Single cell analysis of human foetal liver captures the transcriptional profile of hepatobiliary hybrid progenitors

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The liver parenchyma is composed of hepatocytes and bile duct epithelial cells (BECs). Controversy exists regarding the cellular origin of human liver parenchymal tissue generation during embryonic development, homeostasis or repair. Here we report the existence of a hepatobiliary hybrid progenitor (HHyP) population in human foetal liver using single-cell RNA sequencing. HHyPs are anatomically restricted to the ductal plate of foetal liver and maintain a transcriptional profile distinct from foetal hepatocytes, mature hepatocytes and mature BECs. In addition, molecular heterogeneity within the EpCAM\textsuperscript{+} population of freshly isolated foetal and adult human liver identifies diverse gene expression signatures of hepatic and biliary lineage potential. Finally, we FACS isolate foetal HHyPs and confirm their hybrid progenitor phenotype in vivo. Our study suggests that hepatobiliary progenitor cells previously identified in mice also exist in humans, and can be distinguished from other parenchymal populations, including mature BECs, by distinct gene expression profiles.
In rodents both hepatocytes and biliary epithelial cells (BECs) are derived from a common bi-potent hepatoblast population during liver development. In adult mice, conflicting evidence exists regarding the presence of a distinct bi-potent progenitor capable of regenerating both hepatocytes and BECs. The regenerative potential of the rodent liver has been attributed to hepatocytes, BECs, biliary-like progenitor cells or ‘oval cells’ arising in the ductal region, stem cells located around the central vein and hepatocyte or cholangiocyte de-differentiation into a hybrid bi-potent progenitor. In comparison, the mechanisms of human liver regeneration are poorly characterised. It has been proposed that EpCAM+ human liver stem/progenitor cells reside in the ductal plate (DP) during foetal liver development. After birth these cells localise to the Canals of Hering, where upon severe chronic liver injury they become reactivated forming what is pathologically described as ductular reactions. Despite these findings, the existence of a bi-potent human liver ‘progenitor’ cell remains unclear. This issue is in part due to a substantial overlap in markers between potential progenitor populations, hepatic precursors and mature BECs, challenging the field to define the true transcriptional nature of a bi-potent progenitor phenotype that can be replicated for clinical use. Several recent studies have captured a bi-potent progenitor-like state via small molecule-reprogramming of primary hepatocytes, capable of in vitro hepatic and biliary maturation, imitating a process that has been observed during chronic mouse liver injury. Despite several well-established phenotypic criteria for liver progenitor cells, no benchmark exits that truly distinguishes them from other human hepatic and biliary cells. To facilitate the in vitro development of cell-based therapies for treating liver disease, it is critical to precisely define a liver progenitor cell that accurately captures the developmental origin of human liver parenchyma.

In this study we utilise single-cell RNA sequencing (scRNA-seq) to interrogate the transcriptome of human foetal and adult liver at single-cell resolution. In recent years scRNA-seq has helped identify unreported cell types within populations previously defined as homogenous. Here, we report the transcriptional signature of distinct hepatic cell types in foetal and adult human liver, including a foetal hepatobiliary hybrid progenitor (HHyP) population. Capturing a human hepatic progenitor state in utero provides unparalleled and unexplored insight into the true mechanisms of human liver development. We identify a gene expression profile that can distinguish between foetal HHyPs, foetal hepatocytes and mature BECs. We further identify HHyP-like cells maintained in uninjured adult primary liver tissue. Finally, we FACS sorted HHyPs from freshly isolated human foetal liver and show evidence of hepatic and biliary phenotypes in vivo. Our in depth profiling of previously undefined HHyPs finally provides an accurate template for the human liver progenitor phenotype that will be a valuable roadmap for translating ex vivo hepatic progenitor studies into successful cell-based liver disease therapies.

**Results**

**EpCAM+ cell heterogeneity in human foetal and adult liver by scRNA-seq.** To capture the cellular heterogeneity of human liver during development, we combined a FACS strategy with scRNA-seq. We first sorted by negative selection of red blood cells and immune cells (CD45), and positively selected for EpCAM and NCAM to enrich for potential human liver progenitors. Supplementary Fig. 1. To investigate how foetal human liver populations progress into adult liver we isolated and sequenced EpCAM+ (biliary cells) and EpCAM−/ASGPR1+ (mature hepatocytes) cells from fresh, uninjured adult tissue (Supplementary Fig. 1). In total, 1224 cells were sequenced from human foetal and adult livers. Following stringent quality control (qc), 741 cells were retained for downstream analyses (Supplementary Fig. 1). Sample counts were normalised as transcripts per million (TPM).

To define different populations captured by our FACS strategy, we employed t-Distributed Stochastic Neighbour Embedding (t-SNE) on high-variance genes. We then measured differential gene expression to phenotypically characterise the different cell groups (Fig. 1a, b). We identify several distinct ALB+ cell populations in both the adult and foetal liver single cell analysis. As expected by sorting adult human liver cells by EpCAM expression, nearly all ASGPR1+ cells are identified as ALB+/ASGPR1+/AFP+ mature hepatocytes (Supplementary Data 1). EpCAM+ adult cells express progenitor/BEC markers KRT7 and SOX9, but interestingly most are highly ALB+ as well (Fig. 1b, c, Supplementary Data 2). In foetal liver, two distinct ALB+ expressing populations and several non-hepatic populations transcriptionally resembling stromal, mesothelial and erythroblast cell types were found (Supplementary Fig. 2). Within the ALB− populations we identified a foetal hepatocyte population expressing hepatoblast markers AFP and DLK1, but negative for traditional biliary markers (Fig. 1b, c, Supplementary Data 3). Exclusively within the CD233a+/CD45−/EpCAM+/NCAM+ population of human foetal liver we identify a cluster of cells that closely resembles EpCAM+ biliary cells identified in adult liver, expressing both biliary and hepatic markers which we label here as HHyP cells (Fig. 1b, c). Foetal HHyP cells expressed hepatic genes ALB, APOE, TF and HNF4A, but were also positive for BEC markers KRT7, SOX9 and CD24 (Fig. 1c, d, Supplementary Data 4). Having expected to isolate mature BECs from adult EpCAM+ cells we looked for expression of mature BEC markers recently identified in a comprehensive transcriptomic map of adult human liver. We identify a small ALB− population enriched for mature BEC markers TFF1 and TFF2. This BEC population is transcriptionally distinct from HHyPs which co-express hepatic markers, liver progenitor markers and mature biliary markers (Fig. 1c, d, Supplementary Data 5). Whilst potential BECs in our study share many genes with HHyPs from both foetal and adult human liver, they express a subset of genes enriched in mature human BECs captured by scRNA-seq (Supplementary Fig. 3). Whilst potential BECs in our study share many genes with HHyPs from both foetal and adult human liver, we have identified differences in gene expression that can distinguish BECs from HHyPs (Supplementary Fig. 3). After applying phenotypic labelling to t-SNE analysis we see contribution from multiple donors for each cell type, including rarer populations, demonstrating the robustness of our data set (Fig. 1e, f).

Populations of hybrid bi-potent progenitors have previously been characterised in mice after chronic liver injury. We, therefore, compared the transcription profiles of human ALB+/KRT19+/KRT7+ HHyPs and ALB−/KRT7+/KRT19+ BECs from our data set with mouse hepatocyte-derived proliferative ducts (HepPD) and biliary-derived proliferative ducts (BilPD) from Tarlow et al. Relative to their respective biliary populations, human foetal and adult HHyPs have similar expression patterns to HepPDs, identified in mice as having bi-potent characteristics of liver progenitor cells. Genes, including AHSG, RBP4, SFRP5 and MCAM are enriched in both human HHyPs and mouse HepDs, while mature BEC markers KRT7, MUC1, TSPAN8 and TFF2 are downregulated in both (Supplementary Fig. 4). Thus, it is likely that our single-cell strategy has captured the existence of a hepatobiliary hybrid progenitor population that is transcriptionally distinct from mature BECs and other hepatic cell populations, but similar to hybrid progenitor cells identified in mice after chronic liver injury.
**Identification of a distinct foetal HHyP transcriptional phenotype.** Further in-depth characterisation of the markers defining different liver populations is required to fully understand their role in development and liver regeneration. Foetal hepatocytes and HHyPs demonstrate clear transcriptional distinction. Despite sharing ALB gene expression, foetal hepatocytes express none of the traditional progenitor/BEC markers enriched in HHyPs, including KRT7, SPP1, STAT1, SOX9 and HNF1B. (Fig. 2). Upon t-SNE analysis performed only on ALB+ cells from our study, K-means clustering indicates that adult HHyPs are more closely...
associated with adult mature BECs then foetal HHyPs (Fig. 3a). Studies have suggested uninjured adult liver would not contain progenitor-like cells with a hybrid hepatobiliary phenotype, yet our scRNA-seq analysis on uninjured human adult liver demonstrates HHyPs are both present and negative for many recently identified mature human BEC markers. However, despite the high transcriptional similarity between foetal and adult HHyPs there are some key differences in gene expression (Fig. 3b). Gene set enrichment analysis (GSEA) on foetal HHyP genes enriched over adult identifies 'Stem cell proliferation', 'Developmental cell growth', 'Homophilic cell adhesion via plasma membrane adhesion molecules' and association with 'Extracellular matrix component', suggesting a phenotype associated with progenitor/stem cell-like function and important interactions with the niche environment (Fig. 3c). Genes including TACSTD2 (TROP-2), CLDN4, CLDN10 and KRT7 are enriched in adult HHyPs compared to the foetal HHyP population (Fig. 3d). In contrast, expression of CLDN6 and the transcription factor STAT4 are exclusively detected in foetal HHyPs. MCAM, CDH6 and STAT1 are also enriched in foetal HHyPs over adult (Fig. 3d). We observed several other interesting gene expression patterns, including GPC3 expressed exclusively in foetal parenchymal populations, CXCL2 expressed exclusively in adult parenchymal populations and MUC1 restricted to BECs (Fig. 3d). To further understand how foetal HHyPs related to the current understanding of liver progenitor cells in the field, we looked at the transcriptome of a recent study for human primary hepatocyte-derived liver progenitor-like cells. We identified that many of the top genes enriched in foetal HHyPs are enriched during the transition process from primary hepatocytes to progenitor-like cells, including MCAM, ANXA2, ANXA4, BICC1, SPIN1, TNFRSF12A, STAT1 and ABC3 (Fig. 3e). This suggests that in vitro reprogramming techniques to create progenitor-like cells from mature hepatocytes are moving towards a foetal HHyP-like phenotype.

**TROP-2 expression is restricted to biliary committed cells.** To validate the transcriptional signature of HHyP cells we next employed RNA in situ hybridisation (RNA-ish) and characterised their spatio-temporal regulation in primary human foetal liver tissue. Expression of the HHyP markers CDH6, STAT1, CD24, FGFR2, DCD2 and CTNNND2 that we identified are restricted to the ductal plate (DP) (a layer of cells surrounding the portal tract) in second trimester human foetal liver (Fig. 4a, Supplementary Fig. 5). At this stage ALB is expressed highly in the parenchyma, but absent from intrahepatic bile ducts (BDs) (Supplementary Fig. 5). We confirmed the hepatic phenotype of DP cells by co-expression of the DP marker STAT1 with the hepatic marker HNF4A (Supplementary Fig. 5). We, additionally, observed that CDH6 and STAT1 co-localised with the previously reported human hepatic stem cell marker CLDN3 and biliary marker SOX9 in the DP at the protein level (Fig. 4b, Supplementary Fig. 5). Importantly, these markers were also expressed within foetal BDs alongside CK19, a classical DP marker (Fig. 4c). These results highlight a significant challenge to the field, distinguishing between potential bi-potent liver progenitors and biliary committed cells.

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Fig. 1  ScRNA-Seq analysis of foetal and adult human liver. a Overview of foetal and adult liver FACS strategy. b 2D t-SNE visualisation of single cells isolated from foetal and adult human liver coloured by FACS gating population, shaped by tissue source. c Transcript expression of selected markers overlaid on the 2D t-SNE space of human liver scRNA-seq analysis. Expression is Log10(TPM). d Heat maps of selected gene expression in mature hepatic, foetal hepatic, hybrid hepatobiliary progenitor (HHyP) and mature cholangiocyte cell populations. Gene expression in Log10(TPM). Mean gene expression overlaid on the 2D t-SNE space of human liver scRNA-seq analysis. Expression is Log10(TPM).

Fig. 2  Comparison of foetal hepatic and HHyP scRNA-seq populations. Heat map of selected gene expression in foetal hepatic, foetal hybrid hepatobiliary progenitor (HHyP) and adult HHyP cells. Gene expression in Log10(TPM). TPM transcripts per million.

![Heat map of selected gene expression in foetal hepatic, foetal hybrid hepatobiliary progenitor (HHyP) and adult HHyP cells. Gene expression in Log10(TPM). TPM transcripts per million.](image-url)
which share several markers. Our scRNA-seq analysis identified TROP-2 expression to be restricted to adult progenitor/BEC cells and absent from foetal HHyPs. Furthermore in Tarlow et al., TROP-2 is expressed in biliPDs (biliary progenitors) but not bipotent hepPDs. We, therefore, investigated TROP-2 spatial regulation in human foetal liver, as compared to progenitor markers CDH6 and STAT1. We observed by RNA-ISH that while CDH6 and STAT1 expression were observed in BDs and the DP progenitor zone, TROP-2 expression was anatomically restricted to BDs (Fig. 4d). These findings suggest that TROP-2 is up-regulated...
during biliary lineage commitment. Therefore \( TROP-2 \) is a key marker to distinguish human foetal liver hybrid progenitors from \( TROP-2^+ \) committed BECs present in BDs.

We next investigated whether these in situ findings translated to ex vivo lineage commitment. Previous studies have isolated and expanded progenitor-like cells in vitro from the EpCAM\(^+ \) population of human liver in 3-dimensional (3D) culture systems\(^7,10,21 \), We, therefore, isolated EpCAM\(^+ \) cells from human foetal liver by MACs column purification and generated 3D organoids in matrigel suspension (Fig. 4e)\(^21 \). As expected, organoids grown in layer expansion media were positive for EpCAM, SOX9, CD6 and HNF4\(\alpha \), suggesting they retained a hybrid hepatobiliary phenotype in these conditions (Fig. 4e). To trace lineage commitment, EpCAM\(^+ \) organoids were transferred to either hepatic or biliary differentiation (BD) media\(^13,25-28 \). Consistent with our scRNA-Seq data and in situ staining, organoids became positive for \( TROP-2 \) and \( CK7 \) upon transfer to a BD media, whereas ALB and HNF4\(\alpha \) were not expressed. In contrast, organoids transferred to hepatic differentiation (HD) media expressed ALB, while CD6, \( TROP-2 \) and \( CK7 \) were lost in ALB/HNF4\(\alpha \) expressing structures (Fig. 4f). These findings suggest the expression signature of foetal HHyPs from our scRNA-seq dataset can be utilised to distinguish the profile of a human liver progenitor from biliary and hepatic committed cells ex vivo.

**Adult HHyPs exhibit gene signatures of both hepatic and biliary lineage.** Our findings identified distinct human foetal and adult HHyP populations with key differences in gene expression. To further investigate heterogeneity within the foetal and adult HHyP populations, we examined their lineage potential using the R package MONOCLE\(^29 \). Populations were clustered into 5 ‘pseudo states’ in 2D PCA space (Supplementary Fig. 6). Pseudo state 1 contained all foetal HHyPs, and a sub-population of adult HHyPs (blue), enriched markers DCDC2, CD6, \( STAT1 \) and ANXA13 (Supplementary Fig. 6). Interestingly, we observed distinct adult HHyP clusters enriched for either biliary (Pseudo state 3) or hepatic (Pseudo state 4) lineage markers. Pseudo state 3 revealed an enrichment of ductal marker genes KRT7, KRT23 and \( TROP-2 \), while hepatic transcription factors \( ATF5 \), \( MLXIPL \) and \( CREB3L3 \) were enriched in pseudo state 4 (Supplementary Fig. 6).

We next looked at the spatial expression of enriched pseudo state 1 markers CD6, DCDC2 and ANXA13, alongside \( STAT1 \) in adult uninjured liver by in situ hybridisation (Supplementary Fig. 6). Intriguingly, all markers were expressed in both BDs and at the limiting plate, an embryonic remnant of the DP surrounding the portal mesenchyme, suggesting these cells may be a population of intrahepatic duct residing cells distinct from ALB\(^-\)/\( TFF2 \)/MUC1\(^+ \) BECs identified here and in other studies\(^23 \). This is further supported by our observation that \( TROP-2 \) expression is restricted to foetal liver BDs and not HHyPs localised to the DP (Fig. 4d). \( TROP-2 \) has previously been identified in mouse liver injury as a marker that distinguishes oval cells from BECs\(^{30} \), and only expressed in human cancers in the liver\(^{31} \). Therefore, it was important to confirm that \( TROP-2 \) is highly expressed in the BDs of uninjured adult human liver by in situ (Supplementary Fig. 6).

**FACS isolation and in vivo transplantation of human foetal liver HHyPs.** We next looked to assess the intrinsic hepatobiliary lineage potential of distinct foetal human liver populations in vivo. We used FACS to isolate distinct foetal hepatic cell populations based on their differential surface marker expression, and transplanted each population individually underneath the renal capsule of 10-week-old NOD scid gamma (NSG) immunodeficient mice (Fig. 5a). This approach has previously been used to validate the differentiation potential of mouse and human stem cell populations\(^{32-38} \). We FACS sorted a number of populations to investigate their respective in vivo differentiation potential. We isolated CD235a\(^-\)/CD45\(^-/\)EpCAM\(^+\)/NCAM\(^+\)/MCAM\(^+\) HHyP cells, based on our scRNA-seq analysis of the HHyP phenotype (Fig. 5b). As a control we sorted for CD235a\(^-\)/CD45\(^-/\)EpCAM\(^+\)/NCAM\(^-\)/MCAM\(^-\) cells to assess the in vivo behaviour of non-HHyP EpCAM\(^+\) cells relative to potential HHyPs (Fig. 5c). The xenografts were analysed after 4 weeks of development within the kidney capsule, and were subsequently assessed by H&E and immunofluorescence (IF) staining to determine their expansion capabilities and lineage potential. After 4 weeks, only the HHyP cell population produced clear expanded regions, despite matched cells numbers transplanted between HHyPs and EpCAM\(^+\) only populations. Non-transplanted kidney from matched samples contained no such explant regions (Fig. 5d).

We investigated the presence of hybrid progenitor cells within the foetal HHyP explant by IF staining of hepatobiliary markers identified in our scRNA-seq analysis and validated ex vivo. IF staining of human-specific ALB and the BEC/progenitor marker CK19 within foetal HHyP explant sections revealed the presence of ALB\(^+/\)CK19\(^+\) cells, as well as cells expressing only ALB (Fig. 5e). ALB staining was negative in matched mouse adult liver control, confirming the presence of human cells in the explant. We next looked at markers of hepatic and biliary commitment by IF to assess lineage potential of the FACS sorted foetal HHyP population. The hepatic markers fumarylacetoacetate hydrolase (FAH) and HNF4\(\alpha \) are expressed widely across the explant region of HHyP cells confirming the hepatic potential of foetal HHyPs (Fig. 5f). To confirm biliary lineage potential we co-stained HHyP explants for ALB and \( TROP-2 \), a marker we identified as negative in foetal HHyPs and expressed in mature BECs by scRNA-seq analysis. We identify ALB\(^-/\)TROP-2\(^+\) duct-like structures suggesting that the HHyPs population is also capable of producing mature BECs (Fig. 5e). Finally, we also isolated CD235a\(^-\)/CD45\(^-/\)EpCAM\(^+\)/NCAM\(^+\)/TROP2\(^-\) cells to enrich for HHyPs over biliary lineage committed cells (Fig. 6a, b). As
Fig. 4  TROP2− foetal HHPs are restricted to the ductal plate of foetal liver.  

a RNA-ISH for CDH6 and STAT1 on human second trimester (15–21 pcw) foetal liver ductal plate (DP) and bile duct (BD) regions. Scale bars represent 50 µm.  
b Immunofluorescence (IF) staining of CDH6 (yellow), CLDN3 (magenta) and STAT1 (grey) co-expression in human foetal liver slides. Slides counterstained in DAPI (cyan). DP and BD structures outlined in white. Scale bars represent 25 µm.  
c Immunohistochemistry (IHC) of EpCAM, CK19, CDH6 and STAT1 in BD and DP regions of human foetal liver. Scale bars represent 50 µm.  
d Duplex RNA-ISH for CDH6(red)/STAT1(blue) and TROP-2(red)/STAT1(blue) in foetal liver BD and DP structures. Scale bars represent 50 µm. Scale bars of zoomed in region represent 25 µm.  
e Phase contrast imaging and IF of foetal intra-hepatic organoids (f-IHOs) derived from EpCAM enriched foetal liver cells in liver expansion (LE) media. All structures are counterstained with DAPI (blue). All staining performed between passages 3 and 5.  
f Schematic and IF staining of f-IHOs cultured for 7 days in hepatic differentiation (HD media) and biliary differentiation (BD media). All staining performed between passages 3 and 5. Images representative of n = 3 foetal liver differentiation experiments.
expected few cells were captured from foetal CD235a−/CD45−/EpCAM+/NCAM−/TROP2+ population, likely due to their restriction to forming BDs, thus proving difficult to isolate (Fig. 6c). Again only HHyP cell expansion was observed upon renal capsule transplantation over matched control cells (EpCAM−/NCAM−/TROP2− cells) or BECs (Fig. 6d). We further show that these cells express FAH, confirming their hepatic lineage potential upon in vivo implantation (Fig. 6e). Collectively, these results demonstrate the hepatobiliary hybrid phenotype of foetal HHyPs identified in our scRNA-seq analysis, as captured previously in mouse chronic liver injury studies and small-molecule reprogramming of primary human hepatocytes.
Fig. 5 In vivo lineage potential of human foetal HHyPs. 

a Experimental strategy for isolation and in vivo characterisation of foetal hepatobiliary hybrid progenitors (HHyPs) by transplantation beneath the renal capsules of immunodeficient NOD scid gamma (NSG) mice.

b 2D t-SNE visualisation of single cells isolated from foetal human liver coloured by FACS gating population (left panel) and transcript expression of ALB and MCAM overlaid on the 2D t-SNE space of human foetal liver scRNA-seq analysis (right panel). Expression is Log10(TPM).

c Gating scheme for the isolation of distinct foetal human liver populations based on expression of CD235a, CD45, EpCAM, NCAM and MCAM.

d Hematoxylin and eosin (H&E) staining in tissue cross-sections of implant regions 4 weeks post renal capsule transplantation of human foetal liver FACS populations. Scale bars represent 100 μm.

e Immunofluorescence (IF) co-staining of CK19 (green) and ALB (red) in implant regions of HHyPs post 4 weeks transplantation and matched control mouse adult liver. Slides counterstained in DAPI (cyan). Scale bars represent 25 μm.

f IF staining of FAH (red) and HNF4A (green) in implant region of HHyPs 4 weeks post transplantation. Slides counterstained in DAPI (cyan). Scale bars represent 25 μm.

g IF co-staining of TROP-2 (white) and ALB (red) in implant region of HHyPs 4 weeks post transplantation. Slides counterstained in DAPI (cyan). Scale bars represent 25 μm.

t-SNE t-distributed stochastic neighbour embedding, FACS fluorescence-activated cell sorting, TPM transcripts per million.
Our FACS and scRNA-seq strategy has captured the transcriptional profile of a HHyP population that arises during human foetal liver development. We utilised this profile to clarify the strong overlap in markers between potential progenitor populations and mature BECs and define a gene signature capable of truly distinguishing liver progenitor cells from mature BEC populations. Integrating our foetal data set with recent scRNA-seq and bulk analysis of different hepatic populations, we identify marker sets that exclusively define BEC and progenitor populations, as well as markers associated with both8,16,23. Mature BECs uniquely express the genes CLDN10, CLDN4, CXCL2, LGALS2, MMP7, MUC1, MUC5B, TROP-2, TFPI, TFF2, TFF3 and TSPAN8 as determined by our study and MacParland et al.23 (Fig. 7). CLDN4, MMP7, MUC1, TROP-2 and TSPAN8 interestingly are also enriched in mouse BilPDs over HepPDs suggesting a gene signature of biliary specific lineage8 (Fig. 7).

We further identified a signature defining human hybrid progenitor cells, distinct from mature BECs, immature hepatocytes and mature hepatocytes. Genes, including CAV1, CLDN6, GPRC5B, MAMC, NCAM and STAT4 are restricted to expression in HHyPs (Fig. 7). Several of these genes, including MAMC and CAV1 are enriched in bipotent HepPDs that arise in mice after chronic liver injury8. Our study, therefore, has captured a transcriptional signature of previously undefined human foetal HHyPs, comparable to hepatic progenitor-like cells that arise in mice during liver injury.

Discussion

In this study, we captured the molecular identity of distinct parenchymal and supporting cell populations in both foetal and adult human liver using scRNA-seq. We identified the transcriptional signature of a foetal human liver HHyP population, validated its presence in primary human liver samples, and showed its bi-lineage differentiation potential in vivo. In situ, HHyP cells could be anatomically differentiated from cholangiocytes/BECs that populate intra-hepatic BDs by using markers identified from our transcriptional profiling including TROP-2. Our work defines precise transcriptional changes during hepatic and biliary lineage commitment and provides evidence to suggest that hybrid hepatobiliary progenitor cells previously identified in mice also exist in humans during foetal development.

Previous work has proposed human liver stem/progenitors are bi-potent cells capable of repopulating both hepatocytes and cholangiocytes/BECs during development and injury21,39. These cells were reported to be EpCAM+/NCAM+, reside in the DP of human foetal liver and be retained in the Canals of Herring of normal adult livers10,40. Transcriptionally, they co-express genes associated with both hepatic and biliary lineage. Recent studies have captured similar hybrid-like liver progenitor cells in mouse and human adult liver tissue. In mouse, bi-potent progenitors have been traced from chronically injured hepatocytes8 and mature BECs3,4. In humans, hepatocyte trans-differentiation post injury gives rise to hybrid cells41. Finally, in vitro small-molecule reprogramming of both mouse and human mature adult primary hepatocytes can generate a population of proliferating bipotent liver progenitor cells14–16. Despite validating their in vivo efficacy, little effort has been put into understanding the developmental origin of human liver progenitor cells and therefore how physiologically relevant they may be in a clinical setting. Using scRNA-seq, we identified cells with hybrid hepatobiliary characteristics within the human foetal liver EpCAM+/NCAM+ FACS population. These cells transcriptionally resemble the periportal hybrid progenitors previously identified in mice, being positive for SOX9, HNF1B and KRT19, but also ALB, APOE and TFP6,8,9. HNF4A was also confirmed by in situ mRNA staining. They also express BEC/progenitor markers CD24, CD133, CLDN3, FGFR2, KRT7 and SPPI. How these hybrid cells arise is contentious, having been previously attributed to hepatocyte plasticity8,9, cholangiocyte trans-differentiation9 or representative of a quiescent undifferentiated/reserve state in uninjured postnatal liver10.

We found that HHyPs were distinct from foetal hepatoblasts which are AFP+/DLK+. Interestingly, recent scRNA-seq data on mouse liver was used to propose that hepatocyte and cholangiocyte lineages originate from AFP+/DLK+ hepatoblasts, and not from HHyPs12. However, the authors positively selected for DLK expressing cells, and therefore excluded EpCAM+/DLK− cells. This cell capture strategy may explain the discrepancy with our results. Our cell-sorting protocol on the other hand is likely enriching for progenitors. Accordingly, we captured only a very limited number of mature ALB+/SOX9+ /KRT7+ BECs. Intriguingly, our sorting strategy isolated a population of EpCAM+ cells highly similar to foetal HHyPs, expressing hepatic markers ALB, HNF4A, ALPL and biliary markers SOX9, KRT19 and KRT7. However, these cells were negative for mature BEC markers identified in the recent MacParland et al.23 scRNA-seq study of human liver, including Trefoil factors (TF)1–3 and mucins MUC1, MUC3A and MUC5B. This study however, also identified ALB expression in a subpopulation of BECs23. This raises the possibility that adult HHyPs identified in our study may indeed represent a population of mature BECs distinct from ALB+/SOX9+/KRT7+ BECs. TFFs have been previously reported to be heterogeneously expressed across different intrahepatic BDs3,43. Furthermore, it has recently been reported that adult mice BDs contain distinct BEC populations with differing proliferative capabilities43,44. Our findings suggest that this could be interpreted as revealing BEC heterogeneity in healthy liver. It is possible that these populations and the mechanisms that govern their behaviour regulate the inherent plasticity of parenchymal cell populations towards mature hepatic and biliary lineage in response to injury4,8,14,15,41,46. A key difference between foetal and adult HHyPs is the expression of TROP-2, also expressed in mature BECs. TROP-2 is a closely related family member of EpCAM thought to be absent or weakly expressed in normal liver tissue and enriched only at times of injury30 or cancer31. Our data clearly show this is not the case in humans, with TROP-2 being expressed in adult BDs during normal homeostasis. Our data further suggest that TROP-2 expression marks HHyP commitment towards biliary lineage, consistent with recent mouse data8. While this supports the notion that we have captured distinct populations of adult BECs, our analysis also confirms that adult HHyPs are transcriptionally close to foetal HHyPs validated in vivo for lineage potential and mouse ‘oval cells’. Therefore, we cannot say conclusively that these cells are a specific mature BEC population without further functional validation, a collective problem the field of human liver progenitor studies faces. It is likely, however that, these populations respond differently to different injury stimuli. Understanding the phenotypes of distinct biliary populations is important when considering that the management of hepatic diseases is often hindered by difficulties in identifying their cellular origin. Given the frequent overlap in markers between multiple parenchymal cell types, it is crucial to accurately determine new histological markers.

Using the renal capsule of immunodeficient mice, we demonstrated the proliferative and hepatobiliary hybrid lineage potential of freshly isolated foetal human HHyPs. Investigating true functional stemness of freshly isolated progenitor cells for clonality, bi-potency and rescue was unfeasible in this study due to the low cell numbers obtained post FACS sorting from rare human tissue.
Fig. 7 Collated marker expression in hepatic progenitor populations. Heatmaps of selected marker expression in populations identified in this study with different cohorts of liver progenitor-like cells, biliary epithelial cells (BECs) and primary hepatocytes. Shown is gene expression patterns of publicly available sequencing data from GEO (https://www.ncbi.nlm.nih.gov/geo/) or literature mined. Bulk RNA-seq data sets, include reprogrammed human hepatic liver progenitor cells (hepLPCs-Heps, GSE105019) and primary hepatocytes (pHs, GSE105019) from Fu et al.16 and mouse hepatocyte-derived proliferative ducts (HepPD, Tarlow et al. 2015) and biliary-derived proliferative ducts (BilPD) (GSE55552), Tarlow et al. 2015)8. ScRNA-seq data sets, include human BECs (GSE115469) from MacParland et al.23. Expression graded as high (red) to low (blue) relative to individual data sets.
Recent studies focused on the translational potential of progenitor cells for liver repopulation required ex vivo expansion to produce sufficiently high numbers for transplantation into multiple animals, injured with enough severity such that an environment conducive to engraftment of donor cells is induced. To avoid physiological variables that compound this approach, we elected to implant cells directly after FACS to experimentally match cells we assayed in vivo with those defined through scRNA-seq. This approach also completely removes results obtained as a consequence of ex vivo culture. We chose, therefore, to implant the foetal HHyP cell population into a non-hepatic space previously reported to be permissive for evaluation of the developmental potential of small numbers of human cells. While this assay limits the functional validation of HHyP clonality and functional bi-potency of liver injury studies, this allowed us to investigate proliferative and hepatobiliary lineage potential of well-defined and freshly isolated rare human liver progenitor populations without in vitro expansion prior to transplantation.

Using this approach we show that foetal HHyP within the EpCAM+/-NCAM+/- FACS population previously shown to harbour human foetal progenitor cells are capable of expanding into FAH and HNF4A expressing hepatocytes within the kidney capsule environment. Specifically, EpCAM+/NCAM+/-MCAM+/- FACS isolated human foetal liver HHyPs were capable of expansion within the renal capsule, and shown to be ALB+/CK19+ confirming the hepatobiliary hybrid phenotype of this population. Cells isolated from EpCAM+/-NCAM- foetal human livers did not engraft efficiently beneath the renal capsule. Our scRNA-seq analysis predicts this population is likely enriched for foetal immature hepatocytes, and may reflect their inability to expand in this niche. In our scRNA-seq analysis MCAM is identified as specifically enriching the foetal and adult HHyP phenotype, absent from expression in mature TTF1+/TFT2+/TTF3- BEC populations and from foetal hepatocytes. Furthermore, in mouse chronic liver injury studies, MCAM is enriched in hepatocyte derived proliferative ducts, over those derived from mature BECs alongside AHSG, ALB, CAV1 and RBP.

Interestingly, MCAM (CD146) has previously been reported to be expressed on hepatic stellate cells that closely interact with human hepatic stem cells, as well as many different cell types including aggressive epithelial cancers. MCAM forms homotypic cell–cell interactions, and therefore may play a key role in the niche–progenitor interface and regulating the interactions of foetal HHyPs with cells that comprise the stem cell niche during human liver development. Our in vivo findings validate our initial transcriptional labelling of FACS isolated putative foetal liver progenitors as hepatobiliary in nature. It will be of great interest for future research to investigate the presence of an expanded HHyP phenotype in human hepatobiliary disease tissue for fresh isolation in greater cell numbers to more specifically address questions on clonality, bi-potency and functional rescue potential.

Methods

See cell sorting and cDNA library preparation. All human tissues were collected with informed consent following ethical and institutional guidelines. Freshly isolated adult hepatocytes were obtained from Triangle Research Labs, while foetal livers were obtained from the Human Developmental Biology Resource of University College London. Human foetal tissue was dissociated by Collagenase XI enzymatic dissociation for 25 min at 37 °C with agitation. Samples were stained with the following primary antibodies, CD31 (349104, FITC, mouse; Biologend), CD45 (304050, BV711, mouse; Biologend), EpCAM (324208, APC, mouse; Biologend), NCAM (362524, PE, mouse; Biologend), ASGR1 (563365, PE, mouse, BD Pharmingen), TFRC (334106, PE mouse; Biologend), MCAM (361005, PE, mouse; Biologend).

For library size and total genes the lower tail outliers were excluded. For mitochondrial and ERCC spike in percentage upper tail outliers were excluded. t-SNE, spearman’s rank co-efficient, hierarchical clustering and Student’s t-tests were performed using custom scripts in R. t-SNE was performed on log10(TPM) normalised gene counts. For t-SNE analysis, the top 1000 variable features were used. Lineage trajectory analysis was performed in R using the MONOCLE package.

In situ RNA hybridisation. Foetal and adult human liver samples were fixed in 10% formalin buffer saline (HTS01128, Sigma Aldrich) for 2 days then dehydrated and paraffin wax infiltrated using Excelsior® AS Tissue Processor. After embedding, sections (5 µm) were processed for in situ RNA hybridisation RNA-ISH using the RNAscope 2.5 HD Duplex Assay (Agilent Technologies) and stained with DAPI (D1306, Takara Clontech). RNA was converted into cDNA using SMARTScribe Reverse Transcriptionase (Hs-SCRT2, custom), (All ACD Bio). For Duplex RNA-ISH for transcript expression was performed using RNAscope® 2.5 HD Duplex Assay (322435, ACD Bio) according to manufacturer’s instructions using the following custom probes: STAT1 (Hs-STAT1 469861), C2 channel change). Images were acquired with a Nikon A1 confocal microscope, single cells were imaged using NanoZoomer (Hamamatsu).

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Case–tissue sections. Paraffin-embedded foetal liver tissue was prepared as described for RNA-ISH. After embedding, sections (5 µm) were stained using mouse and rabbit specific HRP/AEC (ABC) Detection IHC Kit (abcam) using antibodies against EpCAM (ab75054, mouse, 1:100; abcam), CK19 (ab52625, rabbit,
EpCAM™ foetal liver cell isolation. Dissociated and filtered foetal liver cells were incubated with anti-CD36 (EpCAM) Microbeads (130-061-101, Miltenyl Biotec) in 0.5% bovine serum albumin (BSA) with 5 U/ml DNaseI (M0303, N.E.B.) and passed through a large cell preparation column (1×300-042-202, number 2, Dynal) for 10 min. Total EpCAM enriched and EpCAM depleted populations were FACS analysed for CD235a and EpCAM on a BD Fortessa cell analyser. For foetal liver intra-hepatic organoid formation (f-IHO) EpCAM enriched cells were pelleted at 300 × g for 5 min. The cell pellet was resuspended in a Matrigel® dome containing ~50,000 cells/dome in one well of a 48-well plate covered in 250 μl of liver expansion (LE) media. Media was changed every 48 h.

Organoid culture conditions. LE media was based on DMEM/F-12, GlutaMAX™ (10565018, Gibco, Life Technologies) supplemented with 1% N2 supplement (17502048, Gibco, Life Technologies), 2% B27 supplement (17504044, Gibco, Life Technologies), 20 mM HEPES (H8634080, Gibco, Life Technologies), 1.25 mM N-Acetylcysteine (A7250, Sigma), 1% penicillin–streptomycin (15140122, Sigma Aldrich), 1% insulintransferrin:serum (TETF) (41400045, Gibco, Life Technologies), and the growth factors: 50 ng/ml EGF (AF-100-15, Peprotech), 500 ng/ml RSPO1 (120-38, Peprotech), 100 ng/ml FGF10 (100-26, Peprotech), 25 ng/ml HGF (90-39, Peprotech), 10 mM Nicotinamide (72540, Sigma), 25 μM RepSox (3742, ToPro), 10 μM Fodrin (T099, ToPro), 61 μM Dexamethasone (1126, R&D Systems), 25 mM 3-Nitrohenic acid R2625, Sigma) and 10 ng/ml Activin-A (ActA, Q-kine). The culture medium was changed every 48 h.

Passaging and staining organoids. To split f-IHOs, Matrigel® was digested with Trypsin-EDTA for 15 min at 37 °C. Cell suspension was centrifuged at 300g for 4 min, washed once with William’s E medium and resuspended in Matrigel® domes as described above. Organoids were typically passaged at a 1:4 ratio. For IF staining of organoids, Matrigel® was dissolved in cell recovery solution (11543560, ThermoFischer scientific) for 30 min at 4 °C and fixed for 30 min in 4% w/v PFA. Blocking was performed for 1 h in 3% donkey serum, 0.3% Triton and 0.1% DMSO. Primary antibodies were used for 4°C overnight at the indicated dilutions: CDH6 (AF2715, sheep, 1:50, RnD Systems), EPCAM (ab17504, mouse, 1:100; abcam), KRT7 (ab9021, mouse, 1:100; abcam), HNF4A (ab92378, rabbit, 1:100; abcam), CK19 (ab52625, rabbit, 1:200; abcam), albumin (A80-129A, goat, 1:100; Bethyl), TROP2 (10428-MM02, mouse, 1:100; Stratech), HNF4A (ab92378, rabbit, 1:100; abcam), KRT7 (ab9021, mouse, 1:100; abcam), albumin (A80-129A, goat, 1:100; Bethyl), TROP2 (10428-MM02, mouse, 1:100; Stratech). DAPI was used at 1:1000 dilution as a counterstain. Alexa Fluor-conjugated secondary antibodies (all 1:500, abcam). Mounted slides were imaged using a NanoZoomer microscope, a Nikon Ti spinning disk confocal microscope (Nikon Instruments London, UK) under a Home Of (7100115, mouse, 1:100; Transduction laboratories) then counterstained with Eosin Y dye (ab146322, abcam). Mounted slides were imaged using a NanoZoomer (Hamasatsu).

Data availability
The authors declare that all data supporting the findings of this study are available within the article and its Supplementary information files or from the corresponding author upon reasonable request. Raw RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession code GSE130473.

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