin by the AhR has been confirmed also in other cell types [112, 116]. Finally, disruption of contact inhibition in WB-F344 cells also leads to downregulation of E-cadherin (and $\beta$-catenin) and reduced cell adhesion, suggesting that an impaired adherens junction function could be one of the consequences of the AhR-mediated disruption of cell proliferation control [111]. All of these findings are in line with the proposed role of the AhR as a regulator of cell adhesion and cell-to-cell communication [48, 49], two key mechanisms establishing and maintaining the contact inhibition of cell proliferation [48, 117, 118]. Nevertheless, it should be stated that the *in vivo*

relevance of these findings remains open and future studies should establish the importance of the AhR-dependent disruption of contact inhibition in the carcinogenic effects of its toxic ligands.

## 6. The Signaling Pathways Regulating Proliferation, Differentiation, and Fate of Adult Liver Progenitor Cells and Their Potential Interactions with the AhR Signaling

The analysis of global gene expression in WB-F344 cells released from the contact inhibition (by persistent toxic AhR ligand, PCB 126) has revealed a significant deregulation of a number of signaling components and transcriptional targets of the signaling pathways that are known to contribute to regulation of liver progenitor cell activation and/or differentiation [119]. An outline of the effects of toxic AhR ligands in this model of rat liver progenitor cell model is provided in Figure 1.

The major affected pathways included downregulation of Wnt/$\beta$-catenin and transforming growth factor-$\beta$ (TGF-$\beta$) signaling pathways, upregulation of ligands of the epidermal growth factor receptor (EGFR), or induction of some genes regulated by transcriptional cofactor Yes-associated protein (YAP) and/or its paralogue, transcriptional coactivator with PDZ-binding motif (TAZ; also known as WWTR1), the effectors of Hippo signaling pathway.

Notably, the global gene expression data suggested that multiple members of Wnt signaling pathway can be deregulated by toxic AhR ligands in liver progenitor cells [119]. Wnt/$\beta$-catenin signaling pathway is a key pathway regulating both development and adult tissue homeostasis. In the absence of Wnt stimulation, $\beta$-catenin binds to cytoplasmic destruction complex, which is formed by tumor suppressor proteins Axin and adenomatous polyposis coli, and kinases belonging to glycogen synthase kinase 3 and casein kinase 1 families. Within this complex, $\beta$-catenin is phosphorylated, ubiquitinated, and consequently degraded via a proteasome [120–122]. The activation of cognate Wnt receptor (Frizzled) and coreceptor (low density lipoprotein 5/6) by Wnts or related ligands inactivates the cytoplasmic destruction complex, thus leading to accumulation and nuclear translocation of $\beta$-catenin. The nuclear $\beta$-catenin forms complexes with LEF (lymphoid enhancer-binding factor)/TCF (T-cell factor) transcription factors and drives transcription of their target genes in a cell context-specific manner [120–122]. Apart from its signaling role, $\beta$-catenin plays also an important structural role in formation of adherens junctions, main epithelial adhesive junctions further contributing to the regulation of $\beta$-catenin activity and turnover [123]. In the liver, Wnt/$\beta$-catenin signaling plays a major role in its embryonic development, early postnatal growth, regeneration, and maintenance of adult liver functions, such as liver zonation (for recent reviews, see [124, 125]). Deregulation of Wnt signaling is also an important factor in liver carcinogenesis [125]. Importantly, a number of reports have indicated that the activity of this pathway controls proliferation and/or differentiation of liver progenitor cells, as well as liver cancer stem cells [40, 126–131].

It is becoming increasingly evident that Wnt/$\beta$-catenin signaling interacts with the AhR at multiple levels [132]. The increased activity of $\beta$-catenin upregulates the AhR expression in various tissues, including mouse liver [133, 134]. Given that $\beta$-catenin activity is high within the pericentral zone [135, 136], this may imply that the cells surrounding the central vein (including the proposed liver stem cells contributing to hepatocyte renewal [38]) could be more sensitive to toxic AhR ligands, because of significantly higher AhR levels in this region, as compared with periportal zone [137]. $\beta$-Catenin plays a major role in the expression of xenobiotic metabolizing enzymes in the liver [133, 136]. These include in particular the CYP1 family enzymes regulated by the AhR, which are involved in bioactivation of numerous environmental carcinogens [45, 138–141]. In rat liver progenitor cells, activation of Wnt/$\beta$-catenin has been found to significantly promote expression of both Cyp1a1 and Cyp1b1 [111] playing a principal role in bioactivation and genotoxicity of the environmental AhR ligands, such as PAHs [45].

Importantly, at the same time, TCDD could block the $\beta$-catenin-dependent signaling in liver progenitor cells. WB-F344 cells are sensitive to the activation of the canonical Wnt signaling by recombinant Wnt ligands, which induce a moderate cell proliferation in this cell model [101, 142]. The activation of AhR by TCDD significantly decreases levels of the active form of $\beta$-catenin (dephosphorylated on S37 and T41 residues) in liver progenitor cells [111]. Downregulation of $\beta$-catenin by activated AhR has also been observed in other cell models [143]. Additionally, TCDD induces dephosphorylation of Dvl (dishevelled) 2 and Dvl3 proteins that play a key role as the branching points regulating both canonical and noncanonical Wnt pathways [144]. In line with this, the sustained AhR activation has been found to reduce expression of a number of Wnt/$\beta$-catenin pathway targets in WB-F344 cells [111, 119].

The AhR-mediated deregulation of Wnt/$\beta$-catenin-dependent transcription in WB-F344 has been also linked with changes in their progenitor phenotype since CK14 and CK19, which are abundantly expressed in oval cells [145, 146], are downregulated by TCDD, while CK8 is simultaneously upregulated, which suggests that progenitor cells exposed to the AhR ligands may progress towards more hepatocyte-like phenotype [111]. This is supported also by the observation that expression of other genes associated with hepatic progenitor cell compartment, such as Kitl and Ncam1, is downregulated in WB-F344 cells exposed to toxic AhR ligands [111, 119]. The inhibitory role of the AhR in this important signaling pathway, contributing to regulation of progenitor cell proliferation and phenotype, is also supported by additional studies indicating that AhR ligands repress production of canonical Wnt ligands and/or repress Wnt/$\beta$-catenin signaling in a variety of tissues and cell models, including embryonic stem cells [143, 147–151]. Together, these results indicate that, apart from modulating the structural role of $\beta$-catenin in adherens junctions, toxic AhR ligands might also block its signaling role in liver progenitors.

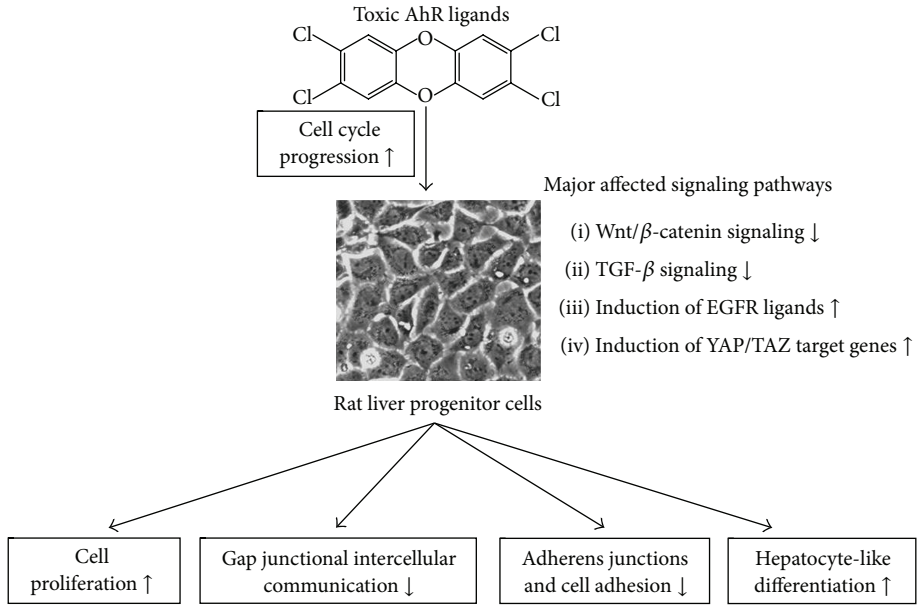


Figure 1: A summary of effects of AhR agonists on deregulation of signaling pathways and liver progenitor cell functions in WB-F344 cell model.

Wnt/$\beta$-catenin signaling is closely connected with the TGF-$\beta$ signaling pathway, since both pathways cross-talk at multiple levels, such as through reciprocal regulation of their ligands or via direct interaction of their signaling effectors within cell nuclei that are involved in transcriptional regulation of common gene targets [152, 153]. The TGF-$\beta$ family of cytokines includes, apart from TGF-$\beta$1, a number of proteins playing important roles in embryonic development, adult tissue homeostasis, and the cancer development, such as bone morphogenic proteins and activins/inhibins. TGF-$\beta$1 is a pleiotropic cytokine inducing a range of effects within liver cells, including regulation of cell migration, invasion, and stemness [154]. TGF-$\beta$1 blocks proliferation and induces apoptosis in mature hepatocytes, while its role in liver progenitor cells is less clear [154, 155]. Some studies have indicated that adult liver progenitor cells could be less sensitive to the TGF-$\beta$1-induced apoptosis and its antiproliferative effects than hepatocytes [156–158]. On the other hand, TGF-$\beta$1 blocks proliferation and promotes apoptosis in oval cell lines derived from DDC-treated mice [17]. Active TGF-$\beta$1 and $\beta$3 proteins are elevated in AhR knockout mice, and this corresponds with increased numbers of hepatocytes undergoing apoptosis, as compared with wild-type mice [159]. Interestingly, the analysis of global gene expression changes in WB-F344 cells revealed that AhR ligands could induce expression of follistatin in rat liver progenitors [119]. Upregulation of follistatin by TCDD has been observed also in additional cell models [160, 161]. This protein directly binds and inhibits activin A, TGF-$\beta$ family member and regulator of the liver homeostasis. Activin A blocks hepatocyte proliferation and induces their apoptosis, while its inhibition via follistatin promotes proliferation and decreases apoptosis in the liver [162]. Since activin A has been shown to induce growth arrest in hepatic progenitor cells [163], its inhibition via the AhR-dependent induction of follistatin might further promote the proliferative effects of toxic AhR ligands in rat liver progenitors. Additionally, AhR ligands have been also observed to downregulate activin receptors, which may pronounce their impact on activin signaling [119].

Growth factor signaling plays an important role in regulation of oval cells response, as it contributes to regulation of their growth, survival, motility, and differentiation [154, 155]. Rat liver progenitor WB-F344 cells are sensitive both to hepatocyte growth factor (HGF) and to epidermal growth factor (EGF), which both stimulate their proliferation and/or protect them from apoptosis [100, 164]. EGFR can be activated, apart from EGF, also by other functionally related ligands, such as TGF-$\alpha$, heparin-binding EGF (HB-EGF), amphiregulin (Areg), and epiregulin. The EGFR ligands are upregulated during oval cell activation and they promote oval cell expansion *in vivo* [165, 166]. Oval cell lines have been also proposed to regulate EGFR signaling also via autocrine mechanism(s) [154]. The treatment of WB-F344 cells with toxic AhR ligands has been found to upregulate several EGFR ligands, including Areg and HB-EGF, in the AhR-dependent manner [119]. Areg is a candidate AhR-responsive gene, which has been found to be induced by AhR ligands (or their mixtures) in the developing ureter *in vivo*, as well as in mouse hepatoma and human oral epithelial cells *in vitro* [167, 168]. Whether expression of Areg is increased in response to TCDD in adult liver progenitor cells also *in vivo* remains to be determined. HB-EGF is another EGFR ligand, which has been shown to contribute to liver regeneration or hepatocarcinogenesis [169–171]. TCDD has been shown to regulate also expression of additional EGFR ligands, such as epiregulin or transforming growth factor-$\alpha$ [172, 173]; however, these have not been found to be upregulated in WB-F344 cells [119]. Taken together, rat liver progenitor cells

are capable of the AhR agonist-inducible production of some EGFR ligands; however, the functional role of induction of these growth factors upon AhR activation is not fully clear.

The Hippo signaling pathway is essential for a proper organ size control, tissue regeneration, and stem cell self-renewal and it can play a significant role in cancer development [174]. In mammals, this pathway consists of a core set of kinases, mammalian Ste2-like kinases 1/2 (Mst1/2), and large tumor suppressor kinases 1/2 (Lats1/2), which control the activity of YAP and TAZ [175]. The establishment of cell-cell contacts leads to activation of Hippo kinases, Mst1/2 and Lats1/2, which then inhibit YAP and/or TAZ activity via their cytoplasmic retention and/or proteasomal degradation [175]. In contrast, activation of cell proliferation is linked with active YAP and/or TAZ being present within the nucleus, where they control the expression of a number of growth-promoting or antiapoptotic genes, including connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61), or survivin [176, 177]. The activity of proteins constituting Hippo pathways overlaps at numerous points with other pathways controlling the activation of adult liver progenitor cells, such as Wnt/$\beta$-catenin signaling [178], and it is a principal regulator of contact inhibition [177, 179]. In the liver, knockout of Hippo pathway components regulating YAP/TAZ activity or overexpression of YAP leads to disruption of liver size control or development of HCC [180]. The Hippo pathway has been also proposed to contribute to bile duct development and to hepatocyte reprogramming to biliary epithelial cells [181, 182]. Toxic compounds such as TCBOPOP have been shown to simultaneously increase liver size and increase YAP levels in liver [183]. Both YAP and TAZ have been shown to modulate plasticity and differentiation of hepatocytes, to control development of cancer stem cells during HCC, or to modulate proliferation of HCC cells, thus indicating a potentially important role for Hippo pathway in hepatocarcinogenesis [181, 184–186].

At present, the information on interactions between the AhR activation and Hippo signaling is very limited. Interestingly, the disruption of contact inhibition in rat liver WB-F344 cells has been found to be associated with induction of some YAP/TAZ transcriptional targets [107, 119]. Survivin, which is regulated by YAP/TAZ [187], has been found to be upregulated by TCDD in undifferentiated human liver HepaRG cells, simultaneously with disruption of cell cycle control and induction of cell proliferation [107]. These results seem to indicate that activation of proliferative signaling in cellular models of liver progenitors could be linked with YAP/TAZ-dependent activation of some target genes of this pathway; however, at present, the *in vivo* relevance of these findings remains unclear. Nevertheless, it is of interest that both TCDD and PCB 126 induce expression of CTGF, another YAP/TAZ transcriptional target, in contact-inhibited rat liver progenitor cells [107, 119]. This protein has been shown to promote hepatocyte-like differentiation of rat liver progenitor cells *in vitro* [188]; this seems to support the observation that AhR ligands reduce expression of progenitor cell markers and increase levels of hepatocyte-like markers in rat liver progenitors [111]. Nevertheless, TCDD has been also found to repress CTGF mRNA in HL1-1 adult human liver stem-like cell line, while simultaneously inducing YAP mRNA in the same cell model [189], thus suggesting that the role of the AhR in regulation of CTGF could be more complex and perhaps cell-specific.

Inflammatory cytokines have been proposed to play a major role in mediating both the hepatotoxicity of TCDD and the TCDD-induced liver tumor promotion [59, 190]. The production of inflammatory cytokines, such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), also contributes to the expansion of oval cells during experimental rodent liver injury [191], since TNF-$\alpha$ is upregulated during oval cell proliferation, which is induced by CDE diet, and elimination of TNF receptor 1 blocks oval cell response in mice [192]. Interestingly, TNF-$\alpha$ has been found to be a major factor supporting the proliferative effects of DLCs in rat liver oval cells, promoting both cyclin A induction and cell cycle progression in WB-F344 cells [193]. This cytokine potentiates the effects of both strong and weak environmental AhR agonists in rat liver progenitor cells [193, 194]. Moreover, activation of inflammatory signaling by this cytokine, namely, the p38 mitogen-activated kinase activity, further supports inhibition of gap junctional intercellular communication [194], as well as metabolic activation of genotoxic AhR ligands via upregulation of CYP1B1 [195, 196]. Together, these data seem to support the scenario, where induction of inflammation by toxic AhR ligands in the liver might further support their procarcinogenic effects (deregulation of cell proliferation, induction of DNA damage) in liver progenitors.

## 7. Conclusions

Toxic AhR ligands have been found to disrupt various functions of liver progenitor cell models *in vitro*, including deregulation of cell proliferation control and cell-to-cell communication, or to alter the activity of signaling pathways relevant for the maintenance of liver homeostasis, activation of oval cell response, or liver carcinogenesis. These include modulations of Wnt/$\beta$-catenin and TGF-$\beta$ pathways and induction of expression of EGF-related growth factors or transcriptional targets of Hippo pathway, which are involved in both regenerative and oncogenic signaling. The available data seem to indicate that, apart from their other well-recognized hepatotoxic effects, the environmental ligands of the AhR may alter the functions of cell populations exhibiting phenotypic features of adult liver progenitor cells (or undifferentiated liver cells), with potential to serve as precursors of hepatocytes and biliary epithelial cells. These results may have implications for the carcinogenic effects of sustained AhR activation in the liver; however, it should be noted that a major limitation of the available data is currently their reliance on the use of *in vitro* models of liver progenitor cells. Future studies should therefore focus on analyzing the impact of the AhR activation on liver progenitors *in vivo*, in order to ascertain the relevance of these findings for experimental chemical carcinogenesis, which may help us to better understand the liver toxicity and carcinogenicity of the AhR ligands, both in the experimental animals and in humans.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Jan Vondráček and Miroslav Machala revised the literature and wrote the paper.

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*Research Article*

# Genome Editing of the *CYP1A1* Locus in iPSCs as a Platform to Map AHR Expression throughout Human Development

**Brenden W. Smith,[1,2] Elizabeth A. Stanford,[1,2,3] David H. Sherr,[3] and George J. Murphy[1,2]**

[1]*Section of Hematology and Oncology, Department of Medicine, Boston University School of Medicine, Boston, MA 02118, USA*
[2]*Center for Regenerative Medicine (CReM), Boston University and Boston Medical Center, Boston, MA 02118, USA*
[3]*Department of Environmental Health, Boston University School of Public Health, Boston, MA 02118, USA*

Correspondence should be addressed to George J. Murphy; gjmurphy@bu.edu

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The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that increases the expression of detoxifying enzymes upon ligand stimulation. Recent studies now suggest that novel endogenous roles of the AHR exist throughout development. In an effort to create an optimized model system for the study of AHR signaling in several cellular lineages, we have employed a CRISPR/CAS9 genome editing strategy in induced pluripotent stem cells (iPSCs) to incorporate a reporter cassette at the transcription start site of one of its canonical targets, cytochrome P450 1A1 (*CYP1A1*). This cell line faithfully reports on *CYP1A1* expression, with luciferase levels as its functional readout, when treated with an endogenous AHR ligand (FICZ) at escalating doses. iPSC-derived fibroblast-like cells respond to acute exposure to environmental and endogenous AHR ligands, and iPSC-derived hepatocytes increase *CYP1A1* in a similar manner to primary hepatocytes. This cell line is an important innovation that can be used to map AHR activity in discrete cellular subsets throughout developmental ontogeny. As further endogenous ligands are proposed, this line can be used to screen for safety and efficacy and can report on the ability of small molecules to regulate critical cellular processes by modulating the activity of the AHR.

## 1. Introduction

The aryl hydrocarbon receptor (AHR) has been studied for decades for its role in environmental toxin induced carcinogenesis [1, 2]. A member of the Per/ARNT/SIM (PAS) family of basic helix-loop-helix (bHLH) transcription factors, the AHR is activated by small-molecule ligands that cause it to be disassociated from a cytoplasmic chaperone complex and translocated into the nucleus [3]. Upon nuclear translocation, the AHR dimerizes with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) and subsequently binds to conserved AHR response elements (AHREs) within the genome [4]. Through this pathway, the AHR affects the expression of multiple gene targets that contain AHREs in proximal regulatory regions [5, 6]. Classic examples of AHR ligands include 2,3,7,8-tetrachlorodibenzodioxin (TCDD) [7], polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) [8]. As part of an adaptive response to the presence of these carcinogens, the AHR significantly increases transcription of cytochrome p450 (CYP450) enzymes, specifically *CYP1A1* [9] and *CYP1B1* [10], that will contribute to the metabolism of these compounds into both toxic and nontoxic intermediates [11]. Concomitant with CYP450 activation, the AHR contributes to its own negative regulation by promoting transcription of the AHR Repressor (AHRR), a competitive inhibitor that prevents dimerization of the AHR:ARNT complex, causing free AHR molecules to be exported into the cytoplasm and subsequently degraded [12].

In the last 10 years, there has been a major paradigm shift following the demonstration that the AHR plays important physiological roles in the absence of environmental ligands [13]. Multiple studies suggest the AHR pathway is important in the development and function of the cardiovascular system [14–17] in the *ahr* knockout mouse without the requirement for experimental ligand exposure. Indeed, it is this same

model organism that displays varied and diverse developmental phenotypes including, but not limited to, reduced liver size, increased portal tract fibrosis [18], decreased fertility [19, 20], a suspected resistance to neurotoxicity [21], and an impairment in the lymphocyte compartment [22]. AHR signaling has since been implicated in multiple aspects of human developmental ontogeny. Recent studies suggest that the AHR plays a critical role in human hematopoietic stem cell (HSC) differentiation [23], substantiating murine studies that show *in vivo* AHR modulation resulting in disruption of HSC growth, senescence, and migration [24]. Our own work reveals the AHR as a modulator of erythroid and megakaryocyte specification from a common bipotent progenitor [25], incorporating an endogenous ligand of the AHR (6-formylindolo(3,2-b)carbazole or FICZ) to produce this result. Observed roles of the AHR in immunity and inflammation [26] as well as the discovery of novel endogenous AHR agonists [13] add to the overwhelming evidence that AHR signaling is endogenously regulated and crucially important throughout development. Compounded with evidence of regulatory cross talk with VEGF and TGF-$\beta$ pathways [27, 28], these studies seem to suggest that multiple roles of AHR signaling have yet to be discovered.

The advent of cellular reprogramming and genome editing has provided platforms to study signaling pathways in diverse and novel ways. Since their discovery in 2006 [29, 30], induced pluripotent stem cells (iPSCs) have been shown to functionally emulate embryonic stem cells (ESCs) [31, 32] by having the capacity to differentiate into all three germ layers of the developing embryo [29, 30]. iPSCs have been specified to multiple cellular lineages, including those derived from endoderm (liver [33–36], pancreas [37–39], and lung [40–42]), mesoderm (hematopoietic cells [25, 43–48], heart [49–51], and kidney [52]), and ectoderm (neurons [53]). The flexibility of an iPSC-based system allows for the study of multiple tissue types. In this way, iPSCs stand to revolutionize the way we study human development, model disease, and eventually treat patients. Additionally, genome editing strategies have been widely used to create genetic knockouts, repair disease-causing mutations, and integrate reporter constructs [54]. Clustered regulatory interspersed short palindromic repeats (CRISPRs) have been identified as an element of bacterial adaptive immunity by which foreign DNA of invading species is incorporated into the host genome and subsequently used as a template upon which CRISPR associated (CAS) endonucleases bind and digest newly infected DNA [55]. The result is targeted cutting of double stranded DNA that is specific and highly efficient. Multiple groups have adapted this technology to mammalian systems to improve upon preexisting methodologies and have confirmed that this tool is highly accessible and amenable to targeted genome editing [56]. Despite the promise of this technology, there remains a paucity of studies that examine the AHR within the context of iPSC directed differentiation [25, 57] or incorporate CRISPR/CAS9 to employ genetic manipulation of AHR signaling [58]. Future work will help reveal the signaling dynamics and interregulatory cross talk of the AHR pathway in differentiated iPSCs within multiple cellular contexts.

Using a CRISPR/CAS9 system for genome editing, we have created an endogenous reporter of AHR activity in an iPSC line by targeting the *CYP1A1* locus. *CYP1A1* is a canonical target of AHR signaling, one that is widely used to report on AHR activity in multiple cell and tissue types *in vivo* and *in vitro*. As a result, its expression is commonly used as a functional output of AHR activity in the absence of ligand exposure. In this report, we show the utility of this cell line in its response to multiple agonists and antagonists and validate its function by observing AHR modulation in the context of both hematopoiesis and hepatic specification.

## 2. Materials and Methods

*2.1. iPSC Generation and Maintenance.* Induced pluripotent stem cells were generated as described previously [59, 60]. Briefly, 4 mL of human peripheral blood was collected into a BD Vacutainer CPT Cell Preparation Tube and centrifuged to produce a buffy coat containing peripheral blood mononuclear cells (PBMCs). The buffy coat was collected and PBMCs were cultured *ex vivo* for 9 days before being transduced with the STEMCCA lentiviral vector. At day 12 of culture, STEMCCA transduced PBMCs were plated onto mouse embryonic fibroblasts (MEFs) and cultured until roughly days 30–40, when fully formed iPSC colonies were identified and separately harvested. Following successive passages onto irradiated MEFs (R&D, #PSC001), colonies were adapted to matrigel-coated tissue culture dishes in the absence of a feeder cell layer. iPSCs were then cultured in mTESR1 media (StemCell Technologies, #05850) for all further passages.

*2.2. Creation of CRISPR/CAS9 Targeted CYP1A1 Reporter iPSCs.* Targeting of the *CYP1A1* locus was achieved by cotransfection of the plasmids described (Figure 1(a)). Confluent iPSC cultures were pretreated with 10 $\mu$M Y-27632 (ROCK inhibitor) for 3 hours in mTESR1 medium. Cells were resuspended in 100 $\mu$L of P3 solution (Lonza) and added to a cuvette for the Lonza 4D Nucleofector at a density of 5e6 cells per cuvette. 2 $\mu$g of the CAS9 vector and 3 $\mu$g of the Donor vector were added to the cell suspension and nucleofected using the CB-150 program. Immediately following nucleofection, cells were resuspended into fresh mTESR1 with 10 $\mu$M Y-27632 and plated onto one 10 cm plate (pretreated with matrigel) and left at 37° in a low oxygen (5% $O_2$) incubator. Cells were allowed to grow for 5 days before clones were selected for puromycin resistance by the addition of 0.7 $\mu$g/mL puromycin (ThermoFisher, #A1113802). Colonies were harvested as they appeared in culture and were passaged and maintained separately before being screened by PCR for the integrated construct.

*2.3. PCR and Sanger Sequencing.* To validate proper targeting of the reporter construct to the *CYP1A1* locus, two PCR products were amplified that flank the 5′ and 3′ ends of the reporter construct (resp.) and include elements of both the integrated cassette and the endogenous locus (Figure 1(c)). PCR was performed using recombinant Taq polymerase (ThermoFisher, #10342) with primers

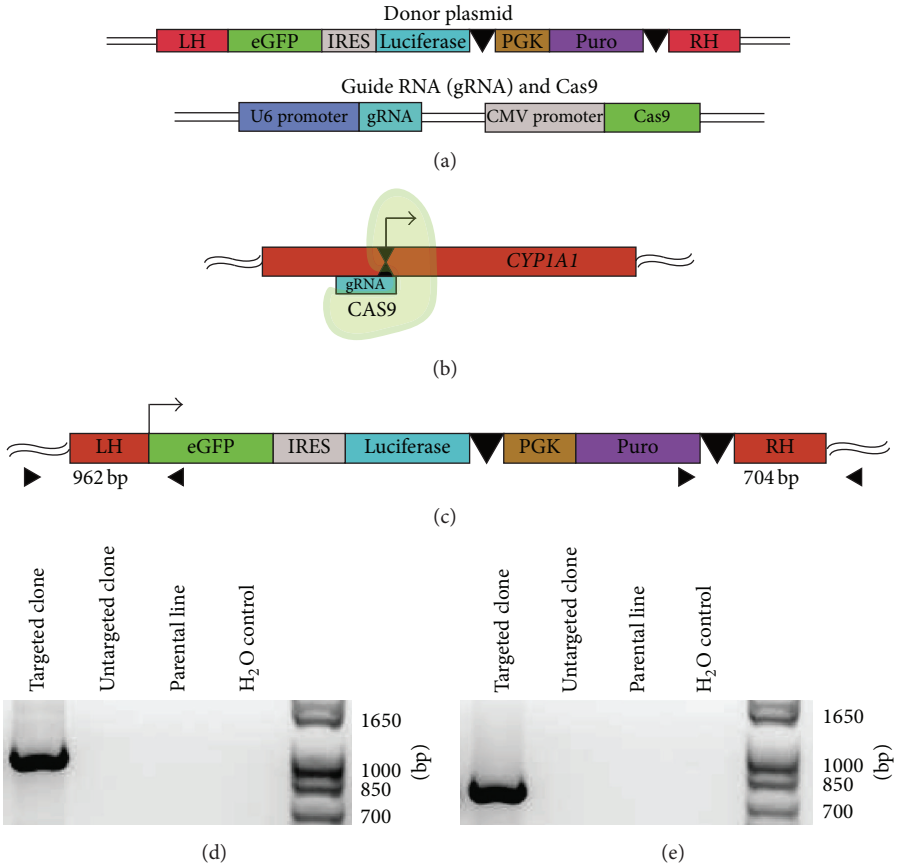


Figure 1: *Construction and validation of CYP1A1 reporter iPSCs.* (a) Two vectors were created to achieve CAS9 targeted digestion at the *CYP1A1* transcription start site and homologous recombination of a reporter construct. The donor plasmid contains a cassette that includes eGFP and luciferase separated by an internal ribosome entry sequence (IRES). Directly downstream of these reporter elements is a puromycin resistance gene (Puro) driven by a constitutive promoter (PGK) and flanked by loxP sites (denoted by black arrowheads). This cassette is flanked by regions that are homologous to the *CYP1A1* endogenous locus (Left Homology, LH; Right Homology, RH) to facilitate homologous recombination. The guide RNA (gRNA) and Cas9 were encoded on the same plasmid, each driven by a separate constitutive promoter (U6 and CMV, resp.). (b) An idealized schematic of Cas9 digestion at the transcription start site (denoted by black arrow) of the *CYP1A1* locus. (c) The integrated reporter construct is expected to specifically target the transcription start site of *CYP1A1*, and a PCR strategy was employed that creates amplicons in the 5′ and 3′ flanking regions of the cassette that include elements from the reporter construct as well as endogenous regions that are not encoded by the donor plasmid. (d) The 5′ amplicon (expected size = 962 bp) was exclusively detected in a properly targeted iPSC clone. (e) The 3′ amplicon (expected size = 704 bp) also could not be amplified in untargeted clones or the parental iPSC line but was detected in a properly targeted iPSC clone.

for the 5′ amplicon (Forward: 5′-ggtgggatttcctgcatcct-3′; Reverse: 5′-cttgtggccgtttacgtcg-3′) and 3′ amplicon (Forward: 5′-cctgcaggatctgatcagataacttcg-3′; Reverse: 5′-caggttgactaggctaagcagttcttg-3′) in separate reactions. PCR products were resolved by agarose gel electrophoresis and were 962 bp and 704 bp, respectively. Bands that appeared to be the proper size by electrophoresis were extracted and purified using the QiaQuick gel extraction kit (Qiagen, #28704) and submitted for Sanger sequencing to Genewiz, Inc. Sequencing data, as well as all homology domain and guide RNA sequences, are available in Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2574152.

*2.4. Generation of Hepatocyte-Like Cells from iPSCs.* iPSC cultures were passaged using Gentle Cell Dissociation (GCD) Reagent (StemCell Technologies, #07174) to obtain a single cell suspension and counted using a hemacytometer. Cells were passaged onto matrigel-coated tissue culture dishes at a cellular density of 3e5 per well of a standard 6-well plate. After 24 hours, mTESR1 was replaced by media provided by the STEMdiff Definitive Endoderm Kit (StemCell Technologies, #05110) and cultured according to manufacturer's instructions for 5 days. At day 5, GCD was used to make a single cell suspension and the cells were passaged at a ratio of 1 : 6 onto matrigel-coated 6 well plates. The media for all subsequent days were an SFD base [61] with ascorbic acid (50 $\mu$g/mL) and monothioglycerol (4.5$e$ − 4 M). Media for days 5 and 6 included Activin A (50 ng/mL), BMP4 (10 ng/mL), FGF2 (10 ng/mL), and VEGF (10 ng/mL). The media for days 7–12, days 13–18, and days 19–25 were adapted directly from a previous manuscript [35]: days 7–12: BMP4 (50 ng/mL), FGF2 (10 ng/mL), VEGF (10 ng/mL),

EGF (10 ng/mL), TGFa (20 ng/mL), HGF (100 ng/mL), and 0.1 $\mu$M Dexamethasone; days 13–18: FGF2 (10 ng/mL), VEGF (10 ng/mL), EGF (10 ng/mL), HGF (100 ng/mL), Oncostatin M (20 ng/mL), Vitamin K (6 $\mu$g/mL), 1.5 $\mu$M gamma secretase inhibitor, 0.1 $\mu$M Dexamethasone, and 1% DMSO; days 19–25: HGF (100 ng/mL), Oncostatin M (20 ng/mL), Vitamin K (6 $\mu$g/mL), and 0.1 $\mu$M Dexamethasone. Cells were kept in a low oxygen (5% $O_2$) incubator throughout the differentiation.

*2.5. Generation of Hematopoietic Progenitor Cells from iPSCs and Treatment with 6-Formylindolo(3,2-b)carbazole (FICZ).* Hematopoietic progenitor cells were derived from induced pluripotent stem cells using our previously published protocol [25]. Briefly, iPSCs seeded on matrigel plates were exposed to cytokine conditions that promoted mesoderm specification, followed by a hemogenic endothelial-like phenotype, and, finally, hematopoietic progenitors that disadhered from the matrigel substrate and were dual positive for CD41 and CD235 (data not shown). At day 7 of the protocol (Supplemental Figure 2), cells were treated with escalating doses of FICZ, at a range of $10e-8$ to $10e-4$ M, and kept in this condition for 5 days. Cells were harvested at day 12, at which point lysates were created for luciferase assays as well as RNA extraction and kept at −80°C.

*2.6. RNA Extraction and Quantitative PCR.* RNA was extracted using the RNeasy Mini Kit (Qiagen, #74104). At the time of harvest, cells were washed with PBS and spun for 5 minutes at 300 ×g, and the pellet was collected in 350 $\mu$L of Buffer RLT. RNA extraction proceeded according to manufacturer instructions. RNA was eluted into 30 $\mu$L of endonuclease-free $H_2O$ and purified with DNAse using the DNA-free DNA Removal kit (ThermoFisher, #AM1906). Once purified, 20 $\mu$L of sample was used to generate cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). RNA samples were quantified using a NanoDrop Lite (Thermo Scientific) Spectrophotometer, and cDNA samples were diluted to 1 $\mu$g/$\mu$L. Quantitative PCR was carried out using the Taqman Universal Master Mix (Thermo Scientific) and primers for *CYP1A1* (Hs01054797_g1) and *β-ACTIN* (Hs99999903_m1) were used. Samples were run in triplicate and, where appropriate, were analyzed by Student's *t*-test to assess significance between groups.

*2.7. Flow Cytometry.* Flow cytometry was performed at days 5, 14, and 25 of hepatocyte specification. For day 5, 3e5 cells were stained per condition, and the C-KIT antibody (Biolegend, #313206) and CXCR4 antibody (Invitrogen, #MHCXCR404) were used at a concentration of 5 $\mu$L per 1e6 cells. Staining was performed on ice for 30 minutes. For days 14 and 25, cells were fixed in 1.6% paraformaldehyde before staining. Primary antibodies for AAT (Santa Cruz, #sc-59438) and FOXA1 (Santa Cruz, #101058) were added at 1 : 100 ratio in Saponin Buffer (2% FBS, 1x Permeabilization Wash Buffer (Biolegend, #421002)) and incubated at room temperature for 30 minutes. Secondary antibodies for AAT (Jackson Immunoresearch, #115-605-205) and FOXA1 (Jackson Immunoresearch, #115-545-206) were added at a dilution of 1 : 500 and incubated at room temperature for 30 minutes. All samples were resuspended in PBS with 0.5% BSA for analysis.

*2.8. Luciferase Assays.* To assess luciferase expression, cells were harvested and counted by hemacytometer to ensure 1e5 cells per 20 $\mu$L of 1x lysis buffer from the commercially available luciferase assay system (Promega, #E1500). Upon sufficient lysis, samples were stored at −80°C until all time points were collected. Samples were then thawed on ice and assayed by adding 20 $\mu$L per well of a 96-well plate, followed by addition of 100 $\mu$L of luciferase assay reagent (Promega) and immediate analysis of luminescence in a Tecan Infinite M1000 microplate reader.

*2.9. Fibroblast Differentiation and Small Molecule Treatment.* iPSCs were seeded on matrigel-coated 12-well plates at a density of 3e5 cells per well and left in mTESR medium for 24 hours. Fibroblast induction media (IMDM, 10% FBS, 2 mM l-glutamine, and 100 $\mu$g/mL primocin (Invivogen, #ant-pm-1)) were added for 2 days, followed by small molecule treatment for exactly 24 hours before cells were harvested for luciferase and qPCR assays. Small molecule AHR modulators were added at the following concentrations: TCDD, 1 nM; CH223191, 10 $\mu$M; benzo[a]pyrene, 1 $\mu$M; benzo[e]pyrene, 1 $\mu$M; FICZ, 10 $\mu$M; indoxyl sulfate, 100 $\mu$M.

*2.10. Image Capture and Analysis.* All images were captured on a Nikon Eclipse TS100 microscope equipped with a Diagnostic Instruments, Inc., model 18.2 Color Masonic Camera. Images were processed using Adobe Illustrator software.

*2.11. Statistical Analysis.* Results are presented as mean ± the standard error of the mean (SEM). Statistical significance was confirmed using the Student *t*-test as indicated.

## 3. Results and Discussion

*3.1. Vector Design and Construction and Validation of a CYP1A1 Reporter iPSC Line.* To create an endogenous reporter of AHR activity in an iPSC line, a CRISPR/CAS9 system was engineered to target the *CYP1A1* locus. A plasmid was created that expresses a reporter cassette of enhanced green fluorescent protein (eGFP) and firefly luciferase bifurcated by an internal ribosomal entry sequence (IRES) to allow for each reporter gene to be expressed on the same transcript (Figure 1(a)). Downstream of this cassette is a puromycin resistance gene (PURO) driven by a constitutive promoter for murine phosphoglycerate kinase (PGK) to allow for antibiotic selection. This reporter sequence does not include a 5′ regulatory region but rather is flanked by homology arms that facilitate recombination directly downstream of the *CYP1A1* transcription start site in the endogenous locus (Supplemental Figure 1B). Using this strategy, reporter expression is exclusively driven by the *CYP1A1* promoter, a regulatory region that includes 10 distinct AHR response elements (AHREs) [62]. A guide RNA sequence (gRNA) was developed using a publically available web resource (http://crispr.mit.edu/)

created and distributed by the Zhang Lab at the Massachusetts Institute of Technology [63]. The guide RNA shares sequence homology with a 23-base-pair region exactly 8 base pairs downstream of the *CYP1A1* start codon (5′-CCCAATCTCCATGTCGGCCACGG-3′) that includes a 3-base-pair protospacer adjacent motif (PAM) sequence that is necessary for CAS9 binding (Figure 1(b); Supplemental Figure 1B). The guide RNA and CAS9 coding regions were included on the same plasmid, separate from the plasmid containing the reporter construct, each with a dedicated constitutive promoter (Figure 1(a)).

Cotransfection of the two engineered plasmids produced a series of puromycin resistant clones which were then screened for the inserted reporter sequence within the *CYP1A1* locus. Validation was accomplished by a PCR strategy that creates two distinct amplicons at the flanking regions of the integrated cassette. Using this strategy, each amplified region contains elements of the *CYP1A1* endogenous locus that is not included in the homology arms as well as elements of the donor sequence (Figure 1(c)). Successful PCR amplification of these regions can only be achieved in properly targeted clones (Figures 1(d) and 1(e)) and Sanger sequencing confirms that each amplicon includes genomic regions of the *CYP1A1* locus as well as elements from the donor construct (Supplemental Figure 1A).

*3.2. CYP1A1 Reporter iPSCs Respond to FICZ in a Dose-Dependent Manner.* To achieve functional validation of the properly targeted clone, we used a previously published, directed differentiation protocol for the production of hematopoietic progenitors of the megakaryocyte and erythroid lineages [25]. Our previous work revealed that activation of the AHR pathway with 6-formylindolo(3,2-b)carbazole (FICZ) in this population causes exponential expansion and increased viability in culture [25]. Having proven this population's responsiveness to AHR agonism, we treated hematopoietic progenitors derived from a *CYP1A1* targeted clone with escalating doses of FICZ for 5 days. FICZ treatment increased transcript expression of *CYP1A1* in a dose-dependent manner, and this result was observed in both the parental iPSC line and the *CYP1A1* targeted clone (Supplemental Figure 2A). These cultures were also assayed for luciferase expression, and unlike *CYP1A1* transcript expression, only the *CYP1A1* targeted line displayed luciferase bioluminescence that increased significantly with each successive FICZ dose (Supplemental Figure 2B). This work confirms that the CRISPR/CAS9 targeted clone faithfully reports on AHR activation through a functional output of luciferase expression.

*3.3. Mapping of AHR Activity throughout Human Hepatocyte Specification Using CYP1A1 Targeted iPSCs.* To fully utilize the *CYP1A1* targeted iPSC line, we differentiated these cells towards the hepatocyte lineage in order to showcase the potential of this reagent to provide a temporal map of AHR activation in a variety of cellular contexts. Multiple studies have reported on the AHR response to environmental ligands in primary liver and have displayed a baseline level of *CYP1A1* expression even in the absence of toxin exposure [64]. Thus, in order to confirm the utility of this cell line, we sought to recapitulate these results in an *in vitro* context using a previously described protocol for directed differentiation to hepatocyte specification [35, 36]. Using this strategy, we successfully produced cells with definitive endodermal markers (CXCR4 and CKIT) after 5 days of differentiation and proceeded to incorporate a cytokine cocktail including FGF2 and Activin A to produce early hepatocyte progenitors at day 14, as indicated by observed dual positivity for Alpha 1 Antitrypsin (AAT) and FoxA1 (Figure 2(a)). Cultures were subsequently exposed to a specified media containing Hepatic Growth Factor (HGF) and Oncostatin M, and at day 25, the AAT+/FoxA1+ population had increased substantially (63.7%) (Figure 2(a)). Micrographs taken at days 5, 14, and 25 of differentiation show the progressive change in cellular morphology of these cells as they formed a homogenous 2D monolayer (day 5) followed by a heterogenous population where polygonal hepatic-like cells began to emerge (day 14) and, finally, an adherent cellular layer dominated by granular, polygonal cells with distinct, sinusoidal-like boundaries (day 25) (Figure 2(b)). The *CYP1A1* reporter clone was differentiated in parallel with the parental iPSC line, and luciferase-dependent bioluminescence was assayed at each time point throughout the hepatic differentiation. Undifferentiated cells (day 0) as well as CXCR4+/CKIT+ definitive endoderm (day 5) produced low levels of *CYP1A1*-driven luciferase, but a marked increase in luciferase expression was observed in hepatocyte progenitors (day 14) and, more significantly, early hepatocytes (day 25) (Figure 2(c)). Interestingly, the discrepancy seen between these two final time points is highly correlated to the relative abundance of AAT+/FoxA1+ dual positive cells within these cultures, suggesting that AHR activation occurs exclusively in this discrete population. The ability of *CYP1A1* targeted iPSCs to faithfully report on patterns of activation previously reported in primary cells is an early indication of the utility of this cell line in mimicking *in vivo* ontogeny and providing an easily accessible model system upon which to study this highly ubiquitous pathway.

*3.4. CYP1A1 Reporter iPSC-Derived Fibroblast-Like Cells Respond to Putative AHR Ligands.* With the evolution of the AHR field, culminating in the description of endogenous roles of the AHR in the absence of classical, environmentally derived ligands, the value of an iPSC clone with the capacity to report on AHR activation is dependent upon its sensitivity to multiple small molecule compounds previously shown to affect AHR signaling [65–67]. To assess the ability of the *CYP1A1* targeted cell line to respond to exogenous and proposed endogenous AHR ligands, we ran a comprehensive chemical screen and assayed for luciferase-dependent luminescence at 24 hours after dosing. Due to the observed lack of AHR dependent luciferase expression in the undifferentiated state (Figure 2(c)) we exposed iPSCs to a simplified media over the course of two days that quickly altered the cellular morphology to a fibroblast-like appearance (Figure 3(a)). These cells had detectable luminescence in the naïve (untreated) condition (Figure 3(b)), whereas undifferentiated cells had an indistinguishable expression

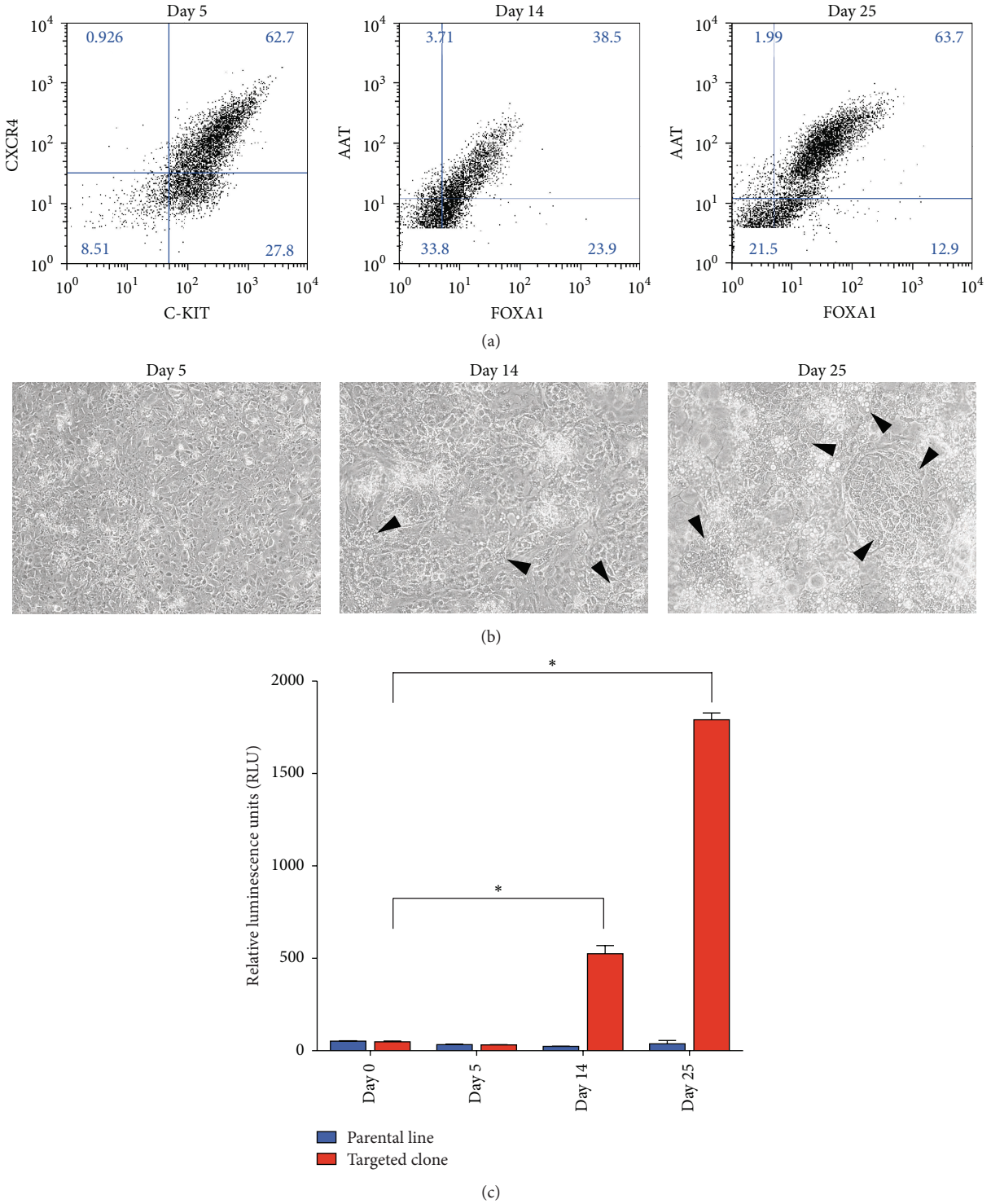


Figure 2: Hepatocyte specification yields luciferase expression in cells derived from *CYP1A1* reporter iPSCs. (a) iPSCs were differentiated towards CXCR4+/C-KIT+ definitive endoderm (day 5) followed by FOXA1+/AAT+ hepatic progenitors (day 14) that grew in number and were the majority of the culture by day 25. (b) Micrographs show homogenous morphology of definitive endoderm cultures (day 5), but by day 14, hepatic-like cells begin to emerge (denoted by black arrowheads) and are observed more frequently by day 25. (c) Concomitant with hepatic specification, luciferase levels significantly increase ($N = 3$, $^{*}P < 0.0005$, Student's $t$-test).

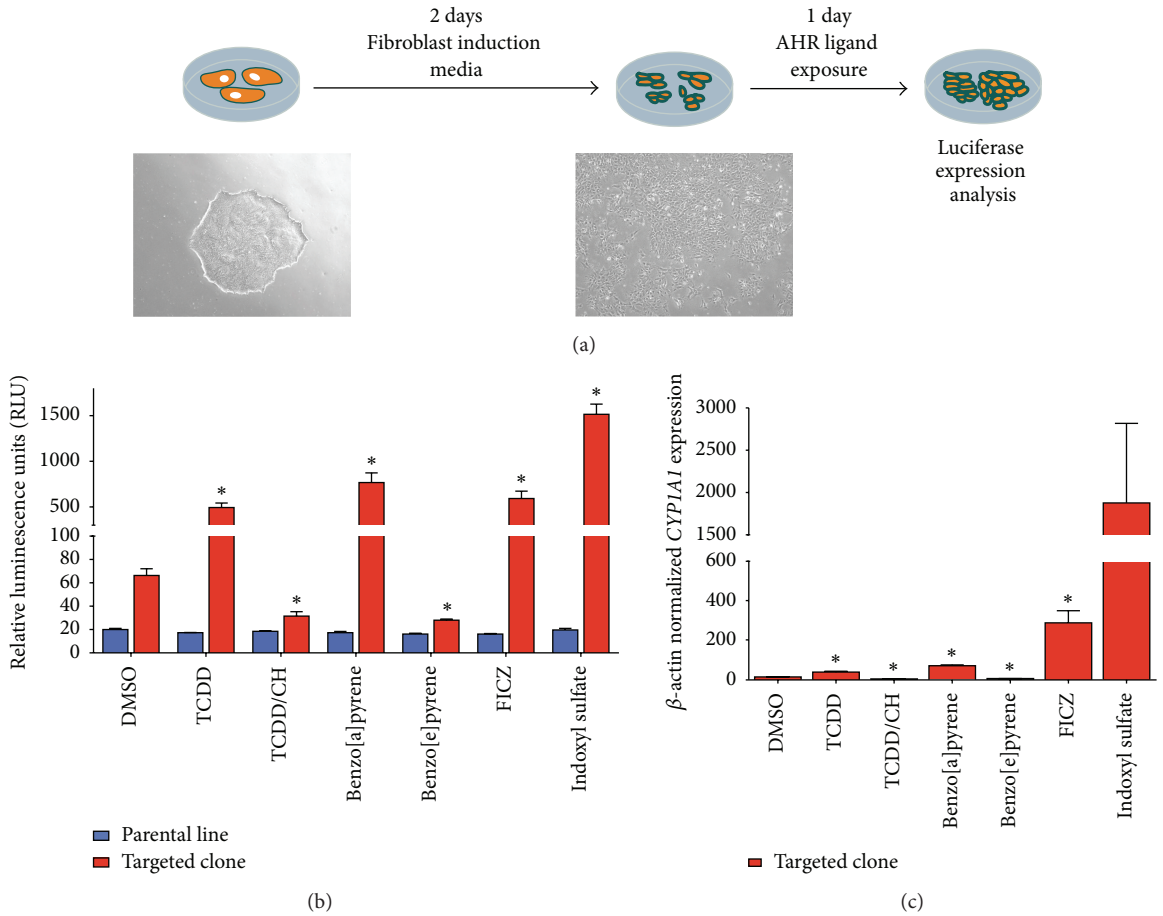


Figure 3: Acute exposure to AHR ligands causes a predictable response in *CYP1A1* reporter iPSCs. (a) Fibroblast-like cells were differentiated from *CYP1A1* iPSCs over the course of 2 days using fibroblast induction media. A compendium of AHR ligands were then added to the cultures for 24 hours before cells were harvested for further analysis. (b) Luciferase expression analysis reveals patterns of activation and inhibition of the *CYP1A1* reporter (targeted clone) as a response to agonist and antagonist treatment. Significance was established by Student's $t$-test for each condition compared to the DMSO control condition ($^*P < 0.01$, $N = 3$) (c) Transcript level expression of endogenous *CYP1A1* in the targeted clone showed similar patterns of modulated expression that positively correlated to luciferase output. Significance was established by Student's $t$-test for each condition compared to the DMSO control condition ($^*P < 0.02$, $N = 3$).

profile to that of the parental iPSC line (Figure 2(c); *day 0 time point*). Our chemical screen incorporated known environmental ligands, including TCDD and benzo[a]pyrene, as well as endogenous agonists' indoxyl sulfate and FICZ, and a potent AHR antagonist, CH223191 (CH). Treatment with DMSO alone established the basal levels of luciferase output, and TCDD induced a significant response that was completely occluded by the presence of CH223191 (Figure 3(b); *TCDD/CH condition*). Both of these compounds are known to affect gene expression in an AHR dependent fashion, giving further credence to the specificity of this AHR reporter system. Further, the polycyclic aromatic hydrocarbon benzo[a]pyrene induced AHR activity while benzo[e]pyrene, a structurally similar compound previously shown to have very little affinity for AHR in the cytoplasm [68], actually seemed to inhibit activity relative to the vehicle control. Additionally, the tryptophan derivatives indoxyl sulfate and 6-formylindolo(3,2-b)carbazole (FICZ) also proved efficacious in this model system. Finally, *CYP1A1* gene expression was assessed and found to be modulated in a similar pattern to that of luciferase expression (Figure 3(c)), further substantiating the hypothesis that luciferase expression reports directly on AHR activation.

## 4. Conclusions

Multiple bioassays have been developed to study AHR signaling in distinct cellular subtypes. These systems' utility has mainly been in the identification of environmental ligands [69] and, recently, the discovery of proposed endogenous ligands that range from the tryptophan derivates FICZ [70, 71] and indoxyl sulfate [72] to bilirubin, a natural product of heme catabolism [73, 74] and the arachidonic acid metabolites prostaglandin G [75] and lipoxin A4 [76]. Perhaps the most widely utilized experimental reporter is the pGudLuc vector, of which multiple iterations have been reported in the literature [69]. This vector was created by incorporating a 482-base-pair segment of the murine *CYP1A1*

promoter with 4 AHREs (also known as Dioxin Response Elements, or DREs) within the mouse mammary tumor virus (MMTV) promoter, with luciferase expression as the functional readout. Since its inception [77], this reagent has been optimized for better stability [69] and used by many groups to assess ligand responsiveness in immortalized cell models [78, 79].

The endogenous reporter of AHR activity described in this work represents a significant technological advancement that is highly specific and accessible to researchers with various expertise. Unlike immortalized cell lines with stable transfection of pGudLuc, this iPSC line has a targeted integrant directly downstream of the *CYP1A1* transcription start site. Despite observations in the literature of off-target CRISPR/CAS9 cutting [80], the functional data presented herein substantiates our hypothesis that little to no off-target integration of the reporter construct exists in this particular case. We hypothesize, then, that this reporter line is isogenic to an in-house iPSC control and can be efficiently differentiated to multiple cellular lineages. Transcript level expression of *CYP1A1* in the targeted line upon FICZ treatment (Supplemental Figure 2) proves that endogenous *CYP1A1* is not knocked-out as a result of genomic integration, making it likely that the reporter construct is hemizygously expressed. This would indicate that this clone has a single integrant in the exact genomic location that AHR:ARNT dimers naturally modulate *CYP1A1* gene expression. This system avoids random integration of an artificial promoter driven construct that could be expressed in multiple genomic locations, affecting endogenous gene expression in unknown ways. It also uses the entire *CYP1A1* promoter to drive expression, utilizing potentially complex interactions and gene expression profiles dependent on distal cis elements that cannot be conveyed by transfection of reporter plasmids. TCDD exposure, for example, is known to affect local chromatin structure in promoting endogenous *CYP1A1* expression [81] and selective ligands may alter AHR:ARNT dimer binding, causing AHRE-independent control of the *CYP1A1* promoter [82]. Indeed, our finding that benzo[e]pyrene inhibits *CYP1A1* expression in fibroblast-like cells (Figure 3) contradicts reports of its lack of affinity towards the AHR and warrants further investigation. Thus, this cell line will be critically important in future studies that implicate novel small molecule compounds as AHR modulators.

iPSC technology continues to be an attractive avenue for basic science to achieve clinically relevant applications. Less than a decade after their inception, iPSCs are being used as a source of cellular therapeutics [82] and have undergone successful gene correction in lines created from primary cells of patient populations [83]. Now, there is the potential to turn iPSC-derived cultures into drug screening tools that can provide early indices of safety and efficacy before patient populations are exposed [84–87]. Given the widely reported role of AHR signaling in hepatotoxicity [88] and carcinogenesis [89], our reporter iPSC line is an optimal tool to reveal potential toxicity of compounds of interest in preclinical phases of development.

As the full extent of AHR pathway dynamics is discovered and the mechanisms of endogenous ligand regulation dominate the literature, it will become paramount to map AHR activation throughout all phases of development. Induced pluripotent stem cells provide an invaluable tool by which to derive distinct cellular subtypes of all three germ layers, and the *CYP1A1* reporter line presented in this work can provide an output of AHR activity that can be observed in every experimental context. Differentiation strategies that mimic *in vivo* ontogeny have the potential to serve as "temporal maps" of AHR activity throughout cytokine driven progression of cells to a distinct lineage. To this end, cellular fate decisions of progenitor populations can be correlated to AHR expression, and terminally differentiated cells can be assayed for AHR activity relative to their specification and function. Multiple novel roles of AHR signaling as well as the identity and dynamics of endogenous ligands have yet to be discovered; the reporter iPSC line described in this work will be invaluable to these studies moving forward.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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