Differences in the Epigenetic Regulation of Cytochrome P450 Genes between Human Embryonic Stem Cell-Derived Hepatocytes and Primary Hepatocytes

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Abstract

Human pluripotent stem cell-derived hepatocytes have the potential to provide in vitro model systems for drug discovery and hepatotoxicity testing. However, these cells are currently unsuitable for drug toxicity and efficacy testing because of their limited expression of genes encoding drug-metabolizing enzymes, especially cytochrome P450 (CYP) enzymes. Transcript levels of major CYP genes were much lower in human embryonic stem cell-derived hepatocytes (hESC-Hep) than in human primary hepatocytes (hPH). To verify the mechanism underlying this reduced expression of CYP genes, including CYP1A1, CYP1A2, CYP1B1, CYP2D6, and CYP2E1, we investigated their epigenetic regulation in terms of DNA methylation and histone modifications in hESC-Hep and hPH. CpG islands of CYP genes were hypermethylated in hESC-Hep, whereas they had an open chromatin structure, as represented by hypomethylation of CpG sites and permissive histone modifications, in hPH. Inhibition of DNA methyltransferases (DNMTs) during hepatic maturation induced demethylation of the CpG sites of CYP1A1 and CYP1A2, leading to the up-regulation of their transcription. Combinatorial inhibition of DNMTs and histone deacetylases (HDACs) increased the transcript levels of CYP1A1, CYP1A2, CYP1B1, and CYP2D6. Our findings suggest that limited expression of CYP genes in hESC-Hep is modulated by epigenetic regulatory factors such as DNMTs and HDACs.

Introduction

Pluripotent stem cells (PSCs) and their derivatives will be valuable in regenerative medicine and for the development and discovery of new drugs. In particular, PSC-derived hepatocytes
have many advantages over primary hepatocytes and hepatocellular carcinoma cell lines, such as their unlimited supply and better functionality, for the in vitro assessment of drug-induced hepatotoxicity[1–7]. Human PSCs can differentiate into hepatocytes that exhibit several liver-specific characteristics, such as the expression of hepatocyte marker genes, albumin (ALB) secretion, glycogen storage, and active cytochrome P450 (CYP) enzymes, which are representative of phase 1 enzymes in drug metabolism [2,8–12]. Although the homogeneity and functional properties of PSC-derived hepatocytes are continually improving, they cannot fully replicate drug metabolism in the liver at present [13]. Most studies have found that low mRNA levels and activities of CYP enzymes were detected in PSC-derived hepatocytes than in adult hepatocytes [13–15].

The developmental stage of the liver is closely correlated with the expression and activities of CYP genes [16–18]. One way in which the expression of CYP genes is controlled during development is epigenetic regulation [19], which refers to genomic modifications that can influence gene expression and cellular phenotypes but do not change the DNA sequence [20]. DNA methylation and histone modifications participate in the regulation of human CYP genes and this has mainly been reported in cancer [19,21,22]. Recent study proves that DNA methylation is associated with variations in hepatic gene expression between fetal and adult human liver [23]. Also, the difference in expression of epigenetic modifier genes, which are responsible for regulating histone and DNA modifications, represents between human embryonic stem cell (hESC)-derived hepatocytes (hESC-Hep) and primary hepatocytes [24]. However, epigenetic regulation of CYP genes during liver development is poorly understood. In this study, we investigated the reduced expression of CYP genes in hESC-Hep and epigenetic differences in regulatory regions around the transcription start sites (TSS) of CYP genes between hESC-Hep and human primary hepatocytes (hPH). Some CYP genes were regulated by inhibition of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) during the differentiation of hESCs into hepatocytes.

Results
Reduced expression of drug-metabolizing enzyme (DME) genes in hESC-Hep

hESCs were differentiated into hepatocytes via definitive endoderm (DE) as detailed in S1 Fig. Approximately 99% of cells differentiated into CXCR4-positive DE cells on day 5 (D5, S2A Fig). At the end of differentiation, Expression of the hepatocyte markers ALB, α-1-antitrypsin (AAT), α-fetoprotein (AFP), and hepatocyte nuclear factor 4 α (HNF4A) was detected in hESC-Hep at the transcript and protein levels (Fig 1A and S2B Fig). Approximately 90% and 81% of hESC-Hep expressed ALB and AAT, respectively, according to fluorescence-activated cell sorting (FACS) (Fig 1B). hESC-Hep could store glycogen in the cytoplasm and take up acetylated-low-density lipoprotein (Fig 1C and 1D). hESC-Hep could also secrete ALB into the culture media and synthesize urea like hPH (Fig 1E and 1F). Moreover, bile canaliculi formation and function in hESC-Hep were verified using 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA). Cells were incubated with carboxy-DCFDA, which was internalized by hepatocytes, cleaved by intracellular esterases, and excreted from bile canaliculi. 5-(and-6)-Carboxy-2',7'-dichlorofluorescein accumulated in bile canaliculi between adjacent cells (Fig 1G). These results demonstrate that hESC-Hep have cellular and molecular characteristics of hepatocytes.

Next, we examined the expression of DME genes in hESC-Hep. The nuclear receptor NR1I3 (CAR), NR1I2 (PXR), and NFE2L2 (NRF2) was significantly expressed in hESC-Hep than in hPH, whereas AHR was highly expressed compared to hPH (Fig 2A). Expression of most DME
Epigenetic Differences of CYP Genes between hESC-Hep and hPH

genes, including those encoding phase I enzymes, phase II enzymes, and phase III transporters, was lower in hESC-Hep than in hPH (Fig 2B–2D). In terms of phase I enzymes, expression of CYP1A1, CYP1B1, and CYP7A1 in hESC-Hep was similar to or higher than that in hPH (Fig 2B). However, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were weakly expressed in hESC-Hep compared to hPH (Fig 2B). Transcript levels of phase II

Fig 1. Differentiation of human embryonic stem cells (hESCs) into hepatocytes in vitro. (A) Expression of hepatocyte marker genes. Genes were examined by real-time RT-PCR in hPH, hESCs, and hESC-Hep (day 20 of differentiation). Data represent mean ± SD from three independent experiments. ** p<0.01, significant values in comparison with hPH;§§ p<0.01, significant values in comparison with hESC-Hep (ANOVA followed by Dunn’s multiple comparison test). (B) Percentages of albumin (ALB)-positive and α-1-antitrypsin (AAT)-positive cells among hESC-Hep. Fluorescence-activated cell sorting analysis was performed 20 days after the onset of differentiation. Black line: isotype control, blue line: primary antibody. (C) Glycogen storage in hESC-Hep. Periodic acid-Schiff staining of glycogen was performed at day 20 of differentiation. Stored glycogen (purple) was observed in the cytoplasm. Nuclei (light blue) were counterstained with hematoxylin. The scale bar represents 100 μm. (D) Acetylated-low-density lipoprotein (Ac-LDL) uptake by hESC-Hep. The ability of cells to take up Ac-LDL was examined at day 20 of differentiation. The scale bar represents 100 μm. (E) ALB secretion from hESC-Hep. The ALB concentration was measured in the conditioned media of hESCs (day 0), hESC-Hep (day 20), and hPH by an enzyme-linked immunosorbent assay using an anti-human ALB antibody. (F) Urea production by hESC-Hep. The amount of urea secreted by hESCs (day 0), hESC-Hep (day 20), and hPH was examined at 0, 24, and 48 hours. (G) Activity of bile canalicular transporter in hESC-Hep. Red arrows indicate cleaved 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, which was excreted into the bile canalicular spaces of cells. The scale bars represent 50 μm.

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enzymes, including UGT2B7, SULT1A1, GSTA, and GSTP1, in hESC-Hep were similar to or higher than the levels in hPH (Fig 2C). hESC-Hep expressed phase III transporters, such as those encoded by ABCC3 and ABCB11 (Fig 2D). These results demonstrate that hESC-Hep have a limited ability to metabolize drugs because most DME genes, especially those encoding major CYP enzymes such as CYP1A2, CYP2D6, and CYP3A4, were not expressed during the differentiation of hESCs into hepatocytes in vitro.

Differences in the epigenetic modifications of CYP genes between hESC-Hep and hPH

In this study, we showed that expression of major CYP genes such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP2E1 was extremely low, whereas CYP1A1 and 1B1 genes were similar and highly expressed compared to hPH, respectively (Fig 2B). To investigate whether epigenetic modifications modulate CYP gene expression during the differentiation of hESCs into hepatocytes in vitro, we studied combinatorial roles of DNA methylation and histone modifications at regulatory regions around the TSS of CYP enzymes in hESC-Hep and hPH. We selected five CYP genes including CYP1A1, CYP1A2, CYP1B1, CYP2D6, and CYP2E1, which have CpG islands in regulatory regions. However, CYP2C9, CYP2C19, and CYP3A4 do not contain CpG islands.
Expression of CYP1A1 and CYP1B1 in hESC-Hep was similar to or higher than that in hPH, whereas CYP1A2 was slightly expressed in hESC-Hep (Fig 2B). Bisulfite sequencing of the CYP1A1 promoter region, which contains 42 CpG dinucleotides, revealed the methylation frequency was 0.0% and 38.6% in hPH and hESC-Hep, respectively (Fig 3A, upper panel). In hESC-Hep, distribution of methylated CpG sites in CYP1A1 was located at position -1353 to -1100 relative to TSS, which includes 18 CpG dinucleotides (Fig 3A, upper panel). In this region, enrichment of active histone mark histone H3 trimethylated at lysine 4 (H3K4me3) in hPH was higher than that in hESC-Hep (Fig 3A, lower panel). By contrast, unmethylated CpG sites (-1099 to -898 relative to TSS) represented similar enrichment patterns of histone modifications between hPH and hESC-Hep (S3A Fig). The promoter region of the CYP1A2 gene, which contains 10 CpG dinucleotides, was completely methylated in hPH and hESC-Hep (Fig 3B, upper left panel). However, the CpG island in the gene body region, which contains 20 CpG dinucleotides, had a methylation frequency of 40.0% and 76.7% in hPH and hESC-Hep, respectively (Fig 3B, upper right panel). H3K4me3 in the gene body region of CYP1A2 likely modulated expression of this gene in hPH (Fig 3B, lower graph). The presence of repressive histone mark histone H3 trimethylated at lysine 27 (H3K27me3) in hESC-Hep was associated with down-regulation of CYP1A2 transcription (Fig 3B, lower graph). The methylation frequency at -1791 to -1362 relative to TSS of CYP1B1, which includes 17 CpG dinucleotides, was 15.0% and 59.5% in hPH and hESC-Hep, respectively (Fig 3C, upper left panel). In this region, H3K4me3 was mainly occupied by H3K4me3 in hPH, while levels of H3Ac and H3K27me3 were similar to those of the IgG controls in hPH and hESC-Hep (Fig 3C, lower graph). However, the CpG island in close to TSS (-435 to -123), which contains 26 CpG dinucleotides, was completely demethylated in hPH and hESC-Hep (Fig 3C, upper right panel). In this region, H3K4me3 was enriched in hPH and hESC-Hep, although H3Ac was only found in hPH (S3B Fig). Therefore, these results indicate that expression levels of CYP1A1, CYP1A2, and CYP1B1 are regulated by DNA methylation and histone modifications in the regulatory region.

The transcript levels of CYP2D6 and CYP2E1 were low in hESC-Hep (Fig 2B). The methylation frequency at the CYP2D6 promoter region, which contains 12 CpG dinucleotides, was 0.0% and 96.3% in hPH and hESC-Hep, respectively (Fig 3D, upper left panel). In the gene body region of CYP2D6, which contains 32 CpG dinucleotides, the methylation frequency was 45.5% and 90.3% in hPH and hESC-Hep, respectively (Fig 3D, upper right panel). The promoter region of CYP2D6 was enriched in H3K4me3 in hPH, and silencing of this gene in hESC-Hep was likely governed by H3K27me3 (Fig 3D, lower graph). Bisulfite sequencing of the CYP2E1 gene body region, which contains 23 CpG dinucleotides, revealed a methylation frequency of 27.4% and 92.6% in hPH and hESC-Hep, respectively (Fig 3E, upper panel). H3Ac and H3K4me3 were enriched in the gene body region of CYP2E1 in hPH, while the levels of H3K27me3 were similar to those of IgG controls in hPH and hESC-Hep (Fig 3E, lower graph). These results show that inhibitory epigenetic regulation of CYP2D6 and CYP2E1 in hESC-Hep is associated with the transcriptional inactivation of these genes. Therefore, our data represents the epigenetic differences in the regulatory regions of five CYP genes between hESC-Hep and hPH. Reduced transcription of CYP1A2, CYP2D6, and CYP2E1 in hESC-Hep may be influenced by DNA methylation and histone modifications.

Transcriptional regulation of CYP genes by inhibition of DNMTs and HDACs

We validated the transcript levels of epigenetic-related genes such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and sirtuins (SIRTs) in hPH and hESC-Hep. DNMT3B, HDAC1, HDAC2, and HDAC3 were highly expressed in hESC-Hep compared to
hPH (S4A and S4B Fig). However, expression of SIRT1 and SIRT3 genes was lower in hESC-Hep than in hPH (S4C Fig). These results represent that epigenetic regulatory mechanism of hESC-Hep differs from that of hPH. To investigate whether DNA methylation directly modulates the expression of these five CYP genes in hESC-Hep, we treated cells with a DNMT inhibitor including decitabine (DAC, 2'-deoxy-5-azacytidine) and RG108 during hepatic maturation and examined changes in DNA methylation and expression of CYP genes. Methylation frequencies in the CYP1A1 and CYP1B1 promoter regions were lower in DAC- and RG108-treated hESC-Hep than in vehicle-treated hESC-Hep (Fig 4A). CYP1A1 was up-regulated at the transcript level in DAC- and RG108-treated cells, whereas expression of CYP1B1 was not affected (Fig 4B). In CYP1A2, transcript level was increased in DAC- and RG108-treated cells, although DNA methylation in two regulatory regions was not significantly changed (Fig 4A and 4B). DNMT inhibition did not seem to affect the methylation frequencies at regulatory regions and transcript levels of CYP2D6 and CYP2E1 compared to DMSO-treated cells (Fig 4A and 4B). However, expression level of CYP2E1 was different between DAC- and RG108-treated cells (Fig 4A). We also investigated transcriptional regulation by DNMT inhibition in hepatocytes derived from human induced pluripotent stem cells (hiPSCs, S5 Fig). hiPSCs were also efficiently differentiated into hepatocytes, which expressed hepatocyte markers and had liver functions including glycogen storage, LDL uptake, secretion of albumin, and synthesis of urea (S6 Fig). Transcriptional activation in CYP1A1 and CYP1A2 was found in...
Fig 4. Transcriptional regulation of CYP genes by inhibition of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) during hepatic differentiation. (A) Methylation frequencies in the promoter and gene body regions of CYP genes were examined by bisulfite sequencing in hESC-Hep treated with DMSO or a DNMT inhibitor (DAC or RG108). Data represent methylation frequencies from two independent experiments. (B) Expression levels of CYP genes were examined by real-time RT-PCR in hESC-Hep treated with DMSO, DAC, or RG108. Data represent mean ± SD from three independent experiments. * p<0.05, ** p<0.01, significant values in comparison with DMSO (ANOVA followed by Dunn's multiple comparison test). (C) Expression levels of CYP genes were examined by real-time RT-PCR in hESC-Hep treated with DMSO or a HDAC inhibitor (1 mM sodium butyrate (SB)) with or without a DNMT inhibitor (DAC or RG108). Data represent mean ± SD from three independent experiments. * p<0.05, ** p<0.01, *** p<0.001, significant values in comparison with DMSO (ANOVA followed by Bonferroni's multiple comparison test).
DAC- and RG108-treated hiPSC-Hep, whereas CYP1B1, CYP2D6, and CYP2E1 were not (S7A Fig). These results indicate that DNA methylation is involved in transcriptional regulation of CYP1A1 and CYP1A2 during the differentiation of hESCs into hepatocytes.

Next, we tested the combined effect of DNMT and HDAC inhibition on the expression of CYP genes in hESC-Hep and hiPSC-Hep. Treatment of sodium butyrate (SB, a HDAC inhibitor) with or without DNMT inhibitors leads to significant increase in transcript levels of CYP1A1 and CYP1B1 in hESC-Hep (Fig 4C). Transcript levels of CYP1A2 and CYP2D6 were increased by co-treatment with SB/DAC and SB/DAC or RG108, respectively (Fig 4C). However, expression of CYP2E1 after co-treatment with HDAC and DNMT inhibitors was similar to DMSO control (Fig 4C). We also found that transcriptional regulation of CYP genes by combined DNMT and HDAC inhibition in hiPSC-Hep was consistent with that in hESC-Hep (S7B Fig). Moreover, expression of CYP1A1, CYP1A2, and CYP1B1 in hESC-Hep were also regulated by treatment with valproic acid (VPA) with or without DNMT inhibitors (S7C Fig).

On the other hand, inhibition of DNMTs and HDACs might not affect the overall impact of hepatocyte differentiation efficiency, although expression of hepatocyte marker genes including ALB and AAT was slightly increased in hPSC-Heps treated with DNMT and HDAC inhibitors (S8 Fig). Taken together, these results show that transcription of CYP genes in hESC-Hep and hiPSC-Hep is influenced by epigenetic modulation via inhibition of DNMTs and HDACs.

**Discussion**

Hepatocytes derived from human PSCs are valuable tools for drug discovery and hepatotoxicity testing. However, DME gene expression was limited in hESC-Hep (Fig 2). hESC-Hep seem to be more similar to fetal liver than to adult liver because they express AFP (Fig 1A and S2B Fig) and the most important DME genes are inactive prior to birth [17,18]. Among DME genes, CYP enzymes play a role in the biotransformation of most drugs [25] and their expression is potentially regulated by epigenetic modifications [19,21,22]. Our study detected low constitutive expression of most CYP genes in hESC-Hep and investigated epigenetic marks, namely, DNA methylation and histone modifications, at the regulatory regions of CYP genes for the first time. The epigenetic regulation of five CYP genes, namely, CYP1A1, CYP1A2, CYP1B1, CYP2D6, and CYP2E1, differed between hESC-Hep and hPH. Moreover, inhibition of DNMTs and HDACs may be involved in the regulation of CYP genes during the differentiation of hESCs into hepatocytes.

CYP1 family genes, including CYP1A1, CYP1A2, and CYP1B1, are induced by aryl hydrocarbon receptor (AHR) signaling in response to polycyclic aromatic hydrocarbon ligands [26–28]. The CYP1A1 and CYP1A2 genes are oriented head-to-head and share a bidirectional regulatory region [29,30]. Unexpectedly, among CYP1 family genes, constitutive expression of CYP1A2 was extremely low in hESC-Hep (Fig 2B). Therefore, our study compared the epigenetic regulations among CYP1 family genes in hPH and hESC-Hep. The transcript levels of genes encoding major CYP enzymes, such as CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP2E1, were much lower in hECS-Hep than in hPH (Fig 2B). CYP3A4 and CYP2C9, which are most abundant and functional in the liver, can be induced by pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [25]. Transcriptional inactivation of CYP3A4 and CYP2C9 in hESC-Hep would be influenced by silencing of the NR1I2 and NR1I3 encoding PXR and CAR, respectively (Fig 2A and 2B). Also, interindividual variation of CYP3A4 expression in fetal and adult human liver is associated with methylation of single CpGs in promoter region, which is approximately 12 kb upstream of TSS [31]. However, the detailed transcriptional regulation of CYP2D6 and CYP2E1 has not been extensively studied. Hence, we focused on the epigenetic regulation of members of the CYP1 family, CYP2D6, and CYP2E1 during hepatocyte differentiation.
CYP1A1 is expressed early in embryogenesis, and CYP1A1 and CYP1B1 are primarily expressed in extrahepatic tissues [32–34]. By contrast, CYP1A2 is constitutively expressed at high levels only in adult liver [35]. Histone deacetylation and DNA methylation influence the constitutive expression of CYP1A1, CYP1A2, and CYP1B1 in a cell type-specific manner [36,37]. In hPH and hESC-Hep, epigenetic modifications involved in transcriptional activation was observed in regulatory regions of CYP1A1 and CYP1B1, although these cells represented different methylation frequency of CpGs and enrichment of H3K4me3 (Fig 3 and S3 Fig). Regulatory regions of CYP1A1 and CYP1B1 include xenobiotic response element (XRE), which is binding sites for the AHR complex [38,39]. Transcriptional activation of CYP1A1 in hESC-Hep is influenced by unmethylated CpG sites into regulatory region, which contains two XRE [39]. Expression of CYP1A1 and CYP1B1 was up-regulated in hESC-Hep by epigenetic regulation via inhibition of DNMTs and HDACs (Fig 4). The methylation status of a CpG island in the second exon of CYP1A2, which contains 17 CpG dinucleotides, is correlated with the hepatic transcript level of this gene [40]. The low expression of CYP1A2 in hESC-Hep was associated with the hypermethylation of CpG sites and H3K27me3 in the second exon and expression of this gene was up-regulated by inhibition of DNMTs and HDACs (Figs 2B, 3B, and 4). However, this up-regulation was not sufficient to induce enzyme activity comparable with that in hPH because CpG sites in the second exon were incompletely demethylated. Moreover, induction of CYP1A2 gene by omeprazole and endogenous AHR agonist (ITE, 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester) did not be altered in the presence of DNMT inhibitors during hepatocyte differentiation (S9 Fig). It seems to be correlated with enrichment of repressive histone mark H3K27me3 in hESC-Hep (Fig 3B). Polycomb group protein EZH2, which is a methyltransferase and contributes methylation of H3K27, interacts with DNMTs and is associated with DNMT activity [41,42].

CYP2D6 and CYP2E1 are poorly expressed in fetal liver but their expression rapidly increases within hours after birth [43–46]. CYP2D6 is associated with the metabolism of more than 50 clinically important drugs [47]. Genetic variations in CYP2D6 have been extensively studied and well characterized, which could have special relevance for revealing CYP2D6 inter-individual variations [48]. The present study revealed that hypermethylation of CpG islands in the promoter and gene body regions of CYP2D6 may be crucial for the down-regulation of this gene in hESC-Hep (Fig 3D). Incomplete demethylation after DNMT inhibition is likely to be associated with enrichment of H3K27me3 in regulatory region like CYP1A2. The transcript level of CYP2D6 was increased after combinatorial inhibition of DNMTs and HDACs (Fig 4C). Methylation of specific 5’ residues of CYP2E1 may be responsible for the lack of expression of this gene in fetal liver [49]. Transcription of CYP2E1 in human lung, kidney, and full-term placenta is regulated by extensive methylation of the first exon and first intron of this gene [50]. Similarly, silencing of CYP2E1 in hESC-Hep was associated with hypermethylation of the CpG island in the first intron (Fig 3E). This regulatory region was occupied by the active histone marks H3Ac and H3K4me3 in hPH, thereby correlating with activation of CYP2E1 (Fig 3E). However, expression of CYP2E1 after DNMT or DNMT/HDAC inhibition did not seem to affect or down-regulate in hESC-Hep and hiPSC-Hep (Fig 4 and S7 Fig). These results suggest that epigenetic modifications, such as DNA methylation and histone modifications, may be closely correlated with the limited transcription of CYP genes, including CYP1A2, CYP2D6, and CYP2E1, in hESC-Hep.

In summary, hESC-Hep have a limited drug metabolism ability, which restricts their use for in vitro hepatotoxicity testing. This is because the majority of CYP genes involved in drug metabolism, including CYP1A2, CYP2C9, CYP2C19, CYP3A4, CYP2D6, and CYP2E1, are lowly expressed in hECS-Hep. DNA methylation and histone modifications of regulatory regions of CYP genes differed between hPH and hESC-Hep. These differences were associated with
inhibitory regulation of CYP genes in hESC-Hep. Inhibition of DNMTs and HDACs increased the transcription of CYP genes in hESC-Hep, but these increased transcripts were not comparable with that in hPH. Further studies are required to improve the expression and activity of CYP enzymes by epigenetic regulations. These findings show that expression of CYP genes is modulated by controlling epigenetic modification enzymes, such as DNMTs and HDACs.

Materials and Methods

Ethics statement

All experiments involving hESCs were 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. CHA-hES15 cell line was received from CHA Stem Cell Institute, CHA University, Korea.

Differentiation of hESCs into hepatocytes

hESCs (CHA-hES15 cell line) were maintained as previously described [51]. hESCs were differentiated into hepatocytes as previously described [52] with some modifications. Briefly, hESCs were cultured for 3 days on Matrigel (Corning Life Science, 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, differentiated cells were cultured in RPMI-1640 containing 0.5 mg/ml BSA, 1× B27, 30 ng/ml fibroblast growth factor 4 (FGF4, Peprotech), and 20 ng/ml bone morphogenetic protein 2 (BMP2, Peprotech) for 5 days, and then cultured in hepatocyte culture medium (HCM, Lonza) supplemented with 20 ng/ml hepatocyte growth factor (HGF, Peprotech) for 5 days. Hepatic maturation was induced by culturing cells in HCM supplemented with 10 ng/ml oncostatin M (Peprotech) and 0.1 μM dexamethasone (Sigma-Aldrich) for 5 days. The culture media was changed daily. After hepatic induction by treatment with FGF4 and BMP2, HCM was supplemented with DNMT inhibitor (5 μM decitabine or RG108, R&D Systems, Minneapolis, MN, USA) and/or HDAC inhibitor (1 mM sodium butyrate, Sigma-Aldrich) and HGF, followed by oncostatin M and dexamethasone. Cells treated with 0.1% DMSO (Sigma-Aldrich) were used as a negative control.

Culture of hPH

BD Gentest Cryo Human Hepatocytes (BD Biosciences, Donor No. HFC 476) were plated in BD hepatocyte culture media according to the manufacturer’s instructions, and experiments were performed 24 hours later.

Characterization of hESC-Hep

Fluorescence-activated cell sorting (FACS). Cells were dissociated by incubation with 0.05% collagenase IV (Invitrogen) at 37°C for 15 minutes followed by incubation with Accutase (Innovative Cell Technologies, San Diego, CA, USA) at 37°C for 15 minutes. Dissociated cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization solution (eBioscience, San Diego, CA, USA) for 1 hour at room temperature (RT). One microgram of mouse anti-ALB (R&D Systems) and rabbit anti-AAT (Abcam, Cambridge, UK) was conjugated using the Zenon R-Phycocerythrin Mouse IgG2a Labeling Kit and the Zenon Alexa Fluor 488 Rabbit IgG
Labeling Kit (Invitrogen), respectively, according to the manufacturer’s instructions. Cells were incubated at RT for 1 hour with each labeled antibody. Cells were also labeled with the isotype control as a negative control. Flow cytometry was performed using BD FACS Calibur (BD Biosciences).

**Immunofluorescence.** Cells were fixed in 4% formaldehyde (Sigma-Aldrich) for 30 minutes at RT, rinsed three times in PBS containing 0.1% Tween 20 (PBST) for 10 minutes, permeabilized in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) for 15 minutes, and blocked for 1 hour in PBS containing 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA). Cells were incubated overnight at 4°C with the following primary antibodies diluted in PBS containing 5% normal goat serum: rabbit anti-ALB (1:50; Dako, Glostrup, Denmark), rabbit anti-AFP (1:200; Dako); rabbit anti-AAT (1:200; Abcam), mouse anti-HNF4A (1:200; Abcam). Cells were rinsed six times in PBST for 10 minutes each. Thereafter, cells were incubated for 1 hour at RT with appropriate secondary antibodies diluted in PBST: Alexa Fluor 488 or 594 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (1:200; Invitrogen). Cells were washed six times in PBST, and mounted in 4’-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich).

**Periodic acid-Schiff (PAS) staining of stored glycogen.** Cells were fixed in 4% formaldehyde for 30 minutes, rinsed three times in PBST for 10 minutes, permeabilized with PBS containing 0.1% Triton X-100 for 15 minutes, and rinsed three times in PBST. Samples were stained using a PAS staining system (Sigma-Aldrich) according to the manufacturer’s instructions and observed under white light using an inverted microscope.

**Acetylated-low-density lipoprotein (Ac-LDL) uptake.** Cells were incubated with 10 μg/ml 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine-labeled Ac-LDL (Life Technologies, Carlsbad, CA, USA) for 5 hours. Red fluorescence was visualized by fluorescence microscopy.

**Enzyme-linked immunosorbent assay (ELISA) of Albumin secretion.** Conditioned medium was collected 24 hours after fresh medium was added and the amount of secreted albumin was measured using a Human Albumin ELISA Quantitation Kit (Bethyl Laboratory, Montgomery, TX, USA) on a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). The mean amount of secreted ALB was measured using 100 μl of conditioned medium from two culture dishes and calculated according to each standard followed by normalization to the protein content. Protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad).

**Urea production.** Conditioned medium was collected 24 hours after fresh medium was added and the amount of secreted urea was analyzed. Urea measurement kits were purchased from BioAssay Systems (Hayward, CA, USA). The experiment was performed according to the manufacturer’s instructions. The amount of secreted urea was calculated according to each standard followed by normalization to the protein content. Protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad).

**Bile canalicular transport.** Cells were washed three times with PBS and incubated with hepatocyte culture medium containing 5 μM 5-(and-6)-carboxy-2’,7’-dichlorofluorescein diacetate (Life Technologies) for 15 minutes. Cells were washed three times and observed by fluorescence microscopy.

**Real-time reverse transcription PCR (RT-PCR)**

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) 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 S1 Table. For comparative quantification, results from real-time PCR were expressed as a relative fold change compared to control cells, after normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ΔCt (ΔACt) value was 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 level was determined using the formula, \(2^{-\Delta\Delta C_t}\).

### Epigenetic analysis

#### Bisulfite sequencing
Genomic DNA was isolated from cells using G-DEX IIc Genomic DNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Korea) according to the manufacturer’s protocol. Bisulfite conversion was performed using the EZ DNA Methylation—Gold Kit (ZYMO RESEARCH, Orange, CA, USA) according to the manufacturer’s protocol. Bisulfite-specific PCR reactions were carried out on a GeneAmp PCR System 9700 (Applied Biosystems) using the following protocol: 95°C for 15 minutes, 50 cycles of 95°C for 20 seconds, 55°C for 40 seconds, 72°C for 30 seconds, and extension at 72°C for 10 minutes. The primer sequences used for PCR are listed in S2 Table. PCR products were purified using the MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology), cloned into pGEM T vector (Promega, Madison, WI, USA), and sequenced using an ABI 3730XL Capillary DNA sequencer (Applied Biosystems). Methylated or unmethylated states of CpG sites were determined from the sequence data by using QUMA (QUantification tool for Methylation Analysis) software [53].

#### Chromatin Immunoprecipitation
Briefly, approximately 1 × 10^6 cells were incubated in cell culture medium containing 1.0% formaldehyde at 25°C for 10 min, and quenched by the addition of 0.125 M glycine for 5 min at 25°C. Cells were harvested by scraping, washed twice in PBS, and three times in ChIP lysis buffer, and resuspended in 200 μl of ChIP lysis buffer containing high salt. Cross-linked chromatin was fragmented by sonication, and pre-cleared with protein A/G PLUS-agarose (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C for 1 h. Each primary antibody was incubated overnight with chromatin at 4°C. Antibodies (Millipore-Upstate, Temecula, CA, USA) used for the ChIP assay were as follows: normal rabbit IgG (#12–370), anti-acetyl-Histone H3 (#06–599), anti-trimethyl-Histone H3 Lys4 (#07–473), anti-trimethyl-Histone H3 Lys27 (#07–449). Immunocomplexes were harvested by incubation with protein A/G PLUS-agarose for 2 h at 4°C. Immunoprecipitates were washed twice with lysis buffer containing high salt, and rinsed four times with wash buffer. Samples were resuspended in elution buffer and incubated at 67°C overnight. DNA samples were isolated using phenol/chloroform extraction, precipitated with ethanol, and resuspended in 50 μl of TE buffer. Quantitative PCR was carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. Triplicate PCR reactions were performed for each sample. The primer sequences used for PCR are listed in S3 Table. ChIP-quantitative PCR results were calculated using the ΔΔCt method. The Ct value of the respective ChIP fraction was normalized against the Ct value of the input DNA fraction (ΔCt). Then, the Ct value of the ChIP fraction was again normalized to the Ct value of the IgG control (ΔΔCt). Fold enrichment of immunoprecipitation was calculated by \(2^{-\Delta\Delta C_t}\).

### Statistical analysis
Data obtained from two or three separate experiments were expressed as mean ± standard deviation (SD), and statistically analyzed by t-test and one-way analysis of variance (ANOVA) using GraphPad Prism software (San Diego, CA, USA). p value lower than 0.05 were considered significant.
Supporting Information

S1 Fig. Methodology used to differentiate human pluripotent stem cells (hPSCs) into hepatocytes. hPSC, human pluripotent stem cell; FGF4, fibroblast growth factor 4; BMP2, bone morphogenetic protein 2; HGF, hepatocyte growth factor; OSM, oncostatin M; Dex, dexamethasone; BSA, bovine serum albumin; SB, sodium butyrate; HCM, hepatocyte culture medium. (TIFF)

S2 Fig. Expression of definitive endoderm and hepatocyte markers. (A) FACS analysis of CXCR4-positive cells was performed 5 days after the onset of differentiation. Blue line: isotype control, red line: primary antibody. (B) Immunofluorescence labeling of albumin (ALB), α-1-antitrypsin (AAT), α-fetoprotein (AFP), and hepatocyte nuclear factor 4 α (HNF4A) was performed at day 20 of differentiation. The scale bar represents 100 μm. (TIFF)

S3 Fig. Histone modifications in regulatory regions of CYP1A1 and CYP1B1 genes. Each diagram shows the locations of the sites of CYP1A1 (A) and CYP1B1 (B) within gene promoters, which were examined by ChIP. ChIP analysis of histone modifications in hPH and hESC-Hep (day 20 of differentiation) is shown in lower graphs. Data validated by real-time PCR are presented as fold enrichment of precipitated DNA associated with a given histone modification relative to a 100-fold dilution of input chromatin. Data represent mean ± SD from two independent experiments. * p<0.05, significant values in comparison with hPH (t-test followed by Wilcoxon matched pairs test). (TIFF)

S4 Fig. Gene expression levels of epigenetic modification enzymes. Expression of genes encoding DNMTs (A), HDACs (B), and Sirtuins (C) was examined by real-time RT-PCR in hPH and hESC-Hep (day 20). Data represent mean ± SD from three independent experiments. * p<0.05, significant values in comparison with hPH (t-test followed by Wilcoxon matched pairs test). (TIFF)

S5 Fig. Characterization of human induced pluripotent stem cells (hiPSCs). (A) Immunofluorescence detection of pluripotency markers including OCT4, SOX2, SSEA4, and TRA-1-60 in hiPSCs was performed at after 4 days culture on feeder cells. Insets show DAPI staining. Scale bar, 100 μm. (B) RT-PCR analysis of endogenous pluripotency marker genes including OCT4, SOX2, CMYC, KLF4, REX1, ECAD, and TERT was examined in hESCs (CHA-hES15), fibroblasts, and hiPSCs. (C) DNA methylation on promoters of pluripotency marker genes including OCT4, REX1, and NANOG was performed by bisulfite sequencing in fibroblasts and hiPSCs. Each row of circles represents the methylation status of each CpG in one bacterial clone. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively. (D) G-banded karyotyping analysis of hiPSCs was performed at passage 31. (E) Teratoma formation of hiPSCs in immunodeficient mice. Hematoxylin and eosin (H&E) staining was performed on formalin-fixed teratoma sections showing ectoderm (a, neural tissue), mesoderm (b, smooth muscle and adipocyte) and endoderm (c, gut) tissues. (TIFF)

S6 Fig. Differentiation of hiPSCs into hepatocytes. (A) Immunofluorescence labeling of ALB, AAT, AFP, and HNF4A was performed at day 20 of differentiation. The scale bar represents 100 μm. (B) FACS analysis of ALB-positive cells was performed 20 days after the onset of differentiation. Blue line: isotype control, red line: primary antibody. (C) Glycogen storage and Ac-LDL uptake in hiPSC-Hep. Periodic acid-Schiff staining of glycogen was performed at day...
20 of differentiation. Stored glycogen (purple) was observed in the cytoplasm. Nuclei (light blue) were counterstained with hematoxylin. The ability of cells to take up Ac-LDL was examined at day 20 of differentiation. The scale bar represents 100 μm. (D) ALB secretion from hiPSC-Hep. The ALB concentration was measured in the conditioned media of hiPSCs (day 0), hiPSC-Hep (day 20), and hPH by an enzyme-linked immunosorbent assay using an anti-human ALB antibody. (E) Urea production by hiPSC-Hep. The amount of urea secreted by hiPSCs (day 0), hiPSC-Hep (day 20), and hPH was examined at 0, 24, and 48 hours.

S7 Fig. Transcriptional regulation of CYP genes by inhibition of DNMTs and HDACs during differentiation of hPSCs into hepatocytes. (A) Expression levels of CYP genes were examined by real-time RT-PCR in hiPSC-Hep treated with DMSO, DAC, or RG108. Data represent mean ± SD. (B) Expression levels of CYP genes were examined by real-time RT-PCR in hiPSC-Hep treated with DMSO or SB with or without DAC or RG108. Data represent mean ± SD. (C) Expression levels of CYP genes were examined by real-time RT-PCR in hESC-Hep treated with DMSO or 2 mM valproic acid (VPA) with or without a DNMT inhibitor (DAC or RG108). Data represent mean ± SD.

S8 Fig. Expression of hepatocyte marker genes by inhibition of DNMTs and HDACs during differentiation of hPSCs into hepatocytes. (A and B) Expression levels of ALB (A) and AAT (B) were examined by real-time RT-PCR in hPSC-Heps treated with DMSO, DAC, RG108, SB, or SB with DAC or RG108. Data represent mean ± SD. (C) Percentages of ALB and AAT positive cells was performed by FACS analysis in hESC-Hep treated with DMSO or SB.

S9 Fig. Transcriptional regulation of CYP1A2 gene by CYP inducer with inhibition of DNMTs. Expression level of CYP1A2 gene was examined by real-time RT-PCR in hESC-Hep (A) and hiPSC-Hep (B) treated with DMSO, DAC, or RG108 at day 15 of differentiation for 5 days and then further treated with 100 μM OME (omeprazole) or 0.5 μM ITE at day 19 of differentiation for 24 hr. Data represent mean ± SD.

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Author Contributions
Conceived and designed the experiments: HJP SY YMH CWS HK. Performed the experiments: HJP YJC JWK HSC II HK. Analyzed the data: HJP HK. Wrote the paper: HJP HK.

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