

Original Research

Single-Cell Transcriptome Analysis Identified Core Genes and Transcription Factors in Mesenchymal Cell Differentiation during Liver Cirrhosis

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Abstract

Background: Mesenchymal cells, including hepatic stellate cells (HSCs), fibroblasts (FBs), myofibroblasts (MFBs), and vascular smooth muscle cells (VSMCs), are the main cells that affect liver fibrosis and play crucial roles in maintaining tissue homeostasis. The dynamic evolution of mesenchymal cells is very important but remains to be explored for researching the reversible mechanism of hepatic fibrosis and its evolution mechanism of hepatic fibrosis to cirrhosis. Methods: Here, we analysed the transcriptomes of more than 50,000 human single cells from three cirrhotic and three healthy liver tissue samples and the mouse hepatic mesenchymal cells of two healthy and two fibrotic livers to reconstruct the evolutionary trajectory of hepatic mesenchymal cells from a healthy to a cirrhotic state, and a subsequent integrative analysis of bulk RNA sequencing (RNA-seq) data of HSCs from quiescent to active (using transforming growth factor $\beta 1$ (TGF- $\beta 1$) to stimulate LX-2) to inactive states. **Results**: We identified core genes and transcription factors (TFs) involved in mesenchymal cell differentiation. In healthy human and mouse livers, the expression of NR1H4 and members of the ZEB families (ZEB1 and ZEB2) changed significantly with the differentiation of FB into HSC and VSMC. In cirrhotic human livers, VSMCs transformed into HSCs with downregulation of MYH11, ACTA2, and JUNB and upregulation of PDGFRB, RGS5, IGFBP5, CD36, A2M, SOX5, and MEF2C. Following HSCs differentiation into MFBs with the upregulation of COL1A1, TIMP1, and NR1H4, a small number of MFBs reverted to inactivated HSCs (iHSCs). The differentiation trajectory of mouse hepatic mesenchymal cells was similar to that in humans; however, the evolution trajectory and proportion of cell subpopulations that reverted from MFBs to iHSCs suggest that the mouse model may not accurately reflect disease progression and outcome in humans. Conclusions: Our analysis elucidates primary genes and TFs involved in mesenchymal cell differentiation during liver fibrosis using scRNA-seq data, and demonstrated the core genes and TFs in process of HSC activation to MFB and MFB reversal to iHSC using bulk RNA-seq data of human fibrosis induced by TGF-*β*1. Furthermore, our findings suggest promising targets for the treatment of liver fibrosis and provide valuable insights into the molecular mechanisms underlying its onset and progression.

Keywords: liver fibrosis; hepatic stellate cells; myofibroblasts; single-cell RNA sequencing (scRNA-seq); monocle; RNA velocity

1. Introduction

Liver fibrosis is the formation of a fibrous scar caused by the accumulation of extracellular matrix (*ECM*) proteins, mainly cross-linked collagen types I and III, that replace damaged normal tissue in most chronic liver diseases [1]. Advanced liver fibrosis can lead to cirrhosis and even hepatocellular carcinoma. Cirrhosis is widespread worldwide and has various causes such as obesity, high alcohol consumption, non-alcoholic fatty liver disease (NAFLD), autoimmune diseases, hepatitis B or C infection, and cholestatic diseases [2]. Liver disease accounts for approximately 2 million deaths worldwide each year, 1 million from complications of cirrhosis, and is now the 11th most common cause of death worldwide [3]. The liver consists of approximately 80% hepatocytes and 20% non-parenchymal cells (NPCs). Liver fibrosis involves complex interactions between multiple lineages of NPCs, which include immune, endothelial, and mesenchymal cells spatially located in scar areas called fibrotic niches [4]. Various populations of mesenchymal cells, such as hepatic stellate cells (HSCs), fibroblasts (FBs), myofibroblasts (MFBs), and vascular smooth muscle cells (VSMCs) contribute to fibrosis after liver injury by producing *ECM* proteins [5]. The major mesenchymal cells populating the liver are HSCs (6%) [6]. In a healthy liver, HSCs are quiescent (qHSCs) and pericyte-like cells, which reside in the space of Disse between parenchymal and endothelial cells, express neural (*LRAT*, *GFAP*, *SYNM*, *SYP*) and lipogenic (*PPARG*, *ADIPOR1*) genes, *BAMBI*,

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NGFR, CEBPB, CEBPA [1,7–9]. Following chronic liver injury, qHSCs are continuously activated (aHSCs), express myogenic markers (ACTA2, MEF2C), and transdifferentiate into MFBs expressing COL3A1, PDGFRB, COL1A1, TIMP1, TGFB1, FAP, acquiring contractile, proinflammatory, and fibrogenic properties [1,7,10,11]. HSCs produce a wide range of cytokines and chemokines [10]. One of the well-characterised cytokines and the main contributor for liver fibrosis is transforming growth factor $\beta 1$ (TGF- β 1). Once activated, TGF- β 1 stimulates the transcription of genes important for fibrogenesis, namely CTGF, leading to the transcription of *ECM* genes. TGF- β 1 overexpression leads to increased matrix deposition [11]. When the damage stimulus is removed, MFBs undergo apoptosis or reverse to inactivated HSCs (iHSCs), acquiring a phenotype similar to, but distinct from qHSCs. iHSCs re-express PPARG and exhibit downregulation of fibrogenic genes (COL1A1, ACTA2, PDGFRB and TIMP1), but fail to express some quiescence-associated genes (ADIPOR1 and GFAP) [9,12]. Vitamin A expression is a key characteristic of qHSCs and is restored in iHSCs. When faced with the fibrogenic stimuli (such as $TGF\beta$), iHSCs rapidly reactivate into MFBs and effectively contribute to liver fibrosis as if they retain a biological memory of being activated [9]. These are the classic results of macro-fibrosis research. However, at present, no clinical drugs directly targeting liver fibrosis and cirrhosis have been approved for market.

Single-cell spatial transcriptome analysis showed that, in addition to HSCs, portal fibroblasts are (COL15A1⁺, ELN^+ , $THYI^+$) are the source of MFBs in the portal area in biliary fibrosis [13–15]. Hepatic fibrosis and cirrhosis are long-term and complex pathological processes in vivo, and the study of their dynamic evolution mechanism is conducive to our understanding of the occurrence and development of these diseases. Previous single-cell studies have focused on the heterogeneity of these mesenchymal cells [4,16], especially HSCs, during disease or injury, and most current studies have focused on exploring the mechanism of qHSCs activation into aHSCs [17-19]. However, the traceability of HSCs and MFBs and the reversal of MFB to iHSC in hepatic fibrosis and the dynamic evolution process of mesenchymal cells remain to be studied. With the development of single-cell RNA sequencing (scRNA-seq), cell trajectory, and cell-cell interaction analysis technology, it is expected that the problem of traceability and dynamic evolution of these cells will be solved.

In this study, we used scRNA-seq to investigate the origin of qHSCs, aHSCs, and MFBs during liver cirrhosis and whether there is a process of MFB reversal to iHSC. According to the pathological mechanism of liver fibrosis, we used TGF- β 1, the most classical inducer of liver fibrosis, to stimulate hepatic stellate cells LX-2, to simulate the occurrence and recovery stage of liver fibrosis *in vitro*, and to demonstrate the conclusion of the scRNA-seq analysis. In addition, the key transcription factors and core genes in-

volved in these processes were explored, which are of great significance for clarifying the pathogenesis of liver fibrosis and identifying potential biomarkers for disease diagnosis and drug development.

2. Materials and Methods

2.1 scRNA-seq Data Availability and Pre-Processing

The scRNA-seq data of three human cirrhotic tissue samples and three healthy liver tissue samples, two mouse healthy liver mesenchymal cell samples and two fibrotic (following chronic CCl₄-induced liver injury) liver mesenchymal cell samples, and one Lin-negative cell sample of the bilio-vascular tree from healthy mouse liver were acquired from the Gene Expression Omnibus (GEO) dataset with accession numbers GSE136103 [4], GSE137720 [20], GSE163777 [14]. Raw data (sra files) were obtained from the NCBI Sequence Read Archive (SRA) database with accession numbers SRP218975, SRP222529, SRP299106. We then extracted reads files from sra files using the SRA Tool Kit v3.0.0 (Bethesda, MA, USA) fastq-dump Tool and obtained the fastq files. We aligned reads to the GRCh38 and mm10 reference genomes as appropriate for the input dataset using the Cell Ranger Single-Cell Software Suite v7.0.0 (Pleasanton, CA, USA) from 10X Genomics, with the functions "cellranger mkfastq" and "cellranger count".

2.2 Human scRNA-seq Data Processing and Analysis

The R package Seurat v4.1.0 (Satija lab, New York, NY, USA) [21] was used to process human liver scRNAseq data (GSE136103). Genes expressed in fewer than three cells in a sample, cells that expressed fewer than 300 genes, and more than 6000 genes, and cells with mitochondrial gene content >20% and ribosomal gene content >50% of the total unique molecular identifier (UMI) count were excluded. After quality control, an expression matrix comprising 52,669 cells and 30,947 genes was obtained. Next, data were normalised using the default parameters of the log-normalisation method, and the top 2000 highly variable genes were identified using the FindVariableFeatures function. Z-score transformation of gene expression was performed using the ScaleData function. The following dimensionality reduction analysis was performed using the RunPCA function. Clustering was based on the 20 most significant principal components (PCs). Then, the batch effect was removed by using R package Harmony v0.1.0 (Center for Data Sciences, Brigham and Women's Hospital, Boston, MA, USA) [22]. Different resolutions were set, and the clustering effect was observed using clustree. Finally, the resolution was set to 0.6, and uniform manifold approximation and projection (UMAP) visualisations were constructed using the same number of PCs as the associated clustering.

We focused on mesenchymal cells, therefore, we extracted subpopulations of mesenchymal cells (cluster 9 and 15) from healthy and cirrhotic samples. Because we were more concerned with the dynamic evolution of mesenchymal cells in cirrhosis, we used the FindClusters function to sub-cluster these mesenchymal cells from healthy and cirrhotic liver separately. Dimensionality reduction analysis was performed using the RunPCA function, and subclustering was based on the nine most important key components. The resolution was set to 0.9 (healthy) and 1.2 (cirrhotic). After subclustering, healthy and cirrhotic mesenchymal cells were visualised with t-distributed stochastic neighbor embedding (t-SNE) using the nine most important PCs.

2.3 Mouse scRNA-seq Data Processing and Analysis

Mouse hepatic mesenchymal cells from two healthy and two fibrotic liver (GSE137720) were analysed using Seurat. Low quality cells (<300 genes/cell, >4500 genes/cell, <3 cells/gene, >7.5% mitochondrion genes, and >25% ribosomal genes) were filtered out from the dataset. After quality control, 12,538 and 10,706 mesenchymal cells were obtained from healthy and fibrotic mouse livers, respectively. Normalisation, z-score transformation, dimensionality reduction, and clustering analysis were similar to those of the human liver scRNA-seq process, except that the resolution was set to 0.5. The t-SNE visualisations were constructed using the nine most significant PCs consistent with the clustering.

The scRNA-seq data of mouse Lin-negative cells of bilio-vascular tree (GSE163777) was analysed in a similar manner. Low-quality cells (<300 genes/cell, >6000 genes/cell, <3 cells/gene, >10% mitochondrion genes, and >25% ribosomal genes) were filtered out from the dataset. After normalisation, z-score transformation, and dimensionality reduction, clustering was performed using the 20 most significant PCs. Finally, the resolution was set to 0.6, and t-SNE visualisations were constructed using the 20 most significant PCs consistent with the clustering. After cell type annotation, we extracted mesenchymal cells for sub-clustering. We used RunPCA to redimensionalise the extracted mesenchymal cells and clustered the 14 most important PCs. Finally, the resolution was set to 0.5, and t-SNE was used for visualisation.

2.4 Identification of Cell Type and Annotation

After obtaining cell clusters, the FindAllMarkers function was used to search for differentially expressed genes in the clusters. We set the log fold-change of the average expression between the two clusters (avg_logFC) >0.5. The *p* value after Bonferroni correction was <0.05. Genes were ranked in ascending *p* values, differentially expressed in each cluster, and cell types were manually identified using liver cell marker genes from previous studies [4,14,20] and marker genes in PanglaoDB, CellMarker, and the Human cell landscape database.

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2.5 Pseudotime Differentiation Trajectory Analysis

The differentiation trajectory of the mesenchymal subpopulations was constructed using the R package Monocle v2.24.1 (Cole Trapnell's lab, Seattle, WA, USA) [23]. The newCellDataSetFirst function was used to build a Monocle object. We used dispersionTable (mean_expression ≥ 0.1 & dispersion_empirical ≥ 1 * dispersion_fit) to select highly variable genes as ordering genes and used in Monocle for clustering. We ran reduceDimension (max_components = 2, method = 'DDRTree') with t-SNE as the reduction method, and ran orderCells under default parameters. Finally, a pseudo-time differentiation trajectory was plotted using the cell trajectory function.

2.6 RNA Velocity-Based Cell Fate Tracing

We verified the Monocle trajectory and its directionality using the velocyto Python package v 0.17.17 (La Manno Lab, Stockholm, Sweden) [24] to estimate the cell velocity from the spliced and unspliced mRNA content. We used velocyto to convert the cellranger result files (output directory) into loom files and merged the loom files of all samples. The merged loom files were used as input for scvelo v0.2.4 (Volker Bergen, Munich, Germany) Python pipeline [25]. The calculation of RNA velocity values for each gene in each cell and embedding of the RNA velocity vector in t-SNE were performed using scvelo and finally visualised with Python v3.8 (Python Software Foundation, Delaware, DE, USA).

2.7 Cell-Cell Interaction Analysis

The R package CellChat v1.5.0 (Department of Mathematics, University of California, Irvine, CA, USA) [26] was used for cell communication analysis, and the Seurat object was exported as the input to CellChat. After the construction of the CellChat object using the create-CellChat function, the ligand-receptor interaction database CellChatDB.human (or CellChatDB.mouse) was set up. The computeCommunProb function was used to calculate the communication probability with default parameters and infer the CellChat network. Function filterCommunication was used to filter groups with fewer than 10 cells. The function subset Communication was then used to save the prediction results of cell communication in the form of a data frame. The computeCommunProbPathway function was used to summarise the communication probability of all ligand-receptor interactions related to each signalling pathway, to calculate the communication probability at the signal pathway level. The integrated cell communication network was evaluated using the aggregateNet function by calculating the number of links or summarising the communication probability. The results of cell-cell communication were displayed using netVisual heatmap function.

2.8 Transcription Factor Analysis

The Python package pySCENIC v0.12.0 (VIB Center for Brain Disease Research, Laboratory of Computational Biology, Leuven, Belgium) [27] was used for transcription factor analysis. First, the gene expression matrix was exported from R and saved as "csv" files, and then the "csv" file was converted into a loom file in Python. The pyscenic grn, pyscenic cistarget, and pyscenic AU-Cell functions were run sequentially for transcription factor analysis and the results were visualised.

2.9 Construction of Human Liver Fibrosis Model

The human hepatic stellate cell line (LX-2) was provided without Mycoplasma contamination from Professor Wang Qingqing's research group at Zhejiang University School of Medicine and was verified as LX-2 cells by the STR technology of Wuhan Punosei Life Technology. To maintain the cells in the same state before dosing, LX-2 cells in the logarithmic growth phase were starved for 24 h (the medium did not contain foetal bovine serum or penicillin/streptomycin). TGF- β 1 at 50 ng/mL was then added for 24 h to construct the liver injury model (stimulate period), and no drug treatment was added to the blank control group (quiescent period). After 24 h, the LX-2 cells were left without treatment for 7 days (recovery period).

2.10 RNA-seq

The stimulate, control, and recovery period samples were repeated three times, and RNA extraction, quality control, library construction, and RNA-seq were performed on nine samples. Total RNA was extracted using TRIzol reagents, RNA integrity was assessed using 1% agargel, and RNA concentration and purity were quantitatively and qualitatively analysed using a nanoophotometer spectrophotometre. RNA volume was 3 µg/ sample, the RNAseq library was constructed using NEBNext UltraTM RNA Library Prep Kit for Illumina, and index codes were added to the attribute sequence of each sample. Following the manufacturer's instructions, we used the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, CA, USA), to cluster samples using index codes on the cBot Cluster Generation System. After clustering, we sequenced the library using the Illumina platform and obtained 150 bp paried-end sequences.

2.11 RNA-seq Data Processing

First, FastQC was used to evaluate Illumina reads. The Fastp software was used to discard low-quality reads. Then, we downloaded the human reference genome (GRCh38) from the Ensembl database and used HISAT2 software for read alignment. Finally, the gene levels were quantitatively analysed using the featureCounts tool to obtain the final standardised matrix. The expression level of each gene was quantified as normalised fragments per kilobase of transcript per million mapped reads (FPKM).

2.12 Statistical Analyses

Unless otherwise stated, statistical analyses were performed using Graphpad Prism v8.0 (GraphPad Software, San Diego, CA, USA). A paired *t*-test was used to compare the differences between the two groups. Differences were considered statistically significant at p < 0.05.

3. Results

3.1 Single-Cell Transcriptomic Profile of the Human Liver

A graphical overview of the study design is shown in Fig. 1A. To examine the heterogeneity of hepatic NPCs, we analysed the hepatic NPC scRNA-seq data obtained from the GEO database (GSE136103). Hepatic NPCs were isolated from three healthy and three cirrhotic human livers. NAFLD was the cause of liver fibrosis in one female patient, and alcohol was the cause of liver fibrosis in the other two male patients (Supplementary Fig. 1A). Leucocytes (CD45⁺) or other NPC (CD45⁻) fractions were FACS-sorted before scRNA-seq [4]. After filtering low-quality cells (Supplementary Fig. 1B,C), the batch effect was removed using Harmony, and cells of the same type, such as endothelial cells, clustered more distinctly (Supplementary Fig. 1D,E). After dimensionality reduction and clustering (clustree is shown in Supplementary Fig. 2A), 52669 NPCs revealed 24 populations (Fig. 1B), and the top marker genes from the 24 cell clusters were identified by Seurat (Supplementary Fig. 2B, Supplementary Table 1). NPCs were represented by a total of 11 distinct cell types (Fig. 1C), which correspond to T cell $(TRAC+IL7R^+)$, innate lymphoid cell (ILC; $PRF1^+FGFBP2^+$), Endothelial cell (VWF^+STAB2^+), phagocyte mononuclear (MP; $ClOB^+MARCO^+),$ Epithelial cell (ALB^+KRT18^+) , mesenchymal cell (COL3A1 + TAGLN + ACTA2 + PDGFRB +),В cell $(CD79A^+MS4A1^+)$, plasma cell $(FCRL5^+JSRP1^+)$, cycling cell (TOP2A+MKI67+), plasmacytoid dendritic cell (pDC; $LILRA4^+PTCRA^+$), mast cell ($TPSAB1^+CPA3^+$) (Fig. 1D). Because the parenchymal cells were not fully removed during centrifugation, a small cluster of epithelial cells remained (Fig. 1C). MPs, Mast cells, ILCs, and T cells displayed high expression levels of CD45 (PTPRC) (Supplementary Fig. 2C). Although all 11 major cell types were present in both healthy and cirrhotic tissues, the proportions of major cell types were different (Fig. 1E). Cell sorting approaches led to loss of fragile cells, particularly hepatocytes and cholangiocytes, and the relative enrichment of more robust NPCs, such as endothelial cells/MPs [28]. As a result, a large number of endothelial cells and MP cells were enriched in the NPCs clustering results, while only a small proportion of mesenchymal cells (the main mesenchymal cells, HSC, accounted for only 6% of the liver).



Fig. 1. Single-cell transcriptomic profile of human liver. (A) Graphic overview of the study design. (B) Clustering 52,669 cells from three healthy and three cirrhotic human livers. (C) The 11 lineages of 52,669 human liver cells, inferred from the expression of marker genes. Right, annotation by injury condition. ILC, innate lymphoid cell; MP, mononuclear phagocyte; pDC, plasmacytoid dendritic cell. (D) UMAP plots of conserved hepatic cell markers. *CD79A*, CD79a Molecule; *MK167*, Marker Of Proliferation Ki-67; *VWF*, Von Willebrand Factor; *ALB*, Albumin; *KRT18*, Keratin 18; *PRF1*, Perforin 1; *TPSAB1*, Tryptase Alpha/Beta 1; *COL3A1*, Collagen Type III Alpha 1 Chain; *ACTA2*, Actin Alpha 2, Smooth Muscle; *TAGLN*, Transgelin; *PDGFRB*, Platelet Derived Growth Factor Receptor Beta; *C1QB*, Complement C1q B Chain; *FCRL5*, Fc Receptor Like 5; *TRAC*, T Cell Receptor Alpha Constant. (E) Bar plot representing the relative contribution of cells from each donor (three healthy livers, three cirrhosis livers) for each cell type.

3.2 Pseudotime Analysis Revealed the Differentiation Trajectories of Healthy Mesenchymal Cells

We focused on mesenchymal cells, and we isolated clusters 9 and 15 (2918 mesenchymal cells) for further analysis. In healthy liver, mesenchymal cells sub-clustered into eight distinct mesenchymal subpopulations (Fig. 2A). We identified the cells in clusters 1, 2, 3, and 7 as VSMCs that displayed high expression levels of *MYH11*, *PLN*, *CNN1*, and *RCAN2*. Cells in clusters 0, 4, and 5 were classified as qHSCs based on their high expression levels of markers

PPARG and *NGFR* [1]. Finally, cells in cluster 6 were annotated as FBs because of their high expression levels of markers *DPT*, *GGT5*, and *MFAP4* (Fig. 2B, **Supplementary Table 2**). Each cluster was assigned a unique name based on the expression of the predominant marker (**Supplementary Fig. 3A**).

To determine the evolutionary trajectory of hepatic mesenchymal cells in healthy livers, we used Monocle 2 for trajectory analysis. This trajectory indicated that FBs differentiated into VSMCs and qHSCs (Fig. 2C). To provide



Fig. 2. Pseudotime analysis revealed the differentiation trajectories of healthy mesenchymal cells. (A) Mesenchymal cells clustered into eight subpopulations in human healthy livers. FB-c6-GGT5, the marker gene of fibroblasts from cluster 6 is GGT5. VSMC, vascular smooth muscle cell; FB, fibroblast; HSC, hepatic stellate cell; tSNE, t-distributed Stochastic Neighbor Embedding. (B) Dot plots showing the expression of mesenchymal cellular marker genes in healthy human livers. (C) A differentiation trajectory of healthy human liver mesenchymal cells inferred by Monocle 2 (pseudotime along differentiation trajectory in inset). (D) Gene expression dynamics along the trajectory of healthy hepatic mesenchymal subtypes. (E) RNA velocity of mesenchymal subtypes in healthy livers, pseudotime (right) detected (purple to yellow). (F) Heatmap showing the TF activity of mesenchymal subtypes in healthy livers. (G) The relative expression of mesenchymal cellular marker genes and transcription factors (TFs) along the differentiation trajectory in healthy livers. (I) Dot plot showing the significance and average expression of ligand-receptor interactions between FB and other cell types in healthy human livers.

further insights into FB differentiation, we identified three sets of differentially expressed genes (DEGs) along the trajectory within branch 1 (Fig. 2D). The first set, consisting of VSMC markers (*ACTA2*, *TAGLN*, *PLN*, and *TPM2*), in-

creased towards the end of trajectory. Meanwhile, the second set, consisting of *HLA-DRA*, *ANK3* and *PCDH9*, were highly expressed in FB and showed an upward trend in the early developmental trajectory. The third set of genes (*PDE3A*, *PLA2G5*, and *CTNNA3*) were highly expressed in HSC and showed an upward trend in the late developmental trajectory (**Supplementary Fig. 3B**).

To validate the developmental trajectory of Monocle 2, we utilized the scVelo pipeline to compute RNA velocity values for each gene in each cell. The analysis predicted that HSCs and VSMCs arose from FBs (Fig. 2E), which is consistent with Monocle2's results (Fig. 2C). Moreover, VSMC-c2-GBP2, VSMC-c3-SEMA4A, and HSC-c4-IGFBP5 exhibited faster velocities in the late pseudo-time than FB-c6-GGT5.

FB differentiation is regulated by a complex network of transcription factors (TFs) that function in conjunction with one another. We used PySCENIC to explore the top five activities in the TF regulatory network. We observed that NFATC2 and NR1H4 displayed high expression and activity levels in the FB regulatory network (Fig. 2F). However, transcriptomics-based TF exploration provides indirect results. As pseudotime increased, the expression of NFATC2, NR1H4, and ZEB2 gradually decreased, along with the expression of FBs-specific genes (IGFBP3, MFAP4, DPT, GGT5, LUM), and the FBs ultimately differentiated into qHSCs and VSMCs (Fig. 2G,H, Supplementary Fig. 3B). During the differentiation of FBs into qH-SCs, the qHSC marker genes RGS5 and PLAT was upregulated. Regarding the differentiation of FBs into VSMC, the expression of ACTA2, TPM2, PLN, and MYH11 were upregulated. Finally, CellChat was used to analyse unbiased interactions of mesenchymal subpopulations with ligands and receptors [26]. CellChat revealed that FB significantly interacted with HSC and VSMC, particularly through the ligand-receptor pair GAS6-AXL (Fig. 2I).

3.3 Pseudotime Analysis Revealed Differentiation Trajectories of Mesenchymal Cells in Cirrhotic Liver

In human cirrhotic livers, mesenchymal cells were sub-clustered into 10 subpopulations (Fig. 3A) based on the expression patterns of marker genes (Fig. 3B, Supplementary Table 3). Cells in cluster 0 and 6 express high levels of MYH11, ACTA2, TAGLN, and CNN1, and were identified as VSMCs. Cells in cluster 1, 5, and 7 expressed high levels of activation markers TAGLN, ACTA2, VIM, A2M, CD36, IGFBP5, PDGFRB, and quiescent marker PPARG, showing characteristics common to both qHSC and MFBs [29], and were annotated as HSCs. Cells in cluster 2, 4, 8, and 9 expressed high levels of PDGFRA, VIM, COL3A1, and COL1A1, were identified as MFBs. The cells in cluster 3 were annotated as mesothelial cells expressing high levels of SLPI and KRT19 (Fig. 3B). Each cluster was renamed based on the specific marker gene expressed (Supplementary Fig. 3C).

Monocle 2 indicated that VSMC-c0-MYH11 and VSMC-c6-ADRA1A were at the beginning of the trajectory, and HSC-c7-PDGFRB was located at the end stage of branch 1. MFB-c8-IGFBP3, MFB-C9-PDGFRA, MFB-

c4-CRABP2, and Meso-c3-SLPI were concentrated at the two terminal stages of branch 2 (Fig. 3C). The top 50 genes related to developmental trajectory in branch 1 were clustered into three groups (Fig. 3D). Genes in cluster 1 were highly expressed during the early stage of trajectory development, such as *MYH11* and *COX412*, which were highly expressed in VSMCs. Genes in cluster 2 (*S100A6*, *TIMP1*, *COL1A1*, *COL3A1*, *COL1A2*, *LUM*) were highly expressed in MFBs; it has been reported that *S100A6* is highly upregulated on activated MFBs [16]. Genes in cluster 3 showed high expression levels in the transitional stage of the developmental trajectory, such as *PDGFRB*, *CD36*, *MEF2C*, and *SOX5*, which were highly expressed in HSCs (Fig. 3D).

The RNA velocity results are mostly consistent with those of the Monocle, indicating that VSMC-c0-MYH11 and VSMC-c6-ADRA1A were in an early developmental trajectory and tended to differentiate into HSC-c5-RGS5 cells (Fig. 3E). A small proportion of HSC-c1-CD36 and most HSC-C7-PDGFRBs were in the late stages of development, whereas some cells tended to develop into MFBc2-LUM and MFB-c8-IGFBP3. Surprisingly, MFB-C9-PDGFRA showed a tendency to transform into HSC-C7-PDGFRB (Fig. 3E); we hypothesised that, as liver damage was interrupted, MFBs underwent apoptosis or reverted to the iHSCs state.

To further determine the primary regulators of mesenchymal subpopulations, we evaluated the top six activities of the TF regulatory network using pySCENIC (Fig. 3F). During VSMC differentiation into HSCs, the expression of the VSMCs marker genes MYH11, ACTA2 and TAGLN decreased gradually, whereas the expression of HSC-specific genes (PDGFRB, IGFBP5, CD36, RGS5, A2M) increased gradually, and the expression of HSC TFs (SOX5, MEF2C, TCF7L2) also increased (Fig. 3G,H, Supplementary Fig. 3C). During the transformation of HSCs to MFBs, the expression of MFB-specific genes (COL1A1, COL3A1, COL1A2, LUM, PDGFRA, TIMP1) and TFs (NR1H4, SOX6, PBX1, ZNF23) increased, whereas the expression of PDGFRB, RGS5, JUNB, and MEF2C was gradually downregulated and almost undetectable in MFBs. Furthermore, we investigated the cell-cell communication mechanisms of HSCs and MFBs. The results indicated that all HSC subtypes, except HSC-c7-PDGFRB, had strong interactions with MFBs through the adhesive ligand-receptor pairs MIF-(CD74 + CD44), MDK-LRP1, and NGF-NGFR (Fig. 3I).

3.4 Dynamic Evolution Trajectory of Mouse Liver Mesenchymal Cells is Similar to that in Humans

Next, we analysed scRNA-seq (GSE137720) data of mouse livers, including two healthy livers and two livers with fibrosis resulting from chronic CCl₄-induced liver injury (**Supplementary Fig. 4A**). Following quality control (**Supplementary Fig. 4B,C**), 12538 and 10706 mesenchymal cells were successfully classified into nine clus-



Fig. 3. Pseudotime analysis revealed the differentiation trajectories of mesenchymal cells in cirrhotic liver. (A) Human cirrhotic hepatic mesenchymal cells clustered into 10 subpopulations. MFB, myofibroblast. (B) Dot plots showing the expression of mesenchymal cellular marker genes in cirrhotic human livers. (C) A differentiation trajectory of cirrhotic human liver mesenchymal cells inferred by monocle. (D) Gene expression dynamics along the trajectory of cirrhotic liver mesenchymal subtypes. (E) RNA velocity of mesenchymal subtypes in cirrhotic livers. (F) Heatmap showing the TFs activity of mesenchymal cells in cirrhotic livers. (G) The relative expression of mesenchymal cellular marker genes and TFs along the trajectory in cirrhotic livers. (H) Schematic illustrating molecular mechanisms of mesenchymal subpopulations differentiation in cirrhotic human livers. (I) Dot plot showing the significance and average expression of ligand-receptor interactions between HSC and MFB in cirrhotic livers.

ters (Fig. 4A) and ten clusters (Fig. 4B), respectively. In healthy livers, clusters 0, 1, 3, and 6 expressing high levels of quiescence markers (*Ecm1*, *Reln*, *Lrat*, *Hgf*, *Rgs5*, *Ngfr*) were annotated as qHSCs, whereas clusters 2, 4, and 7 were annotated as FBs expressing high levels of *Pi16*, *Cd34*, *Mfap4*, *Col1a1*, *Col15a1*. Cluster 5 and 8 were annotated as VSMCs expressing high levels of *Tagln*, *Acta2*, *Cnn1*,

and *Myh11* (Fig. 4C, **Supplementary Table 4**). In the fibrotic livers, cluster 4, 5, and 9 were annotated as VSMCs expressing high levels of *Tagln*, *Acta2*, *Cnn1*, *Myh11*, and *Tpm2*. Cluster 0 and 6 with high expression of *Pdgfra*, *Pi16*, *Fmo2*, *Col1a1*, *Col3a1* were annotated as MFBs, whereas cells in cluster 1, 2, 3, 7, and 8 were annotated as aHSCs expressing high levels of *Pdgfrb*, *Hgf*, *Pth1r*,

Ecm1, *Ngfr*, and *Adamtsl2* (Fig. 4D, **Supplementary Table 5**). We renamed the mesenchymal cell subpopulations based on the markers specifically exp-ressed in each cluster (**Supplementary Fig. 4D,E**).

In healthy mouse livers, Monocle 2 analysis suggested a differentiation trajectory from FBs to VSMCs and HSCs, which is similar to that in humans (Fig. 2C) but with a simpler trajectory, with each cell type distributed more centrally in three branches (Fig. 4E). Furthermore, this trajectory is consistent with the previously reported results of trajectory analysis of mouse Lin-negative cell in biliary tree fragments (Supplementary Fig. 5A-G, Supplementary Table 6) [14]. Pi16, Col15a1, Cd34, Thy1, Clec3b, Fbln2 are highly expressed in FB (Supplementary Table 7), and $Pi16^+$ cells serve as resource fibroblasts [30]; CD34 and *Thy1* are both stem cell markers [14], further supporting FB as the source cell in the trajectory. Genes related to trajectory were clustered into three clusters. Genes in cluster 1 (Col3a1, Mfap4, Pil6, Clec3b) were highly expressed during the early trajectory. Genes in cluster 2 (Tagln, Tpm1, Myh11, Acta2) were highly expressed during the transitional stage, whereas genes in cluster 3 (Pth1r, Ecm1, Lrat, Ngfr, Hgf, Reln) were highly expressed during the final stage (Fig. 4F). The results of RNA velocity analysis in healthy livers (Fig. 4G) are consistent with the results of Monocle (Fig. 4E). HSCs and VSMCs originated from FBs, which coincided with the results of pseudotime analysis in human healthy livers (Fig. 2C–E).

In mouse fibrotic liver, Monocle trajectory analysis indicated that HSCs and MFBs originated form VSMCs (Fig. 4H), which is consistent with pseudotime analysis results from human hepatic mesenchymal cells with cirrhosis (Fig. 3C,E). The first 50 key genes related to cell trajectory were divided into three clusters. Genes in cluster 1 (Pth1r, Stat1, Vipr1 and Eng) showed high expression mainly in the final stage of the trajectory, genes in cluster 2 (Dpt, Lama2, Cd34, Clec3b, Pi16 and Thy1) were highly expressed in the transitional stage of trajectory, and genes in cluster 3 (Des, Tagln, Rgs4) were highly expressed in the early trajectory (Fig. 4I). The results of RNA velocity analysis in mouse fibrotic liver (Fig. 4J) are consistent with the Monocle results (Fig. 4H). Interestingly, MFB-c0-Pdgfra showed a tendency to differentiated into HSC-c1-Pdgfrb, which is consistent with the RNA velocity of human hepatic mesenchymal cells with cirrhosis (Fig. 3E). LAMA2, CCBE1, PDGFRA and NAALADL2, which were highly expressed in the MFBs of human liver, were also highly expressed in the MFBs of the mouse liver (Supplementary Fig. 6A,B). PDGFRB, ARHGAP42, ASAP1, and GUCY1A2, which were highly expressed in human HSCs, were also highly expressed in mouse HSCs (Supplementary Fig. 6C,D). Therefore, we suggest that MFBs and HSCs in mouse fibrotic liver are similar subpopulations as MFBs and HSCs in human cirrhotic livers.

Finally, we examined the molecular mechanisms underlying mesenchymal cell differentiation in the mouse liver. In healthy mouse livers, FBs TFs Zeb1 showed higher activity during the transformation stage of FBs to VSMCs and qHSCs, the expressions of FBs marker genes (Cd34, Pi16) and TFs (Zeb, Jund) continuously decreased, and the expressions of VSMCs marker genes (Acta2, Myh11, Tagln) and TFs (Mef2c, Klf2) gradually increased (Fig. 5A,B). During FBs differentiation into qHSCs, the expressions of Acta2, Myh11 and TFs Mef2c gradually decreased, whereas the expression of qHSCs marker genes (Ngfr, Lrat and Hgf), qHSC-related TFs (Sox5, Nr1h4, Hand2 and Cebpb) were gradually increased (Fig. 5B,C, Supplementary Fig. 7A). Cell-cell communication analysis showed that FBs interacted with HSCs and VSMCs through the Angptl1-(Itga1 + Itgb1) ligand-receptor pair (Supplementary Fig. 7B).

In mouse chronic fibrosis liver, pySCENIC combined trajectory analysis showed that, at the stage of transformation from VSMCs to MFBs and aHSCs, the expression of VSMCs TFs (Mef2c, Thra, Gata4), markers Myh11 and Tagln continuously decreased, the expression of Tagln was the lowest when differentiation to MFBs, and it gradually increased when differentiation into aHSCs (Fig. 5D-F, Supplementary Fig. 7C). In the differentiation from VSMCs to MFBs, the expressions of the MFBs markers Collal, Clec3b, and TF Zeb1 was upregulated, and then downregulated with the differentiation of VSMCs to aH-SCs. In the differentiation from VSMCs to aHSCs, the expression of aHSCs marker genes Hgf and Pth1r was upregulated, and the expression of the TF Irfl and Junb gradually decreased from the beginning of VSMCs differentiation to the lowest level at the MFBs stage, and then gradually increased at the differentiation stage of aHSCs (Fig. 5D-F, Supplementary Fig. 7C). Among the ligand-receptor pairs in which VSMCs interacted with MFBs and HSCs, Fgf1-Fgfr2 was the most significant, followed by Ngf-Ngfr; Gas6-Axl also significant (Supplementary Fig. 7D).

3.6 MFB Exhibited a Reversal to HSC Following Removal of Hepatic Injury Stimulation.

To examine the translatability of the core genes in scRNA-seq result, we used TGF- β 1 to treat human hepatic stellate cells (LX-2 cells) to establish a liver fibrosis model (Fig. 6A). In a previous study, we verified the expression of fibrogenic genes (including *COL1A1*, *ACTA2*, and *FN1*) in unstimulated, stimulated and recovered LX-2 cells using reverse transcription polymerase chain reaction (RT-PCR), and successfully constructed a human liver fibrosis model. Following 24 h of starvation, LX-2 cells were stimulated with TGF- β 1 for a further 24 h to induce the transition of qHSCs into MFB. Subsequently, the TGF- β 1 stimulation was removed, and the cells underwent 7-days recovery period, promoting the reversal of MFB into inactive iHSCs.



Fig. 4. Dynamic evolution trajectory of mouse liver mesenchymal cells is similar to that of humans. (A,B) t-SNE visualisation of mesenchymal cells clustered into nine and 10 subpopulations in mouse healthy and fibrotic livers, respectively. (C,D) Dot plots showing the expression of mesenchymal cellular marker genes in healthy and cirrhotic mouse livers. (E) Trajectory of mouse healthy hepatic mesenchymal cells inferred by Monocle 2. (F,I) Gene expression dynamics along the healthy and fibrotic mouse hepatic mesenchymal cells trajectory. (G,J) RNA velocity of mesenchymal subtypes in healthy and fibrotic livers, pseudotime (right) detected (purple to yellow). (H) A differentiation trajectory of mouse fibrotic liver mesenchymal cells.

In LX-2 cells, after exposure to TGF- β 1, qHSC-specific genes (*PPARG*, *NGFR*, *RGS5*, *CEBPA*, *PLAT*, *RELN*, *CD36*, *IGFBP5*, *GFAP*) and TF *NR1H4* were mostly downregulated in the fibrotic compared to the quiescent stage (Fig. 6B), and MFB-related genes (*COL1A1*, *TIMP1*, *TGFB1*, *FN1*, *PDGFRA*, *IGFBP3*, *ACTA2*, *PDGFRB*, and *COL1A2*) and TFs (*JUNB*, *ZEB1*, *PBX1*, *TCF7L2*, and *NFATC2*) were mostly upregulated (Fig. 6C). This is in agreement with the RNA expression trends from HSC to MFB in pseudotime analysis of human cirrhotic mesenchymal cells (Fig. 3G,H). Among these, *ACTA2* expression was upregulated during the initial stimulus stage. After the 7-

day recovery period, immune cells or inflammation may still be present in this model, which causes the expression of this gene to continue to be upregulated.

After stimulation removal, qHSC-specific genes were upregulated in the recovery stage, and fibrogenic genes were downregulated (Fig. 6B,C). This implies that MFB transdifferentiates into iHSC, which further supports the results of the reversal of MFB to iHSCs in human cirrhosis and mouse liver fibrosis in the pseudotime analysis (Figs. 3E,4J).



Fig. 5. Molecular mechanism of the differentiation of mesenchymal subpopulations in the mouse liver. (A) Heatmap showing the TF activity of mesenchymal cells in healthy mouse livers. (B) Relative expression of mesenchymal cellular marker genes and TFs along differentiation trajectory in healthy mouse livers. (C) Schematic diagram illustrating the molecular mechanism of the differentiation of mesenchymal subpopulations in healthy mouse livers. (D) Heatmap showing the TF activity of mesenchymal cells in mouse fibrotic livers. (E) Relative expression of mesenchymal cellular marker genes and TFs along the trajectory in mouse fibrotic livers. (F) Schematic diagram illustrating the molecular mechanism of differentiation of mesenchymal subpopulations in mouse fibrotic livers.

4. Discussion

In this study, we performed scRNA-seq to determine the differentiation trajectory of mesenchymal cells from healthy and fibrotic/cirrhotic livers, and utilized pySCENIC and CellChat to investigate the molecular mechanism underlying the trajectory. Finally, analysis of Bulk RNA-seq data from HSCs confirmed key genes and transcription factors in process of HSC activation to MFB and MFB reversal to iHSC. In healthy human livers, a pesudotime trajectory showned that FBs differentiated into VSMCs and qH-SCs with downregulation of FB-specific genes (*IGFBP3*, *MFAP4*, *LUM*, *DPT*, *GGT5*) and TFs (*NFATC2*, *NR1H4*, *ZEB2*), and the upregulation of HSC-specific genes (*RGS5* and *PLAT*) and VSMC-specific genes (*ACTA2*, *MYH11*, *TPM2*, and *PLN*) (Fig. 2C–H, **Supplementary Fig. 3B**). In healthy mouse livers, FBs differentiated into VSMCs with upregulation of VSMC-specific genes (*Acta2*, *TagIn*,





Fig. 6. MFB exhibited a reversal to HSC following removal of hepatic injury stimulation. (A) Schematic diagram of the obtention of a human liver fibrosis model using TGF- β 1 to induce LX-2 cells. (B) Quiescent gene (*PPARG*, *NGFR*, *RGS5*, *CEBPA*, *PLAT*, *RELN*, *CD36*, and *IGFBP5*) expression in LX-2 (qHSC), TGF- β 1 stimulate stage (MFB) and TGF- β 1 recovery stage (iHSC) (n = 3). Mean \pm SEM; *p < 0.05; **p < 0.01 (paired *t*-test). (C) Fibrogenic gene (*COL1A1*, *TIMP1*, *TGFB1*, *FN1*, *PDGFRA*, *IGFBP3*, *ACTA2* and *PDGFRB*) expression in LX-2 (qHSC), TGF- β 1 stimulate stage (MFB) and TGF- β 1 recovery stage (iHSC) (n = 3). Mean \pm SEM; *p < 0.05; **p < 0.01 (paired *t*-test).

Myh11) and downregulation of TF *Zeb1*. In contrast, FBs differentiated into qHSCs with upregulation of TFs (*Sox5*, *Nr1h4*, *Hand2*, and *Cebpb*) and HSC-specific genes (*Hgf* and *Lrat*). Finally, FB-specific genes (*Cd34* and *Pi16*)

were not expressed (Fig. 5B–C). The scRNA-seq analysis of healthy mouse Lin-negative cells of bilio-vascular tree fragments (**Supplementary Fig. 5A–G**) showed that HSCs and VSMCs differentiated from FBs [14], which is consis-

tent with our findings. In healthy human and mouse livers, the expression of TFs *NR1H4* and the member of the *ZEB* families (*ZEB1*, *ZEB2*) changed significantly with the differentiation of FBs into qHSCs and VSMCs, and these may be the core TFs driving differentiation.

In the damaged liver, aHSCs, portal vein FBs, and bone marrow-derived MFBs are the major collagenproducing cells [31]. Cell fate mapping and deep phenotyping have demonstrated in experimental models of liver fibrosis that aHSCs and activated portal FBs comprise >90% of the collagen-producing cells, suggesting that these cells are the major sources of MFBs [5]. In a rodent model of liver fibrosis, HSCs differentiate into scar-producing MFBs [32-34]. In human cirrhotic livers, our differentiation trajectory indicated that VSMCs transformed into HSCs (expressing both qHSC and aHSC markers) with downregulation of VSMC-specific genes (MYH11 and ACTA2) and TFs JUNB, upregulation of HSC-specific genes (RGS5, A2M, IGFBP5, CD36, and PDGFRB) and TFs (MEF2C, TCF7L2, and SOX5) (Fig. 3C-H, Supplementary Fig. **3D**). HSCs then differentiated into MFBs with upregulation of MFB-specific genes (COL1A1, COL3A1, TIMP1, and PDGFRA) and TFs (NR1H4, PBX1, and SOX6) and downregulation of HSC-specific genes/TFs (RGS5, A2M, IGFBP5, CD36, PDGFRB, JUNB, and MEF2C) (Fig. 3C-H, Supplementary Fig. 3D). scRNA-seq analysis of human liver mesenchymal cells with cirrhosis previously identified a scar-associated mesenchymal cell characterized by PDGFRA expression, pseudo-temporal ordering and RNA velocity analyses demonstrating a trajectory from human HSCs to SAMes, as well as upregulation of fibrosis genes COL1A1, COL3A1, and TIMP1 and downregulation of RGS5 and IGFBP5 [4] (Supplementary Table 8). Additionally, in a recent study by Wang et al. [35], the same trajectory analysis was conducted on the same scRNA-seq samples from human livers with cirrhosis, revealing the robustness of developmental trajectory from VSMC (MYH11⁺) to RGS5-expressing qHSCs to COL1A1-expressing aHSCs. Similiar to human, we observed that VSMCs differentiated into MFBs with downregulation of Myh11, Tagln, Acta, Mef2c, upregulation of Collal, Col3al, Pdgfra, Pil6, Dpt, Clec3b, and Zeb1 in mouse liver with chronic liver fibrosis (Fig. 5E,F, Supplementary Fig. 7C). VSMCs differentiated into HSCs with upregulation of Pdgfrb, Hgf, Pth1r, Irf1, and Tead1 (Fig. 5E,F). Interestingly, we found that most MFBs tended to differentiate into HSCs in mice, and a similar phenomenon occurred in human livers; however, only a small proportion of MFBs in cirrhotic human livers differentiated to HSCs. We speculated that the different differentiation trajectories of mesenchymal subpopulations between cirrhotic human liver and mouse chronic fibrosis liver mainly result from the fact that mouse model has certain biological differences from the human system, and the pathogenesis of liver fibrosis is complex. Human hepatic cirrhosis is the result of long-term stimulation, whereas the pathogenesis of chronic liver fibrosis (6 weeks CCl₄ treated) in mouse livers is quite different from that in humans, and disease progression is different in both organisms. Aging of human body also affects chronic liver disease, especially the blood circulation and liver fibrosis, in the late stage of chronic liver disease [36]. The most mature and widely used animal models of liver fibrosis are based on adult or young mice and rats. Our analysis revealed that mouse models cannot completely replace human models for more accurate disease course studies. In human and mouse cirrhotic/fibrotic livers, the expression of TFs MEF2C, JUNB, and members of the SOX families (SOX4 and SOX5) changed significantly during the differentiation of VSMC, HSC and MFB, which may be the core TFs driving differentiation. The inhibition of the *MEF2C* signaling pathway can prevent pulmonary fibrosis [37,38].

Fibrotic models are important tools for studying the cellular and molecular mechanisms of liver fibrosis and for developing specific anti-fibrosis therapies. We used a previous constructed human liver fibrosis model (LX-2 treated with TGF- β 1) to examine the core genes and TFs in mesenchymal differentiation. In LX-2 cells exposed to TGF- β 1, qHSC-specific genes (*PPARG*, *NGFR*, RGS5, CEBPA, PLAT, RELN, CD36, IGFBP5, and GFAP) and TF NR1H4 were mostly downregulated in the fibrotic compared to the quiescent stage (Fig. 6B), and MFBspecific genes (COL1A1, TIMP1, TGFB1, FN1, PDGFRA, IGFBP3, ACTA2, PDGFRB, and COL1A2) and TFs (JUNB, ZEB1, PBX1, TCF7L2, and NFATC2) were mostly upregulated. NR1H4 (farnesoid X receptor, FXR) is highly expressed in the liver and has anti-inflammatory and antifibrotic functions. Ligand-activated FXR inhibits NF- κ B-mediated hepatic inflammatory response and protects against liver fibrosis development [39-42]. High expression levels of COL1A2 require the engagement of a farupstream enhancer whose activation is strongly dependent on the AP1 factor JunB [43].

5. Conclusions

Taken together, by performing single-cell and bulk transcriptome analyses on human and mouse hepatic mesenchymal cells, we elucidates the essential genes and regulatory factors involved in mesenchymal cell differentiation trajectories during liver fibrosis. We found that the differentiation trajectory of mouse hepatic mesenchymal cells was similar to that of humans, but there were also differences. Furthermore, our findings suggest promising targets for the treatment of liver fibrosis and provide valuable insights into the molecular mechanisms underlying its onset and progression.

Abbreviations

CCl₄, Carbon tetrachloride; *NR1H4*, Nuclear receptor subfamily 1 group H member 4; *NFATC2*, Nuclear fac-

tor of activated T Cells 2; MYH11, Myosin heavy chain 11; CNN1, Calponin 1; RCAN2, Regulator of calcineurin 2; JUNB, JunB proto-Oncogene, AP-1 transcription factor subunit; *MEF2C*, Myocyte enhancer factor 2C; *PBX1*, PBX homeobox 1; SOX5, SRY-Box transcription factor 5; LRAT, Lecithin retinol acyltransferase; GFAP, Glial fibrillary acidic protein; PPARG, Peroxisome proliferator activated receptor gamma; ACTA2, Actin alpha 2, smooth muscle; COL1A1, Collagen type I alpha 1 chain; TGFBR1, Transforming growth factor beta receptor 1; TIMP1, TIMP metallopeptidase inhibitor 1; COL15A1, Collagen type XV alpha 1 chain; ELN, Elastin; PLN, Phospholamban; RGS5, Regulator of G protein signaling 5; THY1, Thy-1 cell surface antigen; MFAP4, Microfibril associated protein 4; GGT5, Gamma-glutamyltransferase 5; DPT, Dermatopontin; PDGFRA, Platelet derived growth factor receptor alpha; VIM, Vimentin; COL3A1, Collagen type III alpha 1 chain; SLPI, Secretory leukocyte peptidase inhibitor; KRT19, Keratin 19; Ecm1, Extracellular matrix protein 1; Reln, Reelin; Hgf, Hepatocyte growth factor; Vipr1, Vasoactive intestinal peptide receptor 1; Ngfr, Nerve growth factor receptor; PDGFRB, Platelet derived growth factor receptor beta; Colla2, Collagen type I alpha 2 chain; Tagln, Transgelin; Tpm2, Tropomyosin 2; Pln, Phospholamban; Pdgfra, Platelet derived growth factor receptor Alpha; Pi16, Peptidase inhibitor 16; Fmo2, Flavin containing dimethylaniline monoxygenase 2; Col3a1, Collagen type III alpha 1 chain; *Pth1r*, Parathyroid hormone 1 receptor; Adamtsl2, ADAMTS like 2; ASAP1, ArfGAP with SH3 domain, ankyrin repeat and PH domain 1; TCF7L2, transcription factor 7 like 2; Bbx, BBX high mobility group box domain containing; *E2f4*, E2F transcription factor 4; Klf3, KLF transcription factor 3; Glis1, GLIS family zinc finger 1; Mecom, MDS1 and EVI1 complex locus; Etv4, ETS variant transcription factor 4; Cd34, CD34 molecule; Klf2, KLF transcription factor 2; Hand2, Heart and neural crest derivatives expressed 2; Zeb1, Zinc finger E-Box binding homeobox 1; Irfl, Interferon regulatory factor 1; Tead1, TEA domain transcription factor 1.

Availability of Data and Materials

The single-cell RNA sequencing (scRNA-seq) data of human liver cells, mouse mesenchymal cells, and mouse Lin-negative cells of the bilio-vascular tree fragments were downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/, GSE136103, GSE137720, GSE163777) database. Raw data were obtained from the NCBI Sequence Read Archive (SRA) (ht tps://www.ncbi.nlm.nih.gov/sra, SRP218975, SRP222529, SRP299106) database. All data generated or analysed in this study are included in this article. Scripts describing the main steps of the analysis and other relevant data are available from the corresponding authors upon reasonable request.

Author Contributions

HZ, LZ, ZH, and JW conceived and designed this study; XD drafted the manuscript; YM and YW performed the experiments; XD, MW, and HC made substantial contributions to the analysis, acquisition, and interpretation of data. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10. 31083/j.fbl2902062.

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