SUPPLEMENTARY INFORMATION

In vivo transplantation of Intrahepatic Cholangiocyte Organoids with decellularized liver-derived hydrogel support hepatic cellular proliferation and differentiation in chronic liver injury

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METHODS

1. Characterization of decellularization

The decellularized liver tissues were characterized for acellularity and intact matrix proteins as reported in one of our previous studies[1]. Briefly, the decell tissues were fixed with formalin. The fixed tissue samples were embedded in paraffin and sectioned into slices with 5 µm thickness, which were deparaffinized and rehydrated with different concentrations of ethanol followed by brief washing and incubation with DAPI (1:2000) for 2 minutes followed by three PBS washes. The decell tissues were further stained with hematoxylin and eosin (H&E) solution for 2 minutes. After washing and dehydration, H&E stained sections were mounted for imaging. To confirm the presence of matrix proteins, Masson Trichrome (MT) stain specific for the collagen protein, was performed on the paraffin embedded decell tissue. Alcian Blue specific for the glycosaminoglycans (GAGs) and Orcein stain specific for the elastin protein was also performed. DNA content quantification was carried out on per gram of control vs decell tissue to validate acellularity. The tissue samples were crushed with liquid nitrogen and homogenized with trizol. Chloroform was then added to the homogenized tissue mass and centrifuged at 13000rpm for 10 minutes. The middle buffy coat layer was separated and washed with isopropanol and ethanol. The final DNA pellet was resuspended in nuclease free water and quantified using a nano-drop reader.

2. Physical characterization of decellularized liver extracellular matrix hydrogels (DCL)

- **Rheology**

Previous studies have shown that 2-10mg/mL of liver ECM based hydrogels are beneficial in maintaining hepatocytes *in vitro*[2–4]. Therefore, we prepared two concentrations of DCL 4mg/mL and 10mg/mL and characterized them biophysically. The DCL fabricated hydrogels were rheologically characterized using a parallel plate rheometer (Anton Paar, Germany MCR 102) at 37 °C, with a plate diameter of 25mm and plate spacing of 500 µm. The viscosity was examined against increasing the shear rate from 0.01 to 10^2/s (where 's' is rate of shear) to measure the flow behaviour of the respective DCL hydrogels (4mg/mL and10 mg/mL) under the effect of shear. An oscillatory amplitude sweep was performed between 0.01-100% shear strain at constant frequency of 1 Hz to determine the linear viscoelastic region (LVR) of the DCL hydrogels. Subsequently, the viscoelastic characteristics of the DCL hydrogels were investigated using an oscillatory frequency sweep test from 0.1 to 100 Hz at a constant strain of 1%.

- **Tube inversion test**

Tube inversion test was carried out in screw-capped 10 mL vial (diameter: 10 mm) to examine the gelation kinetics of DCL hydrogels at 37 °C. Gelation was suggested to have occurred when a gel-like material was formed which did not demonstrate gravitational flow after 60 minutes.

- **Water uptake ability of DCL hydrogels**

Briefly, the Decellularized Liver ECM hydrogels (DCL hydrogels) were frozen at -20 °C for 24 hours and subsequently lyophilized for 12 hours and their dry weights were recorded. Thereafter, the DCL gels were immersed in PBS (pH 7.4) at 37 °C for 7 days. Each day, the swelled DCL hydrogels were retrieved and carefully blotted to remove excess of fluid from the surface and weighed. Water absorption ability expressed as swelling percentage was calculated using the equation 1.

Swelling % = $[(W_2 - W_1) / W_1]*100\%$ ---------- (1)

where, W_2 is the weight of the samples after 't' hours of soaking and W_1 is the weight of the lyophilized sample at '0' hours.

- ***In vitro* degradation profile of DCL hydrogels**

Briefly, the DCL hydrogels were frozen at -20 °C for 24 hours and subsequently lyophilized for 12 hours and their dry weights were recorded. Thereafter, the DCL gels were transferred into 6 well plates containing degradation media prepared using PBS (pH 7.4) supplemented with 1µg/mL collagenase type I, 20µg/mL sodium citrate at 37 °C in order to resemble physiological plasma conditions. At fixed time intervals of 1, 4, 7, 10, and 14 days, the DCL hydrogels were retrieved and again lyophilized. The decrease in weight was carefully recorded and expressed in terms of percentage of initial mass remaining using equation 2.

Remaining mass % = (W_2/ W_1)* 100 ---------- (2)

where, W_2 is the weight of the samples after 't' hours of incubation in degradation media and W_1 is the weight of the lyophilized sample at '0' hours.

- **Immunocompatibility assessment**

The DCL hydrogels were transplanted into control C57BL/6 mice sub-cutaneously in three different regions (~50µL) and 24 hours later the animals were euthanized, and blood was withdrawn under sterile conditions. The blood was then treated with RBC lysis buffer, incubated for 5-10 minutes and washed with PBS at 3500rpm for 5 minutes to separate the immune cells. Later, the number of cells/ mL of the blood was estimated by adding a cocktail of fluorescent marker tagged Flow-cytometry specific antibodies (APC-Cy7 rat anti-mouse CD8a, AlexaFlour 647 rat anti-mouse F4/80, PE rat anti mouse Ly6C, PE Cy7 Hamster anti mouse CD3, FITC rat anti mouse CD4) (BD biosciences) and incubated to a suspension of 1 million cells. Percent positive cells acquired were quantified using the Flow-Jo software.

3. Preparation of the CCl_4-induced chronic liver injury mice models

For the preparation of CCl_4 induced chronic injury model, intraperitoneal injection of CCl_4 in 8-week-old C57BL/6 mice was given thrice a week for three weeks (1mL/kg body weight). The level of liver damage was estimated with clinical parameters such as aspartate aminotransferase (ALT). The activity of liver enzymes in serum was measured using institutional protocols. Histopathological staining was performed to investigate the liver tissue architecture as mentioned previously[5]. Briefly, liver tissues were fixed with formalin. The fixed tissue samples were embedded in paraffin and sectioned into slices with 5 µm thickness, which were deparaffinized and rehydrated with different concentrations of ethanol followed by brief washing and staining of cell nuclei with hematoxylin solution. After rinsing with distilled water for 5 minutes, the stained samples were incubated in 0.1% HCl-ethanol. The samples were further counterstained with eosin solution for 2 minutes. After washing and dehydration, the stained sections were mounted for imaging.

4. Primary Hepatocyte Isolation and Characterization

Hepatocytes were isolated by non-recirculating collagenase perfusion through the portal vein from C57BL/6 mice as previously described in our study[6]. Isolated primary hepatocytes were counted, and the viability was checked by trypan blue exclusion, later kept on a 24-well collagen-coated plate at a cell density of 3.5×10^4 cells for 24 hours. Besides this, approximately 10^6 were taken as cell suspension and filtered through a 100 µm nylon mesh to remove the cell

clumps, for transplantation. The hepatocytes were resuspended in DMEM on ice prior to transplantation, mixed with the DCL and transplanted within 3hours after isolation. Control hepatocytes were characterized by the expression of albumin on day 2 after isolation. Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and were permeabilized using 0.1% Triton-X-100 in PBS for 5 minutes. Cells were then treated with blocking buffer (1mg/ml BSA in PBS) for 30 minutes and then incubated with primary albumin antibody (1:200 in blocking buffer) at 4 °C overnight. Following three washes with PBS, cells were incubated with rhodamine labeled secondary antibody (1:500 in blocking buffer) at room temperature for 1hour. After washing, cells were stained with DAPI (Life Technologies) and imaged using a fluorescence microscope Invitrogen™ EVOS™ FL Auto Imaging System by Thermo Fisher Scientific (Model: AMAFD1000).

5. Intrahepatic cholangiocyte organoids (ICO) Culture

The isolated Intrahepatic cholangiocytes were mixed with matrigel (50×10^5 cells/ 50 µL) and allowed to solidify at 37 ˚C for 20 to 30 minutes. The ICO were then cultured in complete DMEM media with the growth factors listed below **(Table S1)** along with 1% antibiotic and 1% antimycotic for upto 14 days.

6. Characterization of the ICO

The viability of the cultured ICO was determined by Calcein/PI whole mount staining. The organoids cultured on confocal dishes were washed with PBS (1x - pH 7.4) thrice for 5 minutes each and were incubated with Calcein (1 µM) (Thermofisher scientific) and PI (1 µM) (Thermofisher scientific) for 30 minutes followed by PBS washes, thrice. They were then viewed under a confocal microscope (Leica LAS X) at 494 nm and 547 nm wavelength for Calcein and PI respectively.

The functional assessment of the ICO was determined by immunostaining. Whole mount staining of the ICO was performed on the cultured organoids with GGT marker. The organoids were first washed gently with PBS and fixed with 4% PFA for 30-40 minutes followed by three PBS washes, then they were permeabilized with 0.1% Triton-X-100 in PBS for 5 minutes. they were then treated with blocking buffer (1mg/mL BSA in PBS) for 30 minutes and then incubated with primary albumin antibody (1:200 in blocking buffer) at 4 ºC overnight. Following three washes with PBS, cells were incubated with rhodamine labeled secondary antibody (1:500 in blocking buffer) at room temperature for 1hour. After washing, they were stained with DAPI (Life Technologies) and imaged using confocal microscope (Leica LAS X). H&E and CK19 staining were performed on the cryosectioned organoids, for which the organoids were first picked from the matrigel dome using autoclaved cut-tips and incubated in 4% PFA overnight at room temperature. Later, the organoids were transferred to 20% sucrose solution and incubated at 4 ºC for 2-3 days. Later, the organoids were transferred to cryomolds containing Tissue Tek OCT compound and kept in -20 ºC for freezing. The frozen moulds were then cut in the cryotome as 5 µm thin sections. The sections were then mounted on to glass slides and fixed for further processing.

7. Assessment of engraftment efficiency

To track the successful engraftment of transplanted cells we transplanted GFP labelled Heps and ICO mixed with 10mg/ml DCL (10^6 cells/200µL of DCL) into the subcapsular region of left lateral lobe in chronic liver injury mice models (n=6/ group) and 14 days post transplantation the animals were euthanized. The transplanted liver lobes were then processed and cryosectioned. The processed tissue sections were then imaged with the confocal microscope

(Leica LAS X). The GFP positive area from each field was quantified using ImageJ software by adjusting the threshold of green vs blue signal (DAPI). Three to five fields in each group were quantified and the graphs were plotted with the help of GraphPad Prism.

8. Histological Characterization of transplanted liver lobes

Characterization of the cell-transplanted liver tissues was performed by histological techniques. Small pieces of liver tissues were randomly cut and fixed with 4% formaldehyde, dehydrated, and embedded in paraffin for histological staining. 5µm thick sections deparaffinized and stained with H&E and MT were examined under a light microscope.

9. Immunohistochemistry

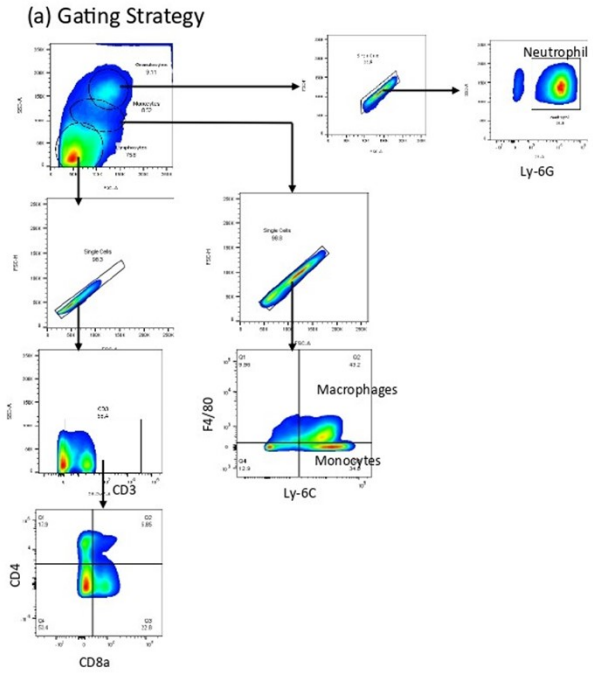
The cell transplanted liver tissue sections were fixed in 10% buffered formalin solution for 24 hours, embedded in paraffin, and thin sections of 4µm thickness were prepared. They were then deparaffinized with xylene followed by gradual hydration with alcohol series. They were rinsed with running tap water thoroughly. Antigen retrieval was performed with Tris EDTA (pH 9). Blocking of endogenous peroxidase was performed in 3% hydrogen peroxide (H_2O_2) containing buffered solution of casein and sodium azide (pH 7.6) for 10 minutes at room temperature to avoid non-specific binding of secondary antibodies. Intrinsic peroxidase was inactivated for 10 minutes with 3% H_2O_2 and rinsed with Tris buffered saline (TBS, 1/15 mol/l, pH 7.6). The sections were incubated overnight at 4 °C with CD31 (Elabscience, 1:200) and Proliferating cell nuclear antigen (PCNA) (1:200, Path n situ), as primary antibodies followed by a reaction for 30 minutes at 20 °C using a biotinylated secondary antibody. After washing, the tertiary antibody was used for 20 minutes. Sections were rinsed first with TBS and then under running tap water. Then, mixed solution of 3, 3-diaminobenzidine tetra hydrochloride (DAB) substrate (in dark) was used for color development (visualization) of the reaction product. Sections were further counterstained with hematoxylin for 1 minute, dehydrated and mounted with Dibutylphthalate Polystyrene Xylene (DPX) and observed under the microscope. Average of 5-6 fields were taken from 2-3 different tissue sections for analysis. Quantification of PCNA positive cells and CD31 positive area were determined with ImageJ software n=5 fields / group.

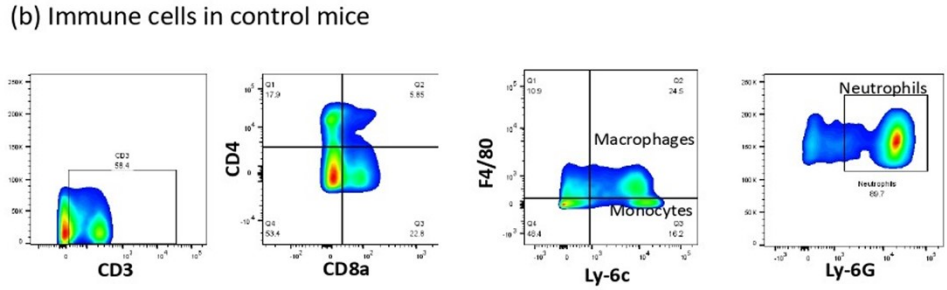
SUPP TABLES

COMPONENT	CAT NO.
1% B27	17504044
1% N2	17502048
30% R-spondin conditioned media from cells	SCC111
L-glutamine (2 mM)	25030081
HEPES (10 mM)	15630130
Nicotinamide (10 mM)	N0636
N-Acetyl-cysteine (1.25 mM)	A9165
HGF (25 ng/mL)	100-39H
Forskolin (10 µM)	6652995
A8301 (5 µM)	9001799
EGF (50 ng/mL)	AF-100-15
FGF10 (100 ng/mL)	100-18B

Table S1- Media composition for ICO culture

SUPP FIGURES





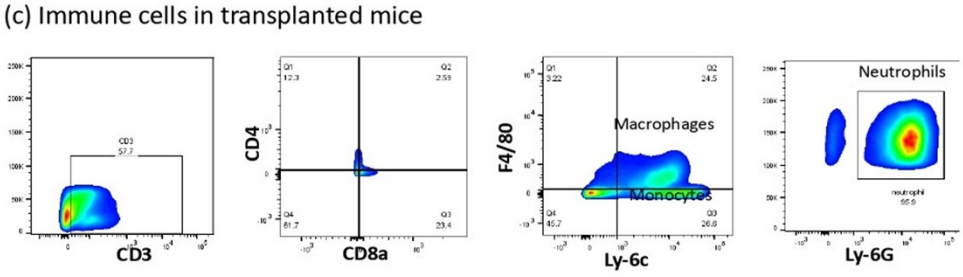


Fig S1. Immunocompatibility assessment of the DCL hydrogels. a) Gating strategy applied to determine the percentage of immune cells in control and transplanted animal's blood circulation post-DCL transplantation. **b)** Representative graphs showing percentage of immune cells in control mice. **c)** Representative graphs showing percentage of immune cells in DCL transplanted mice. All estimates performed in triplicates.

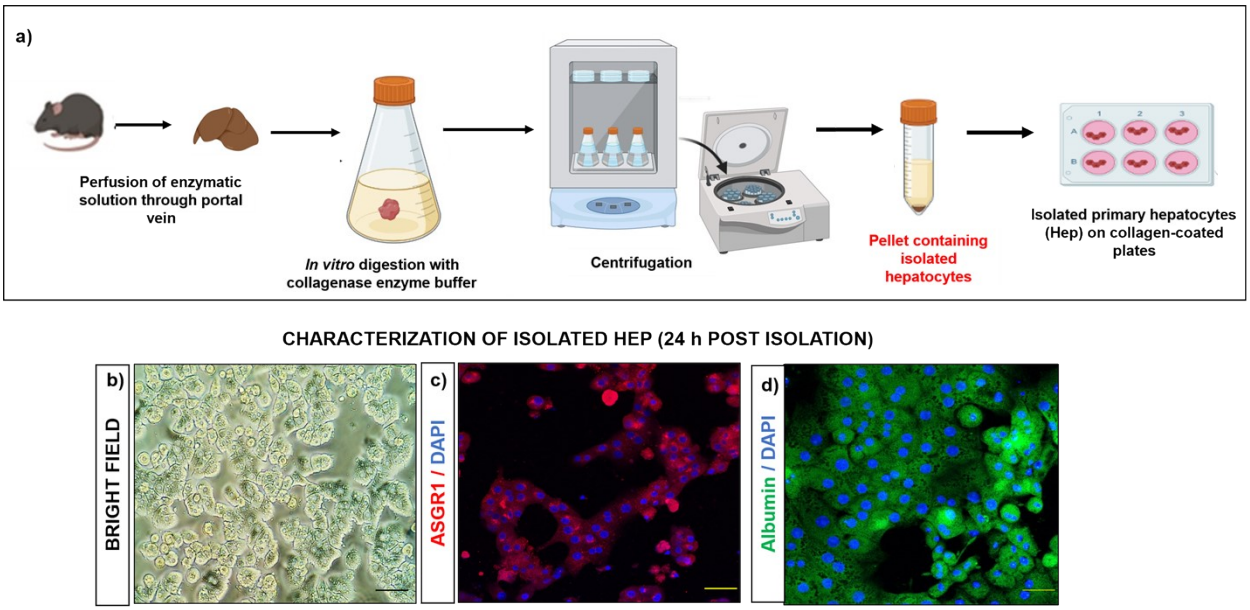


Fig S2. Isolation and characterization of primary hepatocytes. a) Schematic representation of the isolation method used to obtain primary hepatocytes. **b)** Bright-field images of isolated primary hepatocytes, 24 hours post isolation; scale bar 100 µm. **c)** Isolated primary hepatocytes characterized by ASGR1 immunostaining; scale bar 100 µm **d)** Isolated Hep characterized by Albumin immunostaining; scale bar 100 µm.

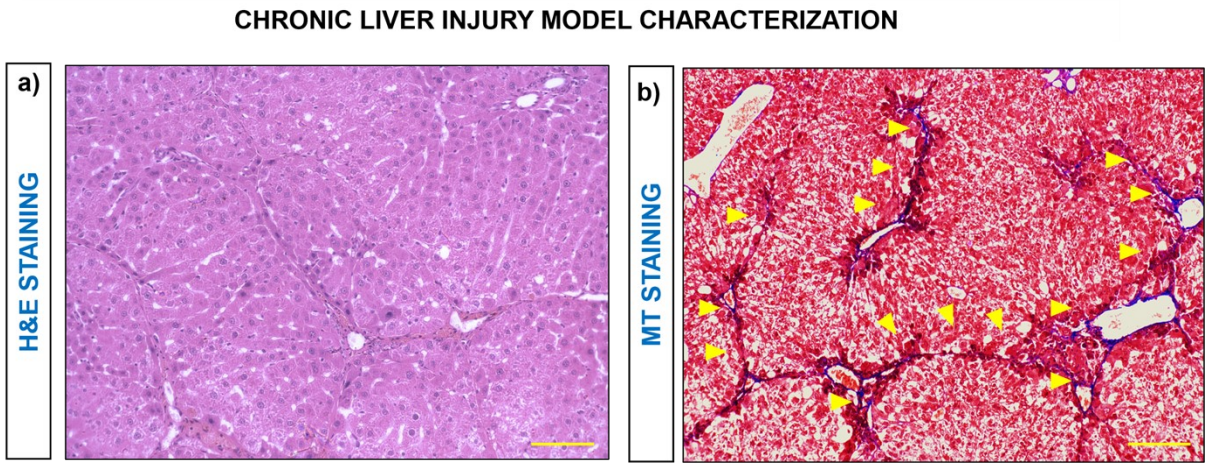


Fig S3. Characterization of chronic liver injury models. a) H&E staining **b**) MT staining obtained from chronic liver injury models showing damaged liver tissue architecture and F2 stage fibrosis after 3 weeks of CCl_4 (1mL/kg B.W thrice a week). arrows showing collagen deposition; scale bar 200 µm

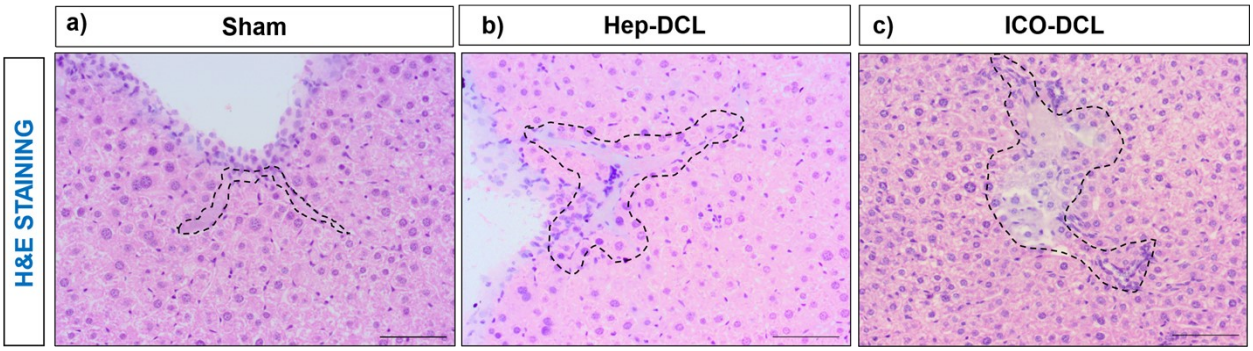


Fig S4. Histological characterization of the transplanted liver lobes two weeks post-transplantation. H&E staining of **a)** Sham **b)** Hep-DCL **c)** ICO-DCL group (dotted lines represent the transplanted material) ; scale bar 200 µm

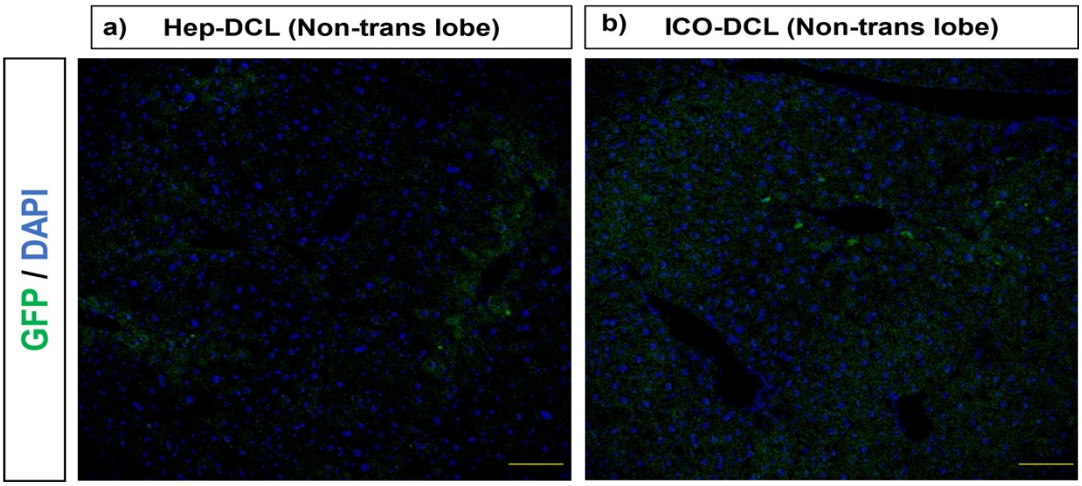


Fig S5. Assessment of cell migration in the non-transplanted liver lobes two weeks post-transplantation. a) Hep-DCL group non-transplanted liver lobe **b)** ICO-DCL group non-transplanted liver lobe; scale bar 200 µm

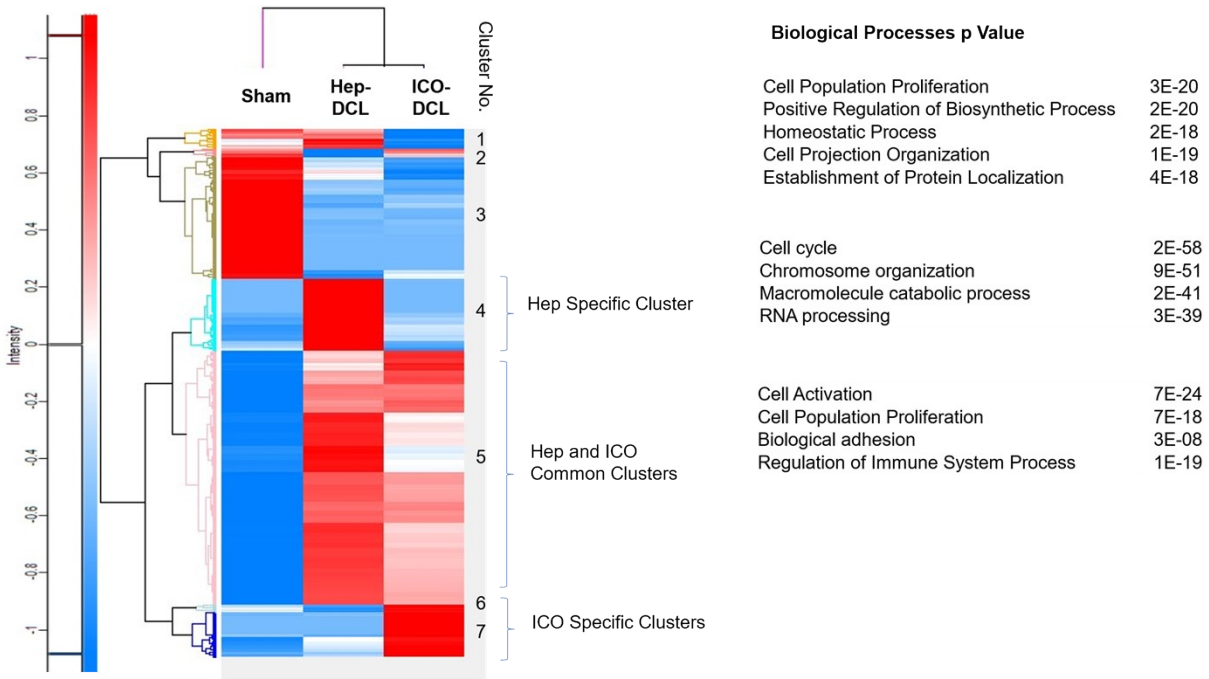


Fig S6. Hierarchical clustering analysis and Gene Ontology-Biological process enrichment analysis showing the major biological pathways in clusters specific for sham, Hep and ICO-DCL clusters (n=3).

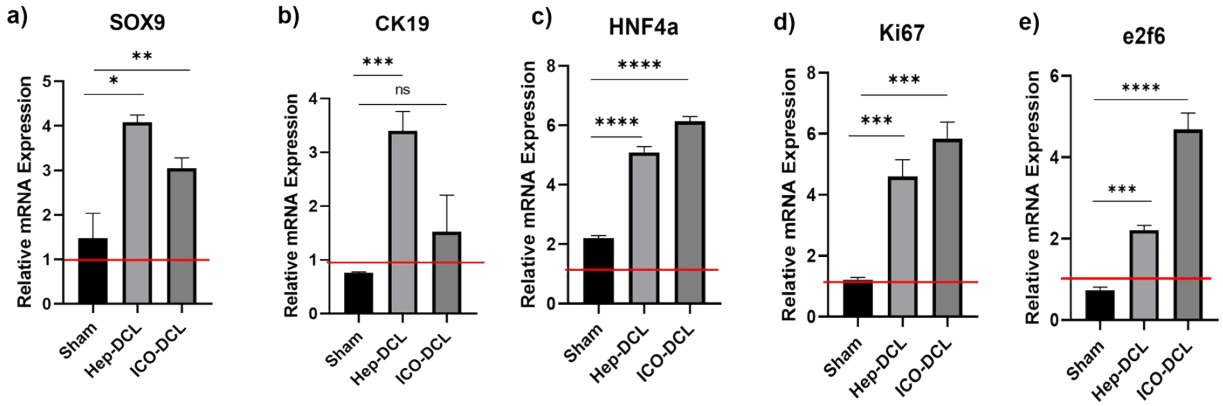


Fig S7. RT-PCR analysis of transplanted liver tissues to validate the RNAseq data. Biliary markers **(a)** SOX9 (SRY-box transcription factor 9), **(b)** Progenitor cell markers**,** CK19 (Cytokeratin 19), **(c)** Hepatocyte differentiation marker, HNF4a; Liver regeneration markers **(d)** Ki67, **(e)** e2f6 (eukaryotic transcription factor 6). Graphs plotted with Graphpad prism; Data represented as sham vs transplanted lobes (normalized with non-transplanted control mice liver represented as red line); Statistical analysis performed by student t-test (**P≤0.001, ***P≤0.0001, *P≤0.01, ****P<0.0001) (n=3).

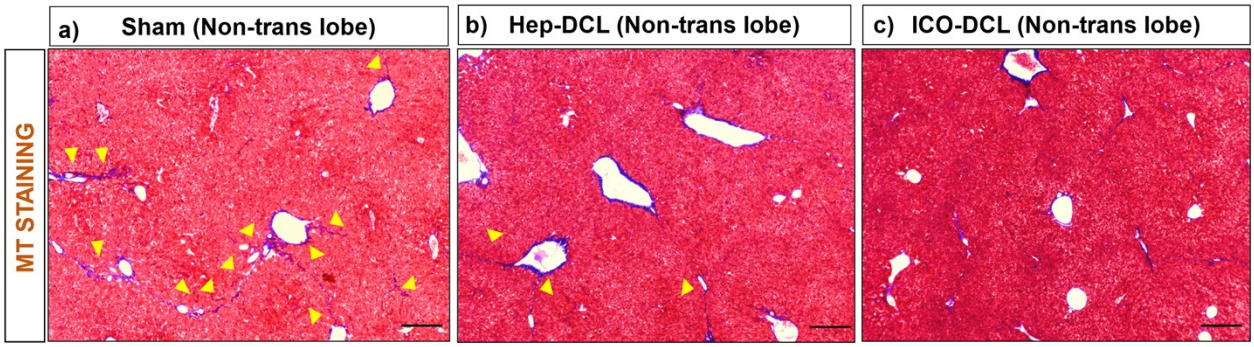


Fig S8. Assessment of fibrosis in the non-transplanted liver lobes two weeks post-transplantation. a) Sham **b)** Hep-DCL group non-transplanted liver lobe **c)** ICO-DCL group non-transplanted liver lobe; scale bar 200 µm (arrows showing collagen deposition)

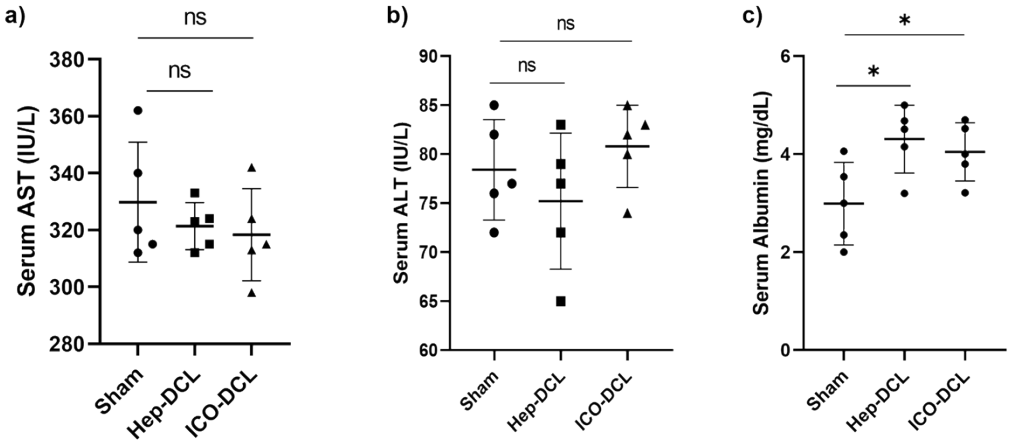


Fig S9. Serum biochemical investigations two weeks post-transplantation. a) Serum AST (aspartate aminotransferase) levels in sham and transplanted animals. **(b)** Serum ALT (alanine transaminase) levels in sham and transplanted animals (P value sham vs Hep-DCL, and ICO-DCL **c)** Serum albumin levels in sham and transplanted animals. Graphs plotted with Graphpad prism; Statistical analysis performed by student t-test (*P≤0.05), (n=5).