Stem Cell Reports
Report

ISSCR

OPEN ACCESS

Functional Blood Progenitor Markers in Developing Human Liver Progenitors

Orit Goldman,[1] Idan Cohen,[1] and Valerie Gouon-Evans[1,*]
[1]Department of Developmental and Regenerative Biology, Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
*Correspondence: gouonevans@gmail.com
http://dx.doi.org/10.1016/j.stemcr.2016.07.008

SUMMARY

In the early fetal liver, hematopoietic progenitors expand and mature together with hepatoblasts, the liver progenitors of hepatocytes and cholangiocytes. Previous analyses of human fetal livers indicated that both progenitors support each other's lineage maturation and curiously share some cell surface markers including CD34 and CD133. Using the human embryonic stem cell (hESC) system, we demonstrate that virtually all hESC-derived hepatoblast-like cells (Hep cells) transition through a progenitor stage expressing CD34 and CD133 as well as GATA2, an additional hematopoietic marker that has not previously been associated with human hepatoblast development. Dynamic expression patterns for CD34, CD133, and GATA2 in hepatoblasts were validated in human fetal livers collected from the first and second trimesters of gestation. Knockdown experiments demonstrate that each gene also functions to regulate hepatic fate mostly in a cell-autonomous fashion, revealing unprecedented roles of fetal hematopoietic progenitor markers in human liver progenitors.

INTRODUCTION

The liver bud in the mouse embryo is formed from the foregut endoderm at around embryonic day 9.5 (E9.5) by the migration into the septum transversum of the fetal liver progenitors, hepatoblasts expressing the hepatic markers α-fetoprotein (AFP), and albumin (ALB). Hepatoblasts proliferate considerably to form the fetal liver mass and finally differentiate in midgestation into either hepatocytes or cholangiocytes based on their proximity to portal veins (Gordillo et al., 2015). In mammals, the fetal liver is also the major site of hematopoiesis (Golub and Cumano, 2013). Murine liver hematopoiesis is initiated at E10 with the colonization of the fetal liver by hematopoietic progenitors migrating from the yolk sac and the region of the aorta-gonad-mesonephros. The fetal liver hematopoietic activity decreases around E15 and disappears shortly after birth.

Surprisingly, few studies on human fetal livers have reported that the hematopoietic progenitor markers CD34, CD117, CD90, CD133, and CD44 are also expressed on a subset of human hepatoblasts and/or precursors of hepatoblasts depending on the embryonic stage examined (Table S1). Most of the EpCAM+ hepatoblasts express CD133 and CD44 in the second trimester of gestation (Schmelzer et al., 2007). Co-expressions of CD117 and AFP or CD117 and ALB in hepatoblasts are detected at around 14 weeks and represent about 2% and 1% of total cells, respectively (Nava et al., 2005). A subset of CD117+ cells that co-express CD34 can turn on the hepatic markers ALB and CK19 when further cultured in vitro suggesting the presence of CD177+CD34+ precursors of hepatoblasts (Nava et al., 2005; Nowak et al., 2005). Similarly, human fetal liver multipotent progenitor cells have been identified from fetal livers from first and second trimesters (Lazaro et al., 2003); they express CD34 and CD44 and differentiate into ALB+ glycogen+ hepatoblasts, CK7+GGT+CK19+ biliary cells, and mesenchymal cells (Dan et al., 2006). These studies indicate that some hematopoietic progenitor markers are surprisingly also expressed on developing hepatoblasts and/or precursors of hepatoblasts in human fetal livers.

Our previous work has established an efficient protocol to generate functional hepatoblast-like cells (referred to as hepatic cells or Hep cells) from human embryonic stem cell (hESC) differentiation cultures that express endoderm and hepatic markers including FOXA2, HNF4α, AFP, ALB, CK18, and EpCAM (Goldman et al., 2013). Here, we characterize expression kinetics of hematopoietic progenitor markers in Hep cells as they specify from the endoderm. We demonstrate dynamic expression patterns for the hematopoietic progenitor markers CD34, CD133, and GATA2 in developing Hep cells, and confirm these findings in vivo with analyses of human fetal livers collected in the first and second trimesters of gestation. Knockdown of CD34, CD133, and GATA2 revealed their impact on hepatic specification of Hep cells mostly in a cell-autonomous fashion. This study highlights the powerful utility of the hESC differentiation system to recapitulate early human hepatic specification and has uncovered the functional impact on hepatic specification and maturation of hematopoietic progenitor markers expressed in human hepatoblasts.

RESULTS AND DISCUSSION

Co-expression of Hematopoietic Progenitor Markers CD34, CD133, and GATA2 in Developing hESC-Derived Hep Cells

Endoderm-derived Hep cells were generated from hESC differentiation cultures as previously described (Goldman et al., 2013). Activin A-induced endoderm cells were

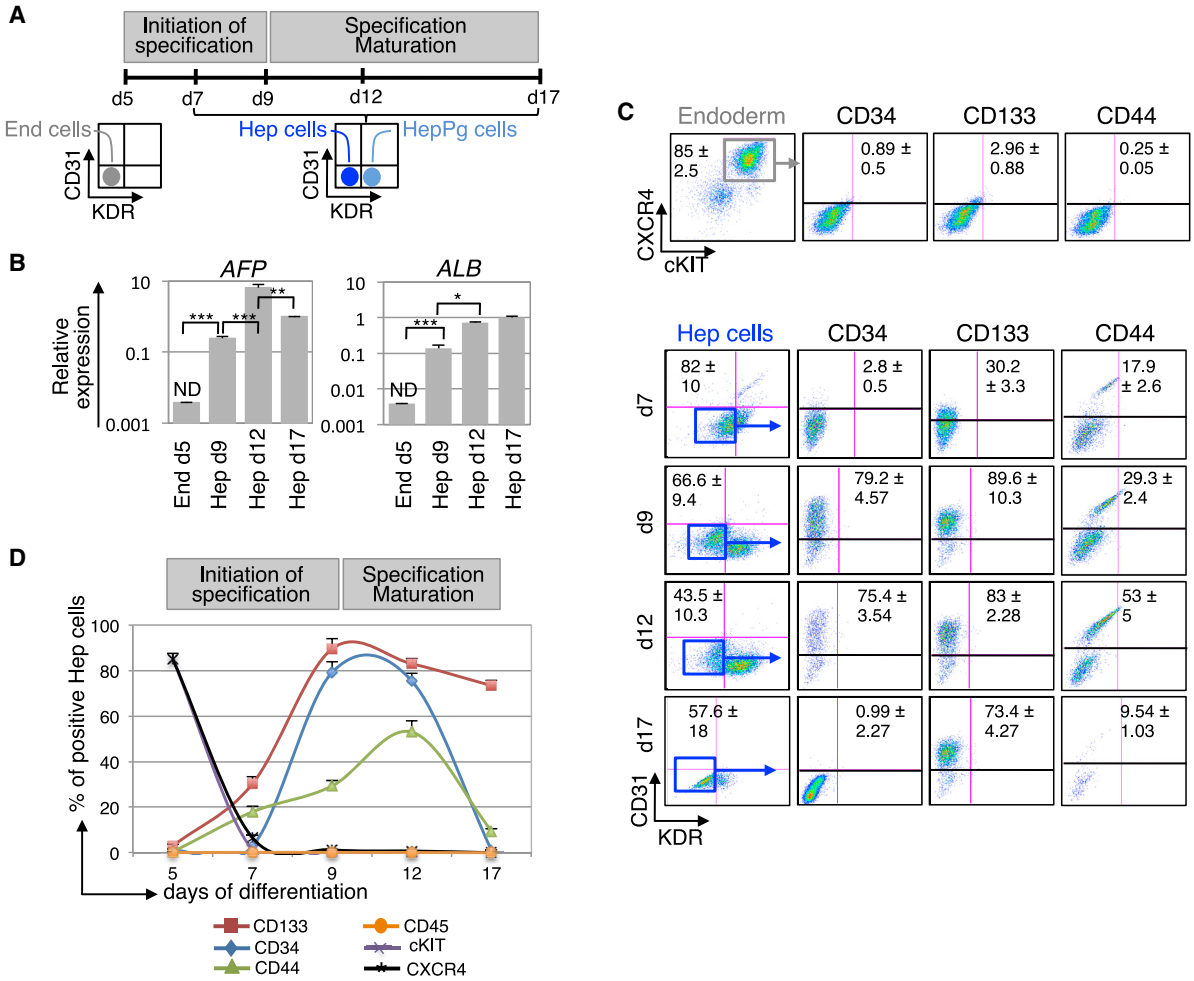

Figure 1. Expression of Cell Surface Hematopoietic Progenitor Markers in Hep Cells

(A) Timeline of generation of day 5 hESC-derived endoderm (End cells) and their progeny Hep and HepPg cells.

(B) Relative transcript levels in purified day 5 End cells and KDR−CD31− Hep cells purified at days 9, 12, and 17. All numbers reflect the mean ± SD for n independent differentiations (n = 3). Transcript levels from day 17 Hep cells were set to 1.

(C) Flow cytometry analysis of day 5 End cells and KDR−CD31− Hep cells from days 7 to 17 (n = 12 for CD31 and KDR and n = 3 for CD34, CD133, and CD44).

(D) Graph summarizing the flow cytometry data shown in (C) and Figure S1B.

*p < 0.05, **p < 0.01, and ***p < 0.001. ND, not detectable (cycle number above 40). See also Figure S1.

purified at day 5 of differentiation within the cell population positive for CXCR4 and cKIT and negative for the mesendoderm marker PDGFRα and the mesoderm marker KDR/VEGFR2/Flk-1. Purified endoderm cells were then cultured in hepatic medium that favors hepatic specification. We previously demonstrated that Hep cells develop concomitantly with an endoderm-derived hepatic progenitor population (referred to as HepPg cells) that surprisingly expresses KDR (Goldman et al., 2013). Hep and HepPg cells have a distinct cell-surface-marker profile with Hep cells being KDR−CD31− and HepPg cells being KDR+CD31− (Goldman et al., 2013) (Figure 1A). Both populations derive from day 5 KDR−CD31− endoderm cells (Figure 1A). At day 7 of differentiation, the KDR−CD31− cell population is heterogeneous and composed of Hep cells and some remaining unspecified endoderm cells. By day 9, new KDR+CD31− HepPg cells developed from the unspecified endoderm cells, and the ratio between KDR−CD31− Hep cells and KDR+CD31− HepPg cells reaches about 50% and remains in this range until day 17 (Goldman et al., 2013). Both Hep and HepPg cells express endoderm (FOXA2, GATA4), epithelial (CK18), and early hepatic commitment (HNF4α) markers, while specific hepatic genes (AFP, ALB, P450 enzymes, α1-antitrypsin) are only present in Hep cells (Goldman et al., 2013). Day 13–16 Hep cells are mature enough to functionally support hepatitis C virus replication

(Goldman et al., 2013). Moreover, HepPg cells have the ability to differentiate into functional Hep cells when grown in aggregates (Goldman et al., 2013). Based on our previous and present extensive characterization of Hep cells, we defined two main stages of hepatic differentiation: initiation of hepatic specification from days 5 to 9, and further specification and beginning of maturation from days 9 to 17 (Figure 1A). Initiation of hepatic specification is illustrated by the detection of *AFP* and *ALB* transcripts in KDR−CD31− Hep cells purified at day 9 (Figure 1B). Expressions of α1-antitrypsin (*AAT*), *CYP3a7* and *CYP3a4* are detected at day 12 and increase with time supporting further Hep cell specification and maturation beyond day 9 (Figure S1A). Hep cell maturation is also indicated with a significant increase of *ALB* levels from days 9 to 12 and decreasing levels of *AFP* from days 12 to 17 (Figure 1B), although day 17 Hep cells remain immature when compared with crude adult human liver samples (Figure S1A).

Given the growing evidence that fetal hepatoblasts from human liver specimens express hematopoietic progenitor markers (Table S1), we investigated whether the hESC-derived hepatoblast-like Hep cells generated in vitro recapitulate a hematopoietic signature as they initiate hepatic specification (days 5–9) and further specify and begin maturation (days 9–17). We examined expression of the hematopoietic progenitor markers CD34, CD133, and CD44 as well as CD45, known to be present on the vast majority of differentiated hematopoietic cells. Day 5 purified CXCR4+cKIT+KDR−PDGFRα− endoderm cells were negative for all markers (Figures 1C, 1D, and S1B). However, expressions of CD34, CD133, and CD44 were rapidly detected in emerging Hep cells as they committed to a hepatic fate to finally peak at day 9 for CD34 and CD133, and at day 12 for CD44 (Figures 1C and 1D). It is noteworthy that almost all Hep cells at day 9 co-expressed both CD34 and CD133. These numbers progressively decreased for CD34 and CD44 as Hep cells began to mature at day 17, while high percentages of CD133+ cells were maintained (Figures 1C and 1D). Given that CXCR4 and cKIT are expressed on hematopoietic progenitor cells (Table S1), we investigated their expression in Hep cells. Expressions of CXCR4 and cKIT were rapidly downregulated by day 7 (Figure S1C), which was consistent with the absence of CXCR4 and cKIT expression in most hepatoblasts from human fetal livers. cKIT expression is indeed restricted to a small subset of CD34+ hepatoblast precursors that are capable of differentiating into ALB+CK19+ hepatoblasts (Nava et al., 2005; Nowak et al., 2005). As expected, CD45, a marker for differentiated hematopoietic cells, was absent on Hep cells (Figure S1C), as also observed in hepatoblasts from human specimens. The hematopoietic progenitor molecular profile on emerging Hep cells was confirmed by quantitative real-time PCR (qPCR) (Figure 2A). Gene expression levels were compared with those from day 5 endoderm cells and from pooled second-trimester (15–22 weeks) human fetal livers that are largely composed of blood cells, and thus served as positive controls for hematopoietic marker expression (Goldman et al., 2013; Schmelzer et al., 2007). Transcript levels for *CD34* and *CD133* peaked at day 9 when Hep cells initiate specification, and paralleled protein levels defined by co-immunostaining on cytospun purified Hep cells (Figure 2B) and by flow cytometry analyses (Figure 1C). It is noteworthy that both CD34 and CD133 were co-expressed in Hep cells purified at days 9 and 12 (Figure 2B).

Given that two of the GATA factors, GATA4 and GATA6, are critical for hepatic fate decision (Gordillo et al., 2015), we tested whether the hematopoietic GATA2 transcription factor was also detected in hepatoblasts. Gata2 is indeed essential for proper mouse fetal hematopoiesis development (Tsai et al., 1994). *Gata2*-deficient embryos are viable at E9.5, yet 67% of the mutant embryos are dead at E10.5 from severe anemia, and none survive beyond E11.5. However, the direct function of Gata2 in the development of hepatoblasts has not been characterized in these studies, most likely due to the early embryonic lethality. To our surprise, GATA2 expression in Hep cells was tightly associated with hepatic commitment. While undetectable in endoderm, *GATA2* levels in purified Hep cells dramatically increased at day 9 and subsequently decreased by day 12, and were finally undetectable at day 17 (Figure 2C). The transient expression of *GATA2* was confirmed at the protein level by immunostaining of cytospun Hep cells (Figure 2D). Given that the vast majority of purified Hep cells expressed CD34 and CD133 at day 9 (Figures 1C and 2B), it is reasonable to conclude that developing Hep cells at day 9 co-express the three hematopoietic progenitor markers CD34, CD133, and GATA2.

Importantly, expressions for CD34, CD133, and GATA2 were quite restricted to Hep cells in hepatic cultures, as very few HepPg cells that arise concomitantly with Hep cells expressed these three markers. Indeed, only 14% of HepPg cells expressed CD34 (at very low levels) at day 7, and these percentages dropped below 1% by day 12 (Figure S1D). In line with low protein expression, transcript levels of *CD34* at day 9 were 119.4-fold greater in Hep cells compared with those in HepPg cells (Figures S2A and S2B). *GATA2* expression was restricted to Hep cells as *GATA2* transcripts were undetectable in HepPg cells (Figure S2B). Although *CD133* transcript levels at day 9 were 4.6-fold greater in Hep cells compared with those in HepPg cells (Figure S2), on average from days 7 to 12, 30% of HepPg cells express low levels of CD133 proteins.

Consistent with a hematopoietic progenitor signature of Hep cells, additional fetal hematopoietic markers including RUNX1, SCA1, THY1, CD109, CD150, and CD41 (Cumano

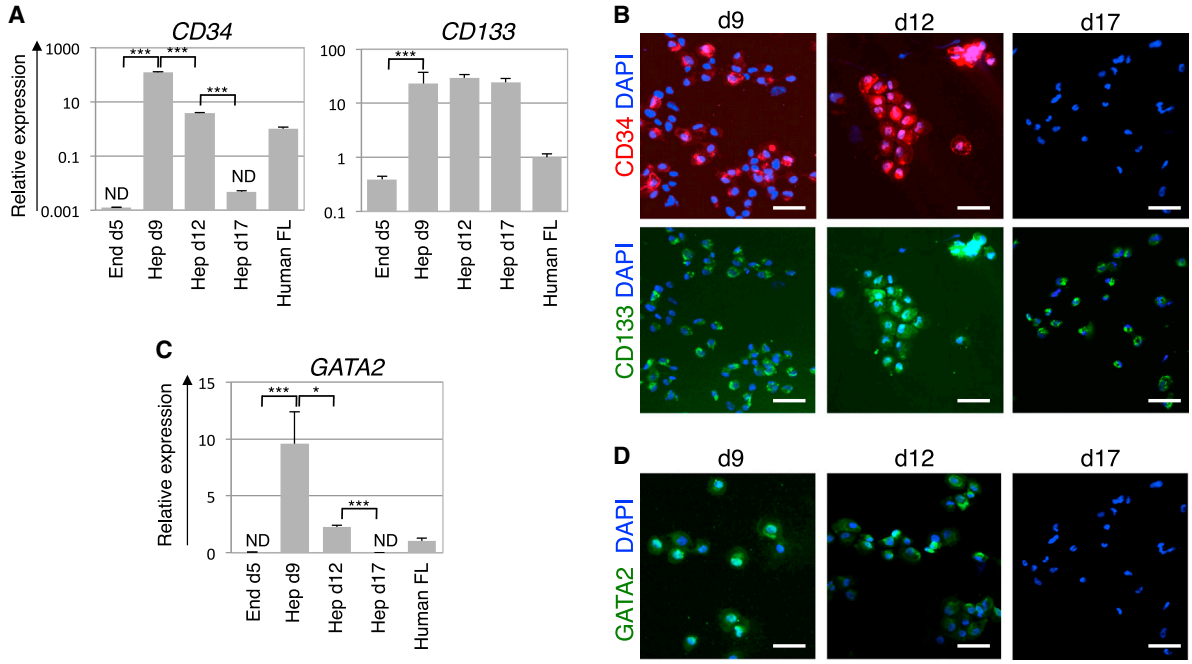

Figure 2. Kinetic of Expression of the Hematopoietic Markers CD34, CD133, and GATA2 in Hep Cells

(A) Relative transcript levels of CD34 and CD133 in purified day 5 End cells and Hep cells purified at days 9, 12, and 17. Transcript levels from human fetal liver sample were set to 1 (n = 3). All numbers reflect the mean ± SD for n independent differentiations.

(B) Co-immunostaining in cytospun Hep cells purified at days 9, 12, and 17.

(C) Relative transcript levels in purified day 5 End cells and Hep cells purified at days 9, 12, and 17. Transcript levels from human fetal liver sample were set to 1 (n = 3).

(D) Immunostaining in cytospun Hep cells purified at days 9, 12, and 17.

*p < 0.05, ***p < 0.001. ND, not detectable. Scale bars, 40 μm. See also Figure S2.

and Godin, 2007) were also detected upon specification of day 9 Hep cells (Figure S2). However, none of these genes had an exclusive expression in Hep cells compared with that in day 5 endoderm cells or day 9 HepPg cells. *RUNX1* expression was greater in both day 5 endoderm and day 9 Hep cells than in HepPg cells. *SCA1*, *THY1*, *CD109*, *CD150*, and *CD41* were highly expressed in both Hep and HepPg cells, while they were undetectable in endoderm cells. Although these markers were present on Hep cells during hepatic specification, they were also expressed in either HepPg or endoderm cells, suggesting that these markers most likely do not participate in the hepatic cell fate decision of Hep cells.

These expression profiles reveal that co-expression of a restricted set of the hematopoietic progenitor markers CD133, CD34, and GATA2 in Hep cells is correlated with hepatic fate decision.

CD133, CD34, and GATA2 Instruct Hepatic Cell Differentiation of Hep Cells Mostly in a Cell-Autonomous Manner

As CD34, CD133, and GATA2 mark quite specifically the vast majority of developing Hep cells, we hypothesized that they are critical in instructing hepatic specification of Hep cells. Knockdowns of CD34, CD133, and GATA2 were performed individually by adding the corresponding pool of small interfering RNAs (siRNAs) every other day on endoderm cells from days 6 to 14 when transcript (Figures 3A and 3B) and protein (Figure S3A) levels of hepatic genes were examined. Knockdown of HNF4α was performed as a positive control for regulating hepatic fate (Parviz et al., 2003). The four knockdowns were very efficient as CD34, CD133, GATA2, and HNF4α transcripts (Figure 3B) and proteins (Figure S3A) were undetectable in the presence of specific siRNAs. We validated the strong specificity of *GATA2* siRNAs against *GATA2* over *GATA4* expression (Figure 3B). Use of specific siRNAs did not alter cell survival or proliferation that could have interfered with hepatic specification and maturation evaluation, as assessed with determination of percentages of live 7AAD− cells and proliferative Ki67+ cells, respectively (Figures S3B and S3C). As expected, none of the knockdowns against CD34, CD133, GATA2, and HNF4α altered expression of the endoderm marker *FOXA2*, whose expression is relatively maintained during liver specification (Figure 3B). In contrast, ablation of HNF4α decreased *AAT, CYP3a4*, and *CYP3a7* transcript

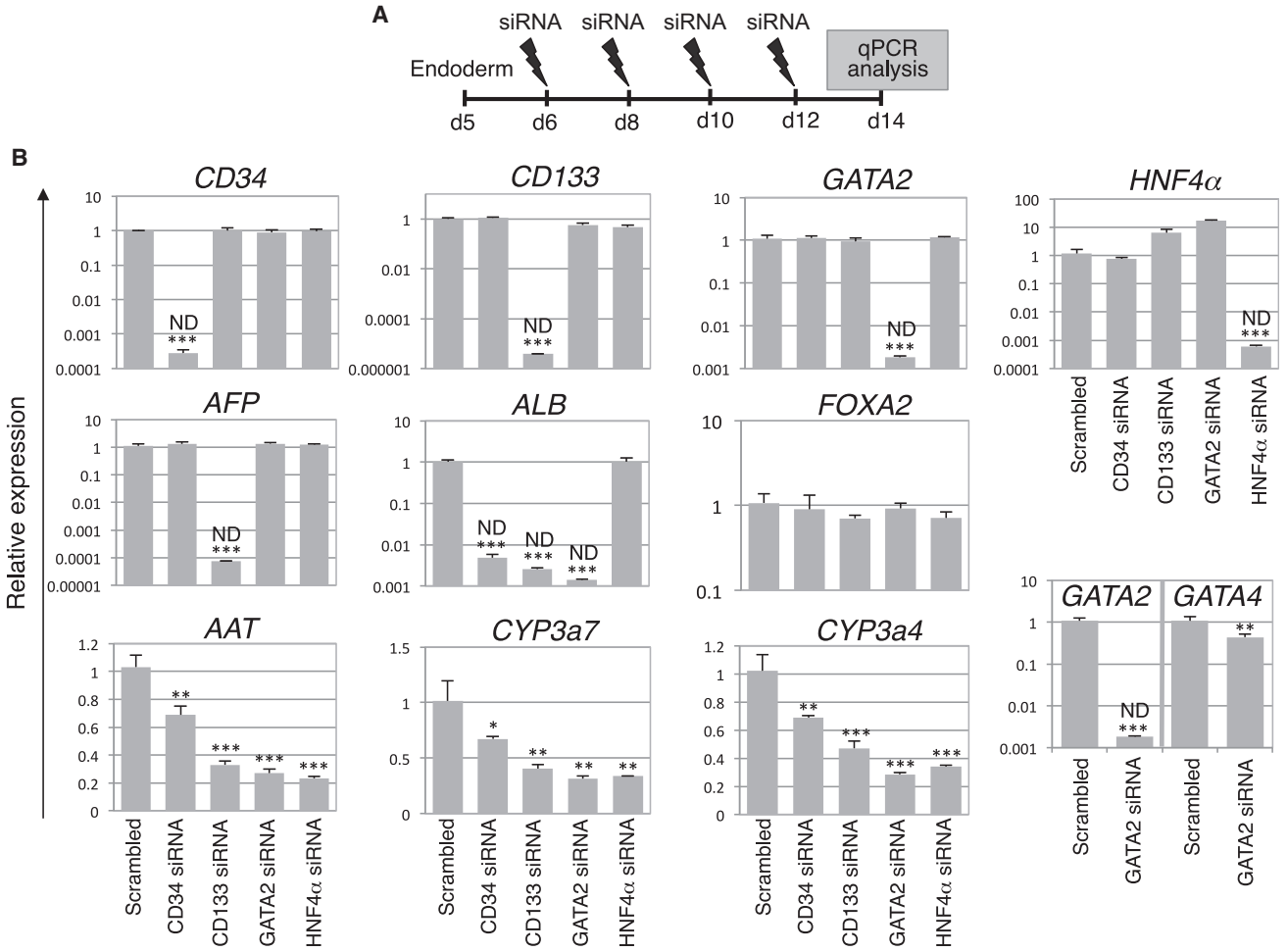

Figure 3. Function of the Hematopoietic Markers CD34, CD133, and GATA2 in Hep Cell Hepatic Specification and Maturation

(A) Protocol of siRNA transfection.

(B) Relative transcript levels in hepatic cultures composed of Hep and HepPg cells collected at day 14 following four treatments of siRNAs. Transcript levels from scrambled siRNA-treated samples were set to 1 (n = 3). All numbers reflect the mean ± SD for n independent differentiations. ND, not detectable (cycle number above 40). Significant statistic differences between the scrambled siRNA condition and specific siRNA conditions are indicated. *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S3.

levels, yet did not affect *AFP* and *ALB* levels (Figures 3B and S3A). These results were consistent with previous studies revealing that deletion of HNF4α during mouse liver development alters morphological and functional differentiation of hepatocytes without affecting hepatic specification (Parviz et al., 2003). A more recent study using hESC hepatic differentiation has, however, indicated that continuous ablation of HNF4α from the pluripotent ESC stage resulted in the absence of expression of AFP and ALB (DeLaForest et al., 2011). The discrepancy of the regulation of AFP and ALB expression by HNF4α may be due to the time difference of HNF4α knockdown, which occurred from the endoderm stage in our study and from the ESC stage in the previous study. Nevertheless, HNF4α knockdown clearly affected hepatic cell fate and thus validated the knockdown strategy for the investigation of hepatic gene regulation by CD34, CD133, and GATA2.

Ablation of *CD34*, *CD133*, or *GATA2* resulted in complete abrogation of ALB transcript levels (Figure 3B) and absence of proteins (Figure S3A). AFP transcript (Figure 3B) and protein (Figure S3A) levels were abrogated in the presence of siRNAs against *CD133* strictly, while they remained the same when *CD34*, *GATA2*, or *HNF4α* levels were ablated. Interestingly, abrogation of *CD34*, *CD133*, or *GATA2* did not affect HNF4α protein expression (Figure S3A) suggesting that Hep cells have not been directed to a different fate, but rather they remained in an early hepatic stage. Since AFP expression occurs earlier than ALB in pluripotent stem cell differentiation cultures, as illustrated in Figure S3A (virtually all Hep cells express AFP, while only a small

percentage of Hep cells is positive for ALB at day 14 in scrambled conditions), these data indicate an earlier function of CD133 on hepatic commitment compared with the role of CD34 and GATA2. The functionality of CD34 on hepatic commitment is reminiscent of the delay in erythroid and myeloid differentiation ability reported in mouse *CD34*-null embryonic stem-cell-derived hematopoietic progenitors (Cheng et al., 1996). GATA2 expression has been mainly reported in the hematopoietic system, and has been involved in the proliferation and maintenance of hematopoietic stem cells and multipotential progenitors (Hsu et al., 2015). Yet, GATA2 has been found expressed in human hepatocellular carcinoma cell lines, and decreased expression of GATA2 promotes proliferation, migration, and invasion of the cell lines in vitro, and correlates with poor differentiation and poor prognosis of hepatocellular carcinoma in patients (Li et al., 2014). Likewise, consistent with our data demonstrating the impact of GATA2 in promoting human Hep cell fate, a recent screen of 137 transcription factors has identified GATA2 as a driver for hepatic differentiation of mouse ESCs (Yamamizu et al., 2013).

Given that day 14 cultures are composed of a mixed culture of Hep and HepPg cells, it was essential to dissect whether CD34, CD133, and GATA2 act in a cell-autonomous manner on Hep cell fate. The restriction of *GATA2* expression in Hep cells validated the cell-autonomous mechanism of GATA2 (Figure S2B). Similarly, the small percentage of HepPg cells expressing CD34 (and moreover at low levels, Figure S1D) supported the hypothesis that CD34 regulates Hep cell fate mostly in a cell-autonomous fashion. Another argument supporting the direct effect of CD34 and GATA2 on Hep cell fate is that ablation of GATA2 or CD34 affects ALB that is specifically expressed in Hep cells but absent in HepPg cells. In day 14 hepatic cultures, ALB protein staining was indeed only present in a subpopulation of cells (Figure S3A) that are Hep cells as *ALB* transcripts were strictly detected in Hep cells from days 9 to 17 and undetectable in HepPg cells (Figure S2B). However, the presence of a significant number of CD133+ HepPg cells (up to 30%) in hepatic cultures suggested that CD133 acts not only directly on Hep cells but also indirectly through HepPg cell activation. AFP immunostaining at day 14 (Figure S3A) indicated that AFP is not only present in Hep cells as expected, but also in HepPg cells that began specification. Initiation of hepatic specification in HepPg cells at day 14 was confirmed by qPCR analyses revealing that *AFP* transcript levels were detected in HepPg cells between days 12 and 17 (Figure S2B). This was not surprising as we previously demonstrated that a more complex 3D culture, that is mimicked when cells reach confluence with time, induces maturation of HepPg cells into AFP-positive cells (Goldman et al., 2013). In fact, complete abrogation of AFP expression in HepPg cells in the presence of siRNAs against CD133 is consistent with both direct and indirect effect of activation of CD133 on at least HepPg hepatic cell fate as only 30% of HepPg cells express CD133.

Knockdown assays reveal that CD133, CD34, and GATA2 expressed on specifying Hep cells instruct hepatic cell differentiation in a strictly cell-autonomous fashion for CD34 and GATA2, and in a partly cell-autonomous fashion for CD133.

Validation of Expression of Hematopoietic Markers in Hepatoblasts in Human and Mouse Fetal Livers

We sought to validate whether the dynamic expression of hematopoietic progenitor markers defined in developing hESC-derived Hep cells in vitro also occurs in vivo. Immunostainings for CD133, CD34, and GATA2 were performed together with ALB or HNF4α staining as a means to identify hepatoblasts/fetal hepatocytes in early first-trimester (FT livers, 7–7.5 weeks of gestation) and second-trimester (ST livers, 18–22 weeks of gestation) human fetal livers (Figures 4 and S4). All human specimens were obtained without identifiers and therefore the proposed research did not meet the definition of human subject research. Virtually all HNF4α+ hepatoblasts/fetal hepatocytes were strongly positive for CD133 in FT livers (Figure 4A, white arrows), and remained positive in ST livers (Figure 4B, white arrows). In FT livers, CD34 was highly expressed in immature endothelial cells as reported previously (Bompais et al., 2004) (Figure 4C, yellow arrows) and was also present in the vast majority of ALB+ hepatoblasts (Figure 4C, white arrows). However, CD34 staining was virtually absent in ALB+ hepatoblasts/fetal hepatocytes in ST livers (Figure 4D, white arrows), but remained present in endothelial cells (Figure 4D, yellow arrows). A similar transient expression pattern was noted for GATA2, with strong expression in nearly all HNF4α+ hepatoblasts/fetal hepatocytes in FT livers (Figure 4E, white arrows), yet with much weaker intensity in ST specimens (Figure 4F, white arrows). No specific spatial pattern of expression of the three markers was observed.

To determine whether the three markers are co-expressed in human hepatoblasts/fetal hepatocytes in vivo, as shown in Hep cells in vitro, co-stainings were carefully quantified from three different FT and ST fetal livers (Figures S4A–S4D). Co-staining revealed that most HNF4α+ hepatoblasts expressed GATA2 in FT livers, and that among the GATA2+ cells 92% were HNF4α+ hepatoblasts (Figures S4A and S4B, white arrows). This suggested that the remaining 8% of GATA2+HNF4α- cells were likely hematopoietic progenitor cells or endothelial cells (Figure 4E, yellow arrowheads), as GATA2 is expressed in both cell types (Lim et al., 2012). Within the GATA2+ cells, 92% were CD133+ and 55% were CD34+ indicating that most hepatoblasts

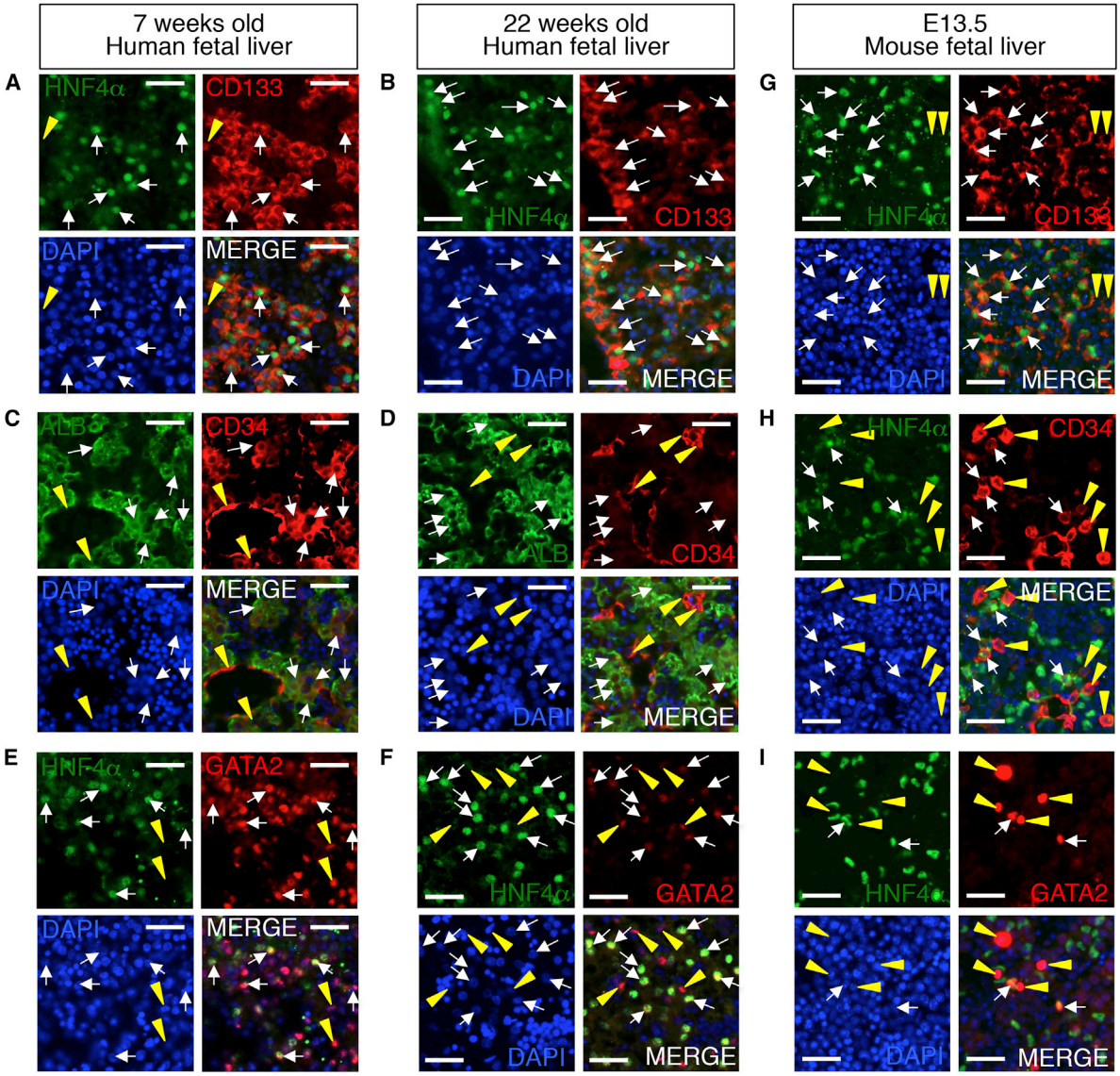

Figure 4. Dynamic Expression of CD133, CD34, and GATA2 in Fetal Hepatoblasts from Human and Mouse Fetal Livers

Co-immunostaining on 7 week (A, C, and E) and 22 week (B, D, and F) old human fetal livers and E13.5 (G, H, and I) mouse fetal livers. To facilitate the visualization of GATA2 expression in 22 week samples (F), intensity of exposure was doubled compared with the intensity used for 7 week samples (E). White arrows indicate selected hepatoblasts positive for the marker analyzed, except for ALB and CD34 co-staining in 22 week old livers in which ALB+ hepatoblasts are negative for CD34. Yellow arrowheads indicate non-hepatoblast cells positive for the marker analyzed. Scale bars, 40 μm. See also Figure S4.

co-expressed GATA2 and CD133, and roughly half co-expressed CD34 in FT livers. In ST livers, GATA2 was still detected in the vast majority of HNF4α+ hepatoblasts/fetal hepatocytes, although at lower levels than in FT specimens (Figures 4F and S4C, white arrows). Quantification of co-staining in ST livers revealed that, within the GATA2+ cells, 77% were HNF4α+ hepatoblasts/fetal hepatocytes. This suggested that the remaining 23% of GATA2+HNF4α− cells were likely hematopoietic progenitors or endothelial cells (Figure 4F, yellow arrowheads). Within the GATA2+ cells, 94% co-expressed CD133, indicating that the majority of hepatoblasts/fetal hepatocytes still co-expressed GATA2 (although at lower levels than in FT) and CD133 in ST livers (Figure S4C, white arrows). Decreasing levels of CD34 and GATA2 and maintenance of high levels of CD133 in hepatoblasts/fetal hepatocytes from the FT to the ST were supported by qPCR analyses performed on crude human fetal liver preparations from FT and ST and compared

with adult human liver samples (Figure S4E). CD34 levels indeed decreased with gestation time, yet not significantly, most likely due to interference with strong CD34 expression in endothelial cells at both time points. Although GATA2 was also present on a subset of hematopoietic progenitors and endothelial cells, transcript levels drastically decreased with time consistent with reduced GATA2 expression in hepatoblasts/fetal hepatocytes during gestation. As expected, CD133 levels were greatly elevated during the whole pregnancy and dropped significantly in adult liver.

In vivo analyses of FT and ST human fetal livers indicate co-expression of CD133, CD34, and GATA2 in the vast majority of developing hepatoblasts from early fetal liver specimens. Interestingly, it is clear that CD34 and GATA2 levels are transiently expressed in human hepatoblasts/fetal hepatocytes from fetal specimens, and this dynamic expression pattern was accurately recapitulated in developing hESC-derived Hep cells in vitro.

To determine whether the hematopoietic profile defined in human hepatoblasts/fetal hepatocytes also characterizes hepatoblasts in the mouse embryo, we examined murine fetal livers from E9.5 to E13.5 embryos. The use of mice in these experiments received Institutional Animal Care and Use Committee approval. We identified subsets of HNF4α+ hepatoblasts co-expressing CD34, GATA2, or CD133 that were the most abundant at E13.5 (Figures 4G–4I, white arrows). Some HNF4α- cells expressing CD133, CD34, and GATA2 were present and most likely represented hematopoietic cells (Figures 4G–4I, yellow arrowheads). There was a significantly greater population of hepatoblasts expressing CD133 than those expressing CD34 or GATA2. Although not all murine hepatoblasts express CD34, GATA2, and CD133 as observed in FT human specimens, these three hematopoietic markers were also detected in a subpopulation of murine hepatoblasts suggesting the conserved function of CD34, GATA2, and CD133 in hepatic specification of the mouse fetal liver.

Conclusion

In conclusion, dissection of the phenotype of developing hESC-derived Hep cells in this study demonstrates a surprising dynamic expression of functional hematopoietic progenitor markers during early hepatic specification of human fetal hepatoblasts. One can wonder about the relevance of the hematopoietic profile in developing Hep cells and whether it represents a generic progenitor cell identity rather than a specific hematopoietic signature. CD34 and CD133 expressions are indeed also shared by other stem and progenitor cells in various tissues including endothelial progenitor cells, mesenchymal stem cells, cancer stem cells, and embryonic stem cells. However, one can also speculate about the existence of a progenitor cell with bipotent hematopoietic and hepatic potential, as was suggested previously with the identification of a hematopoietic progenitor with hepatic potential in the murine fetal liver (Khurana and Mukhopadhyay, 2008), or with the identification of a specialized portal venous endothelium in FT human fetal liver with hematopoietic and hepatoblast potential (Terrace et al., 2010).

The hPSC-Hep cell platform established in this study provides an essential tool that can be used as a surrogate system to study very early human liver specification that is otherwise problematic to study in FT human specimens. Understanding the common molecular profile of developing hematopoietic progenitors and hepatoblasts reveals an unprecedented and partly cell-autonomous role of CD34, CD133, and GATA2 in early hepatic specification and maturation in the developing human liver.

EXPERIMENTAL PROCEDURES

Human Hepatic Differentiation and Cell Sorting

Human ESCs (HES-2) were differentiated into the hepatic lineage as previously described (Goldman et al., 2013). Five days after inducing differentiation, CXCR4+cKIT+KDR−PDGFRα− endoderm cells were isolated with the FACSAria cell sorter (BD Biosciences) and cultured in hepatic media as previously defined (Goldman et al., 2013).

Statistical Analysis

For qPCR, all numbers reflect means ± SD for n independent experiments. Calculations were performed with the paired t test. Significance were defined as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.07.008.

ACKNOWLEDGMENTS

This work was supported by the Black Family Stem Cell Institute, NIH/NIDDK (R01DK087867-01) and the March of Dimes to V.G.E. (FY113-969). The authors are grateful to Drs Georges Uzan and Vanina Jodon de Villenoche for providing us with FT human fetal livers.

Received: May 19, 2015
Revised: July 8, 2016
Accepted: July 10, 2016
Published: August 9, 2016

REFERENCES

Bompais, H., Chagraoui, J., Canron, X., Crisan, M., Liu, X.H., Anjo, A., Tolla-Le Port, C., Leboeuf, M., Charbord, P., Bikfalvi, A., et al.

(2004). Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells. Blood *103*, 2577–2584.

Cheng, J., Baumhueter, S., Cacalano, G., Carver-Moore, K., Thibodeaux, H., Thomas, R., Broxmeyer, H.E., Cooper, S., Hague, N., Moore, M., et al. (1996). Hematopoietic defects in mice lacking the sialomucin CD34. Blood *87*, 479–490.

Cumano, A., and Godin, I. (2007). Ontogeny of the hematopoietic system. Annu. Rev. Immunol. *25*, 745–785.

Dan, Y.Y., Riehle, K.J., Lazaro, C., Teoh, N., Haque, J., Campbell, J.S., and Fausto, N. (2006). Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. Proc. Natl. Acad. Sci. USA *103*, 9912–9917.

DeLaForest, A., Nagaoka, M., Si-Tayeb, K., Noto, F.K., Konopka, G., Battle, M.A., and Duncan, S.A. (2011). HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. Development *138*, 4143–4153.

Goldman, O., Han, S., Sourrisseau, M., Dziedzic, N., Hamou, W., Corneo, B., D'Souza, S., Sato, T., Kotton, D.N., Bissig, K.D., et al. (2013). KDR identifies a conserved human and murine hepatic progenitor and instructs early liver development. Cell Stem Cell *12*, 748–760.

Golub, R., and Cumano, A. (2013). Embryonic hematopoiesis. Blood Cells Mol. Dis. *51*, 226–231.

Gordillo, M., Evans, T., and Gouon-Evans, V. (2015). Orchestrating liver development. Development *142*, 2094–2108.

Hsu, A.P., McReynolds, L.J., and Holland, S.M. (2015). GATA2 deficiency. Curr. Opin. Allergy Clin. Immunol. *15*, 104–109.

Khurana, S., and Mukhopadhyay, A. (2008). Hematopoietic progenitors from early murine fetal liver possess hepatic differentiation potential. Am. J. Pathol. *173*, 1818–1827.

Lazaro, C.A., Croager, E.J., Mitchell, C., Campbell, J.S., Yu, C., Foraker, J., Rhim, J.A., Yeoh, G.C., and Fausto, N. (2003). Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology *38*, 1095–1106.

Li, Y.W., Wang, J.X., Yin, X., Qiu, S.J., Wu, H., Liao, R., Yi, Y., Xiao, Y.S., Zhou, J., Zhang, B.H., et al. (2014). Decreased expression of GATA2 promoted proliferation, migration and invasion of HepG2 in vitro and correlated with poor prognosis of hepatocellular carcinoma. PLoS One *9*, e87505.

Lim, K.C., Hosoya, T., Brandt, W., Ku, C.J., Hosoya-Ohmura, S., Camper, S.A., Yamamoto, M., and Engel, J.D. (2012). Conditional Gata2 inactivation results in HSC loss and lymphatic mispatterning. J. Clin. Invest. *122*, 3705–3717.

Nava, S., Westgren, M., Jaksch, M., Tibell, A., Broome, U., Ericzon, B.G., and Sumitran-Holgersson, S. (2005). Characterization of cells in the developing human liver. Differentiation *73*, 249–260.

Nowak, G., Ericzon, B.G., Nava, S., Jaksch, M., Westgren, M., and Sumitran-Holgersson, S. (2005). Identification of expandable human hepatic progenitors which differentiate into mature hepatic cells in vivo. Gut *54*, 972–979.

Parviz, F., Matullo, C., Garrison, W.D., Savatski, L., Adamson, J.W., Ning, G., Kaestner, K.H., Rossi, J.M., Zaret, K.S., and Duncan, S.A. (2003). Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nat. Genet. *34*, 292–296.

Schmelzer, E., Zhang, L., Bruce, A., Wauthier, E., Ludlow, J., Yao, H.L., Moss, N., Melhem, A., McClelland, R., Turner, W., et al. (2007). Human hepatic stem cells from fetal and postnatal donors. J. Exp. Med. *204*, 1973–1987.

Terrace, J.D., Hay, D.C., Samuel, K., Anderson, R.A., Currie, I.S., Parks, R.W., Forbes, S.J., and Ross, J.A. (2010). Portal venous endothelium in developing human liver contains haematopoietic and epithelial progenitor cells. Exp. Cell Res. *316*, 1637–1647.

Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W., and Orkin, S.H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature *371*, 221–226.

Yamamizu, K., Piao, Y., Sharov, A.A., Zsiros, V., Yu, H., Nakazawa, K., Schlessinger, D., and Ko, M.S. (2013). Identification of transcription factors for lineage-specific ESC differentiation. Stem Cell Rep. *1*, 545–559.