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A single-cell transcriptomic analysis reveals precise pathways and regulatory mechanisms underlying hepatoblast differentiation

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Abstract

How the bi-potential hepatoblasts differentiate into hepatocytes and cholangiocytes remains unclear. Here, using single-cell transcriptomic analysis of hepatoblasts, hepatocytes, and cholangiocytes sorted from E10.5 to E17.5 mouse embryos, we found that hepatoblast-to-hepatocyte differentiation occurred gradually followed a linear default pathway. As more cells became fully differentiated hepatocytes, the number of proliferating cells decreased. Surprisingly, the proliferating and quiescent hepatoblasts exhibited homogeneous differentiation states at a given developmental stage. This unique feature enabled us to combine the single-cell and bulk-cell analyses to define the precise timing of the hepatoblast-to-hepatocyte transition, which occurs between E13.5 and E15.5. In contrast to hepatocyte development at almost all levels, hepatoblast-to-cholangiocyte differentiation underwent a sharp detour from the default pathway. New cholangiocyte generation occurred continuously between E11.5 and E14.5, but their maturation states at a given developmental stage were heterogeneous. Even more surprising, the number of proliferating cells increased as more progenitor cells differentiated into mature cholangiocytes. Based on an observation from the single-cell analysis, we also discovered that the protein kinase C (PKC)/mitogen-activated protein kinase (MAPK) signaling pathway promoted cholangiocyte maturation.

Conclusions—Our studies have defined distinct pathways for hepatocyte and cholangiocyte development in vivo, which are critically important for understanding basic liver biology and developing effective strategies to induce stem cells to differentiate towards specific hepatic cell fates in vitro.

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Data availability: The RNA-seq data have been submitted to the GEO repository. The following link has been created to allow review of record GSE90047 while it remains in private status: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cxehkwyhzipjqn&acc=GSE90047>

Keywords

liver development; cell fate; cell differentiation; single-cell RNA-seq; MAPK

Introduction

The liver comprises multiple cell types, among which hepatocytes and cholangiocytes (biliary duct cells) are the major epithelial cell types, and the hepatic lineages are of endodermal origin. Both hepatocytes and cholangiocytes are derived from the bi-potential progenitors, known as hepatoblasts at the early stage of hepatogenesis.(1)

Once the hepatoblasts have been specified from the foregut endoderm and form the hepatic diverticulum at approximately embryonic day 9.5 (E9.5) in the mouse, they delaminate from the epithelium and migrate into the septum transversum, where they continue proliferating and form the liver bud.(2) Hepatoblast proliferation is regulated by several cell signaling pathways, including the WNT, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), mitogen-activated protein kinase (MAPK) 9, and transforming growth factor (TGF) β pathways.(3) The cell fate decision occurs during liver development when the hepatoblasts start specifying into hepatocytes and cholangiocytes. The transcription factors (TFs) TBX3, CEBP α , PROX1 and HNF4 α have been shown to promote hepatocyte differentiation.(4-7) The segregation of cholangiocytes occurs before E15.5, and these cells undergo morphogenesis to form the biliary duct.(8) The TFs HNF6, SOX4, SOX9 and HNF1 β , as well as several signaling pathways, including Notch, WNT, FGF and TGF β pathways, have been shown to promote cholangiocyte differentiation.(9-15) Despite these findings, the specific pathways and mechanisms underlying the differentiation of hepatoblasts into hepatocytes and cholangiocytes remain unclear.

Cell fate decisions during lineage differentiation are coordinately regulated at the transcriptional level by the activation and repression of specific gene sets.(16) However, due to the lack of cellular markers that can be used to efficiently distinguish and purify hepatoblasts, hepatocytes and cholangiocytes from different stages of the developing fetal livers, the conventional population-based RNA sequencing (RNA-seq) method is inappropriate for investigating the transcriptomic regulation of hepatocytes versus cholangiocytes segregation. This limitation can be circumvented using single-cell RNA-seq, a powerful approach that has been used to identify cell subpopulations, trace the developmental map and decipher the regulatory networks involved in the cell fate decision during organogenesis.(17)

In this study, we have discovered precise pathways and potential mechanisms for the specification and maturation of hepatic lineages by performing single-cell RNA-seq analyses on isolated cells of the mouse hepatic lineages at time points from E10.5 to E17.5. In addition, by combining the single-cell and bulk-cell analyses, we have precisely defined the developmental timing of the hepatoblast-to-hepatocyte transition.

Materials and Methods

Animals

F1 progenies of C57BL/6 and C3H (B6C3F1) mice were used (Supporting Materials and Methods). All experimental animal procedures were approved by the Institutional Animal Care and Use Committees of Peking University. All mice were housed and maintained under specific pathogen-free conditions on a 12-hour day/night cycle at $23 \pm 2^\circ\text{C}$ and received an autoclaved standard diet and water *ad libitum*.

Generation of single-cell RNA-seq libraries

After FACS sorting, single cells were manually picked up under the microscope with a mouth pipette and transferred into 4 μl of cell lysis buffer containing 0.05 μl of a 1:300,000 dilution of ERCC Spike-in RNA (Life Technologies, 4456740). The cDNA was synthesized, amplified (18 cycles) using the Smart-seq2 protocol,(18) and purified using VAHTS DNA Clean Beads XP beads (Vazyme, N411-03). Two nanograms of cDNA were used to prepare the sequencing libraries using the TruePrep DNA Library Prep Kit (Vazyme, TD502) with $0.4 \times$ of the standard reaction volume. PCR amplifications were conducted in 8 cycles. Purification and size-based selection of PCR products were performed to obtain libraries with a peak size of 350 bp.

Results

The single-cell analysis reveals linear and branched pathways for the differentiation of hepatoblasts into hepatocytes and cholangiocytes

To establish a roadmap for the differentiation of hepatoblasts into hepatocytes and cholangiocytes at the single-cell level, we decided to isolate distinct cell populations from mouse fetal livers at different developmental stages and perform single-cell RNA-seq analysis. We used well-characterized cell surface markers to isolate distinct cell populations via FACS sorting. Delta-like (DLK) protein is an authentic hepatoblast cell surface marker that is continually expressed in hepatocytes until the neonatal stages,(19, 20) whereas epithelial cell adhesion molecule (EpCAM) is expressed in early hepatoblasts and differentiated cholangiocytes.(20) To determine the conditions for FACS sorting of the hepatobiliary lineages during liver development, we stained dissociated single fetal liver cells obtained at E10.5, E13.5 and E17.5 with antibodies against DLK and EpCAM (Supporting Materials and Methods). At E10.5, the DLK+ cells in the P1 population primarily co-expressed with EpCAM, whereas the cells in the DLK-low group were EpCAM- (Supporting Fig. S1A). The RT-qPCR analysis confirmed that the P1 cells were hepatoblasts because they expressed high levels of *Alb*, *Afp*, *Foxa2* and *Hnf4a*, but not the cholangiocyte markers *Sox9* and *Krt7* (Supporting Fig. S1B). At E13.5, the cells in P1 population were still DLK+, but the EpCAM level was close to the background. However, we detected a few cells in the P2 population that were EpCAM+. Compared to the cells in the P3 (DLK-/EpCAM-) and P4 (DLK-/EpCAM-low) populations, the cells in the P1 population expressed high levels of *Alb*, *Afp*, *Foxa2* and *Hnf4a*, whereas cells in the P2 population also expressed *Sox9* and *Krt7*, indicating that the P1 population mainly contains hepatoblasts/hepatocytes and the P2 population contains cholangiocytes, and the P3 and P4

populations include other non-endoderm-derived cell types (Supporting Fig. S1A,B). At E17.5, the EpCAM signal in P2 cells became stronger than the signal observed at E13.5. *Alb* was expressed in DLK+ cells (P1) at an extremely high level, whereas *Sox9* and *Krt7* were exclusively detected in P2 cells. Thus, the P1 population must have contained hepatoblasts/hepatocytes, whereas the P2 population contained cholangiocytes (Supporting Fig. S1A,B). Similarly, we identified the gating strategies at E11.5, E12.5, E14.5 and E15.5 for the FACS sorting of the hepatic lineages (Supporting Fig. S1C).

Next, we performed single-cell RNA-seq on these sorted hepatic cells (Fig. 1A). After quality control and ERCC spike-in normalization, 447 cells remained for further analyses, and on average, 7,000-9,000 genes were detected with >1 million mapped reads in each cell (Supporting Fig. S2A-E; Supporting Materials and Methods). To characterize the developmental process and define the populations of cells involved, we performed a principal component analysis (PCA) of all 447 cells. As shown in the PCA plot, most hepatic cells obtained from the early to late developmental stages were located on the mainline along principal component (PC) 1, whereas some formed a distinct branch along PC2 (Fig. 1B,C). Hierarchical clustering of the PC2 higher loading genes divided the cells into two groups (I and II). The cells in the P1 FACS-gated population were exclusively group-I cells. EpCAM+ cells (P2 gating) were mainly group-I cells prior to E12.5, but switched to group-II thereafter. Beginning at E15.5, EpCAM+ cells were mainly observed in group-II (Supporting Fig. S3A,B). Moreover, four distinct clusters were identified among the heterogeneously expressed genes during hepatobiliary development. Cluster 'a' genes were highly expressed in most of the group-I cells from E10.5 to E13.5 but less frequently expressed in group-II cells, as well as in group-I cells from E15.5 and E17.5 (Fig. 1D). According to the Gene Ontology (GO) analysis, cluster 'a' was enriched with genes controlling DNA replication and cell cycle progression, including genes regulating the G1/S and G2/M transitions (Fig. 1E; Supporting Table S1), such as *Foxm1*, *Ccna2*, *Ccnb1*, *Ccne2* and *Cdk1* (Fig. 2A). Based on these results, cells expressing cluster 'a' genes were proliferating. Cluster 'b' genes were highly expressed in the earlier stages of hepatobiliary development and gradually decreased along a pseudo-chronological order (pseudotime) (Fig. 1D). This cluster was enriched with genes involved in cell proliferation and WNT and bone morphogenetic protein (BMP) pathways (Fig. 1E; Supporting table S1), including key genes known to control stem cell proliferation, such as *Id3*, *Lin28b*, *Etv5* and *Lgr5*, (21-24) as well as the hepatoblast expansion enhancing factor *Tbx3* (Fig. 2B). (4) Given the critical roles of WNT signaling and TBX3 in controlling hepatoblast proliferation, (4, 13) we designated the cells expressing high levels of cluster 'b' genes as hepatoblasts. The expression of the cluster 'c' genes in group-I was increased with the developmental pseudotime (Fig. 1D). Cluster 'c' genes were divided into two sub-clusters 'c1' and 'c2'. Cluster 'c1' genes were expressed at an earlier stage of hepatoblast development and gradually increased with hepatoblasts/hepatocyte development, whereas 'c2' genes showed delayed expression. However, as shown in the GO analysis, genes from these two sub-clusters exhibited enrichment in similar GO terms related to hepatocyte functions, such as various metabolic and transmembrane transport processes (Fig. 1E; Supporting table S1). Moreover, cluster 'c' contained hepatocyte-related metabolic regulators such as *Cps1*, *Ppara*, *ApoH*, *Cyp2d10* and *Cyp2d26* (Fig. 2C). (25, 26) Thus, cells expressing high levels of cluster 'c' genes were differentiated

hepatocytes. Notably, the cells at the same developmental time along PC1 tightly clustered together, indicating relative homogeneity at the transcriptomic level (Fig. 1C).

Strikingly, group-II cells exclusively expressed cluster ‘d’ genes that were enriched in cell adhesion, cell migration, epithelial tube morphogenesis and Notch and WNT signaling, which is known to regulate cholangiocyte differentiation(12, 13) (Fig. 1E; Supporting table S1). For example, this cluster includes the TFs *Sox9*, *Sox4* and *Hnf1b* that regulate biliary cell differentiation and morphogenesis,(10, 11) as well as the ductal markers *Spp1* and *Krt7* (Fig. 2D).(8, 27) Therefore, we designated the cells in group-II as differentiated cholangiocytes. Interestingly, a few cholangiocytes were specified as early as E11.5, and new cholangiocytes were continuously branched from the main line along PC1 until E14.5. At E15.5 and E17.5, the cholangiocyte population was completely segregated from the PC1 line (Fig. 1B,C), suggesting that none of cholangiocytes at these stages is newly generated. Taken together, our study has defined a roadmap for hepatobiliary development at the single-cell level. More importantly, our analysis has revealed that cholangiocyte specification follows a path that sharply branches from the linear pathway of hepatoblast to hepatocyte differentiation.

The differentiation states of proliferating and quiescent hepatoblasts are synchronized

Differentiation and proliferation are generally inversely correlated during lineage specification.(28) Researchers have not clearly determined when hepatoblasts begin to differentiate and how they coordinate this process with proliferation. We focused our analyses on hepatoblasts and their progenies from E10.5 to E13.5, excluding the few identified cholangiocytes, to address these questions. According to the PCA plot (Fig. 1B), hepatoblast clusters at different developmental stages move along PC1, suggesting that hepatoblast differentiation starts at a very early stage. The hierarchical clustering analysis enabled the separation of cell cycle regulatory genes into two clusters (Fig. 3A). Cluster-I included the G1/S markers *Ccne1* and *Ccne2*, as well as DNA replication-related genes (Fig. 3A), whereas cluster-II contained the G2/M markers *Cdk1*, *Foxm1*, *Ccna2*, *Ccnb1* and *Ccnb2* (Fig. 3A). Based on the expression patterns of cluster-I and cluster-II genes, we classified the proliferating cells into G1/S (mainly expressing cluster-I genes) and S/G2/M (expressing both cluster-I and -II genes). The cells that did not express cell cycle genes were considered quiescent (G0/G1 phase) (Fig. 3A-C). As shown in the PCA plots, hepatoblast differentiation occurred along PC1, whereas hepatoblasts and their progenies in various phases of the cell cycle are distributed along PC2 (Fig. 3B,C). To determine how hepatoblasts coordinate proliferation and differentiation during lineage specification, we aligned all the cells in the pseudotime order along the direction of hepatoblast development and found that proliferating cells and their corresponding quiescent cells kept pace without an obvious delay along the developmental process (Fig. 3D). Thus, our single-cell analyses revealed that the differentiation states of proliferating and quiescent hepatoblasts are synchronized during early liver development.

Hepatoblast-to-hepatocyte differentiation follows a “default” program

To further explore the mechanism underlying the hepatoblast-to-hepatocyte transition, we analyzed the overall profile of genes upregulated during hepatoblast development to further

explore the mechanism underlying the hepatoblast-to-hepatocyte transition. We performed a hierarchical cluster analysis and identified 383 genes whose expression levels increased gradually from E10.5 to E13.5 (Fig. 4A). Strikingly, the GO analysis revealed that the enriched categories were mainly associated with hepatocyte functions, such as metabolic processes, detoxification and transport (Fig. 4B). Based on these data, hepatoblasts specify into the hepatocyte fate immediately after their differentiation from endodermal progenitors and gradually move towards that fate. We also analyzed the tendency of TFs to be upregulated during hepatoblast/hepatocyte development, and found that their expression started to increase at the beginning of the hepatoblast/hepatocyte developmental process (Fig. 4C,D). In contrast, TFs that were upregulated during the hepatoblast/cholangiocyte transition showed little or no expression in hepatoblasts until the latter started to develop into cholangiocytes (Fig. 4C,D). Thus, the default pathway for hepatoblasts is to differentiate into hepatocytes, but along the way, some hepatoblasts are regulated to differentiate towards the cholangiocyte fate.

Hepatoblast-to-cholangiocyte differentiation follows a regulated branch from the default program

To obtain insights into cholangiocyte specification and maturation, we analyzed the dynamics of gene expression in cells of the cholangiocyte lineage during development. The PCA of all 102 identified cholangiocytes revealed cholangiocyte heterogeneity starting at E13.5 (Fig. 5A). Hierarchical clustering of PC1 genes divided the cells into three groups (I-III) (Fig. 5B). Group-I and group-III cells expressed high levels of cluster PC1-a and PC1-b genes, respectively, while group-II cells expressed both cluster PC1-a and PC1-b genes at relatively low levels (Fig. 5B). The cholangiocytes identified at E11.5 and E12.5 belonged to group-I, whereas E13.5-E17.5 cells were distributed in more than one group. According to GO analyses, cluster PC1-a genes were enriched in terms related to hepatocyte functions, such as metabolic processes, transport and detoxification (Fig. 5C). As these enriched terms were similar to the terms attributed to the upregulated genes during hepatoblast development (Fig. 4A,B), we compared cluster PC1-a genes to those upregulated genes and found that they exhibited 58% overlap (Fig. 5D). Because E11.5 and E12.5 cholangiocytes belong to group-I, we conclude that the cells in this group are newly specified cholangiocytes that still retain significant features of their progenitors. The GO terms enriched in cluster PC1-b included cell adhesion, migration and tube morphogenesis, which are associated with functional cholangiocytes (Fig. 5C). Therefore, we conclude that the cells in group-III are more differentiated cholangiocytes. Moreover, group-I to-III cells represent three developmental stages (I-III) of cholangiocyte early differentiation/maturation. Based on these analyses, the genes contributing to PC1 can be used to distinguish the states of cholangiocyte differentiation. Early-stage cholangiocyte specification involves a gradual silencing of the expression of cluster PC1-a genes that are normally expressed at high levels in hepatoblasts and hepatocytes, whereas cholangiocyte maturation involves inducing the expression of genes required for the structure and function of this cell type.

The GO analysis also showed that genes contributing to PC2 were enriched in terms related to cell cycle regulation (Fig. 5C). Based on the expression of the cell cycle genes identified in Fig. 1D, we defined proliferating and quiescent cells in the three groups of cholangiocytes

(Supporting Fig. S4A). We projected these cells on the PCA plot shown in Fig. 1B and found that cholangiocyte maturation mainly occurred along the PC2 axis (Fig. 5E). Surprisingly, the percentage of proliferating cells increased as cholangiocytes developed from stage-I to stage-III, in sharp contrast to hepatoblast-to-hepatocyte development, during which the proliferation rate steadily decreased (Fig. 5F; Supporting Fig. S4A,B). To verify the proliferation rate determined by single-cell transcriptomic analyses, we collected DLK+ cells at E11.5, E13.5 and E17.5, and EpCAM+ cells at E13.5 and E17.5 for immunostaining using antibody against Ki67, a marker for proliferating cells.(29) Consistently, the percentage of Ki67+ cells gradually decreased during hepatoblast to hepatocyte development (Supporting Fig. S4C,D). Because the transcript of *CK19*, which is expressed in differentiated cholangiocytes, was rarely detected in the single cholangiocyte at stage-I (Supporting Fig. S4E), we considered that E13.5 EpCAM+ cells and E17.5 EpCAM+/CK19- represented less mature cholangiocytes. The percentage of Ki67+ cells significantly increased in more mature E17.5 EpCAM+/CK19+ cells compared with less mature cells (Supporting Fig. S4C,D). Based on these analyses, hepatoblast-to-cholangiocyte differentiation defines a regulated branch of the hepatoblast-to-hepatocyte pathway, and these distinct fate choices involve distinct mechanisms coordinating proliferation and differentiation.

MAPK signaling promotes cholangiocyte maturation

The single-cell analysis not only provides a high-resolution map of hepatoblast differentiation but also offers potential mechanisms that can be experimentally tested. For example, our GO analysis indicated that the MAPK pathway was enriched in more mature (stage-III) cholangiocytes (Fig. 5C). Differential gene expression analyses between the stage-I and stage-III ductal cells identified high levels of expression of 21 MAPK pathway-related genes in stage-III cholangiocytes (Supporting Fig. S5A,B). Many of the genes involved in the canonical RAS-PKC-ERK/MAPK pathway were upregulated during cholangiocyte maturation, such as the ERK1/2 activators *ErbB2/4* and *Fgfr2/3*,(30, 31) as well as the ERK downstream factors *Sor11* and *Wwc1*.(32, 33) To confirm that the MAPK/ERK pathway was activated in the more mature cholangiocytes, we performed immunostaining of the active phosphorylated ERK1/2 (p-ERK1/2) on FACS-sorted E13.5 and E17.5 EpCAM+ cells. E13.5 EpCAM+ cells, most of which belong to the stage-I, were p-ERK- cells, whereas >60% of the more mature E17.5 EpCAM+/CK19+ cells were p-ERK+ (Supporting Fig. S5C,D). Thus, the MAPK/ERK pathway is activated in vivo during cholangiocyte maturation.

To functionally determine whether the canonical MAPK pathway stimulates cholangiocyte maturation, we treated the fetal livers at E12.5 (Supporting Materials and Methods), the time before the fate of hepatoblasts is specified into cholangiocytes, with a PKC agonist, (2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino) benzolactam (TPPB).(34) After 48 hours of culture, the treated liver explants did not show a significant difference in size compared to the controls (Fig. 6A), and the percentages of DLK+ hepatoblasts/hepatocytes between groups were similar (Supporting Fig. S6A). Based on the bromodeoxyuridine (BrdU) incorporation experiments, the proliferation rates of DLK+ cells were indistinguishable between the controls and the TPPB-treated samples (Supporting Fig.

S6B). However, we observed an approximately three-fold increase in the number of EpCAM⁺ cells after the TPPB treatment (Fig. 6B,C), suggesting that PKC activation stimulated the production of EpCAM⁺ cells. PKC is known to stimulate MEK1/2, which, in turn, activates MAPK.(35) To determine whether the TPPB effect was mediated by the MAPK pathway, we treated the E12.5 fetal livers with both TPPB and U0126(36), a MEK1/2 inhibitor. The U0126 treatment abolished the TPPB-induced increase in the number of EpCAM⁺ cells (Fig. 6C). Thus, the PKC-MEK-MAPK pathway is responsible for the expansion of EpCAM⁺ cells.

To determine whether the EpCAM⁺ cells from the TPPB-treated samples were more mature cholangiocytes, we performed single-cell RNA-seq on the EpCAM⁺ and DLK⁺ cells from both TPPB-treated and control samples. The PCA of all previously described hepatic cells (Fig. 1B) and cells from this experiment showed that the DLK⁺ cells from the controls and the TPPB-treated samples were located along the hepatoblast/hepatocyte line, whereas the EpCAM⁺ cells were distributed in the cholangiocyte direction (Fig. 6D). Remarkably, the EpCAM⁺ cells from the TPPB-treated samples and the control samples displayed a significant separation along the PC1 axis (Fig. 6D), suggesting that the TPPB treatment promotes cholangiocyte maturation. This hypothesis was supported by the observation that the cholangiocyte markers *Epcam* and *Sox9* were expressed at high levels in the TPPB-treated cells, whereas the expression of the hepatoblast/hepatocyte marker *Alb* was totally repressed. *Foxa3*, which is downregulated in more mature cholangiocytes (Supporting Fig. S6C), was expressed at a lower level in the TPPB-treated cells (Fig. 6E). Finally, the GO enrichment analysis indicated that the TPPB-treated cells exhibited enrichment in the similar cellular function terms as cholangiocytes compared to the controls (Figs. 1E and 5C; Supporting Fig. S6D). The expression of genes regulating cell proliferation was upregulated in TPPB-treated cells (Supporting Fig. S6D,E), consistent with the finding described above showing that the more mature cholangiocytes exhibit higher proliferation rates (Fig. 5F). Based on our results and analyses, PKC-triggered MAPK activation promotes cholangiocyte maturation.

The hepatoblast-to-hepatocyte transition occurs between E13.5 and E15.5

Because the differentiation of hepatoblasts into hepatocytes occurs progressively, defining the time point of the lineage transition is challenging. However, this definition might be achieved using an unbiased approach, i.e., comparing the similarity between the transcriptomes of hepatoblasts and their progenies at different developmental stages. Although single-cell RNA-seq is an ideal method to profile cell-to-cell variability and reveal the interplay between intrinsic cellular processes, it has limited sensitivity in detecting transcripts, particularly low-abundance transcripts.(37) Considering the relative homogeneity of the cells at the same developmental stages during hepatoblast-to-hepatocyte differentiation at the transcriptomic level (Fig. 1B,C), we adopted bulk-cell RNA-seq to define the timing of the hepatoblast-to-hepatocyte transition. Similar transcriptomic dynamics studies have been performed using the whole fetal liver.(38, 39) However, the fetal liver is also the organ in which hematopoiesis occurs. Hepatoblasts/hepatocytes are only a small fraction of the total fetal liver cells (Supporting Fig. S1A,C), and most of the other cells are blood cells and lymphocytes. Therefore, we performed RNA-seq analyses at the

bulk-cell level in 200,000 sorted DLK+ cells on each day from E10.5 to E18.5 (Supporting Fig. S7A-C; Supporting Materials and Methods). In this study, we identified 4,077 variably expressed genes during hepatoblast/hepatocyte development, a number that is 3 times higher than the number identified using single-cell analyses (Figs. 1D and 7A). The gene expression profiles of the bulk-cell and pooled single-cell RNA-seq showed strong correlations (Supporting Fig. S7B), although the single-cell transcriptomic datasets exhibited increased variability compared with the bulk-cell RNA-seq datasets (Supporting Fig. S7C). We compared our data to the transcriptomic data obtained from the whole liver(39) and found that the hepatic marker *Alb* was expressed at high levels in DLK+ cells, whereas *Gata1*, *Cd41* and *Cd34*, which are specifically expressed in erythrocytes, myeloid progenitors and hematopoietic stem cells, respectively,(40, 41) were robustly detected in the whole-fetal-liver study, but rarely detected in our RNA-seq results (Supporting Fig. S8A). Based on these findings, the transcriptomic data generated from the whole fetal liver are not suitable for defining the timing of the hepatoblast-to-hepatocyte transition.

According to the hierarchical clustering analysis, all time points retained their developmental orders, and the differentially expressed genes were divided into clusters 'a' and 'b' (Fig. 7A). Cluster 'a' genes were mainly downregulated during development, whereas cluster 'b' genes displayed an opposite trend. The expression patterns of these two clusters of genes suggest that hepatoblast-to-hepatocyte differentiation might occur between E14.5 and E15.5. This interpretation is supported by the results of the GO analysis (Fig. 7B). The terms enriched in cluster 'a' included the cell cycle, DNA replication and many cell signaling pathways, such as SMAD, WNT, TGF β , BMP, VEGF and MAPK pathways, as well as histone modification and DNA methylation (Fig. 7B). RT-qPCR was performed to validate the expression patterns of some of these genes, including two DNA methyltransferases, *Dnmt1* and *Dnmt3a*, and two histone modifiers, *Kat2a* (*Gcn5*) and *Kdm5b* (*Jarid1b*). The expression levels of all these genes decreased during hepatoblast/hepatocyte development (Supporting Fig. S8B). Thus, at the hepatoblast stage, cells receive many environmental stimuli to promote cell proliferation and are en route to differentiation. In contrast, cluster 'b' mainly included the terms related to hepatocyte functions, but lacked the cell signaling pathways required to drive proliferation (Fig. 7B), suggesting that cells expressing this cluster of genes have specified into the hepatocyte fate or will reach that stage soon.

Cell fate changes during a developmental process are generally triggered by TFs, and changes in TF expression precede changes in the expression of their target genes, including genes required for cell structure and function. Of the differentially expressed genes shown in Fig. 7A, 222 genes encoded TFs. We then performed hierarchical clustering and differential expression analyses of this pool of genes and identified two clusters a' and b' (Fig. 7C,D). These clusters distinguished the hepatoblasts from the hepatocytes at a time point between E13.5 and E14.5. Taken together, the hierarchical clustering analyses using different gene pools strongly suggest that hepatoblast-to-hepatocyte differentiation begins at E13.5 and is complete by E15.5.

Discussion

Although hepatoblasts are known to be bi-potential hepatic progenitors that give rise to hepatocytes and cholangiocytes, the precise fate map for hepatic lineage specification has not been constructed. In this study, we identified the conditions required to enrich the hepatic lineages and systematically mapped the paths for hepatobiliary development at the single-cell level from E10.5 to E17.5. To our surprise, nascent hepatoblasts have already initiated their differentiation in the hepatocyte direction. This phenomenon suggests that hepatoblast-to-hepatocyte lineage specification is the default process. Hepatoblasts/hepatocytes show homogeneity at the same developmental stages and are “pushed” forward progressively along a linear pathway by differentiation/maturation stimuli. Strikingly, the differentiation states of proliferating and quiescent hepatoblast progenies from the same developmental stages are synchronized. In contrast, hepatoblast-to-cholangiocyte development defines a sharp branch from the main hepatoblast/hepatocyte pathway. Cholangiocyte specification from hepatoblast starts as early as E11.5, becomes more prevalent at E13.5 and is completed at E14.5. Developing cholangiocytes differentiate around the portal vein along the hilum-to-periphery axis; more mature cholangiocytes are located near the hilum and less mature cholangiocytes are located towards the periphery of the lobes.(8) Hence, newly generated cholangiocytes apparently enter the differentiation/maturation path non-synchronously, resulting in the observed heterogeneity in maturation states at the same developmental stage. Thus, our single-cell analysis has established a high-resolution map for hepatobiliary development with surprising features.

Proliferation and differentiation are mutually exclusive processes occurring during development. However, researchers have not determined how different hepatic lineages coordinate these processes during their specification. In this study, we showed that early-stage hepatoblasts maintain a higher proliferation rate, but an increasing number of cells become quiescent during hepatoblast/hepatocyte development. At E17.5, most of the hepatocytes are quiescent. Although most E10.5 to E13.5 hepatoblasts exhibit higher proliferation rate, a small fraction of cells are quiescent at these stages. This quiescent state is presumably important for hepatoblast-to-cholangiocyte fate commitment because reduced hepatoblast proliferation is associated with cholangiocyte differentiation in the *Tbx3*^{-/-} liver. (4) As cholangiocytes become more mature, the number of proliferating cells increases, suggesting that the immature cholangiocytes are transiently non-cycling cells that maintain their ability to re-enter the cell cycle during the maturation process. Thus, the hepatocyte and cholangiocyte lineages must utilize distinct mechanisms to control proliferation and differentiation during their development.

Cholangiocyte specification is coordinately regulated by TGF β , WNT, Notch, and FGF signaling. However, the signals promoting cholangiocyte maturation have remained unknown. In this study, we classified cholangiocytes into different stages based on their maturation states and found that more mature cholangiocytes expressed high levels of genes involved in the PKC/MEK/MAPK signaling pathway. We then showed that this pathway promotes cholangiocyte maturation using an explant system and single-cell analysis. Thus, we have defined a mechanism for cholangiocyte maturation.

In summary, our single-cell studies have established a molecular roadmap for the development of cells in the hepatobiliary lineage in vivo and provided significant insights into the mechanisms of hepatocyte and cholangiocyte segregation, cellular heterogeneity and maturation. Recently, cholangiocytes were induced to differentiate from embryonic stem cells (ESCs) in vitro;(42-44) however, these cells are generally immature. Thus, our findings are important not only for understanding basic cellular mechanisms but also for optimizing the conditions for the induction of ESCs to differentiate into hepatocytes and cholangiocytes in vitro.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BrdU	bromodeoxyuridine
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
GO	Gene Ontology
HGF	hepatocyte growth factor
MAPK	mitogen-activated protein kinase
PC	principal component
PCA	principal component analysis
PKC	protein kinase C
TF	transcription factor
TGF	transforming growth factor
TPM	transcripts per million

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Fig. 1. Single-cell RNA-seq uncovers the roadmap of hepatobiliary lineage development

(A) Schematic workflow of hepatobiliary single-cell RNA-seq. (B) Principal component analysis (PCA) plot of 447 hepatobiliary single-cell transcriptomes across 7 developmental stages identifying hepatoblasts/hepatocytes (circle) and cholangiocytes (triangle). Different stages are color coded. Arrowhead curves indicate hepatoblast/hepatocyte and cholangiocyte paths. (C) Schematic summary of hepatobiliary lineage development based on the PCA plot in (B). Each oval represents the distribution of hepatoblasts/hepatocytes or cholangiocytes of each developmental stage. (D) Hierarchical clustering of 1,761 heterogeneously expressed genes, correlated with the first two PCs (p-value < 5×10^{-15}) identifying clusters 'a-d'. Featured TFs of each cluster are listed on the right. Pseudotime ordering of groups-I and -II is based on the projection of hepatoblasts/hepatocytes on PC1 and cholangiocytes on PC2, respectively. Genes in clusters 'a-c' and 'd' are re-ordered by correlations with PC1 and PC2, respectively. (E) Selected GO terms enriched in clusters 'a-d'.

Fig. 3. Proliferating hepatoblasts develop synchronously with quiescent hepatoblasts
 (A) Hierarchical clustering identifying two clusters (I and II) of cell cycle-related genes and dividing hepatoblasts into three groups with different cell cycle phases. Cluster featured genes are listed on the right. (B, C) The PCA plot of 215 hepatoblasts. Different cell cycle phases (B) or developmental stages (C) are color coded. Solid and empty circles represent quiescent (G0/G1) and proliferating (G1/S and S/G2/M) cells in (B), respectively. (D) The distribution of quiescent (solid circle) and proliferating (empty circle) hepatoblasts from different developmental stages. The x-axis represents the pseudotime ordering of maturation.

Fig. 4. Hepatoblast adopts hepatocyte fate by default
(A) Hierarchical clustering of 383 upregulated genes correlated with the first two PCs in Fig. 3B (p-value < 1×10⁻⁷) during hepatoblast development. Genes are re-ordered by the correlation with PC1. TFs are listed on the right. (B) Selected GO terms enriched in genes identified in (A). (C) The expression dynamics of upregulated TFs across hepatobiliary lineage development. The red line represents the average tendency curve of relative expression levels of 21 TFs upregulated during the hepatoblast-to-hepatocyte transition. The blue line represents 44 TFs upregulated during the hepatoblast-to-cholangiocyte transition. The gray line represents the relative expression curve of an individual TF. (D) The lists of upregulated genes during hepatoblast-to-hepatocyte or hepatoblast-to-cholangiocyte development. Genes bolded have known to be necessary for hepatic development.

Fig. 5. Characterization of cholangiocyte lineage development

(A) The PCA plot of all 102 cholangiocytes. The cells are color coded by the developmental stage. (B) Hierarchical clustering of 261 genes correlated with PC1 in (A) (p -value $< 1 \times 10^{-6}$) identifying two genes clusters ('a' and 'b') and dividing cholangiocytes into three developmental stages (I-III). Cluster featured TFs are listed on the right. Genes are ordered by correlation with PC1. (C) Selected GO terms enriched in clusters 'a' and 'b' identified in (B), and genes related to PC2 in (A). (D) Venn diagram showing the overlap between cluster 'a' genes in (B) and the genes in Fig. 4A. (E) The PCA plot showing the distribution of hepatobiliary cells with different cell proliferation states. Different stages of cholangiocytes and hepatoblasts/hepatocytes are color coded. Individual cells are marked by empty (proliferating) or solid (quiescent) circles. (F) The percentage of proliferating cholangiocytes from stages-I to -III (top) and hepatoblasts/hepatocytes from E10.5 to E17.5 (bottom).

Fig. 6. PKC/ERK/MAPK signaling promotes cholangiocyte maturation

(A) Schematic workflow of the E12.5 liver lobe cultures (top). Liver lobes were treated with DMSO (control), TPPB, U0126 or TPPB+U0126. Images of explants after treatments (bottom) do not show overt differences in morphogenesis between each type of treatment. Scale bar: 200 μ m. (B) FACS gating strategy used to sort and analyze EpCAM+ cells. (C) Statistical analysis of the percentage of EpCAM+ cells observed after treatment. The data are presented as means $\pm$ SEM; n: number of biological replicates; Sample #: the number of cultured liver lobes; ***p-value < 0.001, t-test. (D) PCA plot showing that the TPPB treatment promotes cholangiocyte maturation compared to the control (left). The number of single cells is presented in parentheses. The PC1 values of individual cells in the PCA plot are projected in a box plot (right). ***p-value < 0.001, Wilcoxon rank-sum test. (E)

Expression levels (TPM) of marker genes are projected onto PCA plots (top) and box plots (bottom). ***p-value < 0.001, Wilcoxon rank-sum test.

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Fig. 7. Bulk-cell RNA-seq defines the hepatoblast-to-hepatocyte turning point
(A) Hierarchical clustering analysis of 4,077 variably expressed genes (CV > 0.4) in bulk hepatoblasts/hepatocytes obtained at nine developmental stages. Cluster-featured TFs are listed on the right. The arrow indicates the timing of the hepatoblast-to-hepatocyte transition.
(B) Selected GO terms enriched in clusters 'a' and 'b' shown in (A). (C) Hierarchical clustering of 222 TFs extracted from (A). The arrow indicates the timing of the hepatoblast-to-hepatocyte transition based on TF expression. (D) Volcano plot showing TFs that are highly expressed in hepatoblasts (E11.5) and hepatocytes (E17.5) with an adjusted p-value (padj) < 0.05. The listed TFs have been reported to regulate hepatoblast/hepatocyte development.