Xeno-sensing activity of the aryl hydrocarbon receptor in human pluripotent stem cell-derived hepatocyte-like cells

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Although hepatocyte-like cells derived from human pluripotent stem cells (hPSC-HLCs) are considered a promising model for predicting hepatotoxicity, their application has been restricted because of the low activity of drug metabolizing enzymes (DMEs). Here we found that the low expression of xenobiotic receptors (constitutive androstane receptor, CAR; and pregnane X receptor, PXR) contributes to the low activity of DMEs in hPSC-HLCs. Most CAR- and PXR-regulated DMEs and transporters were transcriptionally down-regulated in hPSC-HLC. Transcriptional expression of CAR and PXR was highly repressed in hPSC-HLCs, whereas mRNA levels of aryl hydrocarbon receptor (AHR) were comparable to those of adult liver. Furthermore, ligand-induced transcriptional activation was observed only at AHR in hPSC-HLCs. Bisulfite sequencing analysis demonstrated that promoter hypermethylation of CAR and PXR was associated with diminished transcriptional activity in hPSC-HLCs. Treatment with AHR-selective ligands increased the transcription of AHR-dependent target genes by direct AHR-DNA binding at the xenobiotic response element. In addition, an antagonist of AHR significantly inhibited AHR-dependent target gene expression. Thus, AHR may function intrinsically as a xenosensor as well as a ligand-dependent transcription factor in hPSC-HLCs. Our results indicate that hPSC-HLCs can be used to screen toxic substances related to AHR signaling and to identify potential AHR-targeted therapeutics.

The need for more predictive in vitro models of the human liver has been highlighted in toxicological studies. The typical hepatotoxicity assessments employing animal models do not adequately provide reliable data on the potential toxic effects of drugs and chemicals on humans as a result of interspecies differences in drug metabolism (or xenobiotic metabolism) and hepatotoxic responses1,2. While in vitro test systems employing human cellular models may overcome the problem of interspecies differences, the use of in vitro human cell line models is still limited. Freshly isolated or cryopreserved primary human hepatocytes (PHHs) are considered the ideal model for in vitro applications. However, the scarcity of fresh resources and tremendous variability in overall cell quality across different cell lots and donors constitute the main limitations3–5.

Recent advances in stem cell technology offer the opportunity to overcome the lack of in vitro models. In vitro-differentiated hepatocytes from human pluripotent stem cells (hPSCs) have emerged as the most attractive alternative source for hepatotoxicity prediction6,7. Several recent investigations show that human embryonic stem cell (hESC)- and human induced pluripotent stem cell (hiPSC)-derived hepatocyte-like cells (HLCs) exhibit predictive sensitivity for distinguishing hepatotoxictants from nontoxic compounds and the functional capacity to generate the relevant bioactive metabolites from known toxic agents8–10. The characteristics of hiPSC-HLCs

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also enable the prediction of inter-individual differences in drug metabolism and susceptibility on the basis of single nucleotide polymorphisms of cytochrome P450 (CYP) genes. These findings of the predictive capacities of hPSC-HLCs wholly rely on the expression and functional activity of drug metabolizing enzymes (DMEs) and related drug transporters. To date, few studies have demonstrated that hPSC-HLCs are comparable to PHHs in terms of the expression and activity of DMEs and drug transporters. However, the precise mechanism underlying how DMEs are induced and constitutively expressed in in vitro-differentiated HLCs remains poorly understood.

In the liver, expression and transcriptional regulation of DMEs and transporters is controlled by epigenetic factors (e.g., DNA methylation, histone modification, and miRNA) as well as by liver-enriched ligand-activated transcription factors. A number of ligand-activated transcription factors, mostly nuclear receptors, exhibit miscellaneous xenobiotic binding capabilities and function as sensors of endogenous toxic metabolites and xenobiotics. These receptors can induce the majority of genes involved in xenobiotic metabolism and transport by recognition of various ligands and are therefore also termed xenobiotic receptors. The major xenobiotic receptors, including constitutive androstane receptor (CAR, NR1I3), pregnane X receptor (PXR, NR1I2), and aryl hydrocarbon receptor (AHR), regulate the induction of a broad spectrum of distinct and overlapping DMEs and transporters in the liver. In particular, the induction profiles of a set of DMEs and transporters are strongly associated with the activation of these xenobiotic receptors. Moreover, each receptor exhibits species-specific differences in its target gene induction responses as well as in binding capability (and/or affinity) to its ligands. As a result of these unique functions, various binding and activation assays employing CAR, PXR, and AHR are widely utilized as useful in vitro screening tools for predicting drug safety and xenobiotic-induced hepatotoxicity.

The purpose of this study was to determine the functional roles of CAR, PXR, and AHR as xenosensors and transcriptional regulators controlling xenobiotic metabolism in hPSC-HLCs. To this end, both hESCs and hiPSCs were differentiated into hepatocytes in which we assessed the mRNA expression and transcriptional activity of CAR, PXR, and AHR. We showed that a marked reduction in CAR and PXR transcript levels, as a result of promoter hypermethylation, led to diminished transcriptional activity in hPSC-HLCs. By contrast, the transcriptional activity of AHR was reproducible in hPSC-HLCs, which was tightly regulated by AHR-specific agonistic and antagonistic xenobiotics.

Results

In vitro differentiation of hESCs and hiPSC into HLCs. To investigate whether xenobiotic receptors are involved in the regulation of DME and transporters in hPSC-HLCs, we first differentiated hESCs and hiPSCs into HLCs in a stepwise manner. Then, hESC- and hiPSC-HLCs were phenotypically and functionally characterized. Transcriptional expression of hepatocyte marker genes including AAT, ALB, AFP, and HNF4A was prominently induced in hESC- and hiPSC-HLCs (Fig. 1a). Also, hESC- and hiPSC-HLCs were positive for AAT, ALB, AFP, and HNF4A (Fig. 1b,c). To determine hepatic differentiation efficiency, the proportion of ALB- and AAT-positive cells was measured by fluorescence-activated cell sorting (FACS). More than 96% of ALB-positive and at least 84% of AAT-positive cells were detected in both hPSC-HLC types (Fig. 1d,e). Furthermore, they exhibited various hepatic functions including glycogen synthesis, low density lipoprotein (LDL) uptake, and albumin secretion (Fig. 1f–j). Although minor differences were observed in marker gene expression and albumin secretion between hESC- and hiPSC-HLCs, the overall results indicate that both hESCs and hiPSCs were efficiently differentiated into hepatocyte-like cells.

Down-regulation of genes regulated by xenobiotic receptors in hPSC-HLCs. Next, we examined gene expression profiles for DMEs and transporters regulated by CAR, PXR, and AHR using microarray analysis. For the selection of target genes, we used Ingenuity pathway analysis to identify all genes directly and indirectly regulated by CAR, PXR, and AHR (Ingenuity® System, www.ingenuity.com). The majority of CAR and/or PXR target genes belonging to phase-I enzymes and transporters in hESC- and hiPSC-HLCs were expressed at levels of less than 10% of adult liver (Fig. 2a). Transcript levels of most phase-II enzymes in hPSC-HLCs were comparable to those in fetal or adult hepatocytes, except glutathione S-transferase pi 1 (GSTP1) gene. Prominent expression of GSTP1 and AFP, which are fetal hepatoblast markers, indicate that both HLCs differentiated from hESCs and hiPSCs still display fetal-like phenotypic characteristics (Figs 2a and 1a). The expression of CAR and PXR genes was also markedly reduced (< 20-fold) in both hESC- and hiPSC-HLCs (Fig. 2a,b). These data imply that transcript level of CAR and PXR genes is closely related to the expression of phase-I enzyme and transporter genes in hPSC-HLCs. Unlike CAR and PXR mRNA levels, AHR mRNA levels in hESC- and hiPSC-HLCs were comparable to those of adult liver and were slightly higher than those of fetal liver, while the expression levels of AHR-target genes varied from 0.0038-fold to 21-fold (Fig. 2c). To confirm the expression profiles of CAR, PXR, and AHR genes, mRNA levels of these receptors were measured during hepatic differentiation by qRT-PCR. CAR and PXR gene expression was significantly down-regulated in hESC- and hiPSC-HLCs (Fig. 3a,b). By contrast, AHR mRNA levels were markedly increased in hPSC-HLCs during hepatic differentiation and were higher on D18 of hepatic differentiation than in adult hepatocytes (Fig. 3c). These expression values of CAR, PXR, and AHR genes were consistent with the expression levels detected by microarray analysis (Fig. 2). Taken together, these results indicate that CAR and PXR genes and their target genes, including phase-I enzymes and transporters, are repressed in hESC- and hiPSC-HLCs.

Epigenetic regulation of the expression of CAR, PXR, and AHR genes in hPSC-HLCs. To determine whether epigenetic modifications are associated with the regulation of CAR, PXR, and AHR genes in hPSC-HLCs, the DNA methylation states of CpG dinucleotides were investigated in the regulatory regions around the transcription start site (TSS) of these receptor genes. Bisulfide sequencing analysis revealed hypermethylation of CpG dinucleotides at the CAR and PXR promoter regions in hESC- and hiPSC-HLCs, but showed
demethylation in PHHs (Fig. 4a,b). The DNA methylation frequency in the CAR promoter region, which includes 11 CpG sites, was 98.2%, 99%, and 10% in hESC-HLCs, hiPSC-HLCs, and PHHs, respectively (Fig. 4a). The PXR promoter region, containing two separated CpG rich regions, was hypermethylated (>90%) in both hESC- and hiPSC-HLCs but was hypomethylated (≤10%) in PHHs (Fig. 4b). A similar methylation pattern was also observed at the PXR exon 3 region (Fig. 4b). In hESCs, hypermethylation of CpG dinucleotides was also observed at the CAR and PXR genes (Supplementary Fig. 1a,b). In contrast to the CAR and PXR genes, the DNA methylation frequency at the AHR promoter region, which includes 63 CpG sites, was less than 2% in PHHs, hESC- and hiPSC-HLCs, and hESCs (Fig. 4c; Supplementary Fig. 1c). AHR transcripts were enriched in hESC- and hiPSC-HLCs relative to PHHs, but transcription of CAR and PXR was marginally activated in hESC- and hiPSC-HLCs (Fig. 4d). These results imply that the magnitude of DNA methylation closely correlates with the transcriptional expression of CAR, PXR, and AHR in hPSC-HLCs.

**Effects of treatment with respective CAR, PXR, and AHR ligands on target gene expression in hPSC-HLCs.** Next, to investigate whether activation of xenobiotic receptors influences the expression of downstream genes, hPSC-HLCs were cultured in the presence of respective ligands specific for CAR, PXR, and AHR. These receptors are ligand-activated transcription factors that regulate the expression of their specific target genes in a ligand-dependent manner [16,17]. Furthermore, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carboxaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO) and [3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]ethenylidene]bis-phosphonic acid tetraethyl ester (SR12813) are specific human CAR and PXR agonists, respectively [21,24,25]. As expected from the above results (Fig. 4), CITCO and SR12813 did not or marginally activate the transcription of CAR and PXR target genes, respectively, including CYP2B6, CYP2C9, CYP3A4, MDR, and UGT1A1, in hESC- and hiPSC-HLCs (Fig. 5a,b). CAR and PXR target gene expression levels were altered at levels less than 3-fold in both hPSC-HLC types after treatment with CITCO (100 nM) and SR12813 (200 nM) (Fig. 5a,b). By contrast, AHR-target genes, such as CYP1A1 and CYP1B1 except CYP1A2, were transcriptionally activated and robustly expressed in hESC- and hiPSC-HLCs in the presence of 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarbonyl...
Acid methyl ester (ITE), which is an endogenous agonist for AHR (Fig. 5a,b). These results suggest that AHR is transcriptionally active in both hPSC-HLC types.

Occupancy of AHR on the promoter of target genes in hPSC-HLCs. AHR mediates myriad toxicological outcomes by sensing a variety of exogenous ligands such as benzo[a]pyrene (BaP), 2,3,7,8-tetrachlorodibenzodioxin (TCDD; also known as dioxin), and 3-methylcholanthrene (3-MC)⁶⁻⁹. Upon ligand binding, the activated AHR binds to a specific DNA recognition site known as the xenobiotic response element...
(XRE), thereby leading to target gene transcription\(^\text{19,30}\). To determine whether BaP, TCDD, and 3-MC mediate transcriptional regulation of \(\text{CYP1A}\) subfamily genes through an AHR-dependent mechanism in hPSC-HLCs, we assessed direct AHR-DNA binding to the XRE via a chromatin immunoprecipitation (ChIP) assay (Fig. 6). DNA fragments isolated from ChIP assays were analyzed by qRT-PCR, and GAPDH and immunoprecipitation with isotype IgG were used as negative controls. Treatment with BaP, TCDD, and 3-MC substantially increased AHR enrichment at the \(\text{CYP1A1}\) promoter region in a time-dependent manner in both hESC- and hiPSC-HLCs (Fig. 6a). AHR enrichment patterns at \(\text{CYP1B1}\) were similar to those observed at \(\text{CYP1A1}\) (Fig. 6c). By contrast, BaP, TCDD, and 3-MC had little effect on AHR recruitment to the \(\text{CYP1A2}\) promoter in hESC- and hiPSC-HLCs (Fig. 6b). To further determine whether AHR recruitment directly affects target gene transcription, we measured of \(\text{CYP1A1}\) and \(\text{CYP1B1}\) transcript levels in the presence of AHR ligands. In hESC- and hiPSC-HLCs, \(\text{CYP1A1}\) and \(\text{CYP1B1}\) transcripts levels were significantly increased after treatment with BaP, TCDD, and 3-MC (Fig. 7). These expression patterns were similar to the AHR enrichment patterns at \(\text{CYP1A1}\) and \(\text{CYP1B1}\) promoter regions (Fig. 6a,c). Thus, ligand-induced AHR recruitment results in AHR-mediated induction of \(\text{CYP1A1}\) and \(\text{CYP1B1}\) in hESC- and hiPSC-HLCs.

**Attenuation of AHR-dependent target gene expression by an AHR antagonist in hPSC-HLCs.** To determine whether the expression of \(\text{CYP1A1}\) and \(\text{CYP1B1}\) induced by toxic ligands is AHR-dependent or not, hPSC-HLCs were cultured with or without the AHR antagonist 6,2′,4′-trimethoxyflavone (TMF) for 6 h in the presence of BaP, TCDD, or 3-MC. In both hESC- and hiPSC-HLCs, TMF significantly inhibited BaP- and TCDD-induced \(\text{CYP1A1}\) and \(\text{CYP1B1}\) transcription, but did not significantly inhibit 3-MC-induced \(\text{CYP1A1}\) and \(\text{CYP1B1}\) transcription (Fig. 8b,c). No significant change was observed for TMF in 3-MC-treated hESC-HLCs (Fig. 8b). By contrast, TMF induced a significant, but slight, increase in \(\text{CYP1A1}\) transcription in 3-MC-treated hiPSC-HLCs (Fig. 8c). The inhibitory effect of TMF on AHR-dependent target gene induction was also examined in PHHs. TMF significantly reduced \(\text{CYP1A1}\) and \(\text{CYP1B1}\) transcript levels in BaP- and TCDD-treated PHHs (Fig. 8a), which was highly consistent with those observed in hPSC-HLCs (Fig. 8b,c). In 3-MC-treated PHHs, although TMF reduced \(\text{CYP1A1}\) and \(\text{CYP1B1}\) transcript levels, it was insignificant and less effective against 3-MC than against BaP or TCDD (Fig. 8a). The inhibitory effect of TMF against 3-MC-induced AHR-target gene transcription varied in hESC-HLCs, hiPSC-HLCs, and PHHs (Fig. 8).
results suggest that 3-MC induces \textit{CYP1A1} and \textit{CYP1B1} transcription via an AHR-independent mechanism. Taken together, our data indicate that the transcriptional activity of AHR was reproducible in both hESC- and hiPSC-HLCs, which was tightly regulated by AHR-specific agonists and antagonists.

**Discussion**

Recent progress in technology and in the understanding of stem cell biology has enabled significant progress towards establishing alternative \textit{in vitro} models based on hPSC-HLCs for drug safety and toxicology applications\(^6,31,32\). Several studies have demonstrated the applicability of hPSC-HLCs for predicting chemical toxicity, inter-individual drug responsiveness, and drug-induced liver injury\(^6,31,33\). Despite these advances, several major limitations associated with hPSC-HLCs, particularly incomplete drug metabolic activity, restrict their application in safety pharmacology and toxicology. Current hepatic differentiation processes yield functionally immature hPSC-HLCs displaying ‘fetal-like’ phenotypic characteristics and markedly reduced expression and activity of major DMEs and drug transporters\(^34–36\). DMEs and drug transporters enriched in hepatocytes play pivotal roles in the metabolism of endo- and xenobiotics as well as in the initiation of xenobiotic-induced hepatotoxicity. Xenobiotic-induced hepatotoxicity is widely accepted to be associated with the excessive formation of reactive xenobiotic metabolites catalyzed by phase-I enzymes, mostly CYP family enzymes, and the impairment of metabolite elimination mediated by phase-II enzymes and transporters\(^37\). Therefore, improvements in the expression and activity of hepatic enzymes and transporters involved in drug metabolism are required for the toxicological applications of hPSC-HLCs. However, the developmental pathways that regulate induction of DMEs and transporters are poorly investigated even though significant changes in DME expression are observed during liver ontogeny in various mammals\(^38–41\).

By contrast, xenobiotic-induced transcriptional regulation of DME and transporters in hepatocytes is relatively well elucidated. The induction of various DMEs and transporters is regulated by several ligand-activated transcription factors, namely, xenobiotic receptors, in response to promiscuous xenobiotics\(^16,20,37\). Despite its significance in xenobiotic metabolism as well as xenobiotic-induced hepatotoxicity, the function of xenobiotic receptors has not been investigated in detail in hPSC-HLCs. In this study, we investigated the function of three xenobiotic receptors, CAR, PXR, and AHR, in the regulation of DME and transporter expression in hPSC-HLCs.

Gene expression analysis revealed a prominent reduction in the transcriptional level of the majority of phase-I enzymes and transporters regulated by CAR and PXR in both hESC- and hiPSC-HLCs (Fig. 2a,b). CAR mRNA levels were much lower in both hESC- and hiPSC-HLCs than in fetal liver as well as adult liver (Fig. 3a), and PXR mRNA was barely detectable (Fig. 3b). These results suggest that the considerably lower levels of DME and
transporter expression may be due to the minimal expression of CAR and PXR. Indeed, neonatal activation of CAR results in long-term epigenetic memory and a permanent induction of CAR target genes in mouse liver. Moreover, PXR activation by microbial byproducts promotes the metabolic maturation of hPSC-HLCs and fetal hepatocytes.

The transcriptional regulatory mechanisms of CAR and PXR have been extensively studied; however, little is known about their expression. Epigenetic modification, for example, DNA methylation and histone modification, was recently shown to be involved in the regulation of the expression and activity of PXR. A comparative analysis of epigenomes by Bonder et al. demonstrated that differential DNA methylation patterns in hepatic metabolism-related genes between human fetal and adult liver were tightly associated with developmental changes in their expression. Thus, we examined the association between DNA methylation and the transcriptional activity of CAR and PXR in hPSC-HLCs. Bisulfite sequencing analysis revealed that promoter hypermethylation resulted in repression of CAR and PXR expression, which was likely associated with diminished transcriptional activity.

Both human CAR and PXR are capable of regulating the expression of various DMEs (e.g., CYP2B6, CYP2C9, CYP3A4, and UGT1A1) and drug transporters such as MDR1. While PXR activation can nonselectively induce CYP2B6 and CYP3A4 expression, CAR preferentially induces CYP2B6 expression. Notably, hESC- and hiPSC-HLCs treated with CITCO and SR12813, selective human CAR and PXR agonists, showed only subtle changes in the expression levels of CAR and PXR target genes. The results indicated that CAR and PXR did not function as xenosensors or transcriptional regulators in both hESC- and hiPSC-HLCs. Taken together, our data suggest that the immature drug metabolism of hPSC-HLCs could be, at least in part, due to the lack of transcriptional activity of CAR and PXR as a result of promoter hypermethylation.

AHR is a member of the HLH (helix-loop-helix)-PER-ARNT-SIM (bHLH-PAS) protein family of heterodimeric transcription factors that function as sensors of extracellular signals and environmental stresses. This xenobiotic receptor is capable of sustained hyperactivation via a number of polycyclic aromatic hydrocarbons (e.g., BaP), planar halogenated aromatic hydrocarbons (e.g., dioxins), and endogenous chemicals, which mediates diverse toxicological responses. The importance of AHR activation in the critical stages of tumorigenesis is an intense focus of academic research. Although extensive in vivo rodent studies have provided valuable insights into AHR-mediated toxicity, interspecies differences in AHR ligand binding affinity between humans and rodents have created a need for reliable human cell models.

In the present study, we demonstrated that the intrinsic AHR function as a xenobiotic sensor, as well as a ligand-activated transcriptional factor, was reproducible in hPSC-HLCs. AHR mRNA expression levels in hESC- and hiPSC-HLCs were comparable to those of PHHs as well as adult liver, and the DNA methylation pattern of the AHR promoter was similar to that in PHHs. Activation of AHR by an endogenous agonist and several toxic substances, including BaP, TCDD, and 3-MC, resulted in prominent induction of its target genes, including CYP1A1 and CYP1B1, in hPSC-HLCs. Moreover, ChIP assays clearly
Figure 6. Ligand-dependent recruitment AHR to CYP1A1 and CYP1B1 promoter regions in hPSC-HLCs. hESC- and hiPSC-HLCs were treated with 1 μM BaP, 10 nM TCDD, 1 μM 3-MC, or DMSO (0.1%) for indicated amounts of time. ChIP assay were performed with primer pairs specific to the XRE-containing promoter regions of CYP1A1 (a), CYP1A2 (b), and CYP1B1 (c) in hESC-HLCS (left panels) and hiPSC-HLCs (right panels) using real-time PCR. Primer pairs that amplify XRE-containing promoter regions as indicated in each diagram (upper panels). Numbers indicate nucleotide positions in relation to the transcription start site (+1, an arrow). Results are mean ± SD (n = 3) and are presented relative to isotype control (IgG = 1). *p < 0.05, **p < 0.01, ***p < 0.001, significant values in comparison with DMSO.
demonstrated direct AHR-DNA binding at the XRE motif in the CYP1A1 and CYP1B1 promoters in response to BaP, TCDD, and 3-MC treatment (Fig. 6a,c). In contrast with CYP1A1 and CYP1B1, constitutive CYP1A2 mRNA expression was barely detectable in hESC- and hiPSC-HLCs (Fig. 2c). AHR agonists, including BaP, 3-MC, and TCDD, did not significantly induce AHR recruitment to the CYP1A2 promoter (Fig. 6b). Our previous study demonstrated that the low expression of CYP1A2 in hESC-HLCs was associated with the hypermethylation of CpG sites and histone modifications such as enrichment of repressive histone mark histone H3 trimethylated at lysine 2752. Therefore, it suggests that these epigenetic modulations are involved in the inhibition of AHR recruitment to the CYP1A2 promoter in hPSC-HLCs.

Recent reports have highlighted the multiple physiological functions of AHR beyond that of a xenobiotic sensor and transcriptional regulator controlling xenobiotic metabolism. These newly identified functional roles of AHR in cancer, inflammation, and adaptive immunity28,53,54 suggest that AHR antagonism may be a potential novel therapeutic target for cancer and immune disease. We have shown that TMF effectively inhibited AHR-mediated transcription of endogenous target genes independent of the cell type, even in the presence of BaP and TCDD (Fig. 8). By contrast, TMF showed different inhibitory effects against 3-MC among PHHs, hESC-HLCs, and hiPSC-HLCs, which could be explained by 3-MC-induced CYP1A1 and CYP1B1 transcription via an AHR-independent mechanism. Recently, several studies indicated that 3-MC directly activates estrogen receptor α, which is capable of modulating AHR-mediated target gene transcription55–57. Taken together, our studies on the effect of AHR agonists and antagonists demonstrated that hPSC-HLCs were capable of reproducing the intrinsic functions of AHR. Furthermore, our data showed no significant differences between hESC- and hiPSC-HLCs in terms of xeno-sensing and transcriptional activity of AHR. Thus, the derivative HLCs from both hESCs and hiPSCs may be a useful alternative model for predicting AHR-mediated toxicity and for identifying potential AHR-targeted therapeutics.

Materials and Methods

Ethics statement. The using hESCs and hiPSCs in the present study was approved by the ethics committee of Korea Institute of Toxicology (approval number: 2013-001) and approval of this research was reported to Korea Centers for Disease Control and Prevention. The methods were carried out in accordance with the approved guidelines.

Hepatic differentiation. The hESCs (CHA-hES15 cell line) and hiPSCs were maintained as previously described58. Furthermore, hESCs and hiPSCs were differentiated into hepatocytes as previously described with some modifications59. Briefly, hESCs and hiPSCs were cultured for 4 days on Matrigel (Corning, Tewksbury, MA, USA) in mTeSR1 (Stem Cell Technologies, Vancouver, Canada). Thereafter, hESCs were incubated in RPMI-1640 (Lonza, Baltimore, MD, USA) containing 0.5 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA), 1 × B27 (Invitrogen, Carlsbad, CA, USA), 50 ng/ml activin A (Peprotech, Rocky Hill, NJ, USA), and
0.5 mM sodium butyrate (Sigma-Aldrich) for 1 day, and then further cultured for 4 days in the same medium except that the concentration of sodium butyrate was reduced to 0.1 mM. After treatment with activin A, cells were cultured in RPMI-1640 containing 0.5 mg/ml BSA, 1 × B27, 10 ng/ml fibroblast growth factor 4 (FGF4, Peprotech), and 10 ng/ml hepatocyte growth factor (HGF, Peprotech) for 5 days. Hepatic maturation was induced by culturing cells in hepatocyte culture medium (HCM, Lonza) supplemented with 10 ng/ml FGF4, 10 ng/ml HGF, 10 ng/ml oncostatin M (Peprotech), and 0.1 μM dexamethasone (Sigma-Aldrich) for 7 days. The culture media was changed daily.

**Characterization of hESC- and hiPSC-HLCs.** FACS, immunocytochemistry, and functional assays were performed as described in the Supplementary Materials and Methods.

**Human liver samples, primary hepatocyte culture, and chemical dosing.** Total RNA obtained from human fetal and adult liver was purchased from Clontech Laboratories (Palo Alto, CA) and Agilent Technologies (Palo Alto, CA). Primary human hepatocytes (BD Gentest™ Cryo Human Hepatocytes, BD Biosciences, Donor No. HFC 476 and 261) were plated in collagen I coated culture plates (Corning) using a Gentest™ High Viability CryoHepatocyte Recovery Kit (Corning) according to the manufacturer's instructions. Experiments were performed 24 h later. Chemicals were purchased from Sigma-Aldrich, Cayman Chemicals (Ann Arbor, MI), or Torcis Bioscience (Ellisville, MO). For CAR, PXR, and AHR activation, human liver samples, primary hepatocyte culture, and chemical dosing were performed as described in the Supplementary Materials and Methods.}

**Gene expression profiling.** Total RNA was isolated from cells using a PureLink RNA Mini Kit (Life Technologies) according to the manufacturer's instructions. RNA integrity was determined with a 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. Gene expression levels were determined using a GeneAmp PCR System, www.ingenuity.com. }

**Quantitative real-time PCR (qRT-PCR).** Total RNA was isolated from cells using a PureLink RNA Mini Kit (Life Technologies) and reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Gene expression levels were measured by real-time RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Relative expression levels were analyzed using a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Triplicate PCR reactions were performed for each sample. The primers used for gene expression analysis are listed in Supplementary Table 1. For comparative quantification, results from real-time PCR were expressed as the relative fold change compared to control cells, after normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ΔCt (SΔCt) values were calculated as the difference between the Ct values of GAPDH and the target. The ΔCt value of control cells was used as the control ΔCt (CΔCt) value. Relative gene expression levels were determined using the formula 2^{-(SΔCt-CΔCt)}.

**Bisulfite sequencing.** Genomic DNA was isolated from cells using a G-DEX Iic Genomic DNA Extraction Kit (iNIRON Biotechnology, Gyeonggi-do, Korea) according to the manufacturer’s protocol. Bisulfite conversion was performed using an EZ DNA Methylation-Gold Kit (ZYMO RESEARCH, Orange, CA, USA) according to the manufacturer’s protocol. Bisulfite-specific PCR reactions were performed on a GeneAmp PCR System 9700.
(Applied Biosystems) using the following protocol: 95 °C for 15 min, 50 cycles of 95 °C for 20 s, 55 °C for 40 s, 72 °C for 30 s, and extension at 72 °C for 10 min. The primer sequences used for PCR are listed in Supplementary Table 2. PCR products were purified using a MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology) and cloned into pGEM-T vector (Promega, Madison, WI, USA), and sequenced using an ABI 3730XL capillary DNA sequencer (Applied Biosystems). The methylation state of the CpG sites was determined from the sequence data by using QUMA (QUantification tool for Methylation Analysis) software.

**Chromatin immunoprecipitation.** ChiP experiments were performed using an EZ ChiP™ chromatin immunoprecipitation kit (Millipore) according to the manufacturer’s protocol. Immunoprecipitation was performed with an AHR antibody (Thermo Fisher Scientific Pierce, PA5-29642). A rabbit IgG antibody (Abcam, ab37415) was used as an isotype control. The purified DNA was analyzed by real-time PCR. Quantitative PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. The primer sequences used for PCR are listed in Supplementary Table 3. Triplicate PCR reactions were performed for each sample. ChiP-quantitative PCR results were calculated using the $\Delta\Delta^{\text{Ct}}$ method. The Ct value of the respective ChiP fraction was normalized against the Ct value of the input DNA fraction ($\Delta^{\text{Ct}}$). Then, the Ct value of the ChiP fraction was normalized to the Ct value of the IgG control ($\Delta\Delta^{\text{Ct}}$). The fold enrichment of immunoprecipitation was calculated using $2^{-\Delta\Delta^{\text{Ct}}}$.

**Statistical analysis.** Data obtained from three independent experiments were expressed as mean ± standard deviation (SD) and statistically analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test and t-test with Mann-Whitney test using GraphPad Prism software (San Diego, CA, USA). A $p$-value < 0.05 was considered significant.

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Supplementary information

Additional Information

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