H3K27me3 Does Not Orchestrate the Expression of Lineage-Specific Markers in hESC-Derived Hepatocytes In Vitro

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SUMMARY

Although pluripotent stem cells can be differentiated into the hepatocyte lineages, such cells retain an immature phenotype. As the chromatin state of regulatory regions controls spatiotemporal gene expression during development, we evaluated changes in epigenetic histone marks in lineage-specific genes throughout in vitro hepatocyte differentiation from human embryonic stem cells (hESCs). Active acetylation and methylation marks at promoters and enhancers correlated with progressive changes in gene expression. However, repression-associated H3K27me3 marks at these control regions showed an inverse correlation with gene repression during transition from hepatic endoderm to a hepatocyte-like state. Inhibitor of Enhancer of Zeste Homolog 2 (EZH2) reduced H3K27me3 decoration but did not improve hepatocyte maturation. Thus, H3K27me3 at regulatory regions does not regulate transcription and appears dispensable for hepatocyte lineage differentiation of hESCs in vitro.

INTRODUCTION

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) are a renewable cell source for the generation of human hepatocytes that could be used for drug toxicity and metabolization studies (Baxter et al., 2010; Mann, 2015; Ulvestad et al., 2013; Zhang et al., 2013; Zhu and Huangfu, 2013). Although many groups (Baxter et al., 2015; Chen et al., 2012; Godoy et al., 2015; Hannan et al., 2013; Shan et al., 2013; Siller et al., 2015; Ulvestad et al., 2013), including ours (Helsen et al., 2016; Roelandt et al., 2012), have generated pluripotent stem cell (PSC)-derived progeny displaying hepatocyte characteristics, these cells are more akin to fetal than post-natal hepatocytes, and are therefore often termed hepatocyte-like cells (HLCs). For instance, HLCs continued to express α-fetoprotein (AFP), a typical fetal hepatocyte marker (Schmelzer et al., 2006).

At the molecular level, cell fate is controlled by epigenetic mechanisms that modulate chromatin structure and thereby control utilization of genetic information (Boland et al., 2014; Meissner, 2010). Changes in chromatin structure are, in part, induced by specific post-translational modifications of nucleosomal and non-nucleosomal histones (Bannister and Kouzarides, 2011; Harshman et al., 2013). Gene activity has been linked to high levels of tri- and dimethylation of histone 3 lysine 4 (H3K4me3/me2) together with binding of RNA polymerase II (POL2RA) at transcriptional start sites (Pokhlokov et al., 2005; Schneider et al., 2004; Schubeler et al., 2004). However, H3K4me3 promoter marking by itself does not predict transcriptional activity (Guenther et al., 2007). In hESCs, for instance, promoters of key developmental control genes are marked with both H3K4me3 and repression-associated histone 3 lysine 27 trimethylation (H3K27me3), resulting in a low transcriptional activity status (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007).

In addition to promoters, enhancers play a critical role in the regulation of tissue-specific gene expression (Heintzman et al., 2009; Ong and Corces, 2012; Wanstad et al., 2012). Enhancer elements are typically located in relatively “accessible” chromatin, i.e., hypersensitive to DNase digestion, often coincide with CpG-poor regions, and act in concert with non-coding RNAs to co-regulate gene expression (Boland et al., 2014; Calo and Wysocka, 2013; Heintzman et al., 2007; Lam et al., 2014; Rada-Iglesias et al., 2011; Stadler et al., 2011; Xie et al., 2013b). Although enhancers are typically decorated with mono-methylated H3K4 marking (H3K4me1), only a fraction of H3K4me1-marked elements are engaged in transcription in a tissue-specific manner (Boland et al., 2014; Calo and Wysocka, 2013; Creighton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011; Visel et al., 2009). In hESCs, poised enhancers harbor H3K4me1 and H3K27me3, while active enhancers are co-marked by H3K4me1 and acetylation of histone 3 lysine...
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27 (H3K27ac) (Calo and Wysocka, 2013; Creyghton et al., 2010; Pekowska et al., 2011).

We hypothesized that mapping histone modifications at promoter and enhancer regions of key pluripotency, hepatic endoderm, and hepatocyte marker genes is likely to provide invaluable information regarding the relevance of epigenetic marking at such regulatory regions during the in vitro hESC-hepatocyte differentiation process that could be used to improve such differentiation protocols. Although a growing number of studies has addressed epigenetic regulation in other cellular contexts, such as for instance β cells (Xie et al., 2013a), neuronal cells (Mikkelsen et al., 2007), or cardiomyocytes (Wamstad et al., 2012), to our knowledge, thus far only one study has been published wherein histone modifications at promoters in the context of hepatocyte differentiation were mapped (Kim et al., 2011).

Here, we examined epigenetic markings in undifferentiated hESCs, hepatocyte nuclear factor α (HNF4A+)-enriched hepatic endoderm cells (HECs), and α1-antitrypsin (AAT+)-enriched HLCs. We also compared the epigenetic status of AAT+ HLCs with uncultured primary human hepatocytes (PHHs). Our studies provide important insights into epigenetic changes that accompany hepatocyte lineage commitment in vitro, and are expected to contribute to the advancement of in vitro generation of mature functional hepatocytes from stem cells.

RESULTS

Alterations in Histone Profile Correlate with Dynamic Changes in Gene Transcription

To address dynamic changes of histone modifications during hepatocyte commitment of hESCs in vitro, we compared histone profiles at promoter and enhancer regions of a selected number of pluripotency, hepatic endoderm, and hepatocyte marker genes throughout differentiation (Figures 1B, 1C, S1A, and S1B). qRT-PCR confirmed the enrichment for transcripts of the selected lineage-specific marker genes in the d8-HNF4A+ or d28-AAT+ cell populations compared with the d8-HNF4A- or d28-AAT- cells, respectively (Figures 1D and 1E). To correlate gene expression with a specific histone profile, we analyzed the enrichment of known active (H3K4me3, H3K4me2, H3K4me1 and H3K27ac) and repressive (H3K27me3) histone marks at promoters and enhancers of the same lineage-specific marker genes by chromatin immunoprecipitation (ChIP)-qPCR in the sorted HNF4A+/HNF4A- and AAT+/AAT- populations (Figures 1F and 1G). To demonstrate the effectiveness and validity of the ChIP-qPCR approach, we first analyzed the histone profile at the promoter region of GAPDH, a housekeeping gene expressed throughout differentiation. As expected, the promoter region was preferentially decorated with H3K4me3/me2 in all cell populations assessed. In contrast, promoters of MYOD1 and HOXD11, not active in hESCs, HECs, or HLCs, were marked with H3K27me3 (Figures S1C and S1D).

H3K27me3 in the Regulatory Regions of Mature Hepatocyte Genes of d28-AAT+ HLCs May Prevent Differentiation to Mature Hepatocytes

We next compared the gene expression and histone profiles at promoters and enhancers of selected hepatic marker genes throughout differentiation (Figures 2A–2E). We also...
compared the gene expression and histone profile in d28-AAT+ sorted cells with PHHs, as this potentially provides important information with which to optimize differentiation protocols (Figures 2A–2E).

As expected, nucleosomes at the GAPDH promoter in hESCs, d8-HNF4A+, and d28-AAT+ cells and PHHs were preferentially marked with H3K4me3/2, while MYOD1 and HOXD11 promoters were marked with H3K27me3 (Figures S2A and S2B).

Transcripts of the transcription factor (TF) HNF4A, which regulates expression of several hepatocyte genes and plays a critical role in liver development, were most highly expressed in d8-HNF4A+ cells, at levels similar to those in PHHs. Levels of H3K27me3 at the HNF4A promoter and enhancer of d8-HNF4A+ cells and PHHs were similar, while active marks (especially H3K27ac; enhancer) were slightly higher in PHHs than in d8-HNF4A+ cells. The lower HNF4A transcript levels in d28-AAT+ compared with d8-HNF4A+ cells correlated with increased H3K27me3 at the HNF4A promoter and enhancer, while levels of H3K4me3/2/1 and H3K27ac were comparable in both cell populations (Figure 2A).

Transcripts for AFP, a typical fetal plasma protein, were highest in d8-HNF4A+ cells and lower in d28-AAT+ cells and PHHs. As observed for HNF4A, a slight increase in active marks at both the AFP promoter and enhancer was observed in d8-HNF4A+ cells compared with hESCs, whereas H3K27me3 levels remained relatively low (Figure 2B). Increased H3K27me3 at AFP promoter/enhancer regions in d28-AAT+ cells accompanied reduced AFP expression, while active marks remained unchanged (Figure 2B). Of note, H3K27me3 levels at the AFP promoter/enhancer regions in PHHs were even lower than in d28-AAT+ cells, even though the expression was lower in PHHs compared with d28-AAT+ cells (Figure 2B).

Transcripts of ALB, an important serum protein synthesized by the liver, were significantly induced throughout differentiation (Figure 2C). No changes in repressive and active marking at the ALB promoter and enhancer were seen between hESCs and d8-HNF4A+ cells. As expected, active marks (H3K4me3 but not H3K27ac) at the ALB enhancer and promoter were higher in d28-AAT+ than in d8-HNF4A+ cells. Unexpectedly, higher levels of H3K27me3 were found at the promoter and enhancer of ALB in 28-AAT+ compared with d8-HNF4A+ cells (Figure 2C).

Transcripts for AAT, another serum protein synthesized by the liver, were also significantly induced throughout differentiation (Figure 2D). In hESCs and d8-HNF4A+ cells, active and repressive marks at the AAT promoter and enhancer were low (Figure 2D). Compared with d8-HNF4A+ cells, in 28-AAT+ cells the AAT promoter was highly enriched for H3K4me3/2 but not H3K27ac, and the AAT enhancer also contained more active marks (H3K27ac and H3K4me3). However, similar to the ALB regulatory regions, H3K27me3 at the AAT promoter/enhancer was significantly higher in d28-AAT+ than in d8-HNF4A+ cells (Figure 2D). Enrichment for active marks at the promoter and enhancer of AAT d28-AAT+ cells and PHHs appeared to correlate best with increased expression, whereby histone methylation was more prominent at the AAT promoter, and histone acetylation at its enhancer. Again, H3K27me3 marking did not correlate with transcriptional activity.

In line with the fact that HLCs remain immature, transcript levels of CYP3A4, responsible for metabolism of ±50% of drugs, were very low in d28-AAT+ cells compared with PHHs. In PHHs, an active chromatin status of the CYP3A4 promoter and enhancer was seen (Figure 2E). In d28-AAT+ HLCs, active marks in the CYP3A4 promoter and enhancer were not significantly different from PHHs. However, levels of H3K27me3 were higher in d28-AAT+ cells compared with PHHs (Figure 2E).

Comparing d28-AAT+ cells and PHHs demonstrated that transcript levels for all lineage-specific genes were 95 times lower (95% CI 62–99) in hESC-derived HLCs compared with PHHs, confirming the immaturity of the HLCs derived in vitro (Figure S2D) (Baxter et al., 2015; Godoy et al., 2015). Furthermore, we found that lower expression levels in d28-AAT+ cells were associated with higher H3K27me3 and lower active marking at both promoters and enhancers of all marker genes tested when compared with the PHHs (Figure S2D). This suggests that inducing a more active histone profile will be necessary to create more mature hESC-derived HLCs in vitro.

Thus, we observed a good correlation between transcriptional activity and active marks at the regulatory regions of hepatic endoderm, and early and more mature hepatocyte
Figure 3. mRNA Transcript, Protein Levels, and Functional Analysis of hESC-Derived HLCs Treated with or without DMSO

(A) Relative gene expression (to GAPDH, log scale) analysis represented in a heatmap of pluripotent, fetal hepatic, mature hepatic, drug transporter, and hepatic TF marker genes in cells treated with or without DMSO, FHs, and PHHs.

(B) Representative FACS plots for AAT (right panels) and isotype controls (left panels) on d28-hESC-derived HLCs obtained in the presence or absence of DMSO.

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marker genes in hESCs, d8-HNF4A+ cells, d28-AAT+ cells, and PHHs. However, higher H3K27me3 marks at promoters and enhancers of ALB, AAT, and CYP3A4 in d28-AAT+ compared with d8-HNF4A+ cells did not correlate with the higher transcript levels for ALB and AAT in d28-AAT+ cells. Immunoblotting confirmed the global increase in cellular H3K27me3 in d28-AAT+ compared with d8-HNF4A+ cells (Figure S2C). Of note, PHHs expressed significantly higher levels of all hepatic marker genes compared with d28-AAT+ cells, which correlated with higher active and lower inhibitory marks at both regulatory regions. Thus, the continued presence of H3K27me3 at regulatory regions of hepatocyte genes during in vitro differentiation may be responsible for suboptimal maturation of HLCs, suggesting that removal of this mark might be needed to attain full HLC maturation.

**DMSO Improves hESC-Derived HLC Homogeneity, but Not HLC Maturation, with Persistent H3K27me3 on Regulatory Regions of Hepatocyte Marker Genes**

As DMSO may improve differentiation of stem cell-derived hepatocytes (Basma et al., 2009; Czyz et al., 2015; Duan et al., 2010; Hay et al., 2008; Kanebratt and Andersson, 2008; Kondo et al., 2014; Szkolnicka et al., 2014; Ulvestad et al., 2010; Hay et al., 2008; Kanebratt and Andersson, 2008; Kondo et al., 2014; Szkolnicka et al., 2014; Ulvestad et al., 2013), we reassessed histone modifications at regulatory regions of hESC progeny from cultures containing DMSO. Addition of 0.6% DMSO throughout the differentiation protocol resulted in increased expression of the mature hepatocyte genes AAT, CYP3A4, CYP2A6, G6PC, PEPCK, and APOA1, and the mature hepatocyte TFs HNF3β, HNF3G, CEBPA, and PROX1 on day 28 (Figure 3A). Nevertheless, the expression profile of a broad panel of fetal markers (AFP, CYP3A7, and GSTR) and mature hepatocyte TFs, genes, and drug transporters of d28-HLCs from DMSO-treated cultures resembled fetal, not mature hepatocytes (Figure 3A), in line with other publications (Baxter et al., 2015; Chen et al., 2012; Godoy et al., 2015; Hannan et al., 2013; Shan et al., 2013; Siller et al., 2015; Ulvestad et al., 2013).

In d28-HLCs, the yield of AAT+ and HNF4A+ cells is ±70% and ±50%, respectively, compared with ±50% AAT+ cells and ±30% HNF4A+ cells in HLCs from cultures without DMSO (Figures 3B and 3C). Addition of DMSO also significantly enhanced CYP3A4 activity by 8- to 10-fold (Figure 3D). To gain insights into the mechanism underlying the increased frequency of HNF4A+ and AAT+ cells on days 8 and 28, respectively, and the increased expression of hepatocyte markers in DMSO-treated cultures, we followed the expression of OCT4, HNF4A, AFP, ALB, AAT, and CYP3A4 throughout differentiation in the presence or absence of DMSO. Improved expression of hepatocyte transcripts in response to DMSO was only seen on days 20 and 28 (Figure 3E).

We next isolated AAT+ cells from DMSO-treated cultures on day 28, and compared their expression profile with AAT+ cells from cultures without DMSO. Transcript levels for nearly all genes tested were not statistically different (Figure 4C). Thus, DMSO did not appear to enhance the maturation of HLCs. However, qRT-PCR (Figure S3B) and FACS/immunocytochemistry (ICC) analysis (Figures S3C and S3D) on d4-progeny demonstrated that DMSO significantly increased the fraction of definitive endoderm cells (CXCR4+/C-kit+ and SOX17+ cells), which might be responsible for the increased homogeneity of d28-HLCs.

We compared the gene expression and epigenetic marks for OCT4, HNF4A, AFP, ALB, AAT, and CYP3A4 in d8-HNF4A+ and d28-AAT+ enriched populations from DMSO-treated cultures, with results obtained in the absence of DMSO (Figures 1 and 2). Differences in transcript levels were not detected in d8-HNF4A+ cells from cultures with or without DMSO (Figure 4A) and in H3K27me3 marks at promoters and enhancers of the hepatic marker genes. A moderate increase in H3K27ac and H3K4me3/2 appeared to be responsible for the increased homogeneity of d28-HLCs. Nevertheless, the results obtained for d8-HNF4A+ cells, in DMSO-treated d28-AAT+ cells neither transcript levels (Figure 4C) nor H3K27me3 enrichment at promoters and enhancers of ALB, AAT, and CYP3A4 (Figure 4D) were altered compared with d28-AAT+ cells from cultures without DMSO. This further indicates that DMSO does not enhance HLC maturation. Moreover, global H3K27me3 levels were higher in d28-DMSO-treated cells (Figure S4C). Interestingly, total H3 levels were lower in d28-AAT+ cells differentiated in the presence of DMSO (Figures S4A and S4B). In addition, H3K27ac and H3K4me3/2 appeared to be lower at the regulatory regions of ALB, AAT, and CYP3A4 of d28-AAT+ cells derived from cultures with DMSO (Figure 4D). However, we detected an increased presence of POL2RA at nearly all

(C) Representative immunofluorescence images for HNF4A, AFP, ALB, AAT (magnification 20 ×; scale bar, 50 μm) on day 20 for cells treated without or with DMSO.

(D) Functional CYP3A4 activity in non-treated (black) or DMSO-treated (white) HLCs.

(E) Relative gene expression (to GAPDH) for OCT4 and hepatic markers at different time points (days 4, 12, 20, and 28) of differentiation with (white) or without (black) DMSO.

Data, except for (C), represent mean ± SEM of n ≥ 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by Student’s t test. See also Figure S3.
gene promoters and enhancers in AAT+ cells derived from DMSO cultures (Figure 4D).

Thus, addition of DMSO during hepatocyte differentiation improved commitment to definitive endoderm, which correlated with a more homogeneous population of d28-AAT+ HLCs. However, none of the transcripts and epigenetic marks at the promoter and enhancer of immature and mature hepatocyte marker genes in d8-HNF4A+ HECs and d28-AAT+ HLCs were affected by addition of DMSO. One exception was the lower levels of active marks in mature hepatocyte gene regulatory regions of DMSO-treated d28-AAT+ cells. However, we observed higher POL2RA levels and lower total H3 levels in nearly all gene promoters and enhancers. Although we cannot formally exclude the possibility that some loci, particularly during early differentiation, are sensitive to global or local alteration of acetylation, this observation may suggest that DMSO affects differentiation in general by altering chromatin accessibility in a more global fashion, as suggested by Lapeyre and co-workers (Lapeyre and Bekhoe, 1974; Stratling, 1976).

**EZH2 Inhibition Does Not Improve In Vitro Hepatocyte Differentiation from hESCs**

As we consistently found high levels of H3K27me3 at promoters and enhancers of d28-AAT+ cells compared with PHHs (Figures 2A–2E), we hypothesized that removal of H3K27me3 might allow maturation of HLCs to a more mature PHH phenotype. As EZH2 is the histone methyltransferase that catalyzes H3K27 trimethylation (Figure 5A) (Verma et al., 2012), we supplemented the culture medium of hESCs and d28-AAT+ cells with an inhibitor against EZH2 (GSK-343, hereafter EZH2i) to reduce H3K27me3 levels, between days 6 and 16 when levels of H3K27me3 marks at marker gene promoters and enhancers increased (Figures 5B, 2A–2E, and S2C). An additional rationale to explore the role of EZH2 was provided by the observation that EZH2i transcripts were higher in d28-hESC progeny than in PHHs. Likewise, mRNA levels of some TFs and functional genes were at significantly lower levels in d28-hESC progeny than in PHHs. Likewise, mRNA levels of some TFs and functional genes were also lower than in hepatocytes from embryos between 20 and 38 weeks.

As epigenetic regulatory mechanisms control DNA-templated activities, including gene transcription, we hypothesized that evaluating the nature and dynamics of hPSC-derived hepatocytes are an attractive, alternative cell system for PHHs to test the safety, efficacy, and metabolization of new chemical entities. However, to date most hPSC differentiation protocols yield cells with phenotypic characteristics of fetal rather than mature hepatocytes. Multiple studies demonstrated that hPSC-derived hepatocytes show inferior drug-metabolizing enzyme activity/expression (e.g., CYP3A4 and CYP2A6), and lower levels of transporter proteins (e.g., NTCP or MRP2), and continue to express AFP, a typical fetal hepatocyte marker (Baxter et al., 2015; Chen et al., 2012; Godoy et al., 2015; Hannan et al., 2013; Shan et al., 2013; Siller et al., 2015; Ulvestad et al., 2013). Consistently, we found that d28-hESC progeny continue to express AFP, and that transcripts for numerous primary hepatocyte TFs and functional genes were at significantly lower levels in d28-hESC progeny than in PHHs. Likewise, mRNA levels of some TFs and functional genes were also lower than in hepatocytes from embryos between 20 and 38 weeks.

As epigenetic regulatory mechanisms control DNA-templated activities, including gene transcription, we hypothesized that evaluating the nature and dynamics of
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epigenetic changes at gene regulatory regions during hepatocyte commitment of hESCs and comparing differences between HLCs and PHHs might identify important hurdles to be overcome to generate more mature hepatocytes from PSCs. Therefore, we investigated histone marking at gene promoters and enhancers of a number of key genes expressed during hepatocyte differentiation. To avoid assessing histone marks in mixed hESC progeny, we isolated pure fractions of d8-HNF4A+ and d28-AAT+ cells. In accordance with an earlier report, histone marks associated with transcriptional activity at the promoters of the HNF4A and AFP genes in d8-HNF4A+ cells correlated with the gene transcriptional status (Kim et al., 2011). We further demonstrated that this correlation was also true for the enhancers. In addition, active H3K4me2/me3 and H3K27ac at ALB and AAT gene promoters and enhancers clearly coincided with increased expression of these genes between days 8 and 28. Surprisingly, we observed that H3K27me3 at promoters and enhancers of hepatoblast/hepatocyte marker genes were significantly higher in d28-AAT+ HLCs than in d8-HNF4A+ HECS, even though transcript levels for these genes increased significantly by day 28, even if they remained significantly lower than in PHHs. These results contradict earlier published results (Kim et al., 2011). A recent study on in vivo endocrine progenitor development also demonstrated gain of H3K27me3 at regulatory regions of genes involved in differentiation and morphogenesis (Xu et al., 2014).

Persistent marking of regulatory gene regions by H3K27me3 despite increased gene transcription supports the notion that H3K27me3 might not be the only determinant of gene activity. A similar finding has been reported recently in the context of transcriptional activation of the immediate-early gene ATF3, in response to serum stimulation: ATF3 induction occurred in the presence of the presumed repressive H3K27me3 and did not require removal of the mark (Prickaerts et al., 2012).

We also compared histone modifications in d28-AAT+ HLCs with those in uncultured PHHs to determine whether specific epigenetic marks might be responsible for the incomplete maturation of HLCs. This revealed that active marks in hepatocyte marker gene promoters and enhancers were similar in d28-AAT+ HLCs and PHHs, while H3K27me3 marks were significantly higher in d28-AAT+ cells compared with PHHs. This suggested that H3K27me3 marking in part explains the apparent inability to generate fully mature hepatocytes from hESCs.

Multiple studies have used DMSO to improve or maintain hepatocyte maturity, by for instance maintaining or increasing expression of nuclear receptors, hepatocyte-specific TFs, and drug-metabolizing enzymes (Kanebratt and Andersson, 2008; Nishimura et al., 2003; Su and Waxman, 2004). Therefore, DMSO has been included in hPSC hepatocyte differentiation cultures (Basma et al., 2009; Czyz et al., 2015; Duan et al., 2010; Hay et al., 2008; Kondo et al., 2014; Szkolnicka et al., 2014; Ulvestad et al., 2013). As DMSO appears to improve PSC-hepatocyte differentiation, we repeated the differentiation protocol in the presence of 0.6% DMSO. DMSO generated, in a much more robust way, definitive endoderm as was reported before (Chetty et al., 2013). This was associated with a significantly more homogeneous population of d28-HLCs. However, stage-specific transcripts for hepatic endoderm markers in d8-HNF4A+ cells and hepatocyte markers in d28-AAT+ sorted cells from cultures with or without DMSO were not significant different, and transcripts in d28-AAT+ cells from DMSO-containing cultures remained significantly lower than in PHHs. H3K27me3 at hepatoblast/hepatocyte marker gene promoters and enhancers were also not reduced in response to DMSO in d8-HNF4A+ cells and d28-AAT+ cells. Consistent with the function of DMSO as an HDAC, H3K27ac marks at the enhancers of marker genes were increased.

Fetal hepatocyte progenitors express EZH2, the histone lysine methyltransferase responsible for H3K27 trimethylation, and EZH2 is required to allow hepatocyte progenitor...
expansion in vivo (Koike et al., 2014). Conversely, knockdown of EZH2 in embryonic murine hepatic progenitors was reported to promote their differentiation to hepatocytes, with an upregulation of several transcriptional regulators of hepatocyte differentiation (Aoki et al., 2010). Similar findings have also been published for endocrine pancreas differentiation (Aoki et al., 2010; Xu et al., 2014). Differentiation of endocrine pancreatic cells to mature β cells in vitro becomes possible after grafting the cells in vivo, and this is associated with complete removal of Polycomb group-mediated repression at stage-specific genes (Xie et al., 2013a). As EZH2 levels were significantly higher in d28-AAT+ cells than in PHHs, we tested whether inhibition of EZH2 would allow further HLC maturation via reduction of H3K27me3. Even if inhibition of EZH2 reduced H3K27me3 at both regulatory regions of hepatocyte genes by 50%, gene expression and protein levels of mature hepatocyte markers did not change. It is thus possible that removing only 50% of the H3K27me3 from HLCs is insufficient to allow full maturation to a PHH state. Alternatively, it is also possible that the impact of the repression-associated H3K27me3 marking may be reinforced by other repressive histone modifications in hESC progeny.

In summary, we demonstrate that the in vitro differentiation of hESC-derived cells to hepatocytes is accompanied by dynamic epigenetic regulation. Although hESC-derived HLC promoter and enhancer decoration with active epigenetic marks resembles that in PHHs, repressive H3K27me3 marks at hepatocyte marker gene promoters and enhancers in HLCs did not reflect histone marking observed in uncultured PHHs. Modulation of promoter and enhancer H3K27me3 marking by inhibition of EZH2 activity did not yield enhanced hepatocyte maturation. This suggests that H3K27me3 is not solely responsible for the lack of maturation of ESC progeny, but may be reinforced by other repressive histone modifications. Future studies will be needed to gain insights into the functional links between signaling events in the hepatocyte environment that support the establishment of an epigenetic state more akin to that of PHHs, thus to optimize differentiation protocols aimed at generating functional hepatocytes from hPSCs.

**EXPERIMENTAL PROCEDURES**

**hESC Differentiation to the Hepatocyte Lineage**
The hESC H9 line (WAO9, WiCell) was cultured on inactivated mouse embryonic fibroblasts (iMEF) as described by Thomson et al. (1998). Experiments were performed with approval from the Medical Ethics Committee (UZ Leuven, Gasthuisberg). Hepatocyte differentiation was done as described earlier with minor adaptations (Roelantd et al., 2013) (Figure 1A). For the DMSO-treated cell culture condition, cells were supplemented from days 0 to 28 to 0.6% DMSO (Sigma-Aldrich). EZH2 inhibition was induced in the cultures by administration of 1 µM GSK-343 (Sigma-Aldrich, SML0766) from days 6 to 16 by changing media completely every 2 days. All growth factors were purchased from PeproTech.

**FACS**
Cells were harvested with 0.05% trypsin-EDTA (d8) or liberase (d28) (Roche), fixed with 1% ice-cold formaldehyde (Fluka) for 10 min at room temperature, and quenched with 0.125 mM glycine for 5 min at room temperature. Fixed cells were permeabilized and blocked with 10% goat serum (Dako)/0.1% saponin/PBS for 45 min at room temperature, followed by incubation with 1 µg/ml/10⁶ cells anti-HNF4A and anti-AAT primary antibodies (Abs) or isotypes in 1% goat serum/PBS for 1 hr at room temperature. Data were analyzed with FACS Diva Software (BD Biosciences). Cells were stored at −80°C.

**RNA Extraction from FACS-Sorted Cells**
Cells were fixed with 1% ice-cold formaldehyde (Fluka) for 10 min at room temperature, and quenched by adding 0.125 mM glycine for 5 min supplemented with 1:125 RNasin Plus RNase Inhibitor (Promega). All the subsequent steps were carried out as described above under FACS. Cells were washed with PBS containing 1:200 RNasin Plus RNase Inhibitor. RNasin Plus RNase Inhibitor (1:60) was added to the permeabilization/blocking, primary, and secondary solutions. Following secondary Ab staining cells were washed twice in PBS, and resuspended in PBS with 0.5% goat serum and 1:100 RNasin Plus RNase Inhibitor. Cells were recovered by centrifugation at 2,200 × g for 15 min at 4°C. To reverse the formaldehyde crosslinking, cell pellets were incubated in 200 µL of 200 mM NaCl, 10 mM Tris- HCl (pH 8), 1 mM EDTA, 1% SDS, and 1:200 RNasin Plus RNase Inhibitor for 2 hr at 65°C (Jeyapalan and Sedivy, 2013). An equal amount volume of TRIzol reagent (Ambion) was added. RNA was extracted according to the TRIzol manufacturer’s instructions.

**ICC for Pluripotent and Hepatic Markers**
Cells differentiated on glass slides were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS (PBS-T), blocked with 0.2% PBS-T supplemented with 5% normal donkey serum (Jackson Laboratory), and stained overnight at 4°C with OCT4 (0.4 µg/mL), HNF4A (5 µg/mL), AFP (4.5 µg/mL), ALB (2.5 µg/mL), AAT (3.95 µg/mL), or the relevant isotype control Abs in Dako diluents (Dako) (Table S1). Immune complexes were detected by incubation with a species-specific AF555-conjugated immunoglobulin G (4 µg/mL, Alexa Fluor, Molecular Probes) for 30 min at room temperature. The nuclei were visualized and the relevant isotype control Abs in Dako diluents (Dako) (Table S1). Immune complexes were detected by incubation with a species-specific AF555-conjugated immunoglobulin G (4 µg/mL, Alexa Fluor, Molecular Probes) for 30 min at room temperature. The nuclei were visualized using Hoechst (Sigma-Aldrich). Signals were detected with an Axioimager.Z1 microscope (Carl Zeiss) using extended focus computation from z stacks. The
percentage of positive cells on days 0, 4, and 8 was manually counted on five representative 10x images. Differently for AFP, ALB, and AAT on day 28, which were also detected surrounding the cells, the percentage of positive signal was based on surface area measurements using the measurement module software (Carl Zeiss). For all pictures, the percentage of positive cells was contoured above the isotype level and three different differentiations were averaged.

**ChiP**

ChiP was performed using 3 x 10^5 or 6 x 10^5 cells per IP, respectively, for histone modifications (H3K27me3, H3K27ac, and H3K4me3/me2/me1) and POL2RA. IPs were processed as described previously (Pistoni et al., 2010). Dynabeads (Life Technologies) were added and DNA purified using a QIAquick PCR purification kit (Qiagen). Purified DNA was analyzed by qPCR. Identification of promoter and enhancer regions was based on literature research and the Encyclopedia of DNA elements (ENCODE) database, available online. Sequences of the primers and ChiP-grade Abs used for ChiP are listed in Tables S2 and S3. The ChIP-qPCR data were presented as percent input/total H3: for the histone modifications we calculated by 2^(-ΔΔCt Histone modification / C0) - 10% input)) /2^(-ΔΔCt total H3 - ΔΔCt 10% input) and for POL2RA we calculated by 2^(-ΔΔCt POL2RA - ΔΔCt POL2RA / C0) - 10% input)) /2^(-ΔΔCt total H3 - ΔΔCt 10% input).

**Statistical Analysis**

Comparisons between two data groups (with n ≥ 3 independent experiments) were analyzed using an unpaired two-tailed Student’s t test (GraphPad Prism 5) (De winter, 2013). p Values of less than 0.05 were considered significant and are indicated in the graphs as *p < 0.05, **p < 0.01, or ***p < 0.001. All data represent the mean ± SEM (n = 3) or mean ± SD (n = 2). To investigate the relationship between cell population (d8-HNF4A+ versus d8-HNF4A− cells and d28-AAT+ cells versus PHHs) and histone enrichment via gene expression levels, a mediation analysis was performed (Krull and MacKinnon, 1999). This involved a regression of gene expression level on cell population, and regression models of each histone modification on gene expression levels. In all models, gene was used as a repeated measure to obtain relationships averaged over all genes, and gene and histone levels were log transformed due to heavy skewness. The indirect effect of cell population on gene expression for each histone modification was then obtained by combining the models using the Sobel method (Krull and MacKinnon, 1999).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.06.013.

**AUTHOR CONTRIBUTIONS**

J.V., M.P., and C.M.V. conceived, designed, and planned the study, generated the data, interpreted the experiments, and wrote the manuscript. M.W. contributed to the maintenance of the hepatic differentiation cultures and analysis of the epigenetic and gene expression profiles. K.E. and V.S. contributed to the immunocytochemical characterization of the differentiation. N.H. contributed to the optimization of differentiation protocol with DMSO. R.B. helped with the interpretation of data. M.N. and E.S. performed the isolation and characterization of the human hepatocyte samples. P.C. and J.W.V. provided scientific discussions, helped with data interpretation, and contributed to writing the manuscript. All of the authors have read and edited the manuscript.

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