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Liver Organoid Promotes Hepatic Differentiation of Human Umbilical Cord Mesenchymal Stem Cells via Hippo Signaling

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Research Article

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Table 1 is available in the supplementary files section

Liver Organoid Promotes Hepatic Differentiation of Human Umbilical Cord Mesenchymal Stem Cells via Hippo Signaling

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Abstract: Human umbilical cord mesenchymal stem cell (hUCMSC)-derived hepatocyte-like cells (HLCs) have broad applications in cell therapy and drug discovery; however, HLCs exhibit an immature phenotype and comprise a heterogeneous population. Thus, our study focused on the potential signaling pathways that promote hepatocyte maturation. Here, we induced hUCMSC differentiation into HLCs using cytokine cocktails and generated liver organoids (LOs) using the self-assembly of HLCs, hUCMSCs, and human umbilical vein endothelial cells. LOs improved the transcript levels of liver-specific proteins synthesized by HLCs. Moreover, the mechanisms underlying this phenomenon have not yet been elucidated. Further investigation revealed that the yes-associated protein (YAP), a key effector molecule of the Hippo signaling pathway, contributes to the regulation of hepatic differentiation in hUCMSCs, further verifying that the hepatic maturation was enhanced while YAP was

phosphorylated and inhibited in the nucleus. Our findings provide new insights into the hepatic differentiation and maturation of stem cells.

Keywords: mesenchymal stem cells; liver organoids; hepatic differentiation; Hippo signaling; yes-associated protein

1. Introduction

Liver diseases have a high prevalence and a poor clinical prognosis worldwide, leading to serious public health problem [1]. Effective treatments are absent throughout the progression of liver diseases. When the disease reaches the stage of cirrhosis or end-stage liver disease, where liver function is irreversibly impaired, the only effective treatment is orthotropic liver transplantation (OLT) [2]. However, OLT has limitations, such as severe post-operative complications, scarcity of liver donors, and surgical difficulty; therefore, the search for alternative therapeutic strategies is a priority.

In 1976, the first preclinical trial of primary human hepatocytes (PHHs) transplantation revealed the use of cell transplantation for the treatment of liver diseases [3]. PHHs synthesize urea, secrete liver-specific proteins, and store glycogen. They have achieved desirable therapeutic effects in clinical trials. However, the number of PHHs derived from adult or fetal livers is limited owing to ethical and moral constraints. Other possible cell sources were also explored. Stem cells (SCs) have self-renewal and multidirectional differentiation potentials. When SCs derived from humans were injected into an animal model of liver disease, disease progression was alleviated and hepatocyte-like cells (HLCs) were detected. HLCs can synthesize and secrete human

albumin (ALB) and human alpha-1-antitrypsin (A1AT), etc. [4, 5], suggesting that the SCs can be differentiated into functional hepatocytes under specific conditions [6].

HLCs differentiated in vitro are not as mature as PHHs [7, 8]. Studies related to hepatic differentiation, especially the developmental pathway from the fetal liver to mature hepatocytes, are limited. Liver organoid (LO) culture models improved the liver function of SCs in vitro [9-11] but the mechanism by which 3D organoids promote the maturation of hepatic differentiation remains unclear. Hippo signaling plays an important role in cell growth, proliferation, apoptosis, and organ size [12]. Hippo pathway activity is closely related to the maintenance of the differentiated hepatocyte [13, 14]. Although YAP is a key effector molecule in the Hippo signaling pathway, YAP is downregulated and hyperphosphorylated during in vitro differentiation[15].

2. Materials and Methods

2.1. Isolation and Expansion of hUCMSCs and Human Umbilical Vein Endothelial Cells (HUVECs)

The hUCMSCs were isolated as previously described, with some modifications[16]. The umbilical cords were collected from healthy donors under sterile conditions. Informed consent was obtained from the patient's mother and family before sampling. This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (approval Number: LDYYLL2022-03). The hUCMSCs were isolated without enzyme solutions and Wharton's jelly pieces were plated on 75-cm^2 culture flasks. The tissue pieces were incubated in DMEM/F12 (01-172-1ACS, BI)

supplemented with 10% fetal bovine serum (FBS; AB-FBS-1050S, ABW) and 1% penicillin- streptomycin solution (03-031-1B, BI) at 37 °C with 5% CO_2 for 2-3 weeks. The medium was changed every week. At 80%-90% confluence, the cells were detached from the flask using trypsin/EDTA solution (C3533-0100, BI). The cells were centrifuged at 800 rpm for 5 min and re-inoculated at a ratio of 1:3.

HUVECs purchased from Zhong Qiao Xin Zhou Biotechnology were cultured in endothelial cell growth medium (EGM; 1001, Sciencell) containing 5% FBS and 1% penicillin- streptomycin solution in incubator at 37 °C with 5% CO_2.

2.2.Identification of hUCMSCs

P3 hUCMSCs were washed with PBS and resuspended at a concentration of 1×10^7 cells/mL. Then cell suspension was then treated with the primary antibodies against FITC-CD73 (344015, BioLegend), PE-CD34 (343605, BioLegend), PE-CD105 (800503, BioLegend), and APC-CD90 (328113, BioLegend), and incubated for 20 min at 4°C in the dark. The cells were washed and resuspended in PBS. Flow cytometry was performed using a flow cytometer (CytoFLEX, Beckman) and analyzed on FlowJo.

2.3.Hepatic Differentiation

Hepatic differentiation of hUCMSCs was performed as previously described, with some modifications[17]. The hUCMSCs (from passage 2 to 8) were seeded in six-well plates coated with 0.1% gelatin (G0040, Solarbio). At 80-90% of confluence, culture medium was switched to IMDM (01-058-1ACS, BI) containing 10 ng/mL of basic

fibroblast growth factor (bFGF; 100-18B, Peprotech) and 20 ng/mL of epidermal growth factor (EGF; AF-100-15, Peprotech) for 48 h. Hepatic differentiation medium was added with IMDM containing 40 ng/mL of hepatocyte growth factor (HGF; 10463-HNAS, Sino Biological), 10 ng/mL of bFGF and 0.61 g/L of nicotinamide (N1651, APExBIO) for 7 days. Hepatic maturation medium was changed with IMDM containing 20 ng/mL of oncostatin M (OSM; 10452-H08H, Sino Biological), 1 μmol of dexamethasone (D-085, Sigma-Aldrich), and 1% insulin-transferrin-selenium (C0345, Beyotime) for 14 days. The medium was changed every 3 days.

2.4.Self-assembly of Complex LOs

The self-assembly of complex LOs was performed as previously described, with some modifications[18, 19]. HLCs (1×10^6), HUVECs (7×10^5) and undifferentiated hUCMSCs (2×10^5) were resuspended together in a mixture of EGM and IMDM (1:1) containing 0.1 μmol of dexamethasone, 10 ng/mL of OSM, and 20 ng/mL of HGF. The cells were plated on pre-solidified Matrigel (356231, Corning) diluted with EGM (1:1) in a 24-well plate. After approximately 6-7 days, the assembled complex LOs were collected for subsequent validation. For in vitro live imaging analysis (Cytation 5, BioTek), lentiviruses expressing GFP (Genechem, Shanghai) were transfected into HLCs and lentiviruses expressing mCherry (Genechem) were transfected into HUVECs.

2.5.Real-time PCR

Total RNA was extracted using the TRIzol reagent (T9108, Takara) and reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific). Quantitative PCR was performed using a QuantiNova SYBR Green PCR Kit (208052, Qiagen) on a Real-time PCR Detection System (CFX96, Bio-Rad). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression. GraphPad Prism 9 was used for the statistical analyses. The experimental data were presented as the mean ± standard deviation and analyzed using unpaired t tests and ordinary one-way ANOVA. P values are represented as asterisks (*) (*P<0.05, ** P<0.01, *** P<0.001). Primer sequences used in this study are listed in Table S1.

2.6.Immunofluorescence Staining

The cells were fixed in 4% paraformaldehyde (G1001, Servicebio) for 30 min and permeabilized with 0.2% Triton X-100 (T8200, Solarbio) for 20 min. Blocking was performed with 10% goat serum (AR1009, Boster) for 1 h. Primary and secondary antibodies were diluted in blocking buffer for staining. Incubation with primary antibodies was performed overnight at 4°C using the following dilutions: mouse anti-human ALB (66051-1-Ig, Proteintech) at 1:200; mouse anti-human A1AT (66135-1-Ig, Proteintech) at 1:200; rabbit anti-human AFP (E-AB-13768, Elabscience) at 1:200; rabbit anti-human YAP1 (GB113975-100, Servicebio) at 1:1500; TRITC-conjugated phalloidin (G1041-100T, Servicebio) at 1:200; mouse anti-human CD31 (GB12064, Servicebio) at 1:200; and mouse anti-human vimentin (GB14167, Servicebio) at 1:200. Secondary antibodies were performed for 1.5 h in the dark with FITC-conjugated goat anti-mouse IgG (GB22301, Servicebio), FITC-conjugated goat anti-rabbit IgG

(GB22303, Servicebio); and CY3-conjugated goat anti-mouse IgG (GB21301, Servicebio). Nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; G1012, Servicebio). For tissue sections, after deparaffinization and dehydration, the remaining protocol was the same as that used for the cells.

2.7.Cytochalasin D and Verteporfin Inhibit YAP Nuclear Translocation

The hUCMSCs were cultured until the cell fusion was 70–80%; the original cell culture medium was removed and replaced with fresh cell culture medium containing 1 μM Cytochalasin D (Cyto D; 22144-77-0, MCE) and 1 μg/mL of verteporfin (VP; 129497-78-5, MCE) and cultured for 18 h. The cells were then collected for the subsequent experiments.

3. Results

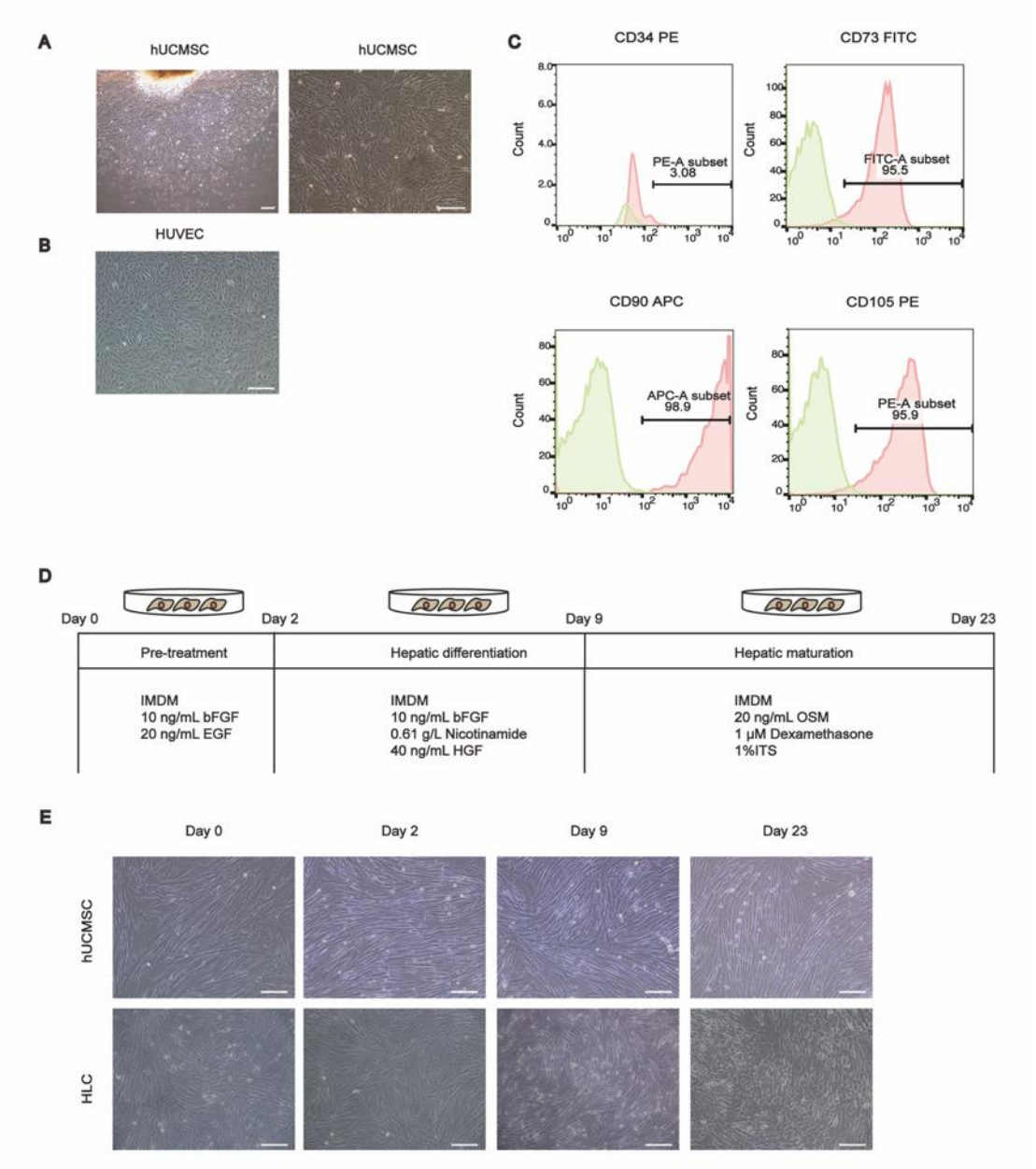
3.1.Generation of the HLCs from hUCMSCs

The morphological features of the hUCMSCs were observed using inverted phase-contrast microscopy. Migrated cells were observed around the umbilical cord tissue blocks after approximately 7 days. The cells resembled fibroblasts (Figure 1a). The markers of these cells were detected using flow cytometry, which revealed that these cells were positive for CD73 (95.5%), CD90 (98.9%), and CD105 (95.9%) but negative for CD45 (3.08%) (Figure 1c). The hUCMSCs-derived HLCs were generated via cytokine induction, as shown in Figure 1d. Differentiation of hUCMSCs occurs in three stages: pre-treatment, hepatic differentiation, and hepatic maturation. Before switching to the maturation medium, the cell morphology was fibroblast-like, and after 14 days

of induction with the maturation medium, the cell morphology and distribution significantly changed, with an ovoid paving-stone distribution. Some of the cells had a binucleate morphology, which was similar to the morphology of hepatocytes (Figure 1e).

Figure 1. Generation of the HLCs through cytokine perfusion (a) Representative pictures of hUCMSCs. (b) Representative pictures of HUVECs. (c) Identification of hUCMSCs with CD34, CD73, CD90, and CD105. (d) Schematic representation showing the protocol to generate the HLCs from hUCMSCs. (e) Sequential morphological alterations of the HLCs during hepatic differentiation stages. Scale bar: 100 μm. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; OSM, oncostatin M; ITS, insulin-transferrin-selenium; HLC, hepatocyte-like cell; hUCMSC, human umbilical cord mesenchymal stem cell;

HUVEC, human umbilical vein endothelial cell



3.2.HLCs Derived from hUCMSCs are Characteristic of Hepatocytes in mRNA and Protein Expression.

As shown in Figure 2a, cytokine induction generated HLCs, which are the hallmarks of mature hepatocytes. The HLCs exhibited roughly time-dependent expression of hepatic markers (ALB, A1AT, and HNF4α), ALB and A1AT mRNA

expression reached a peak at day 23. AFP is more highly expressed in fetal hepatocytes than in adult hepatocytes. Here, the AFP transcript level in HLCs compared to that in hUCMSCs was not statistically significant. Cytochromes (CYP) in the hepatocytes are primarily responsible for drug and chemical metabolism. After hepatic differentiation, HLCs showed higher expression levels of CYP1A1 ($p < 0.05$) and CYP1B1 ($p < 0.001$). Cytokeratin (CK) was used to determine the degree of differentiation in liver malignancies. HLCs showed lower gene expression of CK18 ($p < 0.001$) and CK19 ($p < 0.001$) than those of the hUCMSCs. Consistent with gene expression, the mean fluorescence intensity of specific proteins of ALB (2.9-fold) and A1AT (3.6-fold)

increased in HLCs, which had a higher mean fluorescence intensity of AFP (1.8-fold) than those of hUCMSCs during hepatic differentiation (Figure 2b-e).

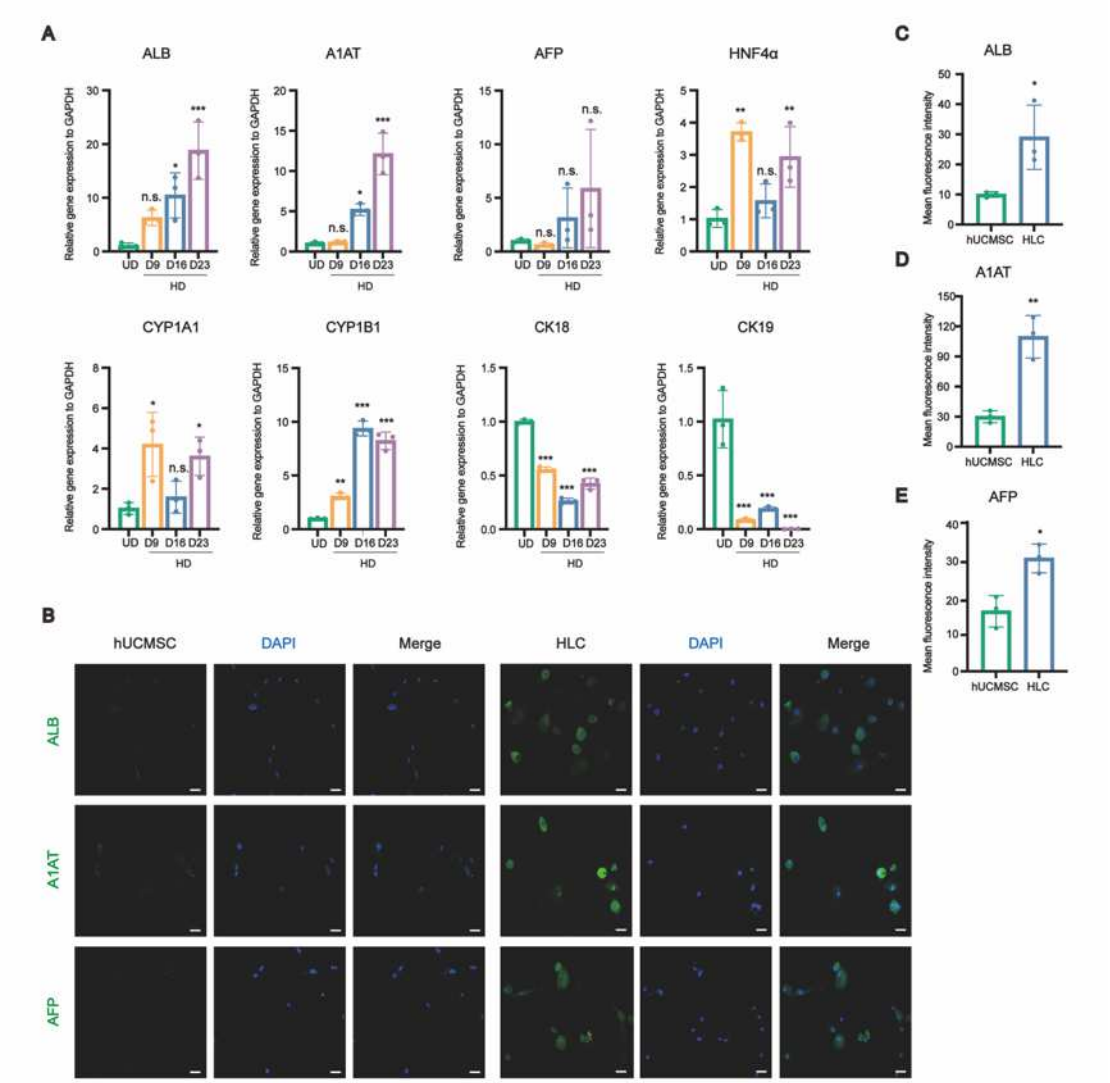


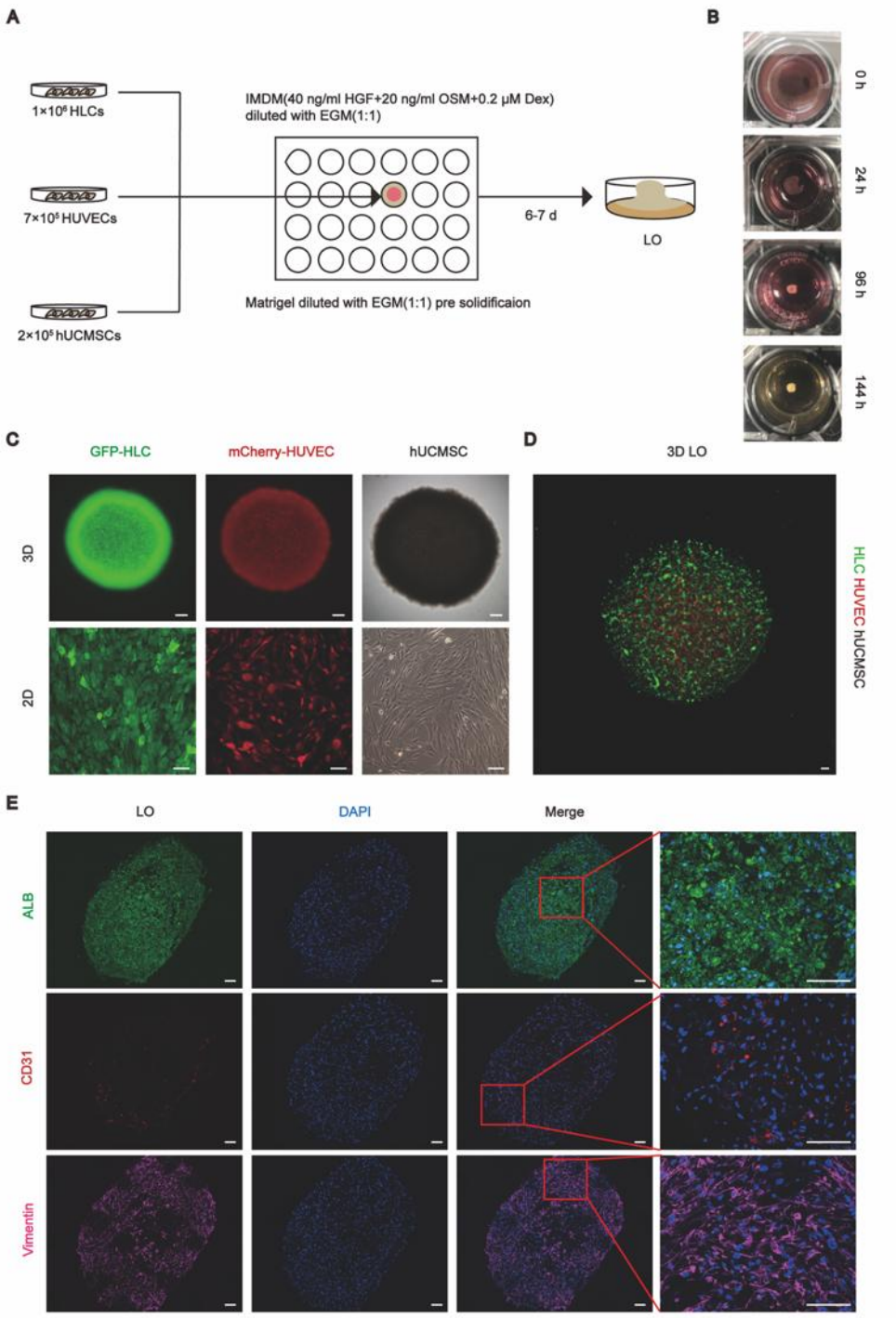
Figure 2. Characterization of the HLCs in mRNA and protein expression. (a) qPCR analysis of hepatocyte gene in the undifferentiated (UD) and hepatic differentiated (HD) cells. (b) Representative immunofluorescence images of ALB, A1AT, and AFP (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) in hUCMSCs and HLCs. (c) Analysis of ALB mean fluorescence intensity using Image J software. (d) Analysis of A1AT mean fluorescence intensity by Image J software. (e) Analysis of AFP mean fluorescence intensity by Image J software. Scale bar: 50 μm. $^{*}P < 0.05$; $^{**}P < 0.01$;

$^{***}P < 0.001$. ALB, albumin; A1AT, alpha-1-antitrypsin; AFP, alpha-fetoprotein; HNF4a, hepatocyte nuclear factor 4 alpha; CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; CK18, cytokeratin 18; CK19, cytokeratin 19; HLC, hepatocyte-like cell; hMUCMSC, human umbilical cord mesenchymal stem cell

3.3.LOs Improved the Maturation and Liver Function of HLCs

The hUCMSCs are adult SCs with limited differentiation potential, therefore, we used three different cell types that self-assembled into liver buds to construct complex LOs (Figure 3a). The HUVECs showed epithelial cell-like growth (Figure 1b). When plated on Matrigel-coated 24-well plates, defined mixtures of these three cell types self-assembled into a compact organoid structures within 144 h (Figure 3b). To determine the individual localization of the three different cell types at the late stages of organoid formation, live cell images were obtained using GFP-HLCs and mCherry-HUVECs (Figure 3c). The results demonstrate that the cells are not simply randomly distributed within the organoid but rapidly start to form structures with a reproducible spatial distribution. HLCs were arranged in closely adherent clusters and moved towards the periphery of the LOs, forming parenchymal areas, whereas HUVECs localized mainly in the central regions (Figure 3d, e). In contrast, hUCMSCs (stained for vimentin) were randomly distributed and formed the skeleton (Figure 3e). We examined the expression levels of ALB ($p < 0.01$), A1AT ($p < 0.05$), AFP ($p < 0.001$), and G6P ($p < 0.001$) in 2D HLCs vs 3D LOs. All of these indicators showed that LOs significantly improved the maturation and liver function of HLCs (Figure 4a).

Figure 3. Generation and identification of HLCs-derived LOs. (a) Schematic representation showing the protocol to generate the LOs. (b) Time-dependent formation of LOs in 24-well plates. (c) Fluorescent pictures of HLCs (green), HUVECs (red), and hUCMSCs in 2D and 3D. (d) HLC (green) and nascent endothelial networks (red) detection inside LOs using live cell imaging. (e) Immunofluorescence-staining for cell-type markers was performed in LOs. ALB is green, CD31 is red, and Vimentin is purple. Scale bar: 100 μm. HLC, hepatocyte-like cell; hUCMSC, human umbilical cord mesenchymal stem cell; HUVEC, human umbilical vein endothelial cell; HGF, hepatocyte growth factor; OSM, oncostatin M; Dex, dexamethasone; EGM, endothelial cell growth medium; LO, liver organoid; ALB, albumin



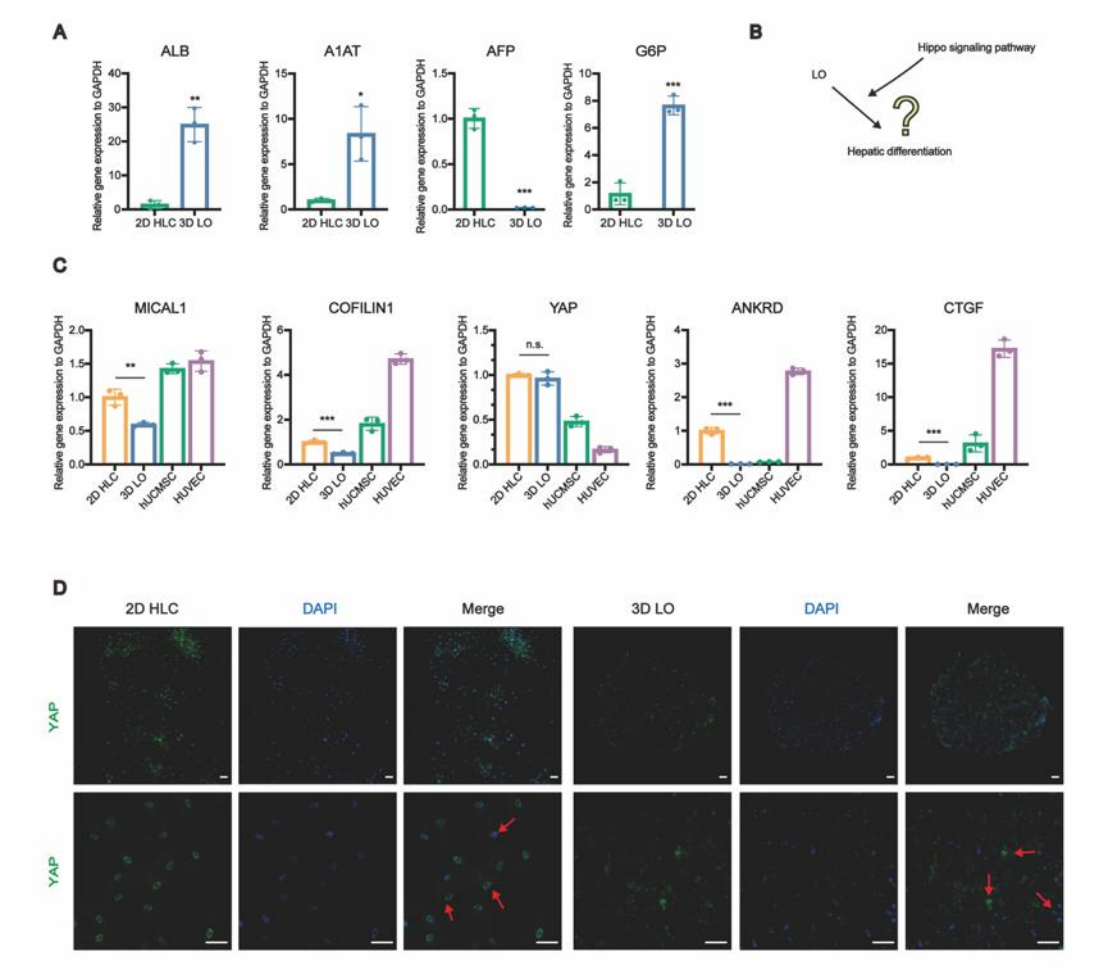
3.4.Hippo Signaling Pathway was Activated in the LOs

Previous studies have suggested that Hippo signaling plays an important role in hepatocyte differentiation[20] (Figure 4b). YAP is a downstream effector molecule of the Hippo signaling pathway that determines whether the Hippo signaling pathway is

activated. We examined the expression of upstream (MICAL1 and COFLIN1) and downstream molecules (ANKRD and CTGF) molecules of YAP in 2D HLCs and 3D LOs. We also found that the gene expression levels of MICAL1 ($p < 0.01$), COFLIN1 ($p < 0.001$), ANKRD ($p < 0.001$), and CTGF ($p < 0.001$) substantially decreased in the LOs. To avoid errors in gene expression due to different cell types, we performed a genetic examination of HUVECs and hUCMSCs (Figure 4c). Although there were no statistically significant differences in the levels of YAP mRNA, previous studies have shown that YAP functions primarily through nuclear translocation and phosphorylation. Immunofluorescence analysis of HLCs and LOs showed that YAP from HLCs aggregated in the nucleus, whereas YAP from LOs lost its nuclear localization and was phosphorylated into the cytoplasm (Figure 4d). These results indicated that LOs enhanced the maturity and live function of HLCs by activating the Hippo signaling pathway.

Figure 4. The effects of LOs on the maturation and Hippo signaling was involved in the process. (a) qPCR analysis of ALB, A1AT, AFP and G6P gene in the HLCs and LOs. (b) Possible mechanism of the mature LOs. (c) Hippo signal pathway-related gene mRNA expression of HLCs and LOs. (d) Representative immunofluorescence images of YAP (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) in HLCs and LOs. Scale bar: 50μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ALB, albumin; A1AT, alpha-1-antitrypsin; AFP, alpha-fetoprotein; G6P, glucose 6 phosphate; MICAL1, molecule interacting with CasL protein; YAP, Yes-associated protein; ANKRD, ankyrin repeat domain; CTGF, connective tissue growth factor; HLC, hepatocyte-like cell; hUCMSC,

human umbilical cord mesenchymal stem cell; HUVEC, human umbilical vein endothelial cell; LO, liver organoid

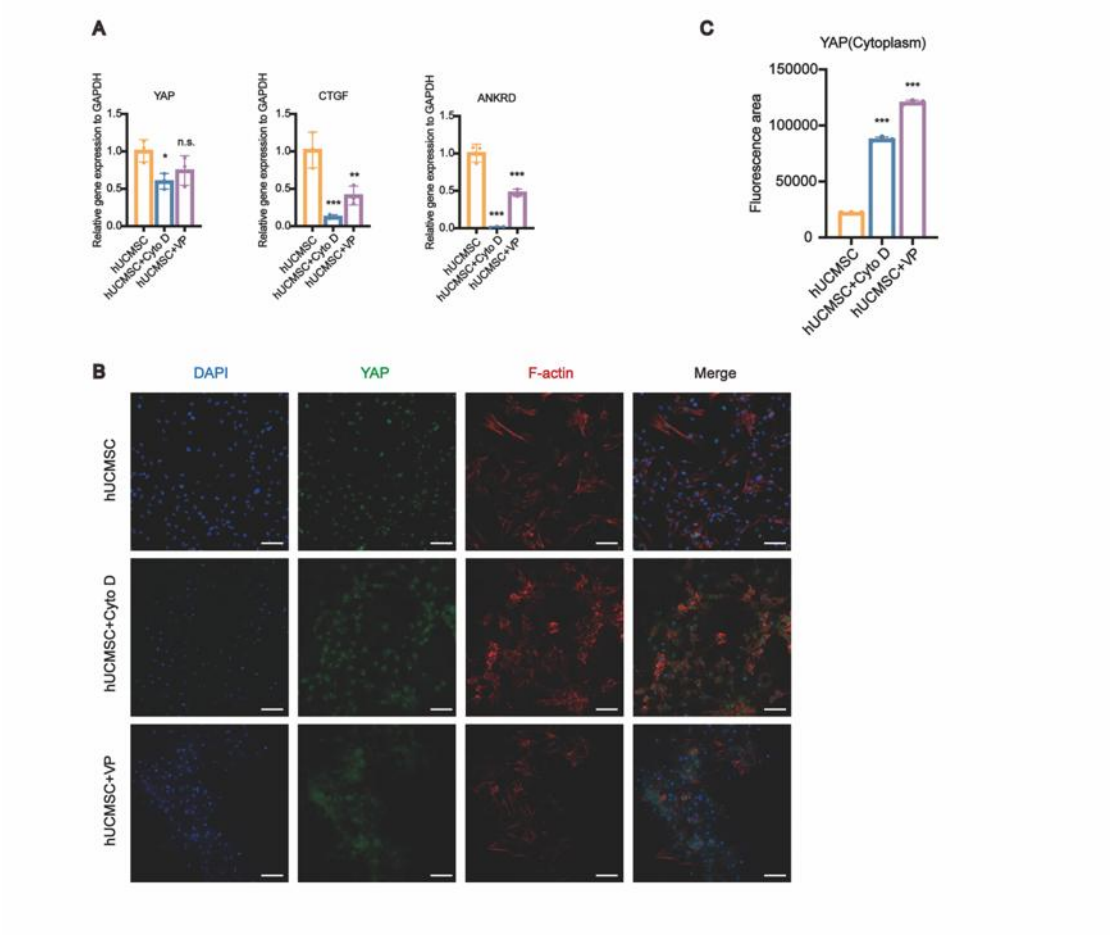


3.5.YAP Phosphorylation Regulated Hepatic Differentiation of the hUCMSCs

The results described above have shown that YAP regulated the hepatic differentiation of cells. To examine this possibility, we treated hUCMSCs with the actin polymerization inhibitor Cyto D and the VP for 18 h. Cyto D (1 μM) disrupts stress fibers and induces YAP phosphorylation, while the cell maintains the original area and loses nuclear YAP localization. VP is a YAP inhibitor that inhibits the binding of YAP to transcriptional enhanced associate domain protein (TEAD), thereby inhibiting its

transcriptional activity. The gene expression levels of CTGF and ANKRD was decreased in both the hUCMSC+ Cyto D and hUCMSC+ VP groups (Figure 5a). Immunofluorescence results showed that the translocation of YAP into the cytoplasm was significantly increased in the hUCMSC+ Cyto D (3.9-fold) and hUCMSC+ VP groups (5.4-fold) (Figure 5b, c), and that YAP phosphorylation was accompanied by varying degrees of F-actin dissociation (Figure 5b). Additionally, consistent with the inhibition of YAP translocation into the nucleus, the mRNA levels of ALB, A1AT, and AFP were calculated in the hUCMSC and hUCMSC+ inhibitor groups. The expression of ALB mRNA was increased in the hUCMSC+ VP ($p < 0.001$), but not in the hUCMSC+ Cyto D group, A1AT was upregulated in both groups, and AFP was only downregulated in the hUCMSC+ Cyto D group ($p < 0.05$) (Figure 6a). Immunofluorescence analysis showed that the mean fluorescence intensities of ALB, A1AT, and AFP in the hUCMSC+ Cyto D group were 1.5-fold, 0.9-fold and 0.8-fold, respectively, compared with those in the control group. Correspondingly, the mean fluorescence intensities of the hUCMSC+ VP group was 1.5-fold, 0.9-fold and 1.2-fold than that of the control group (Figure 6b-e). These results indicated that the hUCMSC differentiation is regulated by the YAP phosphorylation.

Figure 5. The YAP enriched in the nucleus was blocked by inhibitors Cyto D and VP. (a) Relative mRNA expression levels of YAP and downstream in hUCMSCs treated with Cyto D and VP. (b) Representative immunofluorescence images of YAP (green), F-action (red), and 4′,6-diamidino-2-phenylindole (DAPI) (blue) in hUCMSCs treated with Cyto D and VP. (c) Analysis of YAP (cytoplasm) fluorescence area by Image J software. Scale bar: 100 μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. YAP, Yes-associated protein; ANKRD, ankyrin repeat domain; CTGF, connective tissue growth factor; hUCMSC, human umbilical cord mesenchymal stem cell; Cyto D, cytochalasin; VP, verteporfin



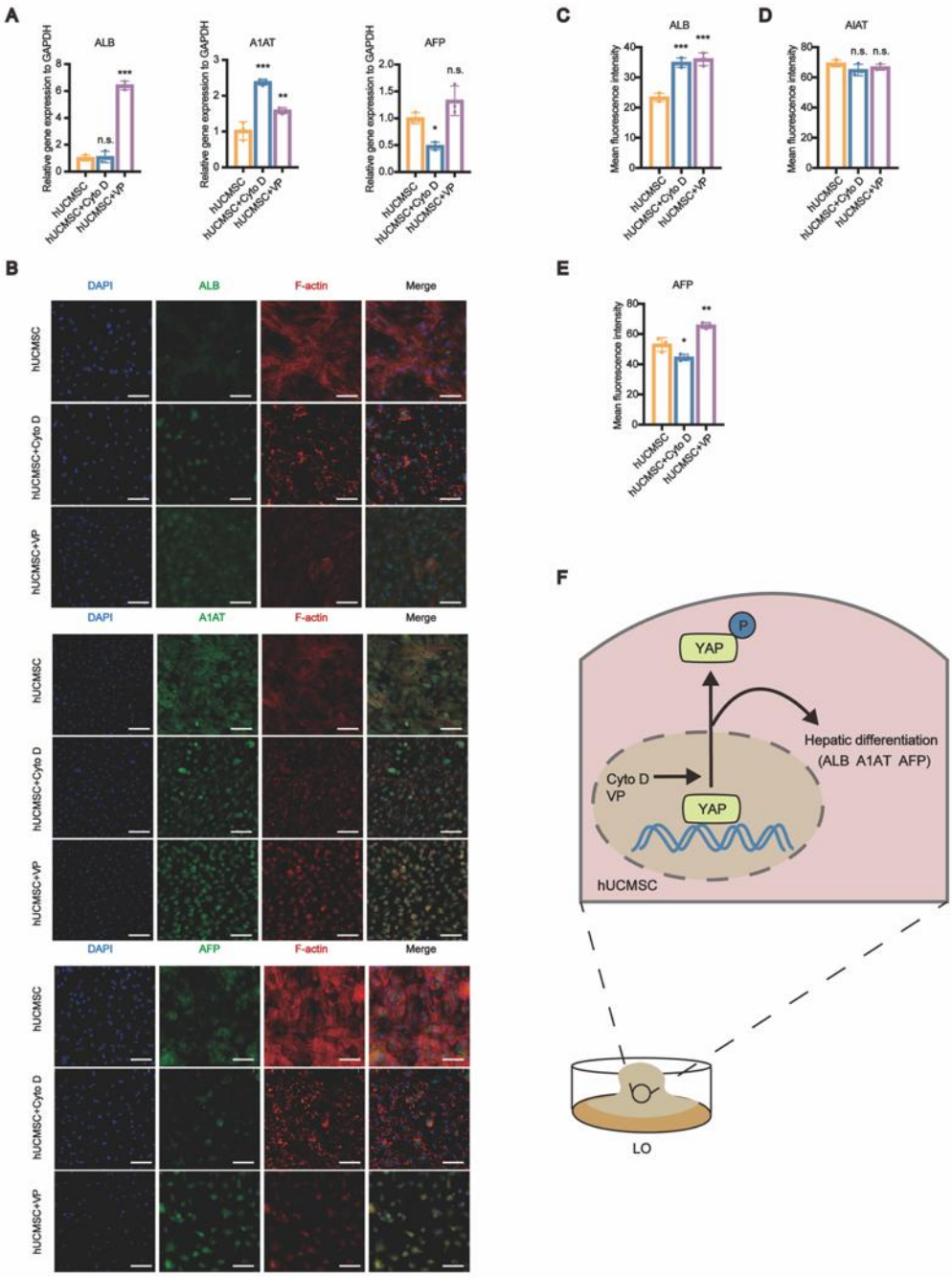


Figure 6. The translocation of YAP into the cytoplasm induce hepatic differentiation of hUCMSCs. (a)qPCR analysis of ALB, A1AT, and AFP genes in the hUCMSCs intervened with inhibitors. (b) Representative immunofluorescence images of ALB, A1AT, AFP (green), F-action (red), and 4′,6-diamidino-2-phenylindole (DAPI) (blue) in the hUCMSCs intervened with inhibitors. (c) Quantification of ALB mean

fluorescence intensity by Image J software. (d) Quantification of A1AT mean fluorescence intensity by Image J software. (e) Quantification of AFP mean fluorescence intensity by Image J software. (f) The signal pathway of hepatic differentiation in hUCMSCs. Scale bar: 100 μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ALB, albumin; A1AT, alpha-1-antitrypsin; AFP, alpha-fetoprotein; hUCMSC, human umbilical cord mesenchymal stem cell; Cyto D, cytochalasin; VP, verteporfin; LO, liver organoid; YAP, Yes-associated protein

4. Discussion

In this study, we showed that LOs promoted the hepatic differentiation of hUCMSCs via Hippo signaling (Figure 6f). HLCs-derived LOs were the closest models to PHHs [2, 11]. Some of the results of this study also showed that LOs can significantly increase the transcriptional levels of the liver-specific proteins, ALB, A1AT, and glycogen storage-related G6P. It is controversial whether the RNA of LOs is the total RNA of the three cell types; hUCMSCs and HUVECs rarely secrete liver-specific proteins; therefore, the fold expression of the hepatocyte characteristic genes in LOs is higher. Our goal was to explore the underlying mechanisms by which LOs promote HLC maturation. Compared with the 2D HLCs, 3D LOs are characterized by the fact that the cells have changed from the original monolayer culture to cell aggregates with a spatial structure [21]. Hippo signaling closely regulate the size and volume of the organ, and is a sensor and mediator of mechanical signaling in the extracellular matrix [22, 23], we expect that activation of the Hippo pathway plays a crucial role in the liver

differentiation of LOs. In addition, signal exchange between multiple cells may also be the reason for the improvement in the LOs. We examined gene expression of the vascular endothelial growth factor (VEGF) signaling pathway in LOs (Figure S1). VEGF secreted by HUVECs can promote the differentiation of liver progenitor cells into mature hepatocytes [24, 25], suggesting that activation of the VEGF signaling pathway may also promote the hepatic differentiation of hUCMSCs.

YAP, as the core of the Hippo signaling pathway, functions through nuclear translocation. YAP is regulated by actin recombination and intercellular adhesion [26]. In 2D cultured cells, YAP was mainly enriched in the nucleus [15], whereas in the LO model, the cytoskeleton changed, the mRNA expression of MICAL1 and COFLIN1 was significantly reduced, and actin recombination induced YAP nuclear translocation. There was no difference in the total expression of YAP; however, LOs reduced the transcription efficiency of CTGF and ANKRD downstream of the nuclear YAP. Immunofluorescence demonstrated that YAP lost its nuclear localization and the proportion of phosphorylated YAP in the cytoplasm was upregulated, thereby increasing the transcription and protein generation in the liver. However, the research methods adopted in this study are limited. Western blot and enzyme-linked immunosorbent assays should be used for protein quantification. In addition, our study did not focus on the transcription factors (TFs) that are regulated by YAP during liver differentiation; therefore, the transcriptomes that regulates TFs are unclear.

Finally, we used the YAP inhibitors, Cyto D and VP, to verify whether Hippo signaling activation promoted the differentiation of hUCMSCs. The results showed that

when nuclear localization of YAP in hUCMSCs was blocked and YAP phosphorylation increased, the synthesis of ALB and A1AT in the cells also increased. In the groups treated with different inhibitors, the mRNA and protein expression of AFP showed opposite results, and the possible mechanism remains to be elucidated. Overall, Cyto D induces YAP phosphorylation through upstream actin recombination, while VP directly inhibits YAP entry into the nucleus, making VP more efficient. As an alternative in vitro model, HLCs exhibit immature phenotypes and comprise a heterogeneous population [27]. Further differentiation of HLCs must be induced before they can be used for medical applications [27, 28]. Based on the signaling pathways involved in liver maturation, many new small molecule compounds have been used to improve the differentiation success rate and shorten differentiation cycles [29, 30]. Our study suggests that inhibitors of the Hippo pathway, especially YAP, can be used to promote liver differentiation.

5. Conclusion

We revealed that LOs enhance the maturity and liver function of HLCs. Moreover, these results demonstrated that YAP, a key effector molecule of the Hippo signaling pathway, is involved in the differentiation of hUCMSCs. In addition, our study provides new molecular targets for hepatic differentiation and maturation of SCs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1,

Author Contributions: Pinyan Liu conceived and designed the experiments, collected and analyzed the data, provided financial support, and prepared the manuscript; Yongcui Mao collected and analyzed the data and prepared the manuscript; Jiayun Wei collected and analyzed the data. Xuekai Hu and Haixia Zhao contributed to data collection. Ye Xie performed the methodology. Jia Yao conceived and designed the experiments, and provided financial support. All the authors have read and agreed to the published version of the manuscript.

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Ethics approval and consent to participate: The human umbilical cord mesenchymal stem cells (hUCMSCs) used in this study were previously established from the patient's mother under the approved project entitled" Promoting effect of umbilical cord mesenchymal stem cell extracellular matrix on hair growth and its mechanism" (approval number LDYYLL2022-03; approved on January 5, 2022). The primary human umbilical vein endothelial cells (HUVECs) used in this study were obtained

from Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd. (HUVEC; DFSC-EC-01), the cells were prepared by the National Stem Cell Transformation Resource Bank. The supplier has confirmed that the collection of theses human-derived cells was performed with prior ethical approval, and all related donors signed written informed consent.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: All scientific content, references, interpretations, and conclusions were independently reviewed, corrected, and finalized by the authors, who take full responsibility for the final manuscript. The authors declare that they have not used AI-generated work in this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ALB	Albumin
bFGF	Basic fibroblast growth factor

Cyto D	Cytochalasin D
EGF	Epidermal growth factor
EGM	Endothelial cell growth medium
HGF	Hepatocyte growth factor
HLC	Hepatocyte-like cell
hUCMSC	Human umbilical cord mesenchymal stem cell
HUVEC	Human umbilical vein endothelial cell
LO	Liver organoid
OLT	Orthotropic liver transplantation
OSM	Oncostatin M
PHH	Primary human hepatocytes
SC	Stem cell
TF	Transcription factor
VP	Verteporfin
YAP	Yes-associated protein

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