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Stage-specific regulation of the WNT/β-catenin pathway enhances differentiation of hESCs into hepatocytes

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Background & Aims: Hepatocytes differentiated from human embryonic stem cells (hESCs) have the potential to overcome the shortage of primary hepatocytes for clinical use and drug development. Many strategies for this process have been reported, but the functionality of the resulting cells is incomplete. We hypothesize that the functionality of hPSC-derived hepatocytes might be improved by making the differentiation method more similar to normal *in vivo* hepatic development.

Methods: We tested combinations of growth factors and small molecules targeting candidate signaling pathways culled from the literature to identify optimal conditions for differentiation of hESCs to hepatocytes, using qRT-PCR for stage-specific markers to identify the best conditions. Immunocytochemistry was then used to validate the selected conditions. Finally, induction of expression of metabolic enzymes in terminally differentiated cells was used to assess the functionality of the hESC-derived hepatocytes.

Results: Optimal differentiation of hESCs was attained using a 5-stage protocol. After initial induction of definitive endoderm (stage 1), we showed that inhibition of the WNT/β-catenin pathway during the 2nd and 3rd stages of differentiation was required to specify first posterior foregut, and then hepatic gut cells. In contrast, during the 4th stage of differentiation, we found that activation of the WNT/β-catenin pathway allowed generation of proliferative bipotent hepatoblasts, which then were efficiently differentiated into hepatocytes in the 5th stage by dual inhibition of TGF-β and NOTCH signaling.

Conclusion: Here, we show that stage-specific regulation of the WNT/β-catenin pathway results in improved differentiation of hESCs to functional hepatocytes.

Keywords: Human embryonic stem cell; Hepatoblast; WNT/β-catenin; Fetal hepatocyte; Functionality.

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Abbreviations: HPSCs, human pluripotent stem cells; GI, gastrointestinal; FGF4, fibroblast growth factor 4; WNT, wingless-type MMTV integration site family; PFG, posterior foregut; HNF, hepatocyte nuclear factor; HHEX, hematopoietically-expressed homeobox protein; AFP, alpha-fetoprotein; PDX1, pancreatic and duodenal homeobox 1; TGF-β, transforming growth factor, beta; BMP4, bone morphogenetic protein 4; SHH, sonic hedgehog; HGF, hepatocyte growth factor; OSM, oncostatin M; HESC, human embryonic stem cell; DE, definitive endoderm; AFG, anterior foregut; NOG, noggin; RA, retinoic acid; VEGF, vascular endothelial growth factor; ALB, albumin; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor.

Introduction

End-stage liver disease is a major health problem affecting over 600 million people world-wide and 30 million within the US and for many of these patients, liver transplantation is the only effective long-term treatment. Given that the supply of cadaveric livers for transplantation is limited, the use of primary fetal or cadaveric hepatocytes directly transplanted or integrated to a bioartificial device has been proposed as an alternative to whole organ transplantation [1,2]. However, it is widely appreciated that it is difficult to culture and amplify primary hepatocytes while preserving their function [3]. Therefore, there is a need for alternative sources of large numbers of functional hepatocytes. Human pluripotent stem cells (hPSC) have the ability to self-renew and proliferate indefinitely *in vitro* and to differentiate into a wide range of cell types, including hepatocytes [4–7], and it has been widely appreciated that such cells might be useful for clinical applications [8–10].

Although a number of methods for direct conversion of one differentiated cell type to another have been described [11], the majority of successful strategies for generating large numbers of differentiated cells attempt to recapitulate the normal developmental progression from the pluripotent to the terminally differentiated state [6,4,12]. In these strategies, pluripotent cells are progressively directed by external signals, first to one of the three germ lineages, and then to more specific downstream cell types. The liver is derived from the endoderm lineage, which also gives rise to the lung, pancreas, and gastrointestinal tract (GI).

Tracing the progression of specification from definitive endoderm (DE) to hepatocyte, the endoderm first gives rise to the primitive gut tube, which is patterned along the anterior-posterior axis as it receives signals from the adjacent mesoderm. Next, the posterior region of the gut is exposed to fibroblast growth factor 4 (FGF4) [13] and wingless-type MMTV integration site family (WNT), again from the mesoderm, and begins to express CDX2, which leads to the formation of the

gastrointestinal tract. Concurrent repression of WNT signaling ventral to this region of development of the GI tract results in specification of the posterior foregut (PFG) expressing hepatocyte nuclear factor 1-beta (HNF1β), HNF4, and hematopoietically-expressed homeobox protein (HHEX), from which the liver and pancreas are derived [14]. In fact, activation and inhibition of the WNT pathway have been used to direct differentiation of hPSCs into intestinal cells [15] and foregut derivatives [16,17], respectively.

Subsequently, at a timepoint corresponding day 8.5 of mouse embryonic development, the hepatic and pancreatic sublineages (characterized by expression of alpha-fetoprotein (AFP) and pancreatic and duodenal homeobox 1 (PDX1), respectively) diverge from a common ventral PFG domain [18]. This process is directed by transforming growth factor beta (TGF-β), bone morphogenetic protein 4 (BMP4), FGF4 and sonic hedgehog (SHH), which are secreted from the surrounding mesoderm [19,18], with FGF4 at this stage being primarily associated with liver development, pancreatic development being characterized by inhibition of BMP4 and SHH signaling, and TGF-β playing a more complex role in both sublineages.

Shortly after the hepatic specification, hepatoblasts delaminate from the diverticulum and migrate through the septum transversum (STM) to form the liver bud. Up until E13.5 in mouse development, TBX3 maintains proliferative hepatoblasts in a bipotent state, capable of becoming both hepatocytes and cholangiocytes [20]. From E13.5, the hepatoblasts that are in contact with the portal vein receive signals from the vascular endothelial cells through direct cell-cell contact, which active the NOTCH pathway, resulting in expression of SOX9 [21]. To our knowledge, this signaling pathway has not been employed in hPSC-to-hepatocyte differentiation strategies but has been employed to differentiate human and mouse bipotent progenitors into hepatocytes [22,23] and direct reprogramming of fibroblast to hepatocyte [24]. Instead, the majority of hepatic differentiation protocols established thus far have used a combination of hepatocyte growth factor (HGF) and oncostatin M (OSM) to drive hepatic progenitors towards a more hepatocyte state [25,5,4,7]. Using this approach, generation of metabolically functional hepatocytes has been achieved, but only exposing the cells to undefined conditions (e.g. fetal bovine serum, matrigel, etc.) for extended periods of time [5,7]. Another isolated study has reported the use of cell dissociation during the differentiation process to promote hepatocyte maturation [26].

Here, we report a new approach to differentiating human embryonic stem cells (hESCs) into functional hepatocytes by refining the steps of our previously described protocol by more closely reproducing the steps involved in normal hepatic development [6]. This new differentiation protocol drives differentiation of hESCs into hepatocytes through 5 stages. During this differentiation process, the cells faithfully express the key transcription factors characteristic to each stage of liver organogenesis. We observe the generation of a proliferative hepatoblast population that expresses both hepatic and cholangiocyte markers, as well as PROX1 and HNF6, which have been shown to be associated with successful generation of functional hepatocytes in a previous report [26]. Key to our strategy, we use inhibition of the NOTCH and TGF-β pathway to direct hepatocyte differentiation from hepatoblasts. The hepatocytes we have generated using this new method express high levels of hepatocyte specific genes, including metabolic genes encoding key metabolic enzymes, and also display the appropriate metabolic activities.

Materials and methods

Culture and differentiation of human pluripotent stem cells

hESCs (H9/WA09 from WiCell, Madison, WI, USA) were maintained as colonies in mTeSR1 medium on growth factor-reduced matrigel (BD Bioscience) and passaged every 5 days using Cell Dissociation Buffer (StemCell Technologies). hiPSCs were generated by reprogramming human dermal fibroblast (HDF) cultures using standard reprogramming factors carried by Sendai virus vectors (Cytotune Kit, from Life Technologies). hiPSC1 was reprogrammed from HDFs from an individual with type 2 Crigler-Najjar Syndrome (Coriell, GMO9551), and hiPSC2 from HDFs from a healthy individual. For differentiation experiments, cells were passaged onto gelatin/FBS coated plates as previously described [6], and differentiation was initiated 48 h after passage. To optimize hepatic differentiation of hESCs, we modified a protocol previous published in [6] by testing several different combinations of factors and small molecules at stages 2, 3, 4, and 5 of differentiation. Details of the differentiation method and supplementary information are described in the Supplementary material.

Isolation and culture of adult primary hepatocytes

In order to obtain the highest possible quality of adult primary hepatocytes, for the freshly isolated human adult hepatocyte (FI-HAH) preparations, donor livers were collected directly from the operating room, the whole liver was perfused with dissociation buffer, and the analysis of the freshly isolated cells was completed within 6 days of isolation, as described in detail in the Supplementary material.

Results

We have reviewed recent discoveries in developmental biology elucidating the signaling pathways involved in normal hepatic development to refine our previously reported protocol for directed differentiation of hESCs to hepatocytes. Our new method consists of 5 stages of differentiation, compared to the previously published 4-stage method, as shown in Fig. 1. Major alterations in the procedure include inhibition of the WNT pathway to pattern DE cells to ventral PFG, the generation of hepatoblasts through activation of the WNT pathway, and finally dual inhibition of TGF-β and NOTCH signaling to promote hepatocyte differentiation from the hepatoblast stage.

Differentiation of hESCs into ventral posterior foregut cells

During the first stage of differentiation, the cells were differentiated to a near homogenous population of DE using a previously published method [6]. The DE cells expressed the typical DE markers SOX17, FOXA2, GATA4, but not the pluripotency-associated markers OCT4 and SOX2 (Fig. 2 and Supplementary Figs. 1, 2). We then wished to optimize the commitment of these DE cells into PFG.

We screened selected growth factors and inhibitors targeting the TGF-β, BMP4, and WNT signaling pathways to identify the best combination for induction of PFG. Specifically, we used the BMP4 antagonist noggin (NOG), which has been previously used to generate anterior foregut (AFG) and PFG [16,27], the TGF-β family member Activin A [12], and the WNT/β-catenin inhibitor IWR-1 [17,16]. As expected, inhibition of BMP4

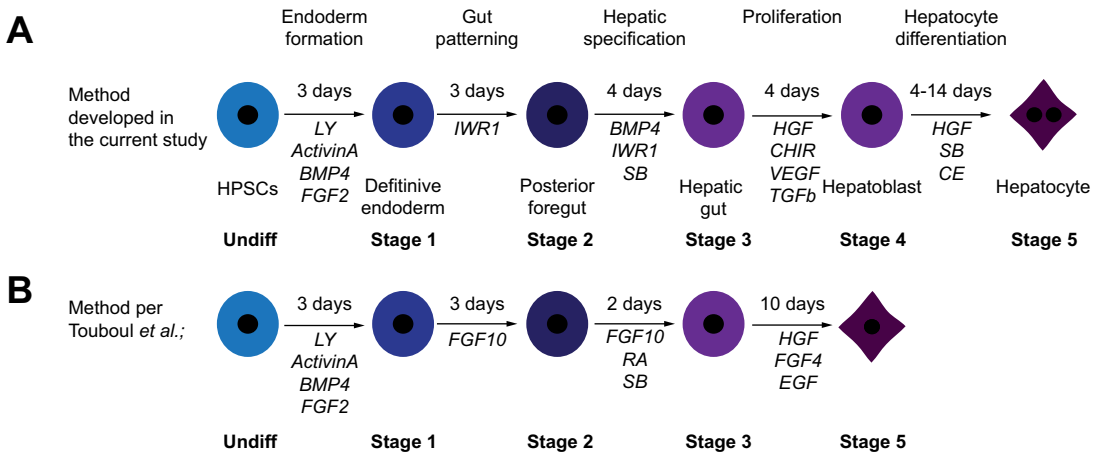

Fig. 1. Schematic representation of the differentiation protocols used to differentiate hESCs into fetal hepatocytes. (A) the method developed in the current study. (B) the previously developed protocol per Touboul *et al.* LY: Ly 294002; SB: SB 431542; CHIR: CHIR99021; CE: Compound E; RA: Retinoic Acid.

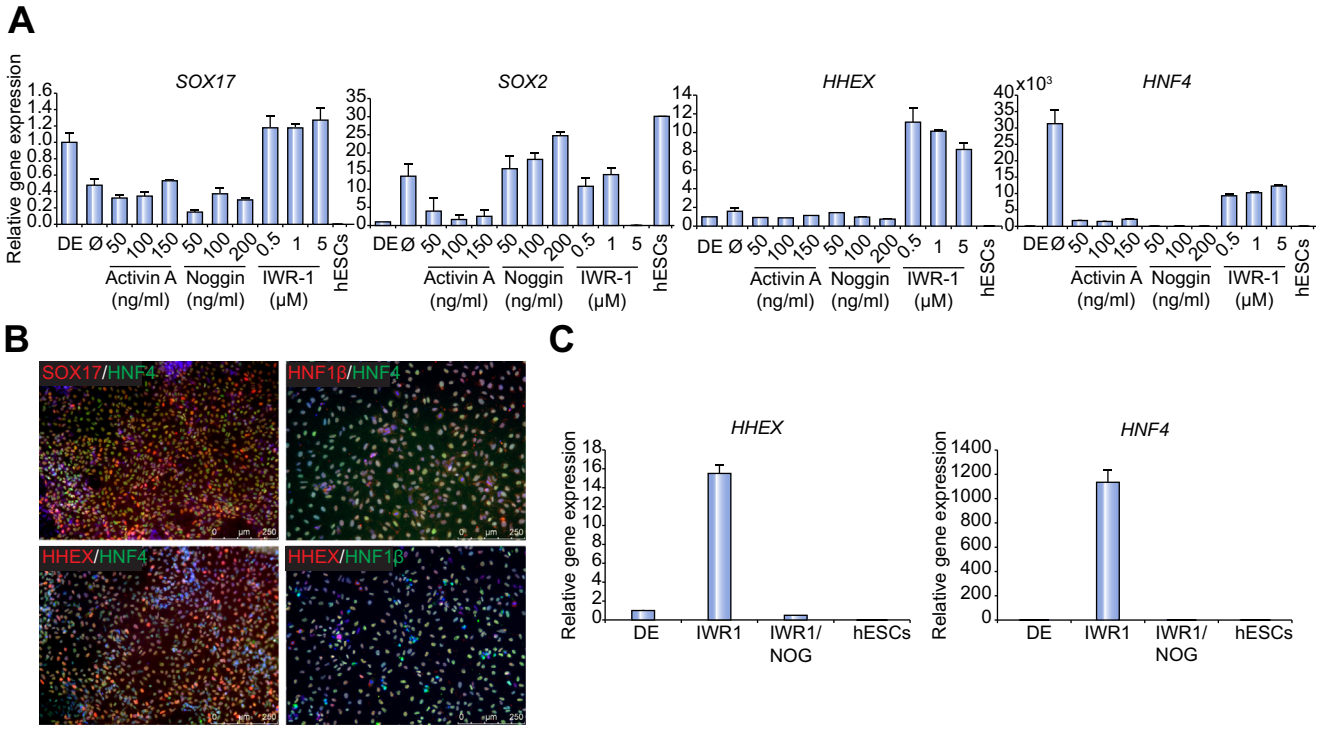

Fig. 2. Establishment of stage 2 differentiation conditions: Differentiation of DE to posterior foregut. (A) qRT-PCR analysis of gene expression of *SOX17*, *SOX2*, *HHEX*, and *HNF4* in undifferentiated hESCs, DE, and DE treated for 3 days in: media without additives (Ø) or in presence of increasing doses of Activin A, Noggin and/or IWR-1. Gene expression levels were normalized to DE. (B) Immunocytochemistry showing expression of the PFG markers HNF4, SOX17, HNF1β after 3 days of treatment of DE cells with IWR-1. (C) qRT-PCR analysis shows expression of *HNF4* and *HHEX* in hESCs, DE cells were treated with IWR-1 or combination of IWR-1/NOG. Gene expression levels were normalized to DE.

signaling by NOG induced expression of the AFG marker *SOX2*, but failed to significantly induce expression of PFG genes *HNF4* and *HHEX* (Fig. 2A). Activin A induced mild expression of *HNF4*; but even at the highest dose, there was only no induction of *HHEX* or *SOX2* (Fig. 2A). IWR-1 significantly improved DE differentiation to PFG, as demonstrated by the expression of the markers HNF4, HHEX, and HNF1β (Fig. 2A–B), while maintaining expression of SOX17 (Fig 2A–B). IWR-1 treated cells also re-expressed *SOX2*, an AFG marker, but did not express the pluripotency transcription factor *OCT4* (Supplementary Fig. 2). Importantly, we noticed that in the absence of treatment, DE cells spontaneously expressed gut marker *HNF4*, but not *HHEX* (Fig. 2A). Moreover, we observed that the addition of BMP4 inhibition to inhibition of the WNT pathway in fact blocked differentiation to PFG (Fig. 2C). Thus, our results showed that inhibition of WNT/β-catenin during differentiation of hESC-derived DE cells is sufficient to induce expression of key PFG transcription factors.

Differentiation of ventral posterior foregut cells into hepatic gut cells

We next addressed how to commit these PFG cells into our third stage hepatic gut cells. At this stage of development, PFG cells have the potential to give rise to the liver and pancreas. We screened growth factors and small molecules targeting the BMP4, TGF-β, and WNT pathways, as well as retinoic acid (RA), which have been previously reported to commit PFG cells to the hepatic lineage *in vitro* and *in vivo* [4,6,16].

We observed that exposure to either BMP4, SB431542 (SB), or IWR-1 in the presence of RA resulted in induction of the hepatic markers *AFP* and albumin (*ALB*), but not the other hepatic markers *PROX1*, *CEBPA* (Fig. 3A). Moreover, there was persistent expression of the DE marker *SOX17*, as well as expression of the hindgut marker *CDX2*, which are undesirable for this stage of hepatic differentiation [18]. We then manipulated pairs of signaling pathways (i.e. BMP4 + SB, BMP4 + IWR-1, and SB + IWR-1) with and without RA, and demonstrated that in the absence of RA, we saw higher induction of the hepatic markers *AFP*, *ALB*, *PROX1*, and *CEBPA*, with low expression of *SOX17* and *CDX2*. The addition of RA to these pairs of factors resulted in reduced expression of the four hepatic markers, and increased expression of *SOX17* and *CDX2*. Finally, the triple combination of BMP4, SB, and IWR-1 results in the best overall induction of the four hepatic markers while reducing expression of the DE and gastrointestinal markers. We showed in a specific comparison of IWR1/SB to IWR1/SB/BMP4 that the inclusion of BMP4 increased the expression of HNF4 by 30% (Supplementary Fig. 3). We note that RA appears not to promote specific differentiation to the hepatic lineage, and instead leads to aberrant expression of the hindgut marker *CDX2* in hepatic cells, which is consistent with a findings reported in a previous study on direct reprogramming methods [28].

Using immunocytochemistry (ICC), we confirmed that cells treated with the combination of BMP4, SB, and IWR-1 co-expressed the hepatic gut marker AFP with both HNF4 and HNF6. GATA4 was co-expressed with HNF4, consistent with an early hepatic progenitor state. We also started to observe clusters of PROX1/HNF6 positive cells (Fig. 3B), with more than 20% of the cells being double positive for these two markers (Fig. 6A).

Generation of bipotent proliferative hepatoblasts

Once the liver domain has been established, the newly formed liver bud, influenced by signals from the surrounding mesenchyme, enters a phase of growth during which hepatoblasts proliferate and remain bipotent [20]. We investigated the reported role of the WNT/β-catenin pathway in this process

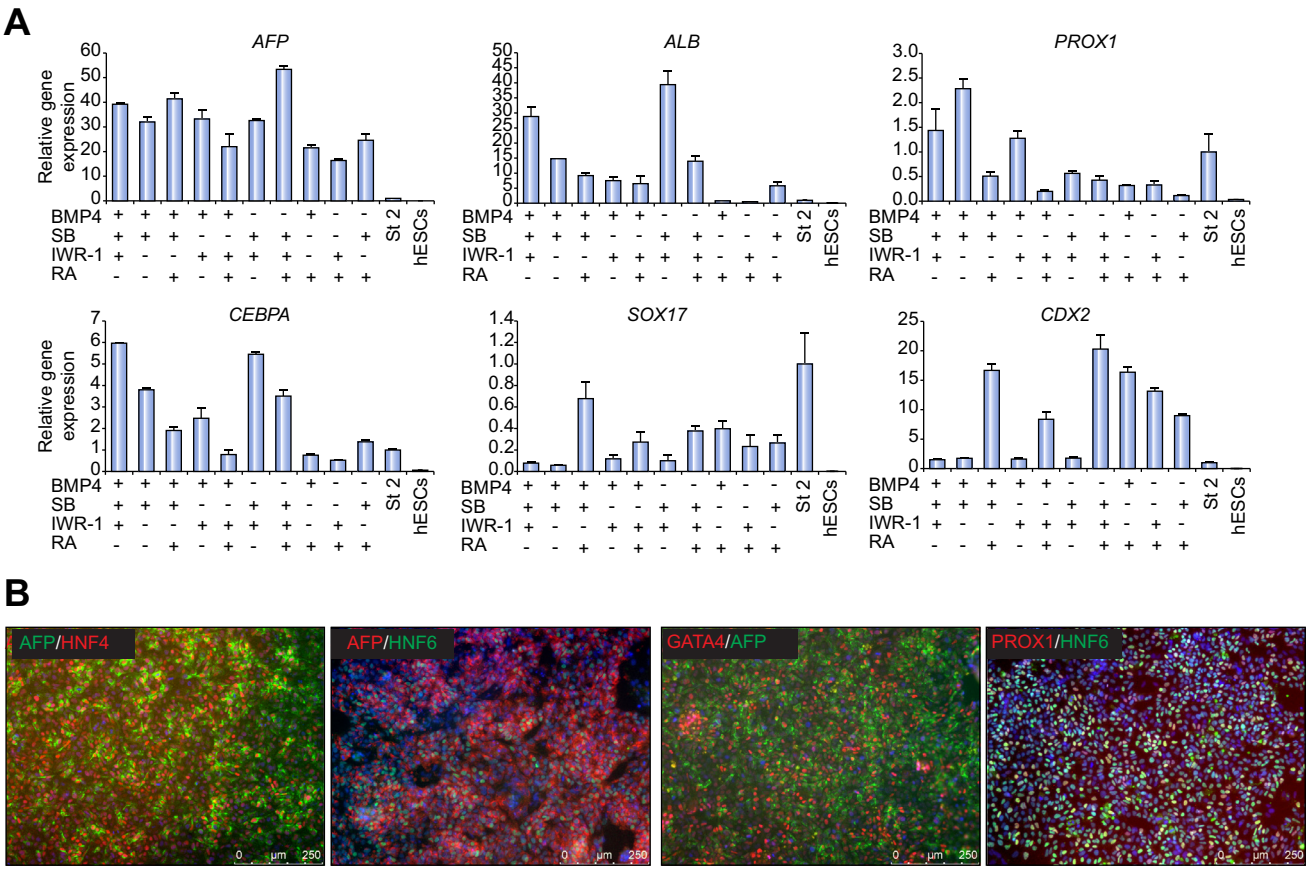

Fig. 3. Establishment of stage 3 differentiation conditions: Induction of hepatic gut from PFG cells. (A) Effect of BMP4, IWR-1, RA and SB on hepatic commitment of PFG cells. PFG cells were exposed for 4 days to various combinations of BMP4, IWR-1, RA and SB. Hepatic and non-hepatic gene expression in the different conditions and in undifferentiated hESCs was quantified by qRT-PCR analysis and normalized to stage 2 IWR-1-treated cells (St 2). (B) Generation of hepatic gut by exposure of stage 2 cells to BMP4/SB/IWR-1 for 4 days was confirmed by immunocytochemistry.

[29], which corresponds to our fourth stage of differentiation, as well as soluble factors which are known to be involved during *in vivo* hepatic growth, including HGF, TGF-β [30], and VEGF [31].

The strongest overall effect we saw was a strong induction of *TBX3*, a member of the T-box gene family, by the GSK3 inhibitor, CHIR 99021(CHIR), which activates the WNT/β-catenin pathway. The addition of HGF, vascular endothelial growth factor (VEGF), or TGF-β did not seem to appreciably modify the effect of CHIR. During liver bud growth, TBX3 expression is maintained among the proliferative hepatoblast population, and restricts early differentiation towards hepatocytes [20]. In the conditions where cells were treated with CHIR, expression of key transcription factors such as *CEBPA* (Fig. 4A and Supplementary Fig. 4) and *HNF4* (data not shown) were maintained at the same level as in stage 3. In the absence of CHIR, cells appeared to differentiate towards a more mature hepatocyte state, with reduced expression of the hepatoblast and cholangiocyte markers *TBX3* and *CK19*, and increased expression of the later hepatic genes *ALB* and *CK18*. Importantly, these cells exhibited features of bipotent hepatoblasts, with co-expression of the early hepatocyte marker AFP and the early cholangiocyte transcription factor SOX9 (Fig. 4B). These cells were also shown to be proliferative by Ki67 staining, with expression of AFP by both qRT-PCR and immunocytochemistry (Fig. 4A and B). This treatment is also, to our knowledge, the first to induce strong co-expression of the transcription factors HNF6 and PROX1 (Fig. 4B), which have recently been shown to be essential for proper maturation of hepatocytes derived from hPSCs [26]. In this previous study, the authors reported that dissociation of the cells during differentiation stimulated expression of these two factors; in our protocol, we achieved induction using the combination of CHIR, TGF-β, HGF, and VEGF without cell dissociation. Our procedure uses physiological cues, rather than a mechanical stimulus, which does not occur during normal liver development, and also can introduce variability in the differentiation process.

Differentiation of hepatoblasts into fetal hepatocytes

We then performed a final screening procedure to develop optimal conditions for differentiation of the bipotent hepatoblasts into fetal hepatocytes. The majority of the established protocols for deriving hepatocytes from hPSCs use HGF and OSM, either as a combination or in a sequential fashion [25,4,26]. HGF is commonly used *in vitro* in primary hepatocyte cultures and OSM has been shown *in vivo* and *in vitro* to promote maturation of primary hepatocytes, particularly in association with glucocorticoids [32]. The TGF-β and NOTCH signaling pathways have been shown to direct the cell fate decision that directs hepatoblasts towards

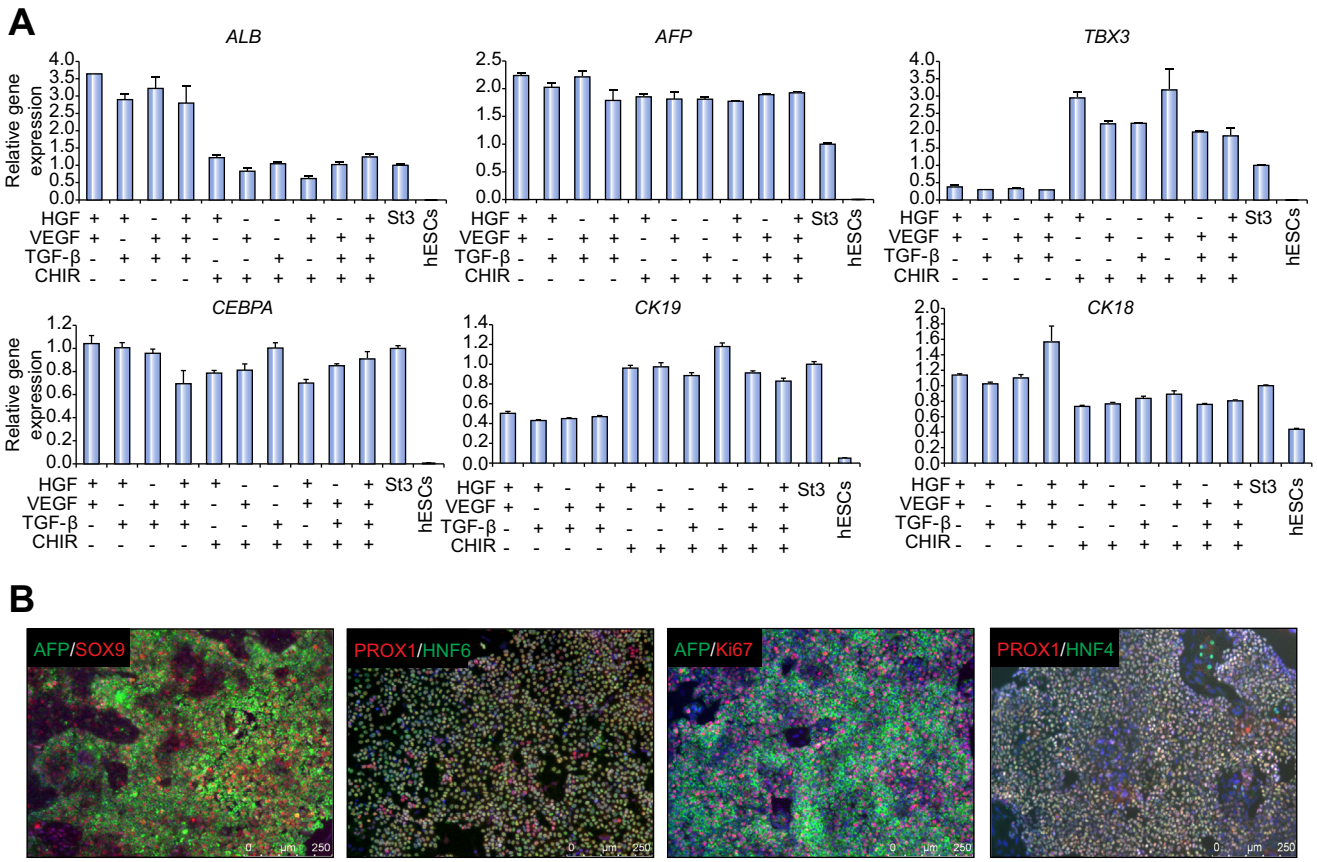

Fig. 4. Establishment of stage 4 differentiation conditions: Generation of hepatoblasts through activation of WNT/β-catenin pathway. (A) Establishment of conditions for differentiation to hepatoblast. Hepatic gut cells were grown for 4 days in presence of different combinations of HGF, VEGF, TGF-β, and CHIR. Gene expression of hepatic and cholangiocytes markers were analyzed by qRT-PCR and normalized to stage 3 cells. (B) Expression of proliferative hepatoblasts markers was detected by immunocytochemistry after treatment with CHIR/TGF-β/HGF/VEGF for 4 days.

the hepatocyte *vs.* the cholangiocyte lineage, with activation of these pathways leading to the cholangiocyte lineage and inhibition favoring the hepatocyte lineage [33,34]. We decided to investigate whether the use of TGF-β and NOTCH inhibitors would promote differentiation of the hepatoblasts into fetal hepatocytes. Since HGF is ubiquitous in the culturing of primary hepatocytes, as well as hepatocytes derived from hPSCs, we maintained the presence of HGF in our cultures at 25 ng/ml in all of our conditions. When cells were cultured in the presence of 25 ng/ml of HGF alone, expression of hepatic (alpha-1-antitrypsin (*AAT*), *AFP*, and *ALB*) markers increased as expected. However, *CEBPA* expression was decreased, indicating that there may be insufficient signaling to allow for clear selection by the hepatoblasts towards the downstream alternative fates (hepatocyte and cholangiocyte); we see further evidence of this with the persistence of *SOX9* (a cholangiocyte marker) expression (Fig. 5A). The addition of SB resulting in improved expression of *CEBPA*, while the inclusion of the inhibitor of NOTCH signaling, Compound-E (CE, 0.5 μM), was associated with decreased expression of *SOX9*. OSM did not appear to influence the expression of any of the tested markers. Therefore, we concluded that the optimal culture condition for the hepatoblast to fetal hepatocyte transition included HGF, SB, and CE. We then demonstrated that this condition resulted in secretion of high levels of albumin (48 μg/ml/day per million cells) (Fig. 5B).

Demonstration that the bipotent hepatoblast stage is dependent on WNT signaling

To show that the induction of hepatoblast markers and the cell proliferation were not simply due to time, and to further demonstrate the role of WNT signaling in this process, we compared *HNF6*, *PROX1*, and *Ki67* expression in stage 3 cells (hepatic gut cells) that were cultured for the same amount of time, but in three different conditions: CHIR, TGF-β, HGF, and VEGF (our stage 4 condition); IWR-1, TGF-β, HGF, and VEGF (our stage 4 condition, but replacing WNT activation by CHIR with WNT inhibition by IWR-1); and HGF, SB, and CE (our stage 5 condition, thus skipping stage 4, similar to previously published methods) (Fig. 6A). These results indicated that both the HNF6/PROX1 double positive population and the Ki67 positive population (indicating the proliferative population) were significantly higher in our stage 4 cells, which was the condition that included WNT activation.

Importance of the hepatoblast stage on cell maturation

We next wanted to examine whether including a proliferative hepatoblast stage (referred to as stage 4 in Fig. 1) would improve the maturation of the cells at the final stage (stage 5 in Fig. 1) of differentiation.

We exposed either stage 3 or stage 4 cells to the same maturation (stage 5) conditions for 14 days. 24 h prior to collecting the cells for analysis, we treated both cultures with TCDD, RIF, and PB, which activate the nuclear receptors AhR, PXR, and CAR, respectively. These receptors, once activated by their ligands, induce the expression of metabolic enzymes and transporters involved in the detoxification of both endogenous and xenobiotic molecules, which is one of the major functions of the liver. After treatment, we verified that these drugs did not negatively impact cell differentiation by monitoring the expression of *ALB* and *AFP*. We also noted that the level of *AFP* expression was significantly lower in the cells that had passed through stage 4 (referred to as "stage 3 → 4 → 5" cells), than in those that had been transitioned directly from stage 3 to stage 5 (referred to as "stage 3 → 5" cells) (Fig. 6B); in Supplementary Fig. 6, we show that the level of expression of *AFP* in the 5-stage protocol peaked at stage 4, and then decreased between stage 4 and stage 5, mimicking the normal decrease in *AFP* expression with hepatic maturation. By qRT-PCR, we observed that the stage 3 → 4 → 5 and stage 3 → 5 cells expressed similar levels of AhR transcript, and

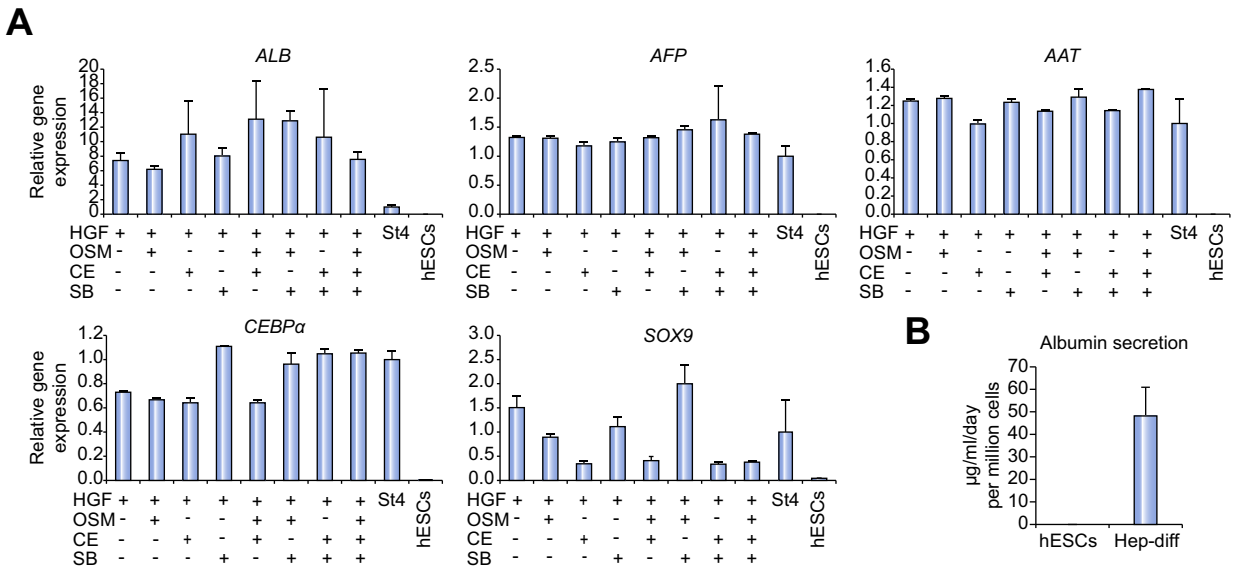

Fig. 5. Establishment of stage 5 differentiation conditions: Differentiation of hepatoblasts into hepatocytes. (A) Hepatoblasts were exposed for 4 days to HGF in combination with oncostatin m (OSM), CE, and SB. qRT-PCR analysis of hepatic (*ALB*, *AFP*, *AAT*, and *CEBPA*) and cholangiocyte (*SOX9*) gene expression. Gene expression data were normalized to stage 4. (B) ELISA analysis showing secretion of albumin into the culture medium by hESCs-derived hepatocytes (compared to undifferentiated hESCs).

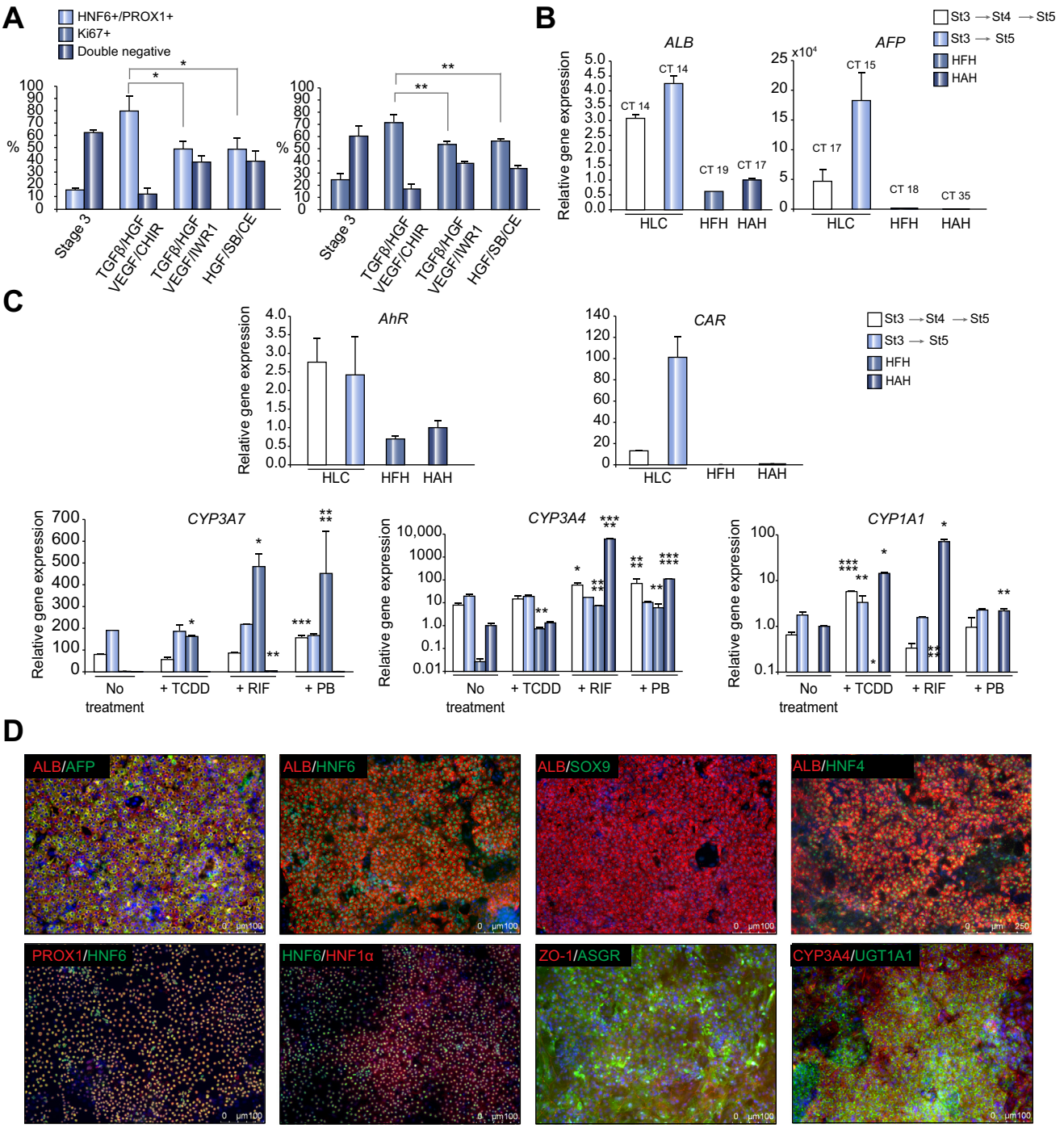

Fig. 6. Importance of the hepatoblast stage for later cell maturation. (A) Effect of WNT/β-catenin activation on the generation of hepatoblasts. FACS analysis performed on stage 3 cells and on stage 4 cells (stage 3 cells exposed for 4 days to: HGF/TGF-β/SB/CHIR; HGF/TGF-β/SB/IWR-1; HGF/CE/SB). Each column represents the average of 3 independent biological replicates. $^{*}p$ <0.02; $^{**}p$ <0.03. (B) and (C) qRT-PCR analysis of hESCs-derived hepatocytes generated by culturing stage 3 (St3 → St5) or stage 4 (St3 → St4 → St5) for 14 days in HGF/CE/SB. Also represented gene expressions of human fetal and adult hepatocytes respectively abbreviated HFH and HAH. Gene expression was normalized to freshly isolated human adult hepatocytes (HAH) (B) Gene expression analysis of the common hepatic markers *ALB* and *AFP* in hESC-derived hepatocytes. (C) Expression of the transcripts for the nuclear receptors *AhR* and *CAR*, *CYP* family members in hESC-derived fetal hepatocytes. $^{*}p$ <10^{-3}; $^{**}p$ <2×10^{-2}; $^{***}p$ <10^{-4}; $^{****}p$ <10^{-2}, $^{*****}p$ <10^{-8}, $^{******}p$ <10^{-6}. (D) Immunocytochemistry for hepatic markers in the hESC-derived hepatocytes generated using the 5-stage protocol.

the stage 3 → 4 → 5 cells expressed lower levels of *CAR* transcript (Fig. 6C). The level of expression of the nuclear receptor *PXR* was very low for both types of cells (data not shown). Although the expression of the transcripts for the nuclear receptors did not point to an advantage for including a hepatoblast stage (stage 4) in our differentiation protocol, we did observe interesting

differences in the induction of downstream targets of these receptors in responds to their ligands. Expression of *CYP3A7* and *CYP3A4* were significantly induced in the stage 3 → 4 → 5 cells, but not the stage 3 → 5 cells, and expression of *CYP1A1* was induced by both TCDD and PB, also only in stage 3 → 4 → 5 cells (Fig. 6C).

We also performed ICC on the stage 3 → 4 → 5 cells to evaluate the expression of typical hepatocyte markers. Consistent with the qRT-PCR data Fig. 6B, we saw near homogenous expression of AFP and ALB. The albumin expressing cells were also positive for HNF6, HNF4 and negative for the cholangiocyte marker SOX9, consistent with their commitment to a hepatic fate [26]. Co-expression of the hepatic transcription factors HNF6, PROX1, HNF1α as well as expression of the cell surface marker asialoglycoprotein receptor (ASGR) and the tight junction protein ZO-1, further support that the cells are moving towards a functional hepatocyte state (Fig. 6D) [35,26,25]. Finally, expression of the phase II enzyme UGT1A1 and the phase I enzyme CYP3A4 could be detected in these cells by ICC following treatment with phenobarbital (PB) (Fig. 6D), but not without induction (data not shown).

We placed these results in the context of primary human fetal hepatocytes (HFHs) and freshly isolated HAHs. We note that the expression of ALB, AFP, AhR, and CAR were in fact higher in the hepatocytes-like cells (HLCs) than in the HFHs or HAHs. The HFHs showed the highest drug-induced expression of CYP3A7, while the HAHs displayed the highest drug-induced expression of CYP3A4 and CYP1A1, consistent with the known developmental time course of expression of these metabolic enzymes. Compared to HLCs and HFHs, HAH cells displayed higher than the HLCs. The intermediate expression of CYP3A7, CYP3A4, and CYP1A1 in the HLCs, with levels between those seen in HFHs and HAHs, suggest that HLCs are not fully mature, but have developed beyond the stage present at 17 weeks gestation (the stage of development at which the HFHs were isolated).

Thus, we have shown that including an intermediate proliferative hepatoblast stage between the hepatic gut and fetal hepatocyte stages results in the production of functional fetal hepatocytes expressing the of the hepatic markers ALB, PROX1, HNF6, AFP, and ASGR, as well as being receptive to induction of the phase I and phase II enzymes CYP3A7, CYP3A4, CYP1A1, and UGT1A1 (Supplementary Fig. 8) by the nuclear receptor ligands TCDD and PB. We evaluated the expression of HNF4, as well as the CYP3A7/CYP3A4 ratio, over the final stages of differentiation, and compared them to HFHs and HAHs. As expected, we found a decrease in HNF4 expression at stage 5 compare to stage 4. HNF4 expression at stage 5 remains higher that in HFHs and HAHs (Supplementary Fig. 7A). *CYP3A7*/*CYP3A4* gene expression ratio shows show *CYP3A7* expression remains higher that *CYP3A4* expression in HLC (Supplementary Fig. 7B). Nevertheless, this ratio confirms the tendency observed with the decrease of *AFP* expression, cells are undergoing towards a more mature state as this ratio is lower than the ratio calculated for HFH.

To obtain a global view of the stage 5 cells differentiated using our final protocol, in the context of normal human liver development, we performed RNA sequencing of undifferentiated hESCs, the stage 5 cells, and human fetal and adult liver. First, we identified transcripts that were differentially expressed between undifferentiated hESCs, fetal liver, and adult liver, and then used CLICK correlation clustering to place the stage 5 cells into this context. The heat map and mean expression profile graphs (Fig. 7) show that the stage 5 cells from the final differentiation protocol are beginning to express typical hepatic transcripts (Cluster 1), which include the transporters of the ABC family, APO lipoprotein, *CYP2E1* and *2C9* and other genes involved in important hepatic functions (e.g. *TAT*, *TDO2*). As expected, the results also indicate that the stage 5 cells are losing expression of number of key pluripotency markers such as *OCT4* and *SOX2* (Cluster 2). Cluster 3 contains transcripts that are mostly highly expressed in fetal liver, with essentially no expression in the undifferentiated and adult liver samples, and low levels of expression in the HLCs. Interestingly, Cluster 4 shows that the stage 5 cells have begun to express markers specific of adult hepatocytes, which are not expressed in the fetal liver samples (*CYP2B6*, *CYP1A2*, *UGTs*). Taken together, these genome-wide gene expression results indicate that the HLCs are likely to be intermediate between the fetal and adult hepatocyte stages. This is consistent with our qRT-PCR and ICC results shown above.

Finally, the function of the stage 5 cells was assessed using a battery of phenotypic assays, including: CYP3A family, CYP1A1 and CYP2B6 activity with and without drug induction using luminescence-based P450-Glo Assays (Promega) with the appropriate substrates (please see Materials and methods for details); Periodic acid-Schiff (PAS) staining to demonstrate storage of glycogen; Oil Red O (ORO) and BODIPY staining to evaluate lipid storage; LDL uptake; and urea production. For the CYP assays, the HLCs demonstrated statistically significant induction with the appropriate drugs, at levels that were comparable to HFHs for CYP1A1 (Fig. 8A and Supplementary Fig. 9). The urea production was also comparable in the HLCs to HFHs (Fig. 8B). It is important to note that freshly isolated adult hepatocytes showed higher basal activity and induction of CYP2B6 and CYP1A1, as well as a higher level of urea production (Fig. 8A and Supplementary Fig. 9). Finally, positive staining for lipid, glycogen, and LDL in the HLCs are shown in Fig. 8C.

To confirm the generalizability of our final hepatic differentiation protocol to human induced pluripotent stem cells (hiPSCs), we differentiated two hiPSC lines (hiPSC1, reprogrammed from HDFs from an individual with type 2 Crigler-Najjar Syndrome; and hiPSC2, reprogrammed from HDFs from a healthy individual), and demonstrated excellent expression of the appropriate stage-specific markers (Supplementary Fig. 10), as wells as inducible CYP activity, urea production, and glycogen and lipid storage (Supplementary Fig. 11).

Discussion

In this study, we present a novel approach to generate fetal hepatocytes from hPSCs. Attempting to better recapitulate the events in normal hepatic development in defined conditions, our new method includes modulation of additional signaling pathways that have not been addressed in previous protocols, and includes an additional stage of differentiation, namely, proliferative hepatoblast. A number of previously published methods have reported differentiation of hPSCs to metabolically functional hepatocytes, but the resulting cells express markedly lower levels of mature hepatocyte markers compared to adult hepatocytes [5,7]. It has been suggested that one of the major deficiencies in existing methods is that they do not include an intermediate stage of differentiation in which the cells express critical transcription

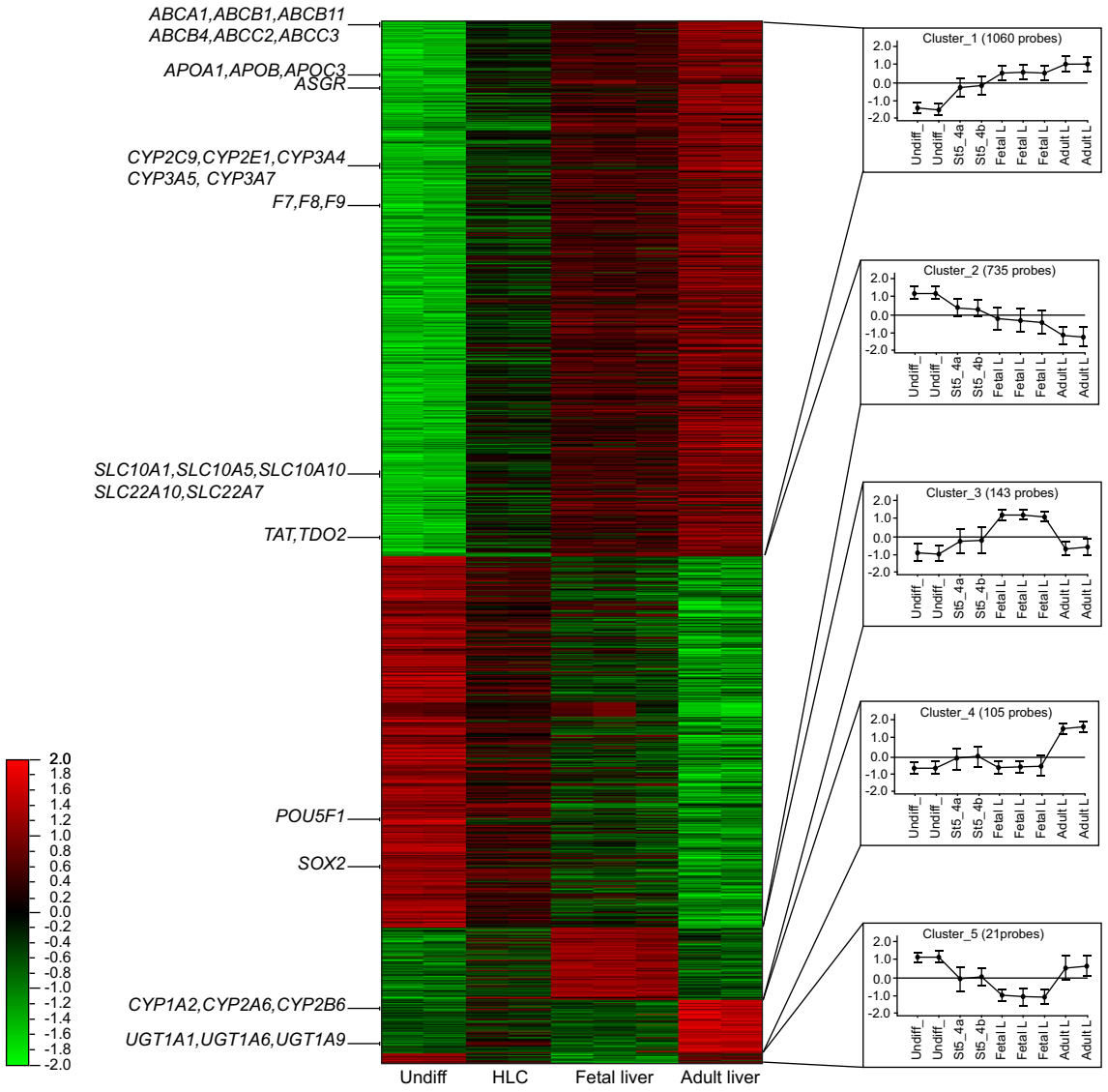

Fig. 7. Comparison of stage 5 hepatocyte-like cells with fetal and adult liver. Transcripts that are differentially expressed between undifferentiated hESC and human fetal liver and/or undifferentiated hESC and human adult liver are shown (*t* test, adjusted *p* value <0.00001, maximum number of counts across all samples >20, absolute fold-change between groups >4). The differentially expressed transcripts were clustered into co-expressed groups using CLICK. Results are shown in the heatmap on the left, as well as the mean expression graphs on the right.

factors necessary for later maturation of the cells, such as PROX1 and HNF6/ONECUT1 [26].

In order to correct these drawbacks, starting with DE generated using the defined method we reported in [6], we optimized conditions for generating PFG cells, which are the common progenitors for the hepatocytes and pancreatic lineages. Still using defined factors, we have shown that inhibition of WNT/β-catenin pathway by the chemical inhibitor IWR-1 was sufficient to induce expression of a panel of genes, including HNF4, HNF1β, and HHEX, which characterize the specification of PFG during early development. Importantly, in contrast to a recent report [16], we did not find it necessary to combine WNT inhibition with retinoic acid and inhibition of FGF4 and BMP4 for efficient induction of PFG; on the contrary, we found that fibroblast growth factor (FGF) inhibition had a dramatic negative effect on HHEX expression.

We then improved conditions for establishing the hepatic gut stage by combining findings from previous studies indicating that stimulation of BMP4 [19,4,26] and inhibition of TGF-β [6,19,16] are important for hepatic specification with knowledge gleaned from developmental biology studies indicating that inhibition of the WNT/β-catenin pathway is important for acquisition of the hepatic and pancreatic domain across diverse species [36,37,14]. Despite the fact that RA has frequently been used to induce hepatic differentiation, our results indicated that it strongly induced expression of the intestinal transcription factor CDX2.

Postulating that incorporating a proliferative hepatoblast stage would promote later maturation, we built upon the observation that loss of WNT/β-catenin activity resulted in liver hypoplasia at E12 of mouse embryogenesis and lethality at E17 [29], we used the GSK3 inhibitor CHIR99021 to activate the

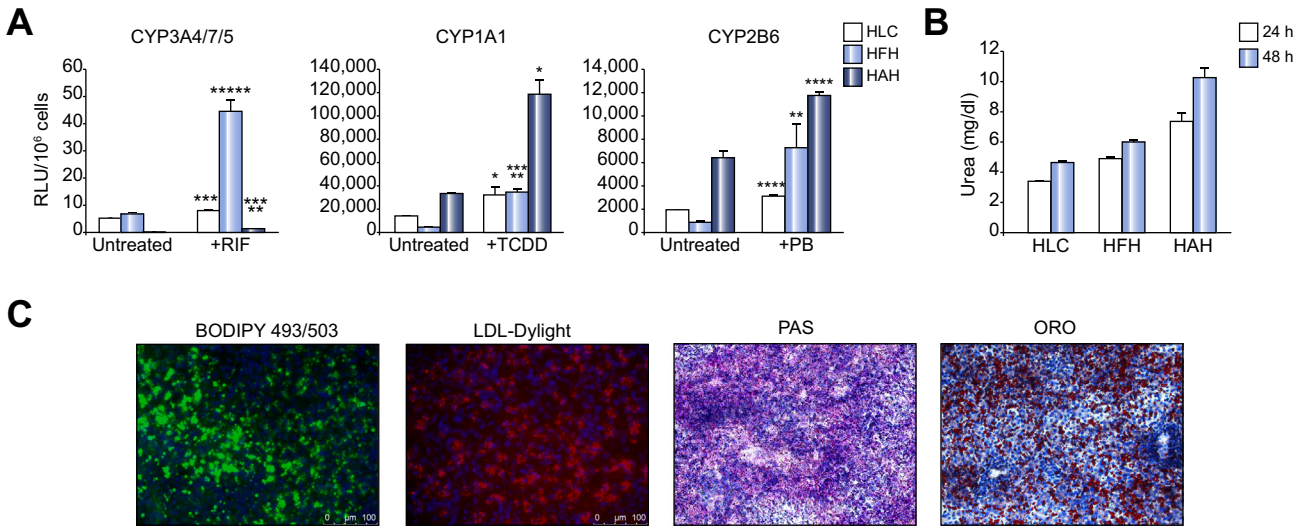

Fig. 8. Functionality of hESC-derived hepatocytes generated using the 5-stage protocol. (A) Activity of CYP family members CYP3A, CYP1A1, and CYP2B6 in untreated hepatocytes-like cells (HLC) and after exposure to RIF, TCDD and PB. Human fetal hepatocytes (HFH) and freshly isolated human adult hepatocytes (HAH) were used as positive controls. The values are indicated as relative light unit (RLU) per million cells and represent the average of 3 independent biological replicates. $^{*}p$ <10^{-5}; $^{**}p$ <10^{-8}; $^{***}p$ <10^{-11}; $^{****}p$ <10^{-3}; $^{*****}p$ <10^{-7}. (B) Production of urea in culture medium by the hESC-derived hepatocytes generated using the 5-stage protocol (HLC) at 24 and 48 h. HAH and HFH were used as positive controls. The values represent the average of 3 independent biological replicates. (C) Oil Red O (ORO) staining and BODIPY 493/503 show storage of lipids by HLCs. Periodic acid-Schiff (PAS) staining show glycogen storage by the HLCs. Uptake of LDL shown by LDL-Dylight staining.

WNT/β-catenin pathway. We found that inclusion of CHIR resulted in increased proliferation and induction of the transcription factors *TBX3*, which has been shown to play a critical role during liver bud formation by maintaining the pool of proliferative hepatoblasts by inhibiting their early differentiation into hepatocytes [20], HNF6 and PROX1, which as we discussed above, are associated with subsequent improved maturation.

In vitro maturation conditions typically include the use of HGF and OSM. However, *in vivo*, bipotent hepatoblasts in contact with the portal veins differentiated into cholangiocytes under TGF-β and NOTCH signaling originating from the endothelial cells of the portal veins [33,38]. In contrast, the cells that mature into hepatocytes do not receive these signals. Here, we have shown that dual inhibition of the NOTCH and TGF-β signaling pathways in the presence of HGF are directed to a hepatocyte fate, with rapid upregulation of ALB expression and maintenance of high levels of expression of CEBPA and HNF1α, and downregulation of the cholangiocyte-associated transcription factor SOX9. After 14 days in these maturation conditions, there was marked induction of several of the major hepatic enzymes via the nuclear receptors CAR, AhR, and PXR. The expression of these enzymes was responsive to induction by TCDD, Rifampicine and/or phenobarbital; this induction is a hallmark of functional hepatocytes (fetal and adult).

Our results indicated that inclusion of the hepatoblast stage in our differentiation protocol led to the strongest induction of these enzymes with the appropriate drugs. Cells that were transitioned directly from the hepatic gut stage into maturations conditions (without passing through the hepatoblast stage) at times showed a higher basal level of expression some of the enzymes, but did not show robust induction. The weak induction of PROX1 and HNF6 in the hepatic gut-to-maturation cells might be due to exposure to the TGF-β inhibitor SB in the maturation conditions, which may promote proliferation and maintenance of any hepatoblast cells that might be present, as was described in a study that reported increased expansion of mouse hepatoblasts, not only by CHIR, but also by a TGF-β inhibitor [22].

Considering our targeted and genome-wide gene expression, immunocytochemistry, and functional results together, we conclude that our HLCs occupy a developmental stage intermediate between fetal and adult hepatocytes. Although our cells are not completely mature, they do express the hallmarks of fetal hepatocytes in terms of expression of key metabolic enzymes and detectable, but still low expression of the nuclear receptor PXR [39,40]. Moreover, the inclusion of the hepatoblast stage was associated with lower gene expression of AFP at the hepatocyte stage, which is consistent with initiation of the transition from fetal to adult hepatocytes (with decreased *AFP* expression and increased *ALB* expression).

In summary, we have developed defined conditions for directed differentiation of hESCs to hepatocytes using a 5 step protocol that exposes the cells to a series of factors mimicking the signals received during normal hepatic development, and shown that the cells follow the progression of the differentiating cells through multiple progenitor stages from DE, through the posterior foregut, hepatic gut, and hepatoblast stages, and finally to fetal hepatocytes. This is the first report demonstrating that regulation of the WNT/β-catenin pathway should be integrated not only for DE commitment, but also at the hepatic gut and hepatoblast specification stages. Although our cells are not fully mature and comparable freshly isolated adult hepatocytes, this work serves as a robust foundation for future studies aimed at defining conditions for further maturation. For example, although we found that inclusion of OSM for 4 days did not appreciably improve maturation, it is possible that longer periods of exposure to this factor may be beneficial. In addition, the ability to generate cells at a bipotent hepatoblast stage will allow development of methods for directing differentiation to the cholangiocyte fate, for which there are as yet few reports [41].

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Conflict of interest

The authors who have taken part to this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contribution

TT contributed to the acquisition of data, its analysis and interpretation; SC and RHT contributed to the acquisition of the drug induction datas and its interpretation; SMC contributed to the generation of the RNAseq libraries; CCT contributed to the analysis and normalization of the RNAseq datas; KS contributed to the generation of the hiPSC lines; TT and LCL were in charge of the overall conception, design of the study and writing of the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.02.028.

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Author names in bold designate shared co-first authorship

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