Expression kinetics of hepatic progenitor markers in cellular models of human liver development recapitulating hepatocyte and biliary cell fate commitment

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Abstract

Due to the limitations of research using human embryos and the lack of a biological model of human liver development, the roles of the various markers associated with liver stem or progenitor cell potential in humans are largely speculative, and based on studies utilizing animal models and certain patient tissues. Human pluripotent stem cell-based *in vitro* multistage hepatic differentiation systems may serve as good surrogate models for mimicking normal human liver development, pathogenesis and injury/regeneration studies. Here, we describe the implications of various liver stem or progenitor cell markers and their bipotency (i.e. hepatocytic- and biliary-epithelial cell differentiation), based on the pluripotent stem cell-derived model of human liver development. Future studies using the human cellular model(s) of liver and biliary development will provide more human relevant biological and/or pathological roles of distinct markers expressed in heterogeneous liver stem/progenitor cell populations.

Keywords: Liver stem cell, liver progenitor, hepatoblast, human liver development, pluripotent stem cells, hepatic differentiation, biliary differentiation, bipotency

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Introduction

For years, embryonic and liver development has been studied thoroughly based on animal models, resulting in progress in the fields of developmental biology as well as regenerative medicine. These studies laid the groundwork for directed differentiation of human pluripotent stem cells (PSC) into hepatocytes, which in turn have augmented our understanding of the signaling processes governing the various stages of liver development and the differentiation stage specific markers.[1–10] While the phenotypic markers for undifferentiated pluripotent stage, endoderm stage, and the mature liver stage are better established, it has been unclear which markers represent multipotent hepatic stem cells or hepatoblast-like bipotent liver progenitor cells. Here, we describe the hepatocytic and biliary commitment of human liver progenitor cells, and the implications of various liver stem/progenitor markers with an emphasis on human stem cell based model of liver development.

Liver development and implicated signaling

Germ-line specification occurs during gastrulation, forming the three germ layers; which give rise to various organs. The liver develops from the anterior definitive endoderm, driven by fibroblast growth factor (FGF) signaling[11] from the adjacent cardiac mesoderm[12,13] and bone morphogenetic protein (BMP) signaling from the septum transversum mesenchyme cells (STM) in the 2–4 somite stage of the embryo.[14] STM cells highly express GATA4, a zinc finger transcription factor, which is critical for the growth of the liver bud.[15,16] The secretion of BMP4, which is also critical for the expansion of the liver and hepatic specification, is regulated by GATA4.[17] In addition, WNT signaling plays a complex role in hepatic development. At the early somite stage in the posterior endoderm, WNT signaling represses the expression of *hhex* (hematopoietically expressed homeobox protein), a vital regulator of hepatic specification in mouse.[18] WNT antagonists in the anterior endoderm are required to relieve WNT signaling, and thus the *hhex* repression to facilitate the endodermal commitment to a hepatic fate.[19] However, in multiple model systems WNT signaling appears to promote hepatogenesis,[19–21] but does not seem to be critical for the process. Forkhead box A1 (FOXA1) and Forkhead box A2 (FOXA2) seem to be especially critical for FGF signaling driven early hepatic specification,[22] however,

the later stages of hepatocyte differentiation following the specification of liver progenitors are independent of FOXA1/2.[23] Since a majority of these reports are based on non-human organism based research studies, knowledge of human liver development and the associated signaling mechanisms is limited.

Identification of human liver stem cells and hepatoblasts

Hepatic stem cells in the human liver are multipotent cells, located in the ductal plates in fetal and neonatal livers, and in the Canals of Hering in pediatric and adult liver.[24] Human hepatic stem cells are reported to express epithelial cell adhesion molecule (EpCAM), CD133, SOX9, cytokeratins (CK) 8/18/19, neural cell adhesion molecule (NCAM), and also markers associated with endoderm such as CXCR4, SOX17, and FOXA2. They do not express alpha-fetoprotein (AFP), intercellular adhesion molecule (ICAM) 1, cytochrome P450s, and only show weak or negligible expression of albumin (ALB).[25,26] These hepatic stem cells have been isolated from donor livers of all ages by dual immunoselection for EpCAM+/NCAM+ cells. In adult human livers, with their inherently scarce population of hepatoblast-like cells, selection for EpCAM+ cells results in isolation of hepatic stem cell population.[25,26] In contrast, immunoselection for EpCAM+ cells from fetal livers results in predominantly hepatoblast population isolation with only a small percentage of hepatic stem cells.[25,26] These isolated hepatic stem cells are capable of self-renewal and differentiate both *in vitro* and *in vivo* into hepatocytes and cholangiocytes, the epithelial cells of bile-duct.[26,27]

The hepatoblast cells within the aforementioned fetal liver bud express AFP and are bipotent, capable of generating hepatocytes and cholangiocytes.[28] These bipotent hepatoblasts have been isolated from human fetal liver (18–20 gestational age) by dual immuno-selection for EpCAM+/ICAM+ cells.[29] In human adult livers, AFP+ hepatocytes have been reported to increase with disease or acute injury.[28,30] Human hepatoblasts and hepatic stem cells share an overlap in their phenotypic markers. They both express EpCAM and both do not express hematopoietic markers (CD45 and CD34) or mesenchymal markers (CD146 and KDR). They are discernable from each other in that hepatoblasts express ICAM1, CK7, AFP and early P450s, while hepatic stem cells express Neural cell adhesion molecule (NCAM) and claudin 3.[24,25,31]

Hepatocytic and biliary commitment of hepatoblast-like bipotent liver progenitors

A delicate balance between several signaling pathways such as the transforming growth factor β(TGF-β), WNT, FGF, and BMP is required for the development of liver.[19,32] In animal liver buds, developing hepatoblasts are exposed to multiple growth signals from various cell sources[33–35] promoting development into hepatocytes and cholangiocytes; the hepatoblasts near the portal vein differentiate and become committed to the cholangiocyte lineage, whereas the hepatoblasts exposed to Oncostatin M differentiate and commit to the hepatocyte fate.[36] Hepatocytes from human PSC-derived hepatoblast-like hepatic progenitors have been generated by others and us (Figure 1) harnessing the above cues,[3,8,37–40] with significantly higher efficiencies than those generated from other cell sources such as primary cells,[40,41] cell lines,[42–44] and mesenchymal stem cells.[45,46] We have also shown both the *in vitro* and the *in vivo* functionalities of human stem cell-derived multistage hepatic cells by demonstrating their potential in disease modeling, drug screening as well as liver engraftment and regeneration.[1,2,7,41]

Cholangiocytes are epithelial cells that line the intra- and extra-hepatic bile-ducts. In humans, around the 8th gestational week, hepatocytes near the portal mesenchyme form the ductal plate, a ring of cells from which cholangiocytes develop.[47] NOTCH pathway is one of the most important pathways driving biliary commitment, by inducing SOX9 expression, which controls bile duct morphogenesis and is thus considered to be the earliest and most-specific marker of biliary cells in the developing liver.[47] SALL4 also plays a key role in biliary commitment of hepatoblasts, by inhibiting their differentiation toward hepatocytes and instead driving cholangiocyte fate.[48] Hepatoblast-like progenitor cells derived from human induced pluripotent stem cell (iPSC), embryonic stem cell (ESC), and HepaRG cells have been differentiated into cholangiocytes by employing different combinations of epidermal growth factor (EGF), interleukin-6, growth hormone (which regulates the insulin-like growth factor pathway),[49] and sodium taurocholate hydrate.[50–52] Others and we have recently demonstrated that these cholangiocytes can develop into functional cysts and biliary ducts in 3D culture conditions.[50,53,54] Recent advances have demonstrated the *in vivo* ability of these human hepatic progenitor cells derived from primary,[40,41,55,56] cell line[42] or hepatic stem cell[57] sources to engraft into animal model livers and differentiate into cholangiocytes that are usually located near or in bile ducts.

Markers for human liver stem and progenitor cells

To identify liver stem cells, hepatoblast-like bipotent progenitors and committed precursors for hepatocytes and cholangiocytes, studies have generated considerable information about the markers that specify early hepatic development, as summarized in the Table 1. Terminology for these reported markers is not very clear and many have used the terms liver stem cells, progenitors, and hepatoblasts without a clear distinction, some have also used the progenitors and precursors interchangeably, as if they have the same capacity. By definition, 1) the term ''liver stem cells'' should ideally be used for the multipotent and self-renewing liver stem cells which can generate hepatocytes, ductal epithelial cells (cholangiocytes), and other cell lineages in liver, 2) ''liver progenitor cells'' should be designated for the bipotent, hepatoblast-like cells which can give rise to both hepatocytes and cholangiocytes, 3) the term ''hepatoblasts'' should be used for fetal (prenatal) bipotent liver progenitor cells, and 4) the term ''liver precursor cells'' should be used for the unipotent hepatocyte- or

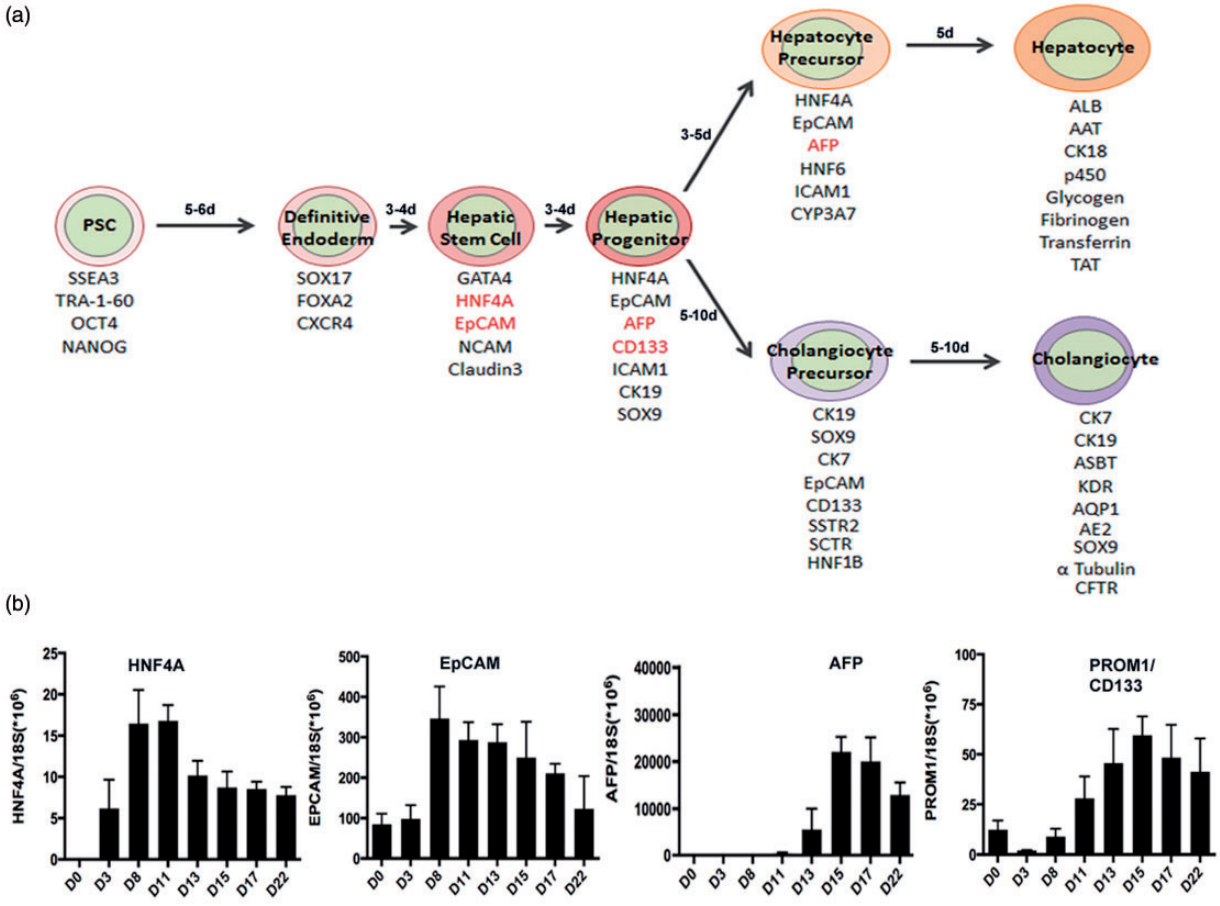

Figure 1 Human iPSC-based *in vitro* model of liver development. (a) Human iPSC-based model of hepatic and bile ductal development depicting the stages passed through during endodermal commitment, liver stem cell and hepatoblast-like liver progenitor formation, hepatocyte- and cholangiocyte- precursor formation and terminal differentiation into mature hepatocytes and cholangiocytes (biliary epithelial cells). Stage-specific markers and time lines are denoted at each stage. (b) Representative expression kinetics of HNF4A, EpCAM, AFP, and CD133 with regards to the differentiation day of an *in vitro* human iPSC-based model of liver development. (A color version of this figure is available in the online journal.)

Table 1 Hepatic stem or progenitor cell markers.

Marker	Reported function in liver	Studied species	References
HNF4A	Early hepatic endoderm, hepatic stem or progenitor marker. Transcription factor controls the expression of various hepatocytic genes, and maintain adult liver function.	Mouse, human	72–74,78,79,82,83
EpCAM	Early hepatic stem or progenitor marker. Roles in cell adhesion, proliferation, differentiation, migration, cell cycle progression and regeneration following ductular reaction in liver.	Mouse, rat, human	26,87,88,89,92,94,95
CK19	Hepatoblast, hepatic progenitor, or cholangiocyte marker. Roles in liver development and regeneration.	Rat, human	118–120
CK7	Ductal precursor, cholangiocytes, or hepatic progenitor marker. Roles in liver development and regeneration.	Rat, human	118–121
AFP	Hepatoblast or fetal hepatocyte marker. Increase in acute liver failure and hepatocellular carcinoma.	Mouse, human	28,99,103,104,105,107
CD133 (PROM1)	Possible hepatic stem or progenitor marker. Roles in fibrosis and cancer stem cell identity maintenance.	Mouse, human	112–115
SOX9	Hepatic progenitor or cholangiocyte marker. Role in regeneration following ductular reaction in liver.	Mouse, human	123–126
NCAM	Immature biliary cells and hepatic stem cell marker Roles in intrahepatic duct development, ductular reactions and liver regeneration	Rat, human	26,135,139,140,141
ICAM1	Hepatoblast marker Role in leukocyte recruitment at inflammatory sites and in liver regeneration	Rat, human	26,150,149,143

cholangiocyte- precursors which have already committed to only one direction in further differentiation potential.

The cells that give rise to hepatic endoderm express key phenotypic markers denoting a hepatocytic cell fate such as ALB, AFP, transthyretin (TTR), retinol binding protein (RBP), and hepatocyte nuclear factor 4A (HNF4a) between the 7 and 11 somite stage of the mouse embryo.[58] Studies have further implicated the role of other transcriptional regulators in later hepatic development, namely Onecut-1 and Onecut-2 that are vital for hepatoblast migration,[59] and Prospero Homeobox 1 (PROX1) which promotes hepatoblast proliferation and also has a role in their migration.[60] The critical regulators of hepatoblast differentiation such as HNF4A and CCAAT/enhancer binding protein (c/EBPa) are highly expressed by migrating hepatoblasts, while the expression of cholangiocyte fate regulators hepatocyte nuclear factors (HNF) such as HNF6 and HNF1β is very low. Oval cells, that are thought to arise from the canals of Hering[61,62] and are bipotent,[63–65] express TROP2 in mice.[66] In mice, Foxl1 has also been proposed as a marker of bipotent liver progenitors.[67] In normal human liver, cholangiocytes express osteopontin,[68] and its expression in liver is increased in response to acute inflammation[69,70] and liver fibrogenesis.[71] Thus, there are a wide variety of suggested markers for liver stem or progenitor cells. Below, we discuss the most relevant and speculated markers for liver stem cells and progenitor cells.

HNF4A has been implicated as a marker for liver stem or progenitor cells in humans[72] and animals,[73,74] and for early hepatic endoderm.[75–77] HNF4A is a nuclear hormone receptor transcription factor and has a critical role in controlling the expression of various hepatocytic genes.[78,79] It is considered to be at the pinnacle of all transcription factors that power hepatocytic differentiation[80] and is vital for hepatocyte differentiation in murine fetal liver,[74,81] and also plays a role in maintenance of liver function in adult.[82,83] HNF4A, along with HNF1A and HNF1b is also implicated in development and functioning of the pancreas.[84,85] Thus, HNF4A is important for the development and functionality of hepatocytes and beta cells. Based on our human iPSC-based model of liver development (Figure 1), HNF4A is highly expressed during the very early hepatic specification stage (i.e., day 8 to day 11 of hepatic differentiation) following definitive endoderm stage and decreases when AFP starts to increase, suggesting that HNF4A might be a marker for early liver stem cells rather than a hepatoblast-like hepatic progenitor.

EpCAM, a transmembrane glycoprotein, has diverse roles in cellular processes such as cell adhesion,[86] proliferation,[87,88] differentiation,[89] migration,[90] and cell cycle progression.[91] In addition, EpCAM has been reported as a marker for human liver stem or progenitor cells.[26,92,93] In humans, hepatoblasts are tethered to the canals of Hering near the portal triads, which are the sites of ductular reaction during regeneration, by exclusively membranous EpCAM expression.[94] Regeneration responses following liver necrosis involve the proliferation of human EpCAM+ hepatic stem and/or progenitor cells.[94] EpCAM+ human fetal stem/progenitor cells are located in ductal plate *in situ*, and on isolation are capable of self-renewal and differentiation into both hepatocytes and cholangiocytes, and further on transplantation are capable of engraftment in the livers of immunodeficient mice.[26,95] In human embryonic liver, bulk of the hepatocytes express EpCAM; however in adult liver, hepatocytes do not express EpCAM,[93] while the bile duct epithelium does.[96] EpCAM is highly expressed in murine and human embryonic stem cells and is down-regulated during spontaneous differentiation (on LIF withdrawal or embryoid body differentiation).[89,97] Concurrently, EpCAM also has an important role in enhancing pluripotency reprogramming and iPSC generation through OCT4 upregulation and a possible suppression of p53-p21 pathway.[98] In line with these findings, our human iPSC-based model of liver development demonstrates that EpCAM which is already expressed at a low level in undifferentiated iPSCs, sharply increases in early hepatic stem/progenitor stage cells and gradually decreases with hepatocytic maturation (Figure 1).

AFP expression is high in fetal hepatocytes, and reduces sharply as they mature to an adult phenotype in mouse liver.[99] Zhx2 is a key transcriptional regulator, which induces the repression of AFP, H19, and GPC3[100,101]; while ZBTB20 has a key role in suppression of AFP during the switch from fetal to adult hepatocyte phenotype.[102] In murine and human liver, AFP has been known as a fetal hepatoblast or bipotent liver progenitor marker.[28,103–105] While AFP expression is high in fetal liver, it is still expressed in adult human liver[106,107] and normal range of detectable AFP in human circulation is <20 ng/mL.[107] In liver cancer, it increases to >400 ng/mL.[108] In our human iPSC-based model of hepatic development, AFP was expressed from hepatic progenitor stage cells to early hepatocyte stage cells and decreased in more mature hepatocytes[2,3,7,8] (Figure 1). Our findings are consistent with previously established data suggesting AFP as a hepatoblast-like progenitor or early hepatocyte marker in human tissue findings described above.

Prominin 1 (CD133) has long been regarded as a primitive hematopoietic and neural stem cell marker,[109] however recent evidence suggests it may also be a cancer stem cell marker in solid cancers such as brain tumors,[110] renal tumors,[111] liver cancer,[112] and colon[113] and prostate carcinomas.[114] Recent evidence suggests that CD133 is also a marker for the oval cells in adult murine liver, which have the gene expression profile and function of bipotent, primitive liver stem cells,[115] CD133, thus has been considered as a liver progenitor marker. However, CD133 is also suggested as fibrosis marker, since hepatic stellate cells express CD133 and are involved in liver fibrosis,[116] especially in biliary atresia-associated liver fibrosis.[117] Therefore, CD133 in human liver may be a marker for a more multipotent progenitor, which produces not only hepatocytes and biliary cells but also hepatic stellate cells. Based on our human iPSC-based model of liver development, CD133 (PROM 1) is expressed in both hepatic stem and progenitor stage cells, and its expression decreased upon further hepatocyte differentiation and maturation (Figure 1).

CK19 and *CK7* have been considered to be indicative markers of biliary differentiation[118] and liver progenitors in both rat and human tissue studies.[119,120] In a human

tissue study, low expression of CK19 is seen in hepatoblasts which rises steadily in differentiating cholangiocytes; while CK7 expression is only detectable when cholangiocytes have already committed to their fate.[118] On the other hand, there have been many reports suggesting that these two cytokeratins are indicative of liver progenitors, regenerating hepatocytes in adult human liver[119–121] or ductal precursors.[118,122] The roles for these markers in liver development or regeneration are seemingly overlapping, and it is not clear if these markers are restricted to ductal precursors or whether they are expressed in subsets of bipotent liver progenitors within the human liver.

SOX9 (Sex Determining Region Y-Box 9) is also regarded as a murine liver progenitor marker.[123] SOX9 expression at E11.5 may be an indication of hepatoblast commitment to a biliary fate, as SOX9 expression is first detected at E11.5 in liver epithelial cells (hepatoblasts) which are located near the portal vein branches, where biliary cells differentiate; while at the later developmental stages SOX9 expression is restricted to the biliary cells in mice.[47] In mice, the reactive ductular cells and many hepatocytic cells near the periportal tracts, where the ductular reaction occurs, are positive for SOX9.[124] In humans with severe nonalcoholic fatty liver disease (NAFLD), the ductular reaction observed in response to the disease is characterized by increased expression of Hedgehog target genes such as SOX9, SPP1, and Jagged-1.[125,126] SOX9 thus seems to have a key role in induction of liver regeneration. Along with ductular progenitors, hepatic stellate cells also express SOX9 in human and rodent liver,[127,128] however, this is a highly debated issue, as some groups have not been able to confirm this. Murine lineage tracing experiments have shown that embryonic SOX9+ cholangiocytes can also give rise to hepatocytes.[123,129] Abnormal expression of SOX9 in fetal human hepatocytes results in ectopic expression of genes encoding components of the extracellular matrix. Also during liver damage, hepatic stellate cells regulated by a SOX9-dependent process, proliferate into myofibroblasts that migrate to the adjoining parenchymal tissue and secrete extracellular matrix components. Activated hepatic stellate cells in turn lead to SOX9 expression and Collagen 1 production which is mediated by TGFβ signaling.[130] SOX9 is also a critical regulator of Osteopontin, and extracellular matrix component, which serves as a biomarker for detecting the severity of liver fibrosis.[128] Overexpression of SOX9 has been noted in hepatocellular carcinoma and is associated with poor clinical prognosis.[131] However, it is not clear how SOX9 is regulated in normal human liver development. It is therefore of vital interest to identify the roles of SOX9 in human liver development and pathogenesis.

NCAM has been known to have roles in morphogenesis, migration and remodeling via cell–cell and cell–matrix interactions in several organs.[132–134] In adult (human and rat) liver, the immature biliary cells present in the reactive bile ductules express NCAM.[120,135,136] These ductules occur in various liver diseases and contribute to an atypical ductular reaction,[137,138] which is modulated by NCAM.[139] In humans, NCAM may have a role in intrahepatic duct development since its patchy expression is observed in the duplicated ductal plates and merging bile ducts from gestational 16th to 40th week. In contrast, ductal plate malformations such as extra-hepatic bile duct atresia and congenital hepatic fibrosis are associated with an overexpression of NCAM.[140] Activated portal fibroblasts observed in the regenerating livers of rats following partial hepatectomy also express NCAM, further supporting the morphogenesis roles of NCAM.[141] Hepatic stem cells have also been known to express NCAM, in addition to EpCAM and Claudin 3.[26]

ICAM-1, a member of the immunoglobulin superfamily,[142] is expressed on the surface of various cells at inflammatory and immune reactive sites,[143] and is induced by pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α.[144] ICAM1 expression has been observed in various inflammatory liver disorders such as human liver allograft rejection,[145] autoimmune liver diseases[146,147] and hepatitis B.[147,148] In mice, ICAM1 also plays a role in liver regeneration through recruitment of leukocytes and triggers the proliferation of hepatocytes via kupffer cell-dependent release of cytokines TNF α and IL-6.[149] Studies have shown that the hepatoblasts isolated from E13 rats were ICAM1+.[150] Human hepatoblasts differentiated from isolated ICAM-fetal hepatic stem cells, have been shown to be express ICAM1, CK19, CD133, EpCAM, and AFP.[26]

Conclusions and perspectives

Due to the limitations of research using developing human embryo or liver tissue and lack of a biological model of human liver development, the roles of the various markers associated with liver stem or progenitor potential and cell fate determination in human liver are currently highly speculative. Based on our human iPSC-based model of liver development, expression of some liver stem markers such as HNF4A and EpCAM increases in early hepatic differentiation stage cells right after the definitive endoderm stage, and decreases with hepatocytic maturation, while the hepatoblast marker AFP is expressed later during the hepatic specification stage and decreases in mature hepatocytes (Figure 1). Therefore, we hypothesize that HNF4A and EpCAM might be markers for more early stage hepatic stem cells, and AFP may be a marker for a later stage or less potent, hepatoblast-like liver progenitors in the human liver development. However, considering the current uncertainty of roles of these known markers for liver stem or progenitor cells (Table 1), and potential heterogeneity of these primitive liver cell subsets, further research warrants extensive biological studies for determining the roles of each marker for identifying liver stem, progenitor or committed precursor cell populations in a human relevant setting. Human iPSC-based *in vitro* hepatocytic and ductal differentiation systems will serve as good surrogate models for mimicking human liver development and for liver disease studies, and may provide a highly human relevant research tool for determining biological functions of many liver stem/progenitor markers; and potentially for discovery of new more reliable stage-specific markers in human liver development and the cell fate determination process associated with liver disease pathogenesis.

Methods

Human iPSC culture, directed hepatic, hepatocytic, and ductal differentiation and quantification of target gene mRNA was performed as previously described.[2,3,7,8,37,54]

Author Contributions: PC and YYJ wrote the manuscript. PC, YYJ, and LT collected the data, and performed data analysis. AD helped in writing the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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