Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 15, Issue 6, 2025, 90
https://doi.org/10.33263/BRIAC156.090

Endothelial and Hepatic Stellate Cells Conditioned Media Promote Hepatocyte-like Cell Differentiation and Functional Maturation of mESCs Cultured on 3D Scaffold

Kshama Kumari [1], Prakash Baligar [1,*]

[1] Amity Institute of Molecular Medicine & Stem Cell Research (AIMMSCR), Amity University, Uttar Pradesh, Noida, India
* Correspondence: pbaligar@amity.edu;

Received: 9.01.2025; Accepted: 27.07.2025; Published: 14.11.2025

Abstract: Hepatocyte transplantation from stem cells holds a promising approach for treating liver failure, but inefficient differentiation into functional hepatocytes limits therapeutic applications. Recent research highlights the role of non-parenchymal cells, such as hepatic stellate cells (HSCs) and human umbilical vein endothelial cells (HUVECs), in supporting hepatocyte function through paracrine signaling. This study investigates the synergistic effects of HUVEC and HSC-conditioned media on the differentiation of mouse embryonic stem cells (mESCs) into hepatocyte-like cells (HLCs) by using a 3D gelatin scaffold. mESCs were cultured on a gelatin scaffold with conditioned media from HUVECs and HSCs (1:1:1), and differentiation was assessed through immunohistochemistry and gene expression analysis. Results showed that mouse embryonic stem cell-derived hepatocyte-like cells (mESC-HLCs) cultured with conditioned media exhibited increased expression of mature hepatic markers, including albumin, cytokeratin 18, and hepatocyte nuclear factor 4α, alongside markers of hepatoblasts and cholangiocytes. Functional assays demonstrated enhanced albumin secretion, creatinine production, and urea synthesis compared to cells treated with single conditioned media or standard differentiation media. These findings suggest that combining scaffold-based culture with niche-mimicking conditioned media effectively enhances hepatocyte differentiation, providing a promising approach for regenerative medicine and drug discovery applications.

Keywords: gelatin scaffold; stem cell; non-parenchymal cell; differentiation; conditioned media; hepatocyte-like cells.

1. Introduction

The liver is primarily an exocrine organ, although it also performs important secondary endocrine functions essential for maintaining physiological homeostasis. It plays a vital role in food metabolism, chemical detoxification, and the synthesis of protein and bile [1,2]. As a result, it is a common site for acute and chronic injuries [3]. Nevertheless, long-term chronic liver illnesses brought on by toxicity, inflammation, viral reactions, and cancer often culminate in liver failure, which is frequently fatal [4]. Currently, the only available treatment for end-stage liver disease is liver transplantation, and the majority of patients perish due to a lack of donor organs and immune rejection. This underscores the urgent need for alternative therapeutic strategies to replace liver transplantation [5]. Cellular transplantation using hepatocytes (HCs) or progenitor

cells has emerged as a promising alternative to allograft hepatic transplantation [6]. However, primary hepatocytes are challenging to culture in vitro because, if not maintained properly, they quickly lose their unique phenotypic and liver-specific functions such as albumin synthesis, urea cycle activity, drug-metabolizing enzyme expression (e.g., cytochrome P450 enzymes), and bile acid secretion. To address these limitations, several studies have focused on optimizing in vitro culture conditions. Strategies such as optimizing cell culture procedures, medium composition with growth factors, cytokines, and essential nutrients have been explored to maintain hepatocyte-specific functions [7,8].

Furthermore, stem cell sources, i.e., induced pluripotent stem cells (iPSCs), ESCs, and mesenchymal stem cells (MSCs), have been explored for generating hepatocyte-like cells. These stem cells offer a renewable and scalable cell source capable of differentiating into functional HLCs, making them a promising candidate for liver disease treatment [9,10]. Despite recent advancements, the differentiation of stem cells into fully mature and functioning hepatocyte-like cells remains a big challenge that must be addressed. Obtaining properly conditioned media derived from non-parenchymal cells (NPCs), such as human umbilical vein endothelial cells and hepatic stellate cells, has emerged as a promising approach to enhance stem cell differentiation into hepatocyte-like cells. Hepatocyte maturation requires the replication of the natural hepatic milieu, which involves complex interactions between hepatocytes and NPC, such as HSCs and endothelial cells. These NPCs play a crucial role by imparting biochemical and physical signals that regulate hepatocyte proliferation, differentiation, and functionality through paracrine signaling and interactions with the extracellular matrix (ECM) [11,12]. In this context, HSC-derived conditioned media comprises key growth factors, such as hepatocyte growth factor (HGF), which support hepatocyte survival, proliferation, and functional maturation [13].

Meanwhile, HUVEC-derived condition media (CM) provides angiocrine factors, such as vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), and fibroblast growth factor (FGF), which are crucial for hepatocyte development, metabolic regulation, and the organization of liver sinusoidal structures [14,15]. Moreover, three-dimensional (3D) scaffolds, such as gelatin, have been shown to imitate the ECM and improve stem cell development. Gelatin scaffolds facilitate cell adhesion, infiltration, and organization, which are necessary for hepatocyte functioning [16]. Gelatin scaffolds combined with HSCs and HUVECs CM can synergistically reconstruct the innate differentiation efficacy and functional maturation of stem cell-derived HLCs. In our previous studies, gelatin-based scaffolds provided a biocompatible 3D microenvironment that supported the differentiation of mESCs into hepatocyte-like cells, promoting cellular proliferation, hepatic lineage commitment, liver-specific functions, and liver regeneration [17]. To enhance this, the present study investigates the combined effect of conditioned media from HUVECs and HSCs within the same 3D scaffold, thereby mimicking hepatic niche-derived paracrine signaling more effectively and improving the hepatic differentiation and maturation of mESC-derived hepatocyte-like cells. We evaluated the outcomes using real-time PCR, immunohistochemistry, and liver function assays, with a focus on key hepatic markers and functions. The novelty of our approach lies in the synergistic integration of endothelial and stellate paracrine cues within a 3D scaffold, a combination that has not been previously explored in mESC models. This method significantly enhanced liver-specific gene expression and functional markers, including increased albumin, creatinine, and urea secretion, compared to 2D cultures or single-CM conditions. By more accurately recapitulating the hepatic microenvironment, this model offers a promising platform for liver tissue engineering and regenerative medicine.

2. Materials and Methods

2.1. Preparation of gelatin scaffold.

Gelatin scaffold preparation was previously described in our study [17]. Briefly, 10% of the gelatin aqueous solution was cross-linked with 0.1% glutaraldehyde. The cross-linked gelatin scaffold was stored at 4°C for 24 hours to attain stability, after which it was sliced into sections. The gelatin scaffold thus obtained was washed with MilliQ water and was acetone-dried, followed by vacuum drying.

2.2. Cell line and cell culture.

2.2.1. Ex vivo expansion of HUVEC and human HSCs.

We purchased HUVEC cells from Lonza and cultured them using the EGM-2 BulletKit. We added the following components to the EBM-2 basal medium, following the manufacturer's instructions: 0.2 ml of fetal bovine serum (FBS), 0.5 ml of vascular endothelial growth factor (VEGF), 0.5 ml of antibiotics (gentamicin, amphotericin B), 0.5 ml of human fibroblast growth factor-beta (hFGFβ-2), 0.5 ml of insulin-like growth factor-1 (R3-IGF-1), 0.5 ml of ascorbic acid, and 0.5 ml of heparin. Following a 24-hour incubation of HUVECs in EGM2, we collected the supernatants in 50 mL centrifuge tubes. The supernatants were centrifuged at 3,000 g for 15 min at 4°C to remove debris. Finally, we obtained the HUVEC conditioned medium (HUVEC-CM) and stored it at –80°C for future experiments. The activated human hepatic stellate cell line LX2, which was given by Dr. Scott L. Friedman, was grown in DMEM medium that had 4 mM L-glutamine, 1% penicillin/streptomycin, and 2% FBS added to it. We cultured hepatic stellate cells (HSCs) in DMEM with 2% FBS for 24 hours and collected the conditioned media as described above. The cell cultures were maintained at 37°C with 5% CO_2 in a CO_2 incubator.

2.2.2. 2D and 3D culture conditions for mESCs.

The R1/E mESC cell line, E14TG2a, was supplied by the group of Dr. Janet Rossant and Dr. John Roder in Toronto, Canada. For culture without feeders, mESCs were grown in DMEM enriched with 20% ESC-grade fetal bovine serum, 100 µM β-mercaptoethanol, 1% penicillin-streptomycin,100 µM non-essential amino acids, and 10,000 U of mouse leukemia inhibitory factor (mLIF). The cells were then kept at 37°C in a 5% CO_2 incubator. The media was changed every day. Trypsin-EDTA was used to dissociate mESCs, which were then used in studies. In both 2D and 3D, the growth conditions were the same. In our previous study, for cell culture experiments, scaffolds were autoclaved and cut into four pieces with an average surface area of 122 mm^2 [17]. The scaffolds were subsequently immersed in a serum-free medium for an overnight period. In every cell culture experiment, any excess media was removed using filter paper and transferred to 12-well plates before the introduction of the cells.

2.3. Hepatic differentiation of mESC with conditioned media of HUVEC and HSC cells.

The mESCs were grown on gelatin scaffolds at a density of 1x10^5 cells per 30µl without the presence of LIF. Iscove's Modified Dulbecco's Medium (IMDM), along with a combination of growth factors (GFs), was used for hepatic differentiation. The mESCs were cultured with

differentiation medium, HUVEC-CM and HSC-CM (1:1:1) consisting of 10 ng ml^{-1} Activin A for three days, 20 ng ml^{-1} bone morphogenic protein-4 (BMP4), and 20 ng ml^{-1} fibroblast growth factor-2 (FGF-2) for four days, and five days with a maturation medium consisting of 10 ng ml^{-1} hepatocyte growth factor (HGF), 20 ng ml^{-1} oncostatin M, 1 mol^{-1} dexamethasone, and 50 mg ml^{-1} insulin transferrin-selenium premix. The media was changed every two days.

2.4. Histological and immunohistochemical analyses.

Fivemicron formalin-fixed and paraffin-embedded sections of the mESC-differentiated hepatocyte-like cells on the scaffold were examined. 5 µm fixed frozen liver tissue sections were used for immunohistochemistry (IHC) analysis. The sections underwent processing using Thermofisher Scientific's Pierce TM DAB Substrate Kit for 3,3′-Diaminobenzidine (DAB) staining. Following deparaffinization and rehydration, epitope retrieval was performed, followed by 3% (v/v) H_2O_2 treatment to quench endogenous peroxidase activity. Sections were stained with, followed by washes with the primary antibody for an hour, followed by three washes. The sections were subsequently washed and treated with HRP-conjugated secondary antibodies, mouse and rabbit (Jackson ImmunoResearch Inc., West Grove, PA) for 1 hour at room temperature. The DAB substrate reaction was followed by a wash and a counterstaining step using Hematoxylin solution on sections. The sections were seen using a Leica Microscope with a 40x objective lens.

For H&E staining,5-µm sections of formalin-fixed scaffold, along with mESC-differentiated hepatocyte-like cells embedded in paraffin, were cut. The samples underwent deparaffinization and rehydration. Epitope retrieval was carried out at room temperature, followed by a wash with PBS. The samples were then stained with Mayer's Hematoxylin solution for 5 minutes. The samples were subsequently rinsed with distilled water and 1X PBS. The slides were immersed in 70% and 90% ethanol consecutively for 1 minute each, followed by staining with alcoholic eosin for 5 minutes. The slides were rinsed with distilled water and then treated with 100% ethanol for 1 minute to remove any remaining impurities. They were then examined using a Nikon Eclipse Ti microscope with a 40x objective lens. The photos were created and manipulated using Adobe Photoshop 6.0. The details of the antibodies used in this study are given in Table 1.

Table 1. List of primary antibodies used in this study, including supplier information, catalogue numbers, and working dilutions.

Name of the antibody	Suppliers name	Catalogue No.	Dilution
anti-mouse albumin	Bethyl laboratories	A90-134A	1: 200
anti-mouse HNF4α	Abcam, Cambridge, UK	ab41898	1: 200
anti-rabbit CK18	Abcam, Cambridge, UK	ab181597	1: 200

2.5. RNA isolation and real-time quantitative polymerase chain reaction (qPCR).

As described above, mESCs were cultured on gelatin scaffolds at a concentration of 1 x 10^5 cells per 30µl in the absence of LIF and cultured with a differentiation medium, HUVEC-CM, and HSC-CM (1:1:1). Total RNA was isolated from mESCs cultured with HUVEC and HSC conditioned media on a 2D and 3D gelatin scaffold. The scaffold was collected at various time intervals (3 scaffolds per time point) using TRIZOL Reagent. cDNA synthesis was done with the Verso DNA synthesis Kit, and 1% agarose gel was used to resolve it. The relative mRNA expression levels were normalized to GAPDH. The experiments were done in triplicate. The primers were designed using Primer Blast software. The list of primers used in the study is given in Table 2.

Table 2. List of gene-specific primers used for quantitative real-time PCR, including forward and reverse primer sequences.

Gene	Forward Primer	Reverse Primer
Collagen Type I α(I) chain (COL1A1)	GACGAGACCAAGAACTGCCC	TTGGTCGGTGGGTGACTCT
α-Smooth Muscle Actin (αSMA)	CAATGGCTCTGGGCTCTGTAA	ACCATCACCCCCTGATGTCT
VEGF	GTACCTCCACCATGCCAAGT	AATAGCTGCGCTGGTAGACG
CD31	AACAGAAACCCGTGGAGATG	GTCTCTGTGGCTCTCGTTCC
FGF2	AGGCCACTTCAAGGCCC	GAAACAGTATGGCCTTCTGTCC
BMP4	CTTCAACCTCAGCAGCATCC	GATGAGGTGTCCAGGAACCA
HGF	AGGAACAGGGGCTTTACGTT	GTCAAATTCATGGCCAAACC
TGFβ	GCCGTGGAGGGGAAATTGAG	GAACCCGTTGATGTCCACTTGC
β- catenin (CTNBB1)	ATCCAAAGAGTAGCTGCAGG	TCATCCTGGCGATATCCAAG
Albumin	GAAGACCCCAGTGAGTGAGC	TCAGAGCAGAGAAGCATGGC
HNF4α	GGGTAGGGGAGAATGCGACT	AACTCCAGGGTGGTGTAGGC
CK18	ATGCCCCCAAATCTCAGGAC	GTTCCTCGCGGTTCTTCTGA
TAT	GAAGGTGAAGCCGAATCCGA	GTCACTTCAGGGTCTGTGGG
TTR	CAGGAAGATGAAGTGAAGC	AGCAGGGGAGAAAAATGAGG
AFP	CCACCAGGAGGAAGTGAAC	ACACCCATCACCAGAGTTTT
CK19	GAGGACGAGGTCACGAAGC	GAGGACGAGGTCACGAAGC
CK7	AGATGAACCGCAACATCAACC	TCAATCTTCTTCACAACCACAGC
HNF1α	TCCCTGGGACCCACGTTCAC	TGCCCGTTGGAGTCGGAACT
HNF1$^{\beta}$	GTCGCTCCAGCAAGAACTCC	GACGGCAGTAACTCCTCCAAG
HNF6	TTACAGCATCCCACAGGCCATCTT	TCTCCCGACCCGACTTGAGTTT
Lgr5	CAGGGCGTTAAGTCCACTGT	CCGAAGTGTGGAGAGGCATT
SOX9	TGGGTCTGCCTGGACTGTAT	CTATCCACGGCACACACACT
Dlk1	CCCAGGTGAGCTTCGAGTG	GGAGAGGGGTACTCTTGTTGAG
GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA

2.6. Liver function test.

The liver-specific functionalities of mESC-HLCs cultured with HUVEC and HSC conditioned media on 3D gelatin scaffolds were investigated by quantifying ALB, urea, and creatinine production in cell culture supernatants. Commercially available diagnostic kits from Erba were used to spectrophotometrically (Spectro UV-1800 Spectrophotometer, Shimadzu) measure the levels of albumin (Cat. No. 120223) at wavelength 630 nm, urea (Cat. No. 120214) at wavelength 340nm, and creatinine (Cat. No. 120246) at wavelength 510 nm. The experiment was done in triplicate.

2.7. Statistical analysis.

All data were reported as the mean ± standard deviation (SD) derived from three independent experiments. The one-way and two-way ANOVA were carried out to assess the statistical significance between the multiple groups. The studies were executed using GraphPad Prism software version 6.2.

3. Results and Discussion

3.1. Gene expression of mRNA through RT PCR analysis.

Gene expression analysis was performed to assess the expression of mESC-derived hepatocyte-like cells cultured with HUVEC and HSC-conditioned media. The increased expression of endothelial markers, such as VEGF and CD31, in the mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC+HUVEC-CM+HSC-CM) compared to mESC-derived hepatocyte-like cells cultured with HUVEC-CM (mESC-HLC+HUVEC-CM) reflects the contribution of HUVEC-derived paracrine factors (Figure 1a).

Similarly, the elevated levels of α-smooth muscle actin (αSMA) and collagen I (COL1A1), markers of activated hepatic stellate cells, in the mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC+HUVEC-CM+HSC-CM) compared to mESC-derived hepatocyte-like cells cultured with HSC-CM (mESC-HLC+HSC-CM) further suggest the involvement of HSC-derived signals in recreating a hepatic niche (Figure 1b). Together, these factors likely contributed to the enhanced differentiation observed in mESC-HLCs when exposed to HUVEC- and HSC-conditioned media.

To determine the synergistic effect of conditioned media on mESC-HLCs with gelatin scaffolds, mediated by the growth factors, we analyzed the expression of key markers involved in hepatic differentiation. Fibroblast Growth Factor-2 (FGF2) and Bone Morphogenetic Protein 4 (BMP4), which are necessary for hepatoblast differentiation, showed increased expression in real-time PCR analysis on the 5th and 7th days in mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC+HUVEC-CM+HSC-CM) compared to mESC-derived hepatocyte-like cells (mESC-HLC), mESC-derived hepatocyte-like cells cultured with HSC-CM (mESC-HLC+HSC-CM), and mESC-derived hepatocyte-like cells cultured with HUVEC-CM (mESC-HLC+HUVEC-CM) (Figure 1c and d).

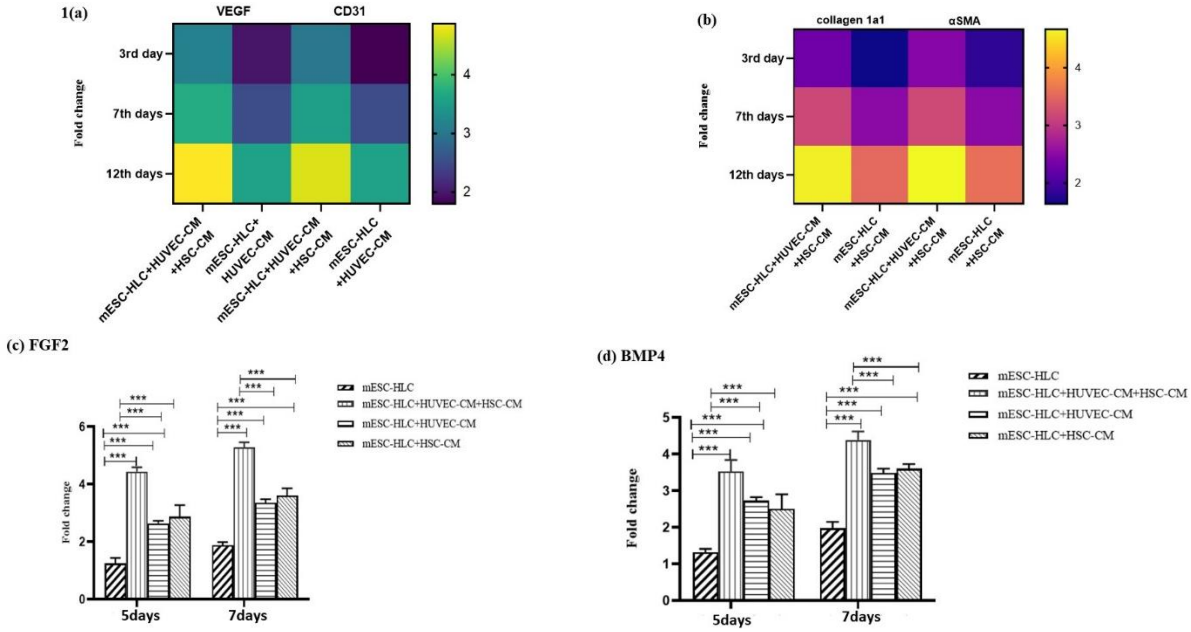


Figure 1. RT-PCR analysis was done to evaluate the fold change of mESC-HLCs cultured with HUVEC and HSC conditioned media on the gelatin scaffold. Results were normalized with GAPDH; (**a)** Heatmap representation of VEGF and CD31 gene expression in mESC-HLCs cultured with HUVEC and HSC conditioned media across three time points (3rd, 7th, and 12th days). Colour intensity corresponds to fold change values as shown by the scale bar (2 to 5), with yellow indicating higher expression and purple lower expression; **(b)** Heatmap representation of fibrogenic marker gene expression (Collagen 1a1 and αSMA) in mESC-HLCs cultured with HUVEC and HSC conditioned media across three time points (3rd, 7th, and 12th days). The color scale represents fold change in expression (range 2–5), with yellow indicating higher expression and purple indicating lower expression. with Real-time; **(c,d)** Upregulation of FGF2 and BMP4 markers at 5th and 7th days in with mESC-HLC+HUVEC-CM +HSC-CMcompared to cellsmESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM). Data represent mean ± standard deviation (SD) from three independent biological replicates (n=3). Statistical analysis was performed using one- or two-way ANOVA; p* < 0.05, p** <, and p*** < 0.001 were considered statistically significant.

Nevertheless, the Wnt-responsive gene LGR5, which is a key marker for hepatic progenitor activity, was noted to have its levels significantly elevated on the 5th and 7th days in mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC + HUVEC-CM + HSC-CM) compared to mESC-HLC, mESC-HLC + HSC-CM, and mESC-HLC + HUVEC-CM (Figure 2a). Additionally, we examined the expression of the Wnt/β-catenin

pathway, which plays a crucial role in liver development, regeneration, and metabolism. The canonical marker β-catenin, associated with the Wnt pathway, was significantly expressed in mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC + HUVEC-CM + HSC-CM) compared to mESC-HLC, mESC-HLC + HSC-CM, and mESC-HLC + HUVEC-CM on the 5th and 7th days (Figure 2b). Transforming growth factor-beta (TGF-β) signalling is known to promote the differentiation of hepatic progenitor cells into functional hepatocytes, which are the main primary functional cells of the liver. Our results showed increased TGF-β expression on 3rd, 5th, and 7th days in mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC + HUVEC-CM + HSC-CM) compared to mESC-HLC, mESC-HLC + HSC-CM, and mESC-HLC+HUVEC-CM. (Figure 2c). Moreover, hepatocyte-growth factor (HGF), which promotes hepatocyte proliferation and leads to an increase in the number of hepatocytes, exhibited increased expression on the 9th and 12th days in mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC-CM (mESC-HLC+HUVEC-CM+HSC-CM) compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM (Figure 2d). These findings suggest a synergistic enhancement of hepatoblast differentiation and liver-specific pathways when mESCs are cultured with conditioned media and growth factors on a gelatin scaffold.

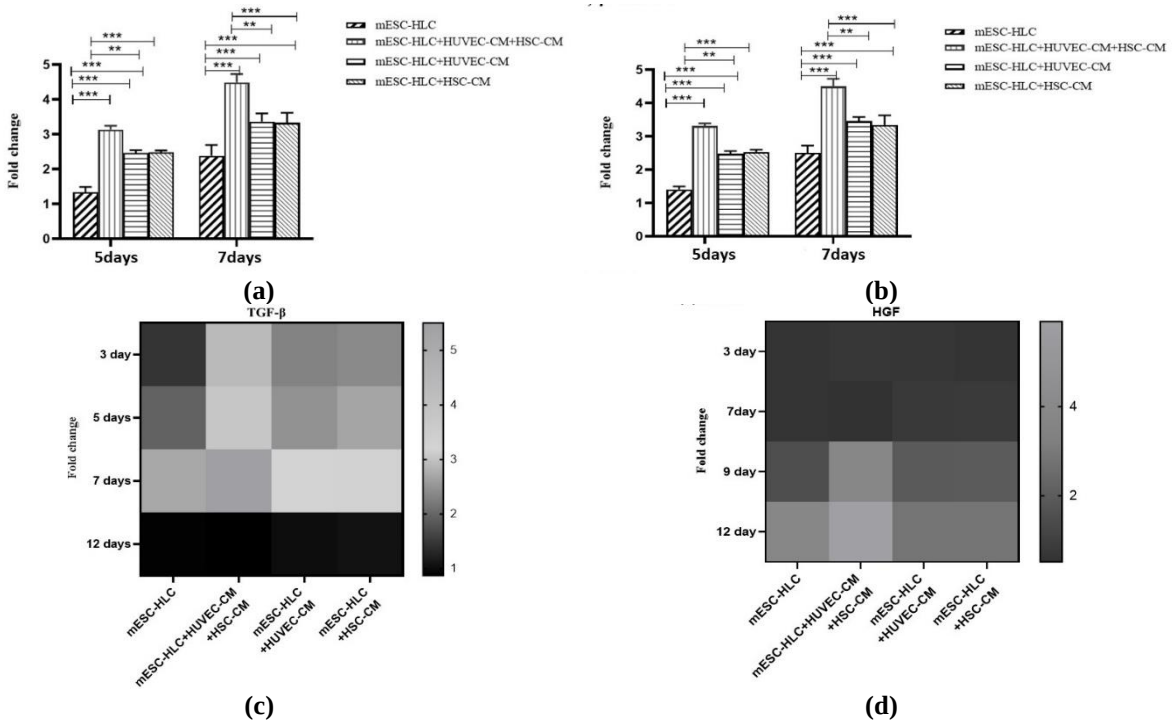


Figure 2. RT-PCR analysis was done to evaluate the fold change of mESC-HLCs cultured with HUVEC and HSC conditioned media on the gelatin scaffold. Results were normalised with GAPDH. **(a,b)** Upregulation of LGR5 and β-catenin marker at 5th and 7th days in mESC-HLC+HUVEC-CM +HSC-CM compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM; **(c)** Heatmap showing temporal expression of TGF-β in mESC-HLCs cultured with HUVEC and HSC conditioned media across three time points (3rd, 5th, 7th, and 12th days). The grayscale color intensity represents fold change in expression, ranging from 1 (black, lowest) to >5 (white, highest). TGF-β levels peaked between days 3 and 7, particularly in cells treated with dual-conditioned media, suggesting early-stage paracrine signaling activity. Expression decreased significantly at the 12th day across all groups; **(d)** Heatmap representation of the HGF gene in mESC-HLCs cultured with HUVEC and HSC conditioned media across three time points (3rd, 5th, 7th, and 12th days). The grayscale color gradient indicates fold change in HGF expression, with lighter shades representing higher expression (up to >4-fold) and darker shades indicating lower expression (~1.5-fold). HGF levels progressively increased from day 9 to day 12, with the dual-conditioned group exhibiting the highest expression, suggesting enhanced paracrine-mediated hepatocyte support. Data represent mean ± standard deviation (SD) from three independent biological replicates (n=3). Statistical analysis was performed using one- or two-way ANOVA; $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

Hepatic differentiation progression was through gene expression studies of mESC-HLC cultured with HUVEC and HSC CM. We evaluated pluripotency markers, as well as early and mature endodermal genes, using real-time PCR. The decreased expression of OCT4, SOX2, and Nanog over a period ranging from 3 hours to 3rd days, indicating the progression of differentiation [17]. Concurrently, increased expression of SOX17 and FOXA2 on the 3rd day confirms the successful induction of definitive endoderm, a crucial step in hepatic differentiation. Interestingly, these genes showed a decline in expression on the 7th and 12th days of differentiation [17]. Interestingly, in this study, we found gradual stimulation of hepatic-related genes, transthyretin (TTR) and albumin, on the 5th and 7th days. Moreover, real-time PCR analysis revealed an upregulation in the expression of markers for hepatic progenitor cells and cholangiocytes in mESC-HLC cultures with HUVEC-CM+HSC-CM on the 5th and 7th days compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM. Leucine-repeat containing G-protein coupled receptor5 (LGR5), cytokeratin 7 (CK7), cytokeratin 19 (CK19), hepatocyte nuclear factor-1β (HNF1β), hepatocyte nuclear factor 1 α (HNF1 α), hepatocyte nuclear factor 6 α (HNF6 α), delta-like 1 homolog (DLK1), and sex-determining region Y-box 9 (SOX9) are some of these markers. (Figures 3 and 4).

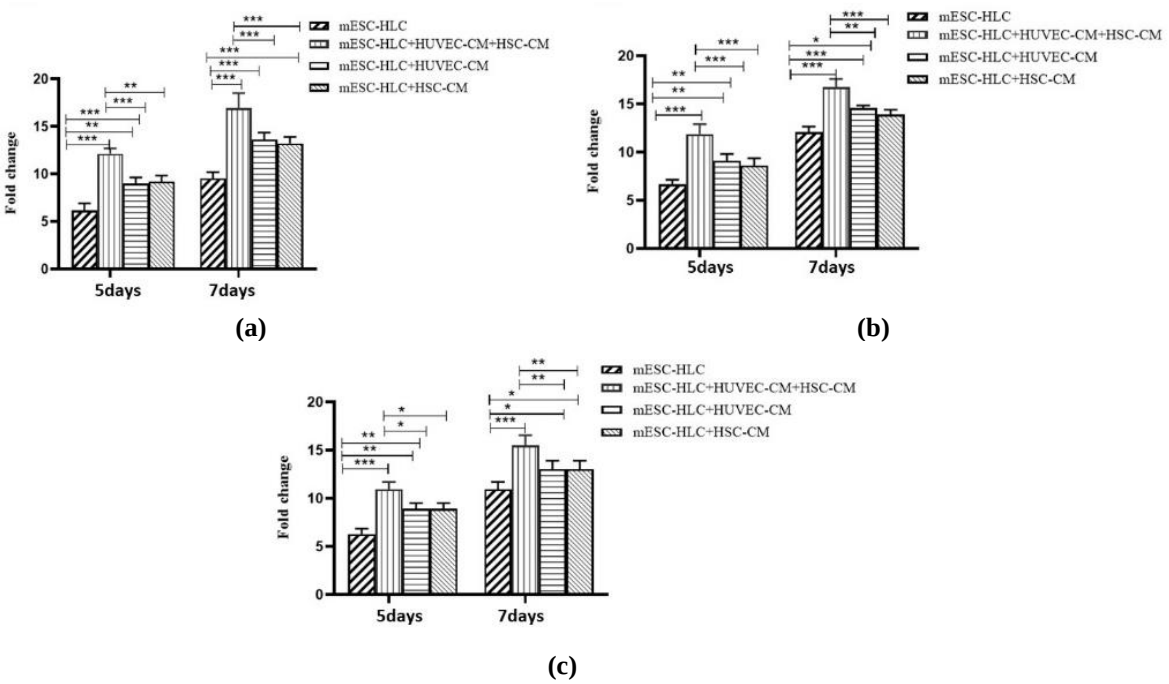
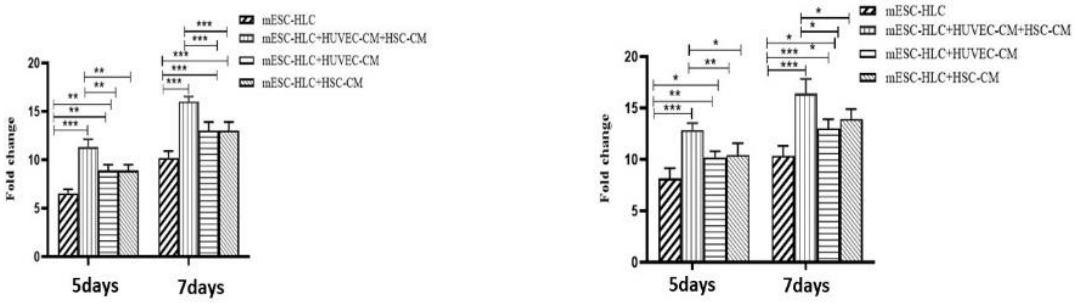


Figure 3. RT-PCR analysis for cholangiocyte marker. There was an upregulation in the expression of **(a)** CK7; **(b)** CK19; **(c)** SOX9 at 5th and 7th days in mESC-HLC+HUVEC-CM+HSC-CM compared to mESC-HLC,mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM. Data represent mean ± standard deviation (SD) from three independent biological replicates (n=3). Statistical analysis was performed using one- or two-way ANOVA; $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.



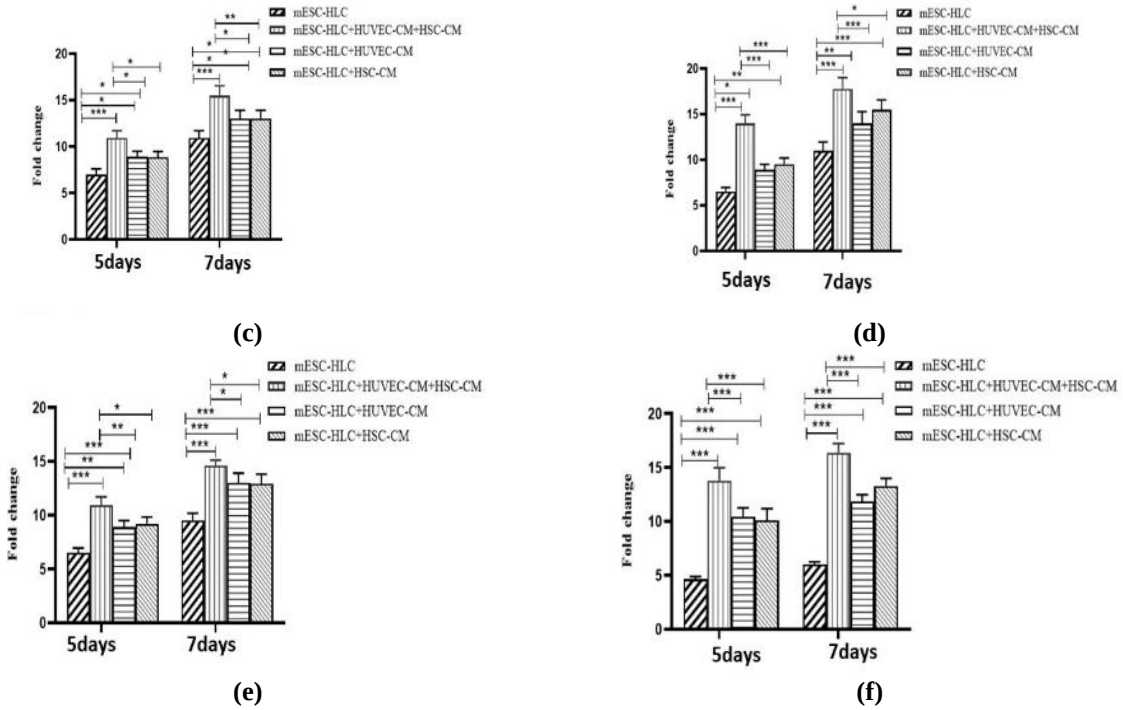
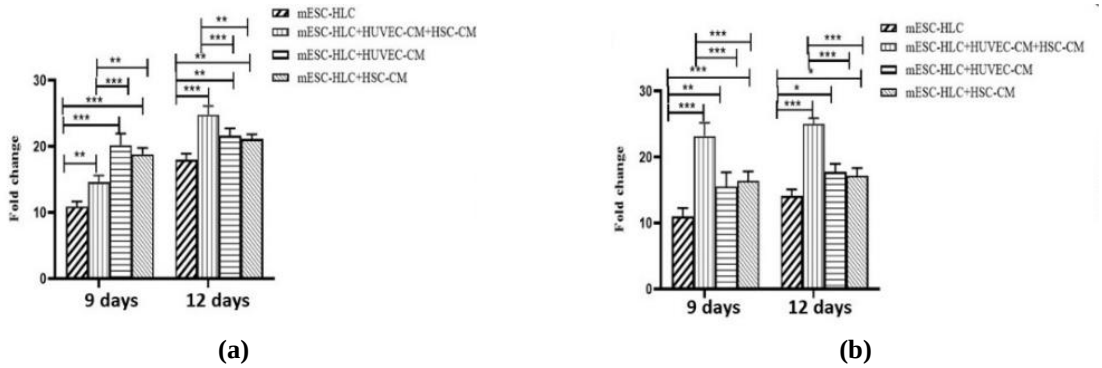


Figure 4. RT-PCR analysis for hepatoblast marker. There was a substantial increase in the expression of **(a)** DLK1; **(b)** HNF1α; **(c)** HNF1β; **(d)** HNF6; **(e)** TTR; **(f)** albumin on the 5th and 7th days in mESC-HLC+HUVEC-CM+HSC-CM compared to cells mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM. Data represent mean ± standard deviation (SD) from three independent biological replicates (n=3). Statistical analysis was performed using one- or two-way ANOVA; $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

We additionally examined the mRNA expression of mature liver markers, including alpha-fetoprotein (AFP), TTR, tyrosine aminotransferase (TAT), hepatocyte nuclear factor 4 alpha (HNF4), and cytokeratin 18 (CK18), on the 9th and 12th days in mESC-HLC+HUVEC-CM +HSC-CM compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM. The overall findings demonstrated a notable rise in the expression of mature hepatic differentiation markers in mESC-HLC+HUVEC-CM +HSC-CM compared to all other condition groups. Furthermore, the results indicated that the gelatin scaffold provided a supportive microenvironment, facilitating differentiation in mESC-derived hepatocyte-like cells cultured with HUVEC and HSC conditioned media (mESC-HLC+HUVEC-CM +HSC-CM). This enhancement was more pronounced compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM, highlighting its potential to promote the differentiation process towards the hepatic lineage and generate mature hepatocyte-like cells. This effect was most evident in mESC-derived hepatocyte-like cells +HUVEC-CM+HSC-CM(mESC-HLC+HUVEC-CM+HSC-CM)compared to all other conditions (Figure 5).



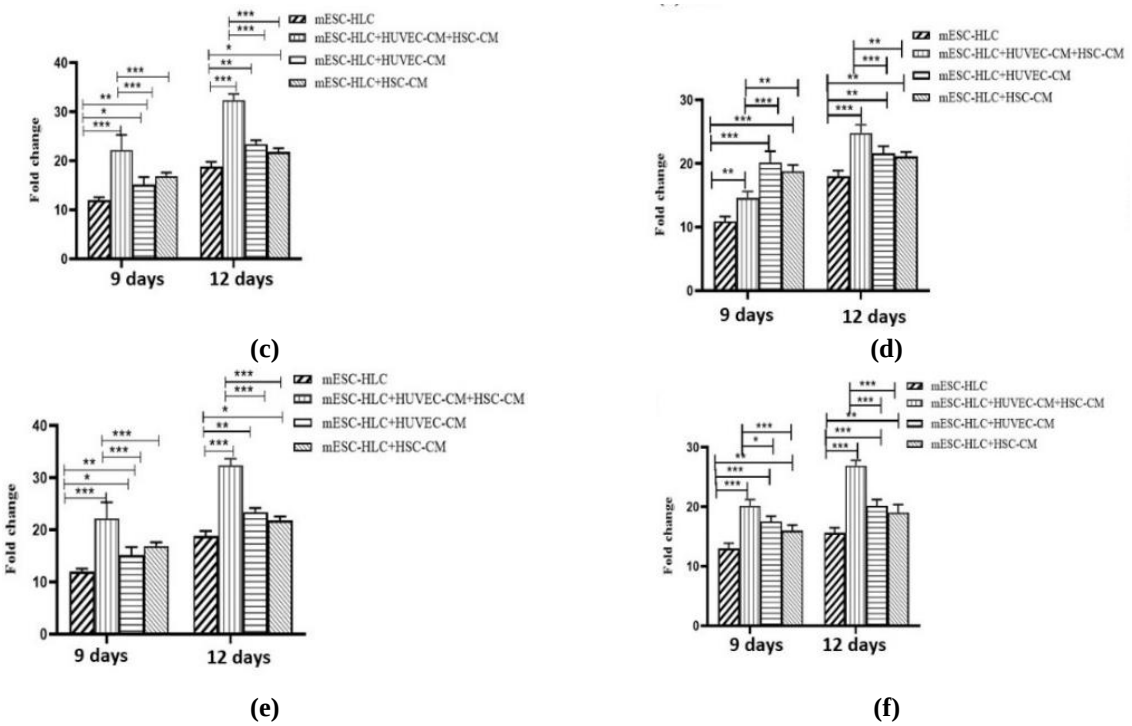


Figure 5. RT-PCR analysis of mature hepatic marker. There was a substantial increase in the expression of **(a)** albumin; **(b)** CK18; **(c)** HNF4α; **(d)** AFP; **(e)** TAT; **(f)** TTR at 9th and 12th days mESC-HLC+HUVEC-CM +HSC-CM compared to mESC-HLCmESC-HLC+HSC-CM, and days mESC-HLC+HUVEC-CM. Data represent mean ± standard deviation (SD) from three independent biological replicates (n=3). Statistical analysis was performed using one- or two-way ANOVA; $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

3.2. Immunohistological analysis.

H&E staining micrographs provide definitive proof by displaying a cross-sectional view of mESC-HLC cultured with HUVEC and HSC conditioned media on the gelatin scaffold.

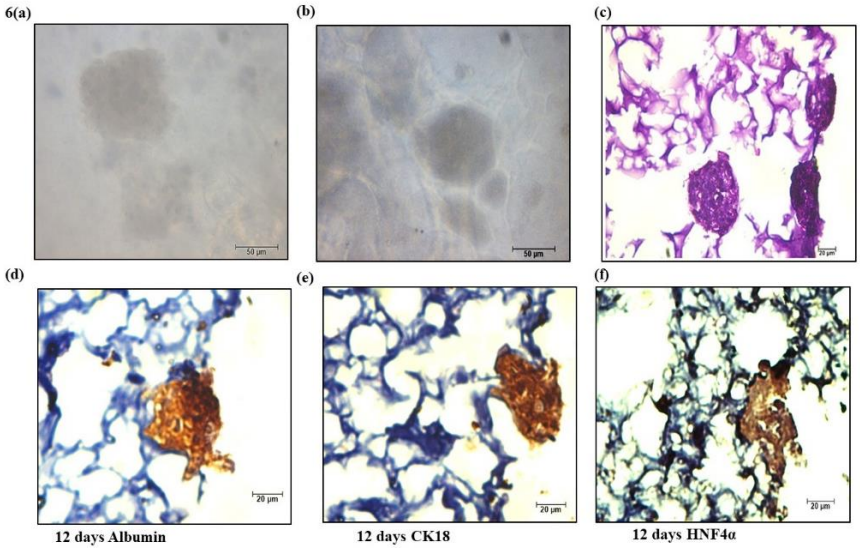


Figure 6. (a,b) Phase contrast images of in mESC-derived hepatocyte-like cells cultured with HUVEC and HSC conditioned media (mESC-HLC+HUVEC-CM+HSC-CM). Scale bar:50 µm; **(c)** H&E-stained micrographs of in mESC-derived hepatocyte-like cells cultured HUVEC and HSC mESC-HLC+HUVEC-CM +HSC-CM on 12th day on gelatin scaffold.Scale bar: 20 µm; **(d-f)** Immunohistochemistry of (mESC-HLC+HUVEC-CM +HSC-CM: DAB staining study indicates positive expression of albumin, HNF4α, and CK18 at day 12, counterstained with Hematoxylin and Eosin solution. Scale bar: 20 µm.

The findings indicated that the cells were in a healthy state and had successfully penetrated inside the scaffold. Additionally, we conducted an analysis of infiltrated cells using hepatic-specific markers. Our findings revealed a positive expression of Albumin, HNF4α, and CK18 at 12 days, as confirmed by DAB staining (Figure 6).

3.3. Liver function tests.

Albumin, urea, and creatinine production were assessed in the culture supernatant to ascertain the effect of conditioned media from HUVEC and HSC on in vitro differentiated mESCs on a 3D gelatin scaffold. The amount of creatinine, urea, and albumin secreted over 12th days period was markedly greater in mESC-derived hepatocyte-like cells cultured with HUVEC-CM and HSC conditioned media (mESC-HLC + HUVEC-CM + HSC-CM) compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM. This indicates enhanced liver-specific functionality and highlights the potential of combining conditioned media with 3D gelatin scaffolds to improve differentiation and functional efficiency (Figure 7).

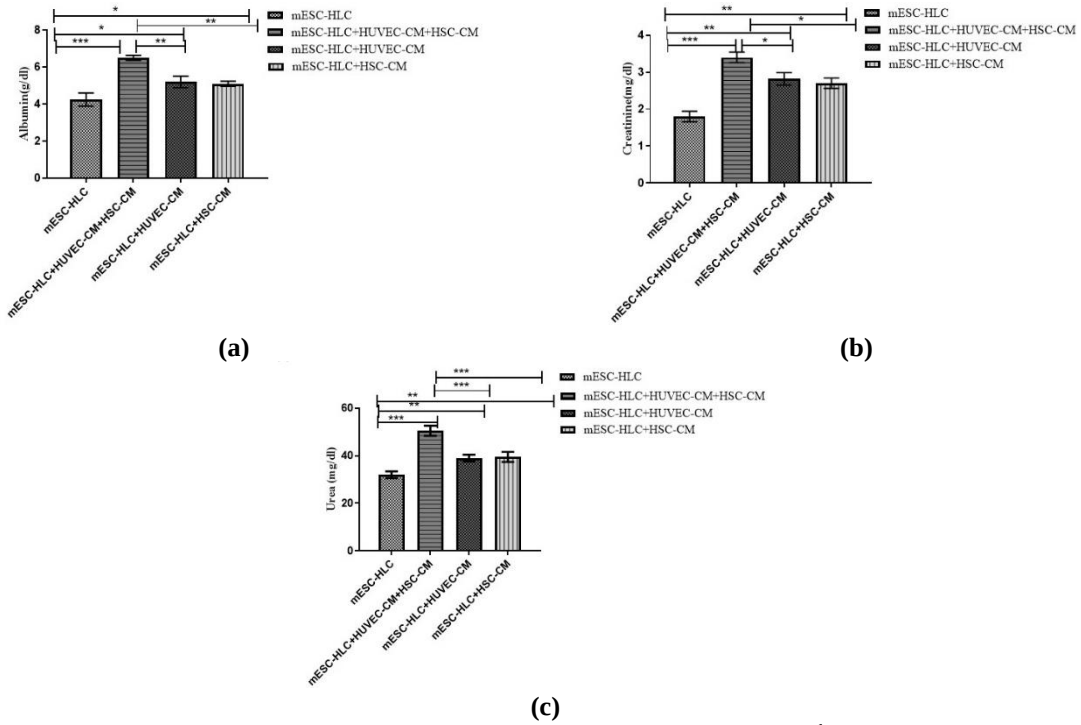


Figure 7. The liver function tests. The conditioned media were collected after 12th dayss of culture **(a)** albumin; **(b)** creatinine; **(c)** urea. There was an increased expression in the secretion of albumin, creatinine, and urea production on the 12th day in mESC-HLC+HUVEC-CM+HSC-CM compared to mESC-HLC, mESC-HLC+HSC-CM, and mESC-HLC+HUVEC-CM. Data represent mean ± standard deviation (SD) from three independent biological replicates (n=3). Statistical analysis was performed using one- or two-way ANOVA; p* < 0.05, p** < 0.01, and p*** < 0.001 were considered statistically significant.

3.4. Discussion.

The current study demonstrates that HUVEC and HSC conditioned medium (CM) exhibit synergistic effects on the differentiation of mESC-HLCs cultured on gelatin scaffolds. Under these conditions, gene expression analysis revealed clear evidence of increased hepatic differentiation and liver-specific functioning. The findings highlight the significance of endothelial and stellate cell-derived signals in promoting hepatocyte maturation and liver-specific function. The gene expression analysis conducted in this study provides insights into the role of HUVEC and HSC-conditioned media in supporting the differentiation of mESC-derived hepatocyte-like cells

(HLCs). It is well known that conditioned media from non-parenchymal cells can improve the microenvironment for cell growth by facilitating biochemical cues, thereby stabilizing the hepatocyte phenotype and functions [18,19].

Conditioned media from endothelial and hepatic stellate cells are known to recapitulate essential microenvironmental signals. HUVECs secrete angiocrine factors, like VEGF, FGF, and TGFβ, which play vital roles in promoting hepatocyte proliferation, survival, and metabolic activation [15,20]. In this study, increased expression of endothelial markers, such as VEGF and CD31, in mESC-HLCs cultured with HUVEC-CM and HSC-CM compared to those cultured with HUVEC-CM alone underscores the critical contribution of HUVEC-derived paracrine factors. These are key markers involved in angiogenesis and vascular development, and their upregulation suggests that HUVEC-conditioned media help to reconstruct elements of the hepatic vascular niche, which is essential for liver functionality [15,21]. VEGF not only promotes angiogenesis but also enhances hepatocyte survival and polarization by stabilizing intercellular junctions and organizing the cytoskeleton. FGF2, a potent mitogen, drives proliferation and differentiation of hepatic progenitors [22,23]. The combined presence of VEGF and FGF2 in HUVEC-conditioned media likely facilitates early hepatic lineage commitment and induces vascular mimicry within the scaffold, ultimately reinforcing liver-specific metabolic function [24,25].

Previous studies have shown that endothelial cells comprise about 50% of non-parenchymal cells and play a critical role in improving the hepatic functions of hepatocytes. Jung *et al*. found that FGF1 and FGF2, produced from the cardiac mesoderm, are necessary for differentiation into AFP and TTR-positive tissue by using a mouse embryo tissue culture model [26]. Rossi *et al*. found that BMP2, BMP4, and BMP7, released by the cardiac mesoderm and septum transversum mesenchyme, are required to induce the expression of the albumin gene [27,28]. The amount of haematopoietically expressed homeobox and prospero-related homeobox1 in hepatoblasts was consistently decreased when the activity of BMP receptors or FGF receptors was inhibited using dominant-negative versions of these receptors [29]. Gouon-Evans *et al*. have revealed that BMP4 and FGF2 are essential for the development of hepatoblasts from definitive endoderm cells generated from mouse embryonic stem cells. These investigations have led to the conclusion that BMP and FGF signals are also necessary for the development of hepatoblasts from ESCs and iPSCs [30]. In this regard, our study revealed an increased expression of FGF2 and BMP4 on the 5^{th} and 7^{th} days in mESC-derived hepatocyte-like cells cultured in the HUVEC and HSC conditioned media compared to other conditioned groups. Moreover, we observed an increased expression of hepatoblast and cholangiocyte markers, which ultimately promoted hepatocyte cell proliferation and maintained liver-specific hepatocyte functions.

Additionally, TGF-β secreted by HUVECs plays a significant role in the differentiation of mESCs into hepatocyte-like cells. TGF-β signaling, through its interaction with hepatic progenitors, promotes the activation of signaling pathways that drive hepatic lineage commitment and hepatocyte maturation [31,32]. This differentiation process is crucial for the development of functional liver-like cells, as TGF-β stimulates the expression of hepatocyte markers and promotes the structural organization necessary for liver function. The synergistic presence of TGF-β in the conditioned media likely reinforces hepatocyte differentiation by modulating key molecular pathways, further supporting the regenerative capacity of the system.

Similarly, the elevated expression of α-smooth muscle actin (αSMA) and collagen I (COL1A1), markers of activated hepatic stellate cells, in mESC-HLCs cultured with HUVEC-CM and HSC-CM compared to those cultured with HSC-CM alone, highlights the critical contribution of HSC-derived signals. These markers are involved in ECM remodeling and liver regeneration,

reflecting the role of HSC-conditioned media in simulating the hepatic microenvironment [33]. The upregulation of these markers can be attributed to the combined effects of HUVECs and activated HSCs in the culture system. HUVECs secrete TGF-β, a fibrogenic factor that can activate HSCs, driving their role in extracellular matrix production and fibrogenesis. This interaction between HUVECs and activated HSCs likely leads to the elevated expression of αSMA and COL1A1, suggesting fibrogenic activation, though this process is also part of normal tissue remodeling [34,35]. Although the presence of these markers is concerning regarding fibrosis, it is essential to note that αSMA and COL1A1 are also involved in regenerative processes during tissue healing and remodeling. Therefore, the expression of these markers in our system may reflect a balance between differentiation, regeneration, and fibrosis, as these processes can coexist, especially in the context of liver tissue regeneration [36]. Therefore, we observed enhanced expression of TGF-β on 3rd, 5th, and 7th days in mESC-HLC+HUVEC-CM +HSC-CM compared to other conditioned groups. Together, these findings demonstrate that the combined influence of HUVEC- and HSC-conditioned media contributes to creating a hepatic niche that enhances the differentiation and maturation of mESC-derived hepatocyte-like cells. The synergistic effect of HUVEC-CM and HSC-CM in improving hepatic differentiation is likely due to the interplay of paracrine factors secreted by HUVECs and HSCs.

Our study also highlighted the role of the Wnt/β-catenin pathway in hepatobiliary development, adult hepatic homeostasis, and restoration [37]. Mutations in its important regulatory genes and abnormal Wnt/β-catenin signaling contribute to the initiation and advancement of several liver disorders, such as cancer and hepatobiliary tumours [38-40]. β-catenin is crucial for the proliferation, survival, and maturation of hepatoblasts [41,42]. In our study, we found a significant increase in the expression of the Wnt-responsive gene and specific marker for hepatoblast cells, Lgr5 and β-catenin, on the 5th and 7th days in mESC-derived hepatocyte-like cells cultured with HUVEC and HSC conditioned media (mESC-HLC+HUVEC-CM +HSC-CM) compared to other conditioned groups. Moreover, activation of the Wnt/β-catenin pathway, evidenced by the upregulation of Lgr5 and β-catenin, supports the hypothesis that dual-CM conditions activate signaling that enhances stem cell commitment and hepatic fate acquisition. Wnt signaling plays a pivotal role in hepatoblast expansion and functional restoration in adult liver models [43,44]. However, its dysregulation is linked to hepatocellular carcinoma and biliary tract malignancies, necessitating further investigation into long-term outcomes [45].

HSCs are closely associated with hepatocytes and play a crucial role in liver regeneration as part of the hepatic progenitor cell niche [46-48]. During liver regeneration, HSCs in the perisinusoidal region release transforming growth factor-alpha (TGF-α), epidermal growth factor (EGF), and hepatocyte growth factor (HGF), which promote hepatocyte proliferation. It regulates cell growth, migration, survival, and angiogenesis, while also being essential for hepatocyte survival, proliferation, and ECM remodeling [49,50]. Therefore, incorporating HSCs' conditioned media can help preserve the phenotypes and functions of hepatocytes. A pleiotropic cytokine called HGF plays a crucial role in the differentiation of hepatocytes from stem and progenitor cells [51]. The signaling pathways triggered by HGF facilitate hepatocyte-specific activities by increasing the expression of key hepatic transcription factors, such as albumin, HNF4, and cytokine 18, which are markers of fully mature hepatocytes [52-53]. Studies have reported that supplementing HGF to stem cell cultures enhances the graftability and long-term viability of hepatocyte-like cells [54-55]. HGF-mediated signaling has previously been shown to enhance hepatocyte-specific functions, including albumin secretion, urea cycle activity, and cytochrome P450 enzyme expression [56]. In line with these findings, our dual-CM group exhibited higher

functional output, including increased secretion of albumin, urea, and creatinine, suggesting a more mature hepatocyte phenotype. Together, these factors enhance the differentiation and functional maturation of mESC-derived HLCs, mimicking the complex interactions of the liver niche.

Studies have demonstrated that co-culturing human iPSCs with HUVECs during hepatic differentiation can enhance the expression of hepatic markers and functions, indicating the supportive role of endothelial cell-derived factors in hepatic maturation [57]. Additionally, the research group has indicated that the presence of HSCs in co-culture systems contributes to the maintenance of hepatocyte function and phenotype. HSCs secrete various growth factors and cytokines that influence hepatocyte differentiation and function, thereby creating a microenvironment conducive to hepatic maturation [58-59]. These findings align with our study, and we observed an increase in the expression of late-stage hepatic markers, including albumin, TTR, TAT, HNF4, and CK18. Furthermore, we also reported that mESC-HLCs with HUVEC and HSC conditioned media exhibited increased secretion of albumin, urea, and creatinine. Together, these findings suggest that soluble factors present in the conditioned media from HUVECs and HSCs may facilitate the differentiation and maturation of HLCs, enabling them to functionally resemble mature hepatocytes. Although this study used mESCs as a model, the translational potential of these findings to human therapy remains limited. Mouse and human ESCs differ significantly in signaling dependencies and epigenetic regulation [60-61]. Therefore, validating these results using human pluripotent stem cells (hPSCs) and clinically compliant scaffolds will be essential for moving toward regenerative applications.

4. Conclusion

In summary, this study demonstrates that the integration of HUVEC-CM and HSC-CM with a gelatin scaffold effectively promotes the differentiation and functional maturation of mESC-derived HLCs. The enhanced expression of hepatic markers and improved liver-specific functions highlight the efficacy of this approach. Furthermore, the gelatin scaffold creates an optimal microenvironment that facilitates cell attachment, growth, and proliferation of hepatocyte-like cells.

Additionally, hepatocyte-specific genes can be preserved for a longer period, which helps mimic natural liver architecture and development. These findings suggest the potential of this approach for liver tissue engineering and regenerative medicine.

While the study's findings are intriguing, it is essential to note that the study was conducted exclusively in vitro using mESCs. This limits the immediate translational applicability, as in vivo conditions and human-specific responses may vary. Therefore, future studies should focus on validating these findings through carefully designed in vivo studies with relevant animal models of liver injury. These investigations would be crucial in determining the therapeutic potential, integration capacity, and safety of the generated hepatocyte-like cells. Additionally, a deeper understanding of the molecular pathways activated by HUVEC-CM and HSC-CM during the differentiation process may uncover novel targets to further refine and enhance hepatic maturation. Furthermore, long-term studies are necessary to evaluate how effectively these HLCs maintain their functionality under physiological stress over time. Adapting this culture approach to human pluripotent stem cells, along with the use of a clinically approved scaffold, would enhance its translational potential. These developments have the potential to contribute meaningfully to personalised liver regenerative therapies and offer new treatment avenues for patients suffering from end-stage liver diseases.

Author Contributions

Conceptualization, P.B. and K.K.; writing—original draft preparation, K.K.; data curation, P.B. and K.K.; writing—review and editing, P.B. and K.K. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

This work was funded by the Science and Engineering Research Board (SERB) (ECR/2016/001508).

Acknowledgments

This work was supported by the Early Career Research Award (SERB-ECR/2016/001508) from the Science and Engineering Research Board (SERB).

Conflict of Interest

The authors declare no financial or non-financial conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Definition
mESCs	Mouse Embryonic Stem Cells
HLCs	Hepatocyte-Like Cells
HUVEC	Human Umbilical Vein Endothelial Cells
HSC	Hepatic Stellate Cell
CM	Conditioned Media
ECM	Extracellular Matrix
HGF	Hepatocyte Growth Factor
TGF-β	Transforming Growth Factor-beta
VEGF	Vascular Endothelial Growth Factor
FGF	Fibroblast Growth Factor
DE	Definitive Endoderm
IHC	Immunohistochemistry
DAB	3,3′-Diaminobenzidine
RT-PCR	Real-Time Polymerase Chain Reaction
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
BMP4	Bone Morphogenetic Protein 4
FGF2	Fibroblast Growth Factor 2

Abbreviation	Definition
LGR5	Leucine-rich Repeat-containing G-protein Coupled Receptor 5
CK18	Cytokeratin 18
CK19	Cytokeratin 19
CK7	Cytokeratin 7
AFP	Alpha-Fetoprotein
TTR	Transthyretin
TAT	Tyrosine Aminotransferase
HNF4α	Hepatocyte Nuclear Factor 4 Alpha
HNF1α	Hepatocyte Nuclear Factor 1 Alpha
HNF1β	Hepatocyte Nuclear Factor 1 Beta
HNF6	Hepatocyte Nuclear Factor 6
DLK1	Delta-like 1 Homolog
SOX9	Sex-determining Region Y-box 9
COL1A1	Collagen Type I Alpha 1 Chain
αSMA	Alpha-Smooth Muscle Actin
ANOVA	Analysis of Variance
PBS	Phosphate Buffered Saline
LIF	Leukemia Inhibitory Factor
IMDM	Iscove's Modified Dulbecco's Medium
SD	Standard Deviation

References

1. Khoury, T.; Rmeileh, A.A.; Yosha, L.; Benson, A.A.; Daher, S.; Mizrahi, M. Drug Induced Liver Injury: Review with a Focus on Genetic Factors, Tissue Diagnosis, and Treatment Options. *J. Clin. Transl. Hepatol.* **2015**, *3*, 99-108, https://doi.org/10.14218/jcth.2015.00007.
2. Fritsche, K.; Ziková-Kloas, A.; Marx-Stoelting, P.; Braeuning, A. Metabolism-Disrupting Chemicals Affecting the Liver: Screening, Testing, and Molecular Pathway Identification. *Int. J. Mol. Sci.* **2023**, *24*, 2686, https://doi.org/10.3390/ijms24032686.
3. Andrade, R.J.; Aithal, G.P.; Björnsson, E.S.; Kaplowitz, N.; Kullak-Ublick, G.A.; Larrey, D.; Karlsen, T.H. EASL Clinical Practice Guidelines: Drug-induced liver injury. *J. Hepatol.* **2019**, *70*, 1222-1261, https://doi.org/10.1016/j.jhep.2019.02.014.
4. Devarbhavi, H.; Asrani, S.K.; Arab, J.P.; Nartey, Y.A.; Pose, E.; Kamath, P.S. Global burden of liver disease: 2023 update. *J. Hepatol.* **2023**, *79*, 516-537, https://doi.org/10.1016/j.jhep.2023.03.017.
5. Terrault, N.A.; Francoz, C.; Berenguer, M.; Charlton, M.; Heimbach, J. Liver Transplantation 2023: Status Report, Current and Future Challenges. *Clin. Gastroenterol. Hepatol.* **2023**, *21*, 2150-2166, https://doi.org/10.1016/j.cgh.2023.04.005.
6. Hashemi Goradel, N.; Darabi, M.; Shamsasenjan, K.; Ejtehadifar, M.; Zahedi, S. Methods of Liver Stem Cell Therapy in Rodents as Models of Human Liver Regeneration in Hepatic Failure. *Adv. Pharm. Bull.* **2015**, *5*, 293-298.
7. Liu, Y.; Li, H.; Yan, S.; Wei, J.; Li, X. Hepatocyte Cocultures with Endothelial Cells and Fibroblasts on Micropatterned Fibrous Mats to Promote Liver-Specific Functions and Capillary Formation Capabilities. *Biomacromolecules* **2014**, *15*, 1044-1054, https://doi.org/10.1021/bm401926k.
8. Kaur, I.; Vasudevan, A.; Rawal, P.; Tripathi, D.M.; Ramakrishna, S.; Kaur, S.; Sarin, S.K. Primary Hepatocyte Isolation and Cultures: Technical Aspects, Challenges and Advancements. *Bioengineering* **2023**, *10*, 131, https://doi.org/10.3390/bioengineering10020131.
9. Luo, Q.; Wang, N.; Que, H.; Mai, E.; Hu, Y.; Tan, R.; Gu, J.; Gong, P. Pluripotent Stem Cell-Derived Hepatocyte-like Cells: Induction Methods and Applications. *Int. J. Mol. Sci.* **2023**, *24*, 11592, https://doi.org/10.3390/ijms241411592.
10. Afshari, A.; Shamdani, S.; Uzan, G.; Naserian, S.; Azarpira, N. Different approaches for transformation of mesenchymal stem cells into hepatocyte-like cells. *Stem Cell Res. Ther.* **2020**, *11*, 54, https://doi.org/10.1186/s13287-020-1555-8.
11. Mata-Martínez, E.; Ramírez-Ledesma, M.G.; Vázquez-Victorio, G.; Hernández-Muñoz, R.; Díaz-Muñoz, M.; Vázquez-Cuevas, F.G. Purinergic Signaling in Non-Parenchymal Liver Cells. *Int. J. Mol. Sci.* **2024**, *25*, 9447, https://doi.org/10.3390/ijms25179447.

12. Shu, W.; Yang, M.; Yang, J.; Lin, S.; Wei, X.; Xu, X. Cellular crosstalk during liver regeneration: unity in diversity. *Cell Commun. Signal.* **2022**, *20*, 117, https://doi.org/10.1186/s12964-022-00918-z.
13. Chen, F.; Wang, H.; Xiao, J. Regulated differentiation of stem cells into an artificial 3D liver as a transplantable source. *Clin. Mol. Hepatol.* **2020**, *26*, 163-179, https://doi.org/10.3350/cmh.2019.0022n.
14. Al-Ghadban, S.; Walczak, S.G.; Isern, S.U.; Martin, E.C.; Herbst, K.L.; Bunnell, B.A. Enhanced Angiogenesis in HUVECs Preconditioned with Media from Adipocytes Differentiated from Lipedema Adipose Stem Cells In Vitro. *Int. J. Mol. Sci.* **2023**, *24*, 13572, https://doi.org/10.3390/ijms241713572.
15. Ding, B.-S.; Nolan, D.J.; Butler, J.M.; James, D.; Babazadeh, A.O.; Rosenwaks, Z.; Mittal, V.; Kobayashi, H.; Shido, K.; Lyden, D.; Sato, T.N.; Rabbany, S.Y.; Rafii, S. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature* **2010**, *468*, 310-315, https://doi.org/10.1038/nature09493.
16. Bhatt S, S.; Krishna Kumar, J.; Laya, S.; Thakur, G.; Nune, M. Scaffold-mediated liver regeneration: A comprehensive exploration of current advances. *J. Tissue Eng.* **2024**, *15*, 20417314241286092, https://doi.org/10.1177/20417314241286092.
17. Kumari, K.; Tandon, S.; Ghosh, S.; Baligar, P. Gelatin scaffold ameliorates proliferation & stem cell differentiation into the hepatic like cell and support liver regeneration in partial-hepatectomized mice model. *Biomed. Mater.* **2023**, *18*, 065022, https://doi.org/10.1088/1748-605x/ad04fd.
18. Ma, Y.; Hu, L.; Tang, J.; Guo, W.; Feng, Y.; Liu, Y.; Tang, F. Three-Dimensional Cell Co-Culture Liver Models and Their Applications in Pharmaceutical Research. *Int. J. Mol. Sci.* **2023**, *24*, 6248, https://doi.org/10.3390/ijms24076248.
19. Unal, A.Z.; Jeffs, S.E.; West, J.L. 3D Co-Culture with Vascular Cells Supports Long-Term Hepatocyte Phenotype and Function In Vitro. *Regen. Eng. Transl. Med.* **2018**, *4*, 21-34, https://doi.org/10.1007/s40883-018-0046-2.
20. Rafii, S.; Butler, J.M.; Ding, B.-S. Angiocrine functions of organ-specific endothelial cells. *Nature* **2016**, *529*, 316-325, https://doi.org/10.1038/nature17040.
21. LeCouter, J.; Moritz, D.R.; Li, B.; Phillips, G.L.; Liang, X.H.; Gerber, H.-P.; Hillan, K.J.; Ferrara, N. Angiogenesis-Independent Endothelial Protection of Liver: Role of VEGFR-1. *Science* **2003**, *299*, 890-893, https://doi.org/10.1126/science.1079562.
22. Lee, C.; Kim, M.-J.; Kumar, A.; Lee, H.-W.; Yang, Y.; Kim, Y. Vascular endothelial growth factor signaling in health and disease: from molecular mechanisms to therapeutic perspectives. *Signal Transduct. Target. Ther.* **2025**, *10*, 170, https://doi.org/10.1038/s41392-025-02249-0.
23. Geervliet, E.; Terstappen, L.W.M.M.; Bansal, R. Hepatocyte survival and proliferation by fibroblast growth factor 7 attenuates liver inflammation, and fibrogenesis during acute liver injury via paracrine mechanisms. *Biomed. Pharmacother.* **2023**, *167*, 115612, https://doi.org/10.1016/j.biopha.2023.115612.
24. Korff, T.; Augustin, H.G. Integration of Endothelial Cells in Multicellular Spheroids Prevents Apoptosis and Induces Differentiation. *J. Cell Biol.* **1998**, *143*, 1341-1352, https://doi.org/10.1083/jcb.143.5.1341.
25. Wechman, S.L.; Emdad, L.; Sarkar, D.; Das, S.K.; Fisher, P.B. Chapter Two - Vascular mimicry: Triggers, molecular interactions and in vivo models. In Advances in Cancer Research, Tew, K.D., Fisher, P.B., Eds.; Academic Press: **2020**; Volume 148, pp. 27-67, https://doi.org/10.1016/bs.acr.2020.06.001.
26. Jung, J.; Zheng, M.; Goldfarb, M.; Zaret, K.S. Initiation of Mammalian Liver Development from Endoderm by Fibroblast Growth Factors. *Science* **1999**, *284*, 1998-2003, https://doi.org/10.1126/science.284.5422.1998.
27. Rossi, J.M.; Dunn, N.R.; Hogan, B.L.M.; Zaret, K.S. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev.* **2001**, *15*, 1998-2009, https://doi.org/10.1101/gad.904601.
28. Tsaytler, P.; Liu, J.; Blaess, G.; Schifferl, D.; Veenvliet, J.V.; Wittler, L.; Timmermann, B.; Herrmann, B.G.; Koch, F. BMP4 triggers regulatory circuits specifying the cardiac mesoderm lineage. *Development* **2023**, *150*, dev201450, https://doi.org/10.1242/dev.201450.
29. Shin, D.; Shin, C.H.; Tucker, J.; Ober, E.A.; Rentzsch, F.; Poss, K.D.; Hammerschmidt, M.; Mullins, M.C.; Stainier, D.Y.R. Bmp and Fgf signaling are essential for liver specification in zebrafish. *Development* **2007**, *134*, 2041-2050, https://doi.org/10.1242/dev.000281.
30. Gouon-Evans, V.; Boussemart, L.; Gadue, P.; Nierhoff, D.; Koehler, C.I.; Kubo, A.; Shafritz, D.A.; Keller, G. BMP-4 is required for hepatic specification of mouse embryonic stem cell–derived definitive endoderm. *Nature Biotechnology* **2006**, *24*, 1402-1411, https://doi.org/10.1038/nbt1258.
31. Wang, M.-K.; Sun, H.-Q.; Xiang, Y.-C.; Jiang, F.; Su, Y.-P.; Zou, Z.-M. Different roles of TGF-β in the multi-lineage differentiation of stem cells. *World J. Stem Cells* **2012**, *4*, 28–34, https://doi.org/10.4252/wjsc.v4.i5.28.

32. Pettinato, G.; Lehoux, S.; Ramanathan, R.; Salem, M.M.; He, L.-X.; Muse, O.; Flaumenhaft, R.; Thompson, M.T.; Rouse, E.A.; Cummings, R.D.; Wen, X.; Fisher, R.A. Generation of fully functional hepatocyte-like organoids from human induced pluripotent stem cells mixed with Endothelial Cells. *Sci. Rep.* **2019**, *9*, 8920, https://doi.org/10.1038/s41598-019-45514-3.
33. Yin, C.; Evason, K.J.; Asahina, K.; Stainier, D.Y.R. Hepatic stellate cells in liver development, regeneration, and cancer. *J. Clin. Invest.* **2013**, *123*, 1902-1910, https://doi.org/10.1172/JCI66369.
34. Yoshimatsu, Y.; Watabe, T. Emerging roles of inflammation-mediated endothelial–mesenchymal transition in health and disease. *Inflamm. Regen.* **2022**, *42*, 9, https://doi.org/10.1186/s41232-021-00186-3.
35. Giarratana, A.O.; Prendergast, C.M.; Salvatore, M.M.; Capaccione, K.M. TGF-β signaling: critical nexus of fibrogenesis and cancer. *J. Transl. Med.* **2024**, *22*, 594, https://doi.org/10.1186/s12967-024-05411-4.
36. Zuñiga-Aguilar, E.; Ramírez-Fernández, O. Fibrosis and hepatic regeneration mechanism. *Transl. Gastroenterol. Hepatol.* **2022**, *7*, 9, https://doi.org/10.21037/tgh.2020.02.21.
37. Zhao, H.; Ming, T.; Tang, S.; Ren, S.; Yang, H.; Liu, M.; Tao, Q.; Xu, H. Wnt signaling in colorectal cancer: pathogenic role and therapeutic target. *Mol. Cancer* **2022**, *21*, 144, https://doi.org/10.1186/s12943-022-01616-7.
38. Matsumoto, S.; Kikuchi, A. Wnt/β-catenin signaling pathway in liver biology and tumorigenesis. *In Vitro Cell.Dev.Biol.-Animal* **2024**, *60*, 466-481, https://doi.org/10.1007/s11626-024-00858-7.
39. Perugorria, M.J.; Olaizola, P.; Labiano, I.; Esparza-Baquer, A.; Marzioni, M.; Marin, J.J.G.; Bujanda, L.; Banales, J.M. Wnt–β-catenin signalling in liver development, health and disease. *Nat. Rev. Gastroenterol. Hepatol.* **2019**, *16*, 121-136, https://doi.org/10.1038/s41575-018-0075-9.
40. Gajos-Michniewicz, A.; Czyz, M. WNT/β-catenin signaling in hepatocellular carcinoma: The aberrant activation, pathogenic roles, and therapeutic opportunities. *Genes Dis.* **2024**, *11*, 727-746, https://doi.org/10.1016/j.gendis.2023.02.050.
41. Tan,X.; Yuan, Y.; Zeng, G.; Apte, U.; Thompson, M.D.; Cieply, B.; Stolz, D.B.; Michalopoulos, G.K.; Kaestner, K.H.; Monga, S.P.S. β-Catenin Deletion in Hepatoblasts Disrupts Hepatic Morphogenesis and Survival during Mouse Development. Hepatology 2008, 47, 1667–1679, https://doi.org/10.1002/hep.22225.
42. Goel, C.; Monga, S.P.; Nejak-Bowen, K. Role and Regulation of Wnt/β-Catenin in Hepatic Perivenous Zonation and Physiological Homeostasis. American Journal Of Pathology 2021,192,4-17, https://doi.org/10.1016/j.ajpath.2021.09.007.
43. Russell, J.O.; Monga, S.P. Wnt/β-Catenin Signaling in Liver Development, Homeostasis, and Pathobiology. *Annu. Rev. Pathol.: Mech. Dis.* **2018**, *13*, 351-378, https://doi.org/10.1146/annurev-pathol-020117-044010.
44. Yang, W.; Yan, H.-X.; Chen, L.; Liu, Q.; He, Y.-Q.; Yu, L.-X.; Zhang, S.-H.; Huang, D.-D.; Tang, L.; Kong, X.-N.; Chen, C.; Liu, S.-Q.; Wu, M.-C.; Wang, H.-Y. Wnt/β-Catenin Signaling Contributes to Activation of Normal and Tumorigenic Liver Progenitor Cells. *Cancer Res.* **2008**, *68*, 4287-4295, https://doi.org/10.1158/0008-5472.CAN-07-6691.
45. Zhao, Z.; Cui, T.; Wei, F.; Zhou, Z.; Sun, Y.; Gao, C.; Xu, X.; Zhang, H. Wnt/β-Catenin signaling pathway in hepatocellular carcinoma: pathogenic role and therapeutic target. *Front. Oncol.* **2024**, *14*, 1367364, https://doi.org/10.3389/fonc.2024.1367364.
46. Friedman, S.L. Hepatic Stellate Cells: Protean, Multifunctional, and Enigmatic Cells of the Liver. *Physiol. Rev.* **2008**, *88*, 125-172, https://doi.org/10.1152/physrev.00013.2007.
47. Kitto, L.J.; Henderson, N.C. Hepatic Stellate Cell Regulation of Liver Regeneration and Repair. *Hepatol. Commun.* **2021**, *5*, 358–370, https://doi.org/10.1002/hep4.1628.
48. Thompson, A.I.; Conroy, K.P.; Henderson, N.C. Hepatic stellate cells: central modulators of hepatic carcinogenesis. *BMC Gastroenterol.* **2015**, *15*, 63, https://doi.org/10.1186/s12876-015-0291-5.
49. Efimova, E.A.; Glanemann, M.; Liu, L.; Schumacher, G.; Settmacher, U.; Jonas, S.; Langrehr, J.M.; Neuhaus, P.; Nüssler, A.K. Effects of Human Hepatocyte Growth Factor on the Proliferation of Human Hepatocytes and Hepatocellular Carcinoma Cell Lines. *Eur. Surg. Res.* **2004**, *36*, 300-307, https://doi.org/10.1159/000079915.
50. Suzuki, A.; Hayashida, M.; Kawano, H.; Sugimoto, K.; Nakano, T.; Shiraki, K. Hepatocyte growth factor promotes cell survival from Fas-mediated cell death in hepatocellular carcinoma cells via Akt activation and Fas-death-inducing signaling complex suppression. *Hepatology* **2000**, *32*, 796–802, https://doi.org/10.1053/jhep.2000.17738.
51. Molnarfi, N.; Benkhoucha, M.; Funakoshi, H.; Nakamura, T.; Lalive, P.H. Hepatocyte growth factor: A regulator of inflammation and autoimmunity. *Autoimmun. Rev.* **2015**, *14*, 293-303, https://doi.org/10.1016/j.autrev.2014.11.013.

52. Wang, J.; Huang, D.; Yu, H.; Cheng, Y.; Ren, H.; Zhao, Y. Developing tissue engineering strategies for liver regeneration. *Eng. Regen.* **2022**, *3*, 80-91, https://doi.org/10.1016/j.engreg.2022.02.003.
53. Varghese, D.S.; Alawathugoda, T.T.; Ansari, S.A. Fine Tuning of Hepatocyte Differentiation from Human Embryonic Stem Cells: Growth Factor vs. Small Molecule-Based Approaches. *Stem Cells Int.* **2019**, *2019*, 5968236, https://doi.org/10.1155/2019/5968236.
54. Kopp, J.B. Hepatocyte growth factor: Mesenchymal signal for epithelial homeostasis. *Kidney Int.* **1998**, 54, 1392–1393, https://doi.org/10.1046/j.1523-1755.1998.00126.x.
55. Gupta, S.; Sharma, A.; Rajakannu, M.; Bisevac, J.; Rela, M.; Verma, R.S. Small Molecule-Mediated Stage-Specific Reprogramming of MSCs to Hepatocyte-Like Cells and Hepatic Tissue for Liver Injury Treatment. *Stem Cell Rev. Rep.* **2024**, *20*, 2215-2235, https://doi.org/10.1007/s12015-024-10771-x.
56. Zhang, W.; Li, W.; Liu, B.; Wang, P.; Li, W.; Zhang, H. Efficient generation of functional hepatocyte-like cells from human fetal hepatic progenitor cells in vitro. *J. Cell. Physiol.* **2012**, *227*, 2051-2058, https://doi.org/10.1002/jcp.22934.
57. Freyer, N.; Greuel, S.; Knöspel, F.; Strahl, N.; Amini, L.; Jacobs, F.; Monshouwer, M.; Zeilinger, K. Effects of Co-Culture Media on Hepatic Differentiation of hiPSC with or without HUVEC Co-Culture. *Int. J. Mol. Sci.* **2017**, *18*, 1724, https://doi.org/10.3390/ijms18081724.
58. Krause, P.; Saghatolislam, F.; Koenig, S.; Unthan-Fechner, K.; Probst, I. Maintaining hepatocyte differentiation in vitro through co-culture with hepatic stellate cells. *In Vitro Cell.Dev.Biol.-Animal* **2009**, *45*, 205-212, https://doi.org/10.1007/s11626-008-9166-1.
59. Coulouarn, C.; Corlu, A.; Glaise, D.; Guénon, I.; Thorgeirsson, S.S.; Clément, B. Hepatocyte–Stellate Cell Cross-Talk in the Liver Engenders a Permissive Inflammatory Microenvironment That Drives Progression in Hepatocellular Carcinoma. *Cancer Res.* **2012**, *72*, 2533-2542, https://doi.org/10.1158/0008-5472.can-11-3317.
60. Yagi, M.; Yamanaka, S.; Yamada, Y. Epigenetic foundations of pluripotent stem cells that recapitulate *in vivo* pluripotency. *Lab. Investig.* **2017**, *97*, 1133-1141, https://doi.org/10.1038/labinvest.2017.87.
61. Schnerch, A.; Cerdan, C.; Bhatia, M. Distinguishing Between Mouse and Human Pluripotent Stem Cell Regulation: The Best Laid Plans of Mice and Men. *Stem Cells* **2010**, *28*, 419-430, https://doi.org/10.1002/stem.298.

Publisher's Note & Disclaimer