Constitutive Activation of the Human Aryl Hydrocarbon Receptor in Mice Promotes Hepatocarcinogenesis Independent of Its Coactivator Gadd45b

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ABSTRACT

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), or dioxin, is a potent liver cancer promoter through its sustained activation of the aryl hydrocarbon receptor (Ahr) in rodents. However, the carcinogenic effect of TCDD and AHR in humans has been controversial. It has been suggested that the inter-species difference in the carcinogenic activity of AHR is largely due to different ligand affinity in that TCDD has a 10-fold lower affinity for the human AHR compared with the mouse Ahr. It remains unclear whether the activation of human AHR is sufficient to promote hepatocellular carcinogenesis. The goal of this study is to clarify whether activation of human AHR can promote hepatocarcinogenesis. Here we reported the oncogenic activity of human AHR in promoting hepatocellular carcinogenesis. Constitutive activation of the human AHR in transgenic mice was as efficient as its mouse counterpart in promoting diethylnitrosamine (DEN)-initiated hepatocellular carcinogenesis. The growth arrest and DNA damage-inducible gene 45b (Gadd45b), a signaling molecule inducible by external stress and UV irradiation, is highly induced upon AHR activation. Further analysis revealed that Gadd45b is a novel AHR target gene and a transcriptional coactivator of AHR. Interestingly, ablation of Gadd45b in mice did not abolish the tumor promoting effects of the human AHR. Collectively, our findings suggested that constitutive activation of human AHR was sufficient to promote hepatocarcinogenesis.

Key words: aryl hydrocarbon receptor; hepatocarcinogenesis; humanized mice; growth arrest and DNA damage-inducible gene 45b; transcriptional regulation.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is considered as both a complete epigenetic carcinogen and a potent tumor promoter (Murray et al., 2014). Unlike a genotoxic carcinogen, TCDD has been shown to be particularly effective during the promotional stage of carcinogenesis in the two-stage initiation-promotion model, in which a genotoxic carcinogen diethylnitrosamine (DEN) was applied to initiate genetic alterations, followed by TCDD exposure (Safe et al., 2013). Using mouse models that express various Ahr alleles, the mouse Ahr has been shown to play a key role in TCDD-induced promotion of liver tumors. For example, mice that express the Ahrb allele with a low binding affinity for TCDD showed a dramatically decreased liver tumor
formation compared with mice that bear the Ahrb allele with a high binding affinity when exposed to both the initiation event (DEN) and the promotion event (TCDD) (Kennedy et al., 2014). The tumor promoting activity of Ahr in the liver was independently verified in transgenic mice that express the constitutively activated Ahr (CA-Ahr). The liver tumor prevalence and multiplicity in the CA-Ahr transgenic mice were much higher than that in wild-type mice upon the DEN exposure (Moennikes et al., 2004).

Although the International Agency for Research on Cancer (IARC) has classified TCDD as a “Group 1 human carcinogen” (IARC, 1997), this definition has been controversial for several decades due to insufficient and inconsistent evidence of its carcinogenicity in humans (Cole et al., 2003). In several follow-up epidemiologic studies on TCDD-exposed populations, the association between the increase of total cancer incidence and TCDD exposure was concluded to be weak (Cole et al., 2003). The major inter-species differences in chemical recognition and transcriptional regulation between the human and rodent AhRs also limit the extrapolation of the hepatocarcinogenic effect of Ahr from rodents to humans.

The species differences in the ligand-dependent activation of AHR has been known for decades, which is attributed to the sequence divergence in their ligand-binding domains. The human AHR has a 10-fold lower affinity for TCDD compared with the rodent high-affinity Ahb allele, which results in a much lower toxicity in humans associated with TCDD exposure (Harper et al., 1988). Sequence analysis revealed a critical alteration in the ligand-binding domain that is responsible for the variation in TCDD response between the human and mouse AhRs (Ema et al., 1994). Moreover, there is only a 58% similarity in the AHR C-terminal transactivation domain between human and mouse receptors, suggesting that AHR may regulate a distinct subset of the hepatocarcinogenic effect of Ahr from rodents to humans.

The major inter-species differences in chemical recognition and transcriptional regulation between the human and rodent AhRs also limit the extrapolation of the hepatocarcinogenic effect of Ahr from rodents to humans. Moreover, there is only a 58% similarity in the AHR C-terminal transactivation domain between human and mouse receptors, suggesting that AHR may regulate a distinct subset of the hepatocarcinogenic effect of Ahr from rodents to humans.

DNA microarray analysis on TCDD-treated hepatocytes from WT C57BL6/J mice and humanized AHR C57BL6/J mice showed dramatic species differences in AHR-regulated gene induction and repression (Flaveny et al., 2010). The structural and functional differences between the human and mouse AhRs underscore the importance of using humanized AHR transgenic mice to address the species specificity of AHR responses in vivo.

The growth arrest and DNA damage-inducible gene 45 (Gadd45b) belongs to the small-molecule (~18 kDa) Gadd45 family of inducible proteins that play important roles in diverse biological processes including stress response, survival, senescence, and apoptosis (Sheikh et al., 2000). More recently, Gadd45b was reported to be a constitutive androgen receptor (CAR)-responsive gene and a CAR coactivator (Tian et al., 2011). CAR is a xenobiotic nuclear receptor and a nongenotoxic tumor promoter that mediates the hepatocarcinogenic effect of pheno-barbital in mice (Huang et al., 2005; Yamamoto et al., 2004). AHR is also a xenobiotic receptor. It is unclear whether Gadd45b is an AHR responsive gene and if so, whether Gadd45b coactivates AHR and plays a role in the hepatocarcinogenic effect of AHR.

In this study, we demonstrated that constitutive activation of human AHR in transgenic mice was as efficient as mouse Ahr in promoting DEN-initiated hepatocarcinogenesis. Gadd45b was identified as a transcriptional target of AHR, as well as an AHR coactivator that potentiates the transcriptional activity of AHR.

MATERIALS AND METHODS

Mice

The generation of CA-AHR and CA-Ahr transgenic mice has been described in our previous reports (Lee et al., 2010; Lu et al., 2015). Gadd45b−/− mice (Lu et al., 2004) in the C57BL/6J background (stock number: 013101) from the Jackson Laboratory (Bar Harbor, Maine) were self-crossed to generate the homozygous Gadd45b−/− mice. Genotyping for the Gadd45b null allele was performed by PCR using primers (10936: GCAAC CCCCAGTACCTTGGAA; 10937: CCTGCGAGAGAAGAGGTG; 01M7996; CTTCCATTGTACGCTCCG) provided by the Jackson Laboratory. CA-AHR transgenic mice were then crossed with Gadd45b−/− mice to generate the CA-AHR-Gadd45b−/− mice in the C57BL/6J background. Ahr knockout (Ahr−/−) mice in the C57BL/6J background were purchased from Taconic (Hudson, New York). WT CD-1 male mice were purchased from Charles River Laboratories.

Study approval

The Central Animal Facility of the University of Pittsburgh is fully accredited by AALAC. All procedures were performed in accordance with relevant federal guidelines and with the approval of the University of Pittsburgh IACUC committee.

Animal Treatment

Six-week-old male and female WT, CA-AHR, CA-Ahr, Gadd45b−/−, and CA-AHR-Gadd45b−/− mice (n = 6–11 for each group) were injected intraperitoneally with a single dose of DEN (90 mg/kg of body weight) dissolved in saline and sacrificed at 9 months after DEN injection. An equal volume of saline was injected to the vehicle control mice. For gene regulation analysis, 10-week-old male C57BL/6J WT, Ahr+/−, and Gadd45b−/− mice were daily gavaged with TCDD (10 μg/kg body weight) or the vehicle control corn oil for 4 days before sacrifice. Hydrodynamic liver transfections were performed as described previously (Liu et al., 1999). Briefly, 10-week-old male CD-1 WT mice were treated with a single gavage of TCDD (10 μg/kg body weight) or corn oil 7 days before the hydrodynamic transfection of the pCMX-Flag-Gadd45b plasmid or the pCMX-Flag empty plasmid into the mouse livers. CD-1 mice were used for hydrodynamic transfections as described (Liu et al., 1999), because their tail veins were easy to be isolated for the injections.

Histology and Immunohistochemistry

For H&E staining, the tumor-bearing liver tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. To detect the Ki67 and Gadd45b proteins by immunohistochemistry, consecutive sections were performed as described (Liu et al., 1999) in the C57BL/6J background (stock number: 013101). The major inter-species differences in the ligand-dependent activation of AHR between the human and rodent AhRs limit the extrapolation of the hepatocarcinogenic effect of Ahr from rodents to humans.
Serum Biochemistry Analysis

Serum ALT and AST levels were measured by using commercial assay kits from Stanbio Laboratory (Boerne, Texas).

Co-Immunoprecipitation and Western Blot Analysis

To study the interaction between AHR and Gadd45b proteins, 293T cells in 6-well plates were cotransfected with pCMX-HA-AHR and pCMX-Flag-Gadd45b plasmids, and treated with 3-MC (2 µM) for 24 h. Cells were lysed in the NP-40 lysis buffer supplemented with protease inhibitors (1:200) and incubated with the anti-HA antibody at 4°C for overnight, followed by incubation with protein G agarose beads at 4°C for 1 h. Cells were then washed with the NP-40 lysis buffer and subjected to Western blot analysis. Protein samples were resolved by SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane, and blotted with antibodies. The primary antibodies used include those against HA-tag (cat no. 26183, Pierce), Flag-tag (cat no. F7425, Sigma), VP16 (cat no. sc-7545, Santa Cruz), and Gal4-DBD (cat no. sc-510, Santa Cruz).

Electrophoretic Mobility Shift Assay, Chromatin Immunoprecipitation Assay, Transient Transfection, and Luciferase Reporter Assay

Electrophoretic mobility shift assay (EMSA) was performed using 32P-labeled oligonucleotides and receptor proteins prepared by the TNT in vitro transcription and translation method (Zhou et al., 2006). Chromatin immunoprecipitation (ChIP) assay for the Gadd45b promoter was performed in WT CD-1 mice (n = 4 for each group) whose livers were hydrodynamically transfected with the pCMX-Flag-CA-AHR plasmid or the pCMX-Flag empty vector (Zhou et al., 2006). Cells or liver lysates were immunoprecipitated with the anti-Flag or anti-lgG control antibody (Sigma). The recovered DNA was analyzed for the recruitment of AHR to the mouse Gadd45b gene promoter (nt -1286 to -1277) by real-time PCR. For luciferase reporter assay, the promoter regions of the mouse Gadd45b gene promoter (nt -1286 to -1277) were PCR-amplified and cloned upstream of the luciferase gene. CV1 cells were transfected in triplicate with the reporter construct together with the AHR or CA-AHR expression vector in 48-well plates. For the coactivation analysis, Huh7 cells in 48-well plates were cotransfected with pCMX-AHR (50 ng/well) constructs, together with the AHR-responsive pGud-Luc reporter gene. When necessary, cells were treated with 3-MC (2 µM) for 24 h before luciferase assay. The transfection efficiency was normalized against the β-galactosidase activities from a cotransfected CMX-β-galactosidase vector.

Mammalian Two-Hybrid Analysis

To assess the AHR-Gadd45b interaction in vivo, fusion constructs containing the Gal4 DNA-binding domain (DBD) upstream of full-length or deletion mutants of Gadd45b (amino acids 1–160, 1–125, 1–92, 93–160, and 126–160) (Tian et al., 2011), and the fusion vector containing the herpes simplex virus VP16 activation domain downstream of the full-length Ahr were cotransfected into 293T cells in 48-well plates, along with a thymidine kinase luciferase reporter containing the Gal4-binding site upstream activating sequence (UAS) tk-UAS. The pCMX-Gal4 and pCMX-VP empty plasmids were used as controls. The luciferase reporter activity of tk-UAS was normalized against the β-galactosidase activities from a cotransfected CMX-β-galactosidase vector.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol and subjected to reverse transcription with random hexamer primers and Superscript RT III enzyme (Invitrogen). SYBR Green-based qRT-PCR was performed with the ABI7500 System. Data were normalized against the cyclophilin control.

Statistical Analysis

Statistical significance between the means of two groups was analyzed using an unpaired Student’s t test, and analysis of variance (ANOVA) for the comparison among the means of 3 or more groups. Differences were considered statistically significant at p < .05.

RESULTS

Transgenic Activation of Human AHR Promotes DEN-Initiated Hepatocarcinogenesis

The role of human AHR activation in hepatocarcinogenesis has been controversial. To determine whether activation of human AHR is sufficient to promote liver cancer, we subjected the liver-specific CA-AHR transgenic mice to DEN-initiated liver tumor development. As outlined in Figure 1A, transgenic mice overexpressing the constitutively activated human AHR (CA-AHR) in the liver were generated by crossbreeding the FABP-tTA transgenic mice and the TetRE-CA-AHR mice as we have described previously (Lu et al., 2015). The FABP-tTA transgene expresses the tetracycline transcriptional activator (tTA) under the control of the liver-specific fatty acid-binding protein (FABP) gene promoter, whereas the TetRE-CA-AHR transgene expresses CA-AHR under the control of the tetracycline response element (TetRE). CA-AHR was constructed by deleting the minimal ligand-binding domain of AHR (Lu et al., 2015). The liver-specific expression of CA-AHR was confirmed at both mRNA and protein levels, without affecting the expression of endogenous AHR as we have reported previously (Lee et al., 2010; Lu et al., 2015). The tumor-promoting effect of the CA-AHR transgene was compared with that of the CA-Ahr transgene. The CA-Ahr transgenic mice were produced with the same strategy, except that the mouse Ahr cDNA was used in constructing the transgene (Lee et al., 2010).

WT, CA-AHR, and CA-Ahr mice were subjected to DEN injection, and the mice were sacrificed 9 months after. At the end of the experiment, the liver to body weight ratios in male CA-AHR and CA-Ahr mice were 5.3 ± 1.13 and 6.6 ± 3.03, respectively, significantly higher when compared with 4.1 ± 0.66 in male WT mice (Table 1). No liver tumors were found in male WT mice and their livers appeared to be normal (Figure 1B). The CA-Ahr transgenic male mice exhibited a tumor incidence of 89% with their tumor multiplicity and nodule size summarized in Table 1, and these results were consistent with a previous report in which the CA-Ahr transgene was under the control of the mouse immunoglobulin heavy chain gene promoter (Meenikes et al., 2004). Interestingly and surprisingly, we found the CA-AHR transgenic mice also exhibited heightened sensitivity, characterized by 100% of tumor incidence and enhanced nodule multiplicity and size (Table 1). Gross appearance showed one or multiple tumors on the surface of the CA-AHR mouse livers, with the largest nodule more than 10 mm in diameter.
These results suggested that genetic activation of human AHR was as efficient as mouse Ahr in promoting DEN-initiated liver tumor formation in the male mice. Females are known to have a lower risk for liver cancer, a notion that is also supported by animal studies using the DEN-induced mouse liver tumor model (Naugler et al., 2007). Indeed, our female transgenic mice showed lower tumor incidence (33% in the CA-AHR group and 37% in the CA-Ahr group, respectively) and multiplicity compared with their male counterparts. The liver weight to body weight ratio in female CA-AHR and CA-Ahr mice were $5.2 \pm 0.70$ and $5.0 \pm 0.73$, respectively, still significantly higher compared with $4.1 \pm 0.24$ in WT mice. In the following experiments, we mainly focused on the liver-promoting effect of human AHR in male mice.

The tumor promotion by AHR was further analyzed under the microscope by H&E staining. At the histological level, the liver architecture became trabecular and solid in both the CA-AHR and CA-Ahr mice, whereas the WT mice displayed a normal liver structure (Figure 1C, top). Within the nodule area, hepatocytes displayed obvious degeneration and atypia, including enlarged and hyperchromatic nuclei, prominent nucleoli, and mitosis (Figure 1C, bottom), which are characteristic for hepatocellular carcinogenesis. The protein expression of Ki67 is strictly associated with cell proliferation, and the fraction of Ki67-positive tumor cells (the Ki67-labeling index) is often correlated with the clinical score of carcinoma (Scholzen and Gerdes, 2000). Therefore, we detected the Ki67 protein expression in the tumor regions by immunohistochemistry (Figure 1D). A
Table 1. Liver Tumors in DEN-Treated Male and Female WT, CA-Ahr, and CA-AHR Mice

| Gender | Genotype | LW/BW (%) | Number | Tumor Incidence (%) | Multiplicity 1-3 mm | 3-7 mm | >8 mm |
|--------|----------|-----------|--------|---------------------|---------------------|--------|-------|
| Male   | WT       | 4.1 ± 0.66 | 11     | 0 (0%)              | 0 (0)              | 0 (0)  | 0 (0) |
|        | CA-AHR   | 5.3 ± 1.33* | 10     | 10 (100%)           | 3.2 (32)           | 2.9 (29)| 0.3 (3) |
|        | CA-Ahr   | 6.6 ± 3.03* | 9      | 8 (89%)             | 6.1 (55)           | 2 (18) | 0.7 (6) |
| Female | WT       | 4.1 ± 0.24 | 11     | 0 (0%)              | 0 (0)              | 0 (0)  | 0 (0) |
|        | CA-AHR   | 5.2 ± 0.70* | 10     | 3 (33%)             | 0.4 (4)            | 0.3 (3) | 0.1 (1) |
|        | CA-Ahr   | 5.0 ± 0.73* | 8      | 3 (37%)             | 2 (16)             | 0.4 (3) | 0.2 (1) |

Livers were harvested 9 months after the DEN treatment, and tumors on the liver surface were counted. Results are presented as means ± standard deviation (SD). The number of mice in each group are shown in the table. The tumor incidence (%) represents the ratio of the number of mice with tumors to the total number of mice in each group. The tumor multiplicity was calculated from the total tumor countings (denoted in the brackets) with varying tumor sizes divided by the total mouse numbers in each group. n = 8–11 for each group.

*p < .05; **p < .01. CA-Ahr versus WT or CA-AHR versus WT.

Activation of Human AHR Increases Inflammation and Impairs Liver Function Upon the DEN Treatment

A connection between chronic inflammation and hepatocellular carcinogenesis has long been proposed. Inflammatory cells and cytokines generated in the tumor microenvironment are a major contributing factor to tumor growth, progression, and immunosuppression, (Balkwill and Mantovani, 2001). Like many other tumor promoters and nongenotoxic carcinogens, inflammatory cytokines play an important role in TCDD-mediated liver tumor promotion (Kennedy et al., 2014). To determine whether activation of human AHR sensitized the DEN-treated mice to inflammatory responses (Naugler et al., 2007), we measured the hepatic mRNA expressions of Il-6 and Tnf-a. The expression of Tnf-a, but not Il-6, was significantly elevated in the livers of DEN-treated CA-AHR mice at the end of the 9-month treatment (Figure 2A). Common liver functional tests, including the measurements of serum ALT and AST levels, can be used to predict hepatocellular carcinogenesis risk in the general population (Figure 2B). Serum levels of ALT and AST were significantly increased in DEN-treated CA-AHR and CA-AHR transgenic mice at the end of the 9-month treatment (Figure 2B), indicating an increased sensitization to impaired liver function by AHR activation upon the DEN treatment.

Activation of Human AHR Increases Inflammation and Impairs Liver Function Upon the DEN Treatment

AHR Activation Induces the Expression of Growth Arrest and DNA-Damage-Inducible 45 β

Comparisons of gene expression profiles have provided important insights in identifying genes that are associated with clinicopathologic features of hepatocellular carcinogenesis. In particular, dysregulation of cell proliferation is a fundamental aspect of cancer, and this can be caused by alterations in the expression of cell cycle-related genes, such as Cyclin D, Cyclin E, and Cyclin-dependent kinase (Cdk) families including Cdk1, Cdk2, Cdk4, and Cdk6 (Deshpande et al., 2005). Among these cell cycle-related genes, the expression of Cyclin D1 and Cdk1 was upregulated by AHR activation in DEN-treated mice (Figure 3A).

The expression pattern of cell cycle-related genes was consistent with the increased hepatocarcinogenesis in the CA-AHR transgenic mice.

Of note, we found the expression of Gadd45b, a signal molecule inducible by external stress and UV irradiation, was highly induced in DEN-treated CA-AHR and CA-Ahr transgenic mice (Figure 3A). Gadd45b has been reported to promote liver regeneration after partial hepatectomy (Papa et al., 2008), and dysregulation of Gadd45b was observed in several types of solid tumors (Qiu et al., 2004; Wang et al., 2012). To examine the expression and subcellular localization of the Gadd45b protein in CA-AHR mouse livers that bear tumors, we collected their liver tissues from tumors and tumor-surrounding areas and measured their mRNA and protein levels. We found a significantly increased hepatic mRNA expression of Gadd45b in both the tumor and nontumor tissues from CA-AHR mice compared with
the WT mice (Figure 3A). The induction of Gadd45b at the protein level was confirmed by immunohistochemistry, with the expression of Gadd45b protein detected in both tumors and the tumor-surrounding areas (Figure 3B). Gadd45 has several isoforms, but the induction was Gadd45b specific, because the expression of Gadd45a and Gadd45g was not induced by TCDD or the CA-AHR transgene (Supplementary Figure 2).

**Gadd45b Is an AHR Target Gene**

To better understand the regulation of Gadd45b by AHR, we profiled the expression of cell cycle-related genes and Gadd45b in unchallenged CA-AHR transgenic mice, in order to determine whether the regulation is secondary to the DEN treatment and subsequent hepatocarcinogenesis. The mRNA expression of Gadd45b in 6-week-old naïve CA-AHR mice showed a dramatic increase, whereas the expression of Cyclin D1 and Cdk1 in the same mice was unchanged (Figure 4A). These results suggested that the induction of Cyclin D1 and Cdk1 might be secondary to the tumor formation, whereas the Gadd45b upregulation is likely an AHR-dependent effect. Indeed, the induction of Gadd45b was also observed in WT mice acutely treated with TCDD, but this induction was abolished in Ahr−/− mice (Figure 4B). Interestingly, the basal mRNA expression of Gadd45b in the liver of Ahr−/− mice was increased compared with the WT mice (Figure 4B). Although the mechanism for this increased basal expression of Gadd45b remains to be understood, this result was reminiscent of the induction of the PXR target gene Cyp3a11 in a PXR knockout mouse line (Staudinger et al., 2001). The expression of GADD45b was also induced in human primary hepatocytes treated with the human AHR ligand 3-MC (Figure 4C), suggesting the induction was conserved in human liver cells. These results, together with the Gadd45b induction in CA-AHR mice, strongly suggested Gadd45b as an AHR target gene.

To directly test whether Gadd45b is an AHR target gene, we cloned the mouse Gadd45b gene promoter and evaluated its regulation by AHR. Inspection of the Gadd45b gene promoter revealed 3 putative dioxin response elements (DREs) (Figure 4D). EMSA showed that only the DRE1 was bound by the AHR-ARNT heterodimer or the CA-AHR-ARNT heterodimer (Figure 4E). To confirm the recruitment of CA-AHR onto the Gadd45b gene promoter, we performed ChIP assay on cells transfected with Flag-CA-AHR. As shown in Figure 4F, CA-AHR was significantly recruited to the DRE1-flanking region on the Gadd45b gene.

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**Figure 3.** AHR activation induces the expression of Gadd45b. Mice were i.p. injected with a single dose of 90 mg/kg DEN at 6-week old and sacrificed after 9 months. Tumor bearing liver tissues and the surrounding nontumor tissues were collected. A, Hepatic mRNA expressions of Gadd45b, c-Myc, and cell cycle-related genes in liver tissues from tumor-surrounding/nontumor (N) and tumor (T) areas in CA-Ahr mice, CA-AHR mice, and WT mice were measured by real-time PCR. B, The protein expression of Gadd45b in liver tissues from tumor-surrounding/nontumor (N) and tumor (T) areas in CA-AHR mice and WT mice was analyzed by immunohistochemistry. Arrows in (B) indicate positive staining. n = 5–7 for each group. *p < .05; **p < .01, all compared with WT. Results are presented as means ± standard deviation (SD).
promoter. The transactivation of the Gadd45b gene promoter by AHR was evaluated by luciferase reporter assays. The DRE1-containing 1.33-kb Gadd45b gene promoter was transactivated by AHR in the presence of the AHR agonist 3-MC, whereas this activation was abolished when the region containing DRE1 was deleted (Figure 4G).

Gadd45b Functions as an AHR Coactivator

The Gadd45b protein contains two LXXLL (where L is leucine and X is any amino acid) signature motifs that are often seen in nuclear receptor coactivators (Heery et al., 1997). Indeed, Gadd45b has been reported to directly bind to the xenobiotic nuclear receptor CAR and facilitate its transcription activity.
As a member of the bHLH/PAS family of transcription factor, AHR has also been reported to recruit several coactivators, such as SRC-1 and p300/CBP, and to enhance its own transcriptional activity (Hankinson, 2005). To determine whether Gadd45b can coactivate AHR, we cotransfected Gadd45b and AHR expression plasmids with the AHR reporter construct pGud-Luc in human hepatoma Huh7 cells. Gadd45b dose-dependently induced the pGud-Luc reporter activity with or without the 3MC treatment, and this coactivation effect was even more pronounced in cells that were cotransfected with the AHR expression vector (Figure 5A). In vivo, hydrodynamic overexpression of Gadd45b in the mouse liver induced the basal expressions of Cyp1a1 and Nrf2, two typical Ahr target genes (Figure 5B). Transfection of Gadd45b also enhanced TCDD-responsive induction of the Ahr target genes Cyp1a2, Cyp1b1, Tiparp, and Nrf2 (Figure 5B). To determine whether Gadd45b is required for Ahr activity, we treated WT and Gadd45b−/− mice with TCDD. The expression of Ahr target genes Cyp1a2, Cyp1b1, and Tiparp remained efficiently induced by TCDD in Gadd45b−/− mice (Figure 5C), suggesting that Gadd45b is not required for ligand-dependent activation of Ahr. These observations suggested that Gadd45b is sufficient but not necessary in facilitating AHR-mediated transcriptional activity.

Coactivators often function by interacting with their target receptors. We first used co-immunoprecipitation analysis to
examine the binding between the HA-tagged AHR and Flag-tagged Gadd45b and the enhancement of the interaction by the treatment of 3-MC (Figure 5D). We then used mammalian 2-hybrid assay to further demonstrate the AHR-Gadd45b interaction and to map the Gadd45b domain that interacts with AHR. In this experiment, Gadd45b was fused to the Gal4-DBD construct (Gal4-Gadd45b) and Ahr was fused to the VP16 activation domain of herpes simplex virus (Ahr-VP). The fusion constructs were transfected with the Gal4 reporter gene thymidine kinase upstream activation sequence (tk-UAS) in 293T cells. As shown in Figure 5E, co-transfection of Ahr-VP and Gal4-Gadd45b full-length (FL) significantly activated the UAS-mediated transcription, whereas deletion of amino acids 93–160 of Gadd45b (Gal4-Gadd45b 1–92) abolished this activity. These results suggested that amino acids 93–160 of Gadd45b are required for the AHR-Gadd45b interaction.

Gadd45b Is Not Required for AHR-Promoted Hepatocellular Carcinogenesis

To determine whether Gadd45b is required for the tumor-promoting effect of AHR, we crossbred the CA-AHR mice with Gadd45b−/− mice to generate AHR transgenic mice deficient of Gadd45b that were termed CA-AHR-Gadd45b−/− mice. Both the male and female CA-AHR-Gadd45b−/− mice were treated with DEN and sacrificed after 9 months. The tumor incidence in male CA-AHR-Gadd45b−/− mice was 100% (Table 2 and Figure 6A). The liver to body weight ratio in male CA-AHR-Gadd45b−/− mice was not different from that of male CA-AHR mice (Table 2). Although the average number of small nodules (<3 mm) appeared to be higher in CA-AHR-Gadd45b−/− mice, these mice developed fewer large-sized nodules (>3 mm) (Table 2). Similar to that in male CA-AHR mice, the tumors found in the male CA-AHR-Gadd45b−/− mice displayed mitotic activity, apoptosis, and trabecular growth pattern (Figure 6B).

Immunohistochemistry analysis revealed that the tumor cells in male CA-AHR-Gadd45b−/− mice were positive for proliferation markers Ki67 and PCNA with the Ki67 labeling index (12.2%) similar to that of the CA-AHR transgenic mice (13.1%) (Figure 6C). The Ki67 mRNA expression was not different between male CA-AHR and CA-AHR-Gadd45b−/− mice (data not shown). Analysis of mRNA expression of Tnf-α and Il-6 revealed little difference between male CA-AHR and CA-AHR-Gadd45b−/− mice (Figure 6D). There were no significant differences in the serum ALT and AST levels between male CA-AHR and CA-AHR-Gadd45b−/− mice (Figure 6E). These results suggested that Gadd45b is not required for the liver tumor-promoting effect of AHR in males.

The female CA-AHR-Gadd45b−/− mice showed lower tumor incidence and multiplicity than their male counterparts (Table 2). The presence of proliferating tumors in both the male and female CA-AHR-Gadd45b−/− mice was confirmed by H&E staining (Supplementary Figure 3A) and Ki67 immunostaining (Supplementary Figure 3B). Although the tumor incidence and the average number of small nodules (<3 mm) appeared to be higher in female CA-AHR-Gadd45b−/− mice compared with female CA-AHR mice, no large-sized nodules (>3 mm) were observed in female CA-AHR-Gadd45b−/− mice (Table 2). Interestingly, male Gadd45b−/− mice also developed hepatocellular carcinoma, although the tumor incidence was lower than male CA-AHR mice (Supplementary Table 1). The tumor development in Gadd45b−/− mice was confirmed by histological analysis (Supplementary Figure 4). The tumor incidence in Gadd45b−/− mice was in agreement with our previous report that the Gadd45b−/− mice showed an increased proliferative response compared with WT mice (Tian et al., 2011).

DISCUSSION

The extrapolation of the carcinogenic effect of AhR from animal studies to humans has been challenging and is confounded by several factors. First, the primary structure of the AhR protein shares limited similarity between human and mouse, conferring distinct ligand-binding affinity and varied transcriptional activity. Second, humans are relatively resistant to the toxic effects of a class of dioxin chemicals represented by TCDD. Additionally, the epidemiological evidence supporting the carcinogenic effect of TCDD in human populations has been considered inadequate and limited (Cole et al., 2003).

The goal of this study is to clarify whether activation of human AHR can promote hepatocarcinogenesis. For this purpose, we utilized transgenic mice expressing the constitutively activated AHR whose activation bypassed the requirement of an AHR agonist. This strategy allowed us to focus on the effect of the transcriptional outcome of AHR activation, rather than the species difference in the ligand efficiency, on the liver tumor-promoting effect of AHR. The same strategy has been used to study the carcinogenic activity of Ahr (Moennikes et al., 2004), as well as the role of Ahr and AHR in hepatic steatosis (Lee et al., 2010), steatohepatitis (He et al., 2013), and high-fat diet-induced metabolic liver disease and insulin resistance (Lu et al., 2015). Interestingly and surprisingly, CA-AHR transgenic mice developed DEN-initiated liver tumors as efficiently as the CA-AHR transgenic mice that express the constitutively activated mouse Ahr. These results seemed contradictory to the previous report that replacement of the mouse Ahr gene with human AHR cDNA conferred to mice a decreased sensitivity to TCDD-induced toxic effects such as fetal teratogenesis (Moriguchi et al., 2003). The comparative studies using the AHR knock-in humanized mice might be largely due to the distinct affinities of mouse and human receptors to TCDD. Our model excluded the potential confounder of inadequate activation of human AHR by TCDD. Limited protein expression of the human AHR protein in the AHR knock-in humanized mice was also believed to have contributed to their attenuated response to TCDD (Moriguchi et al., 2003). One limitation of the humanized CA-AHR transgenic mouse model is that the human receptor may recruit coactivators and corepressors differently in the human hepatocytes compared with the mouse hepatocytes. The lack of ligand-binding domain in the CA-AHR construct may also lead to differential coregulator recruitment compared with the wild-type receptor. Although the hepatic expression of the CA-AHR transgene might be different from that of the endogenous Ahr gene (Lu et al., 2015), the expression levels of AHR target genes, including Cyp1a1, Cyp1a2, and Cyp1b1, in the CA-AHR transgenic mice were similar to those observed in the TCDD-treated WT mice (Supplementary Figure 5). These results suggested that the transcriptional activity of AHR in our genetic model is comparable to that in the pharmacological model, which supports the relevance of our transgenic model to the chronic exposure to dioxin compounds.

The identification of Gadd45b as a novel AHR target gene is intriguing. A link between cell apoptosis and cancer has long been proposed. Whether a hepatocyte proliferates or dies in response to a genotoxic stress such as DEN will dictate the cell fate during carcinogenesis. Gadd45b is an anti-apoptotic factor that belongs to the Gadd45 family of inducible proteins that play important roles in diverse biological processes including...
stress response, survival, senescence, and apoptosis (Sheikh et al., 2000). A possible role of Gadd45b in hepatocellular carcinogenesis and hepatocyte proliferation has been suggested by our establishment of Gadd45b as a CAR-responsive gene and a CAR coactivator (Tian et al., 2011). CAR, a xenobiotic nuclear receptor, is also a nongenotoxic tumor promoter that mediates the tumor promoting effect of phenobarbital or its derivative TCPOBOP in hepatocellular carcinogenesis in mice. Another xenobiotic receptor pregnane X receptor (PXR) was also reported to stimulate the expression of Gadd45b expression and then interact with the protein after it is expressed (Kodama and Negishi, 2011).

We found that Gadd45b was highly induced in DEN-challenged CA-AHR mice and established that Gadd45b is a novel AHR target gene. Moreover, we have provided both in vitro and in vivo evidence that Gadd45b can function as a coactivator of AHR. These results led to our hypothesis that the expression of Gadd45b might have been a driver for the tumor-promoting activity of AHR, because the effect of AHR on the

Figure 6. Gadd45b is not required for AHR-promoted hepatocellular carcinogenesis. A, Representative gross appearance of livers of male and female CA-AHR-Gadd45b<sup>−/−</sup> mice 9 months after the DEN injection. B, H&E staining of liver sections of DEN-treated male CA-AHR-Gadd45b<sup>−/−</sup> mice. Dashed line denotes the nodule area (top). Dotted line denotes abnormal liver cell plates that are 3+ cells thick (middle). Arrowheads indicate apoptotic cells (bottom). C, Immunohistochemical staining of Ki67 and PCNA in DEN-treated male CA-AHR and CA-AHR-Gadd45b<sup>−/−</sup> mice. Arrows indicate positive staining. Magnification, 400×. D, The hepatic mRNA expression of Il-6 and Tnf-a from tumor-surrounding (N) and tumor (T) areas in male CA-AHR mice and CA-AHR-Gadd45b<sup>−/−</sup> mice. n = 8–15 for each group. E, The serum levels of ALT and AST in male CA-AHR mice and CA-AHR-Gadd45b<sup>−/−</sup> mice. n = 8–15 for each group. Results are presented as means ± standard deviation (SD).
expression of oncogene and cell cycle-related genes was secondary to the tumor development. However, ablation of Gadd45b in CA-AHR mice did not affect the tumor incidence, although the average number of nodules greater than 3 mm was lower in male and female CA-AHR-Gadd45b−/− mice. These results suggested that Gadd45b is not indispensable for the tumor-promoting effects of AHR, but the loss of Gadd45b may have slowed down the tumor growth in vivo. It is also possible that the loss of Gadd45b is compensated by other coactivators of AHR. Nevertheless, the mechanism by which AHR promoter hepatocarcinogenesis remains to be clearly defined. Interestingly, the in vivo requirement of Gadd45b in the tumor-promoting effect of CAR is also yet to be established.

In summary, we showed that genetic activation of human AHR in transgenic mice was efficient to promote DEN-initiated hepatocarcinogenesis. Cautions need to apply when extrapolating these results to human situations, because we cannot exclude the possibility that the species-specific cellular environment other than or on top of the species origin of the AhR receptor may also be important for the tumor-promoting effect of AHR in the liver.

**SUPPLEMENTARY DATA**

Supplementary data are available at Toxicological Sciences online.

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