Functional maturation of iPSC-hepatocytes in extracellular matrix — A comparative analysis of bioartificial liver microenvironments

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Supplemental Materials and Methods:

Western blot analysis; growth factor, albumin, and AFP detection

Growth factor content was determined in both the native and decellularized ECM scaffolds, as well as the 0.25 mg/ml growth factor reduced Matrigel® overlay and the type 1 collagen used to make sandwich and PLLA-collagen environments, as previously described [1]. The concentration of basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) in urea–heparin extracts was evaluated with the respective enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Minneapolis, MN) per the manufacturer's protocols.

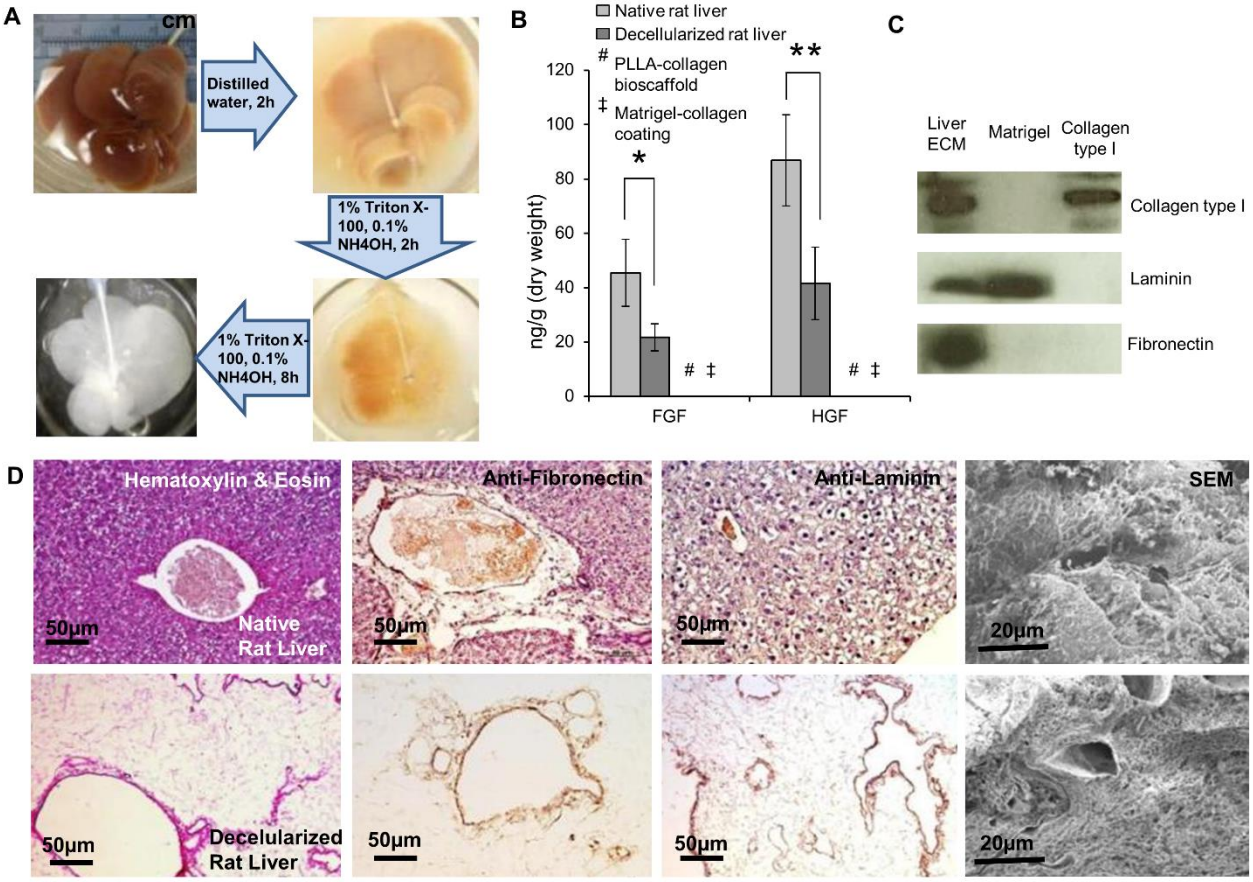
The concentration of human albumin and alpha fetoprotein (AFP) secreted into the culture medium was determined in the culture media collected from each flask containing scaffolds or from each well of the sandwich control group before medium exchange. Samples were centrifuged at 500g to separate debris. Albumin and AFP were measured by an albumin ELISA kit (Bethyl Laboratories, Montgomery TX) or an AFP human ELISA kit (Abcam, Cambridge, MA), respectively. Albumin and AFP synthesis is expressed as the amount of albumin or AFP produced per 10^6 cells per day using the total cell number per bioscaffold at each time point obtained from the Picogreen measurement of cell density.

The presence of ECM-associated proteins was determined by western blot analysis of individually homogenized scaffolds or Matrigel and type 1 collagen in lysis buffer (Thermo Scientific, Pittsburgh, PA) containing 2% Halt phosphatase inhibitor (Thermo Scientific, Pittsburgh, PA) and 5% protease cocktail inhibitor (Sigma Aldrich, St. Louis, MO). Protein concentrations were quantitated and normalized using a bicinchoninic acid assay (Sigma Aldrich, St. Louis, MO). Primary antibodies for laminin B2 gamma 1, fibronectin, or type 1 collagen (all at 1:2000, Abcam, Cambridge, MA) were used. Bound antibody was revealed with goat anti-mouse IgG (HRP) or goat anti-rabbit IgG (HRP) (1:10000, Abcam, Cambridge, MA)

and developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Biosciences, Pittsburgh, PA).

Supplemental Figures and Legends:

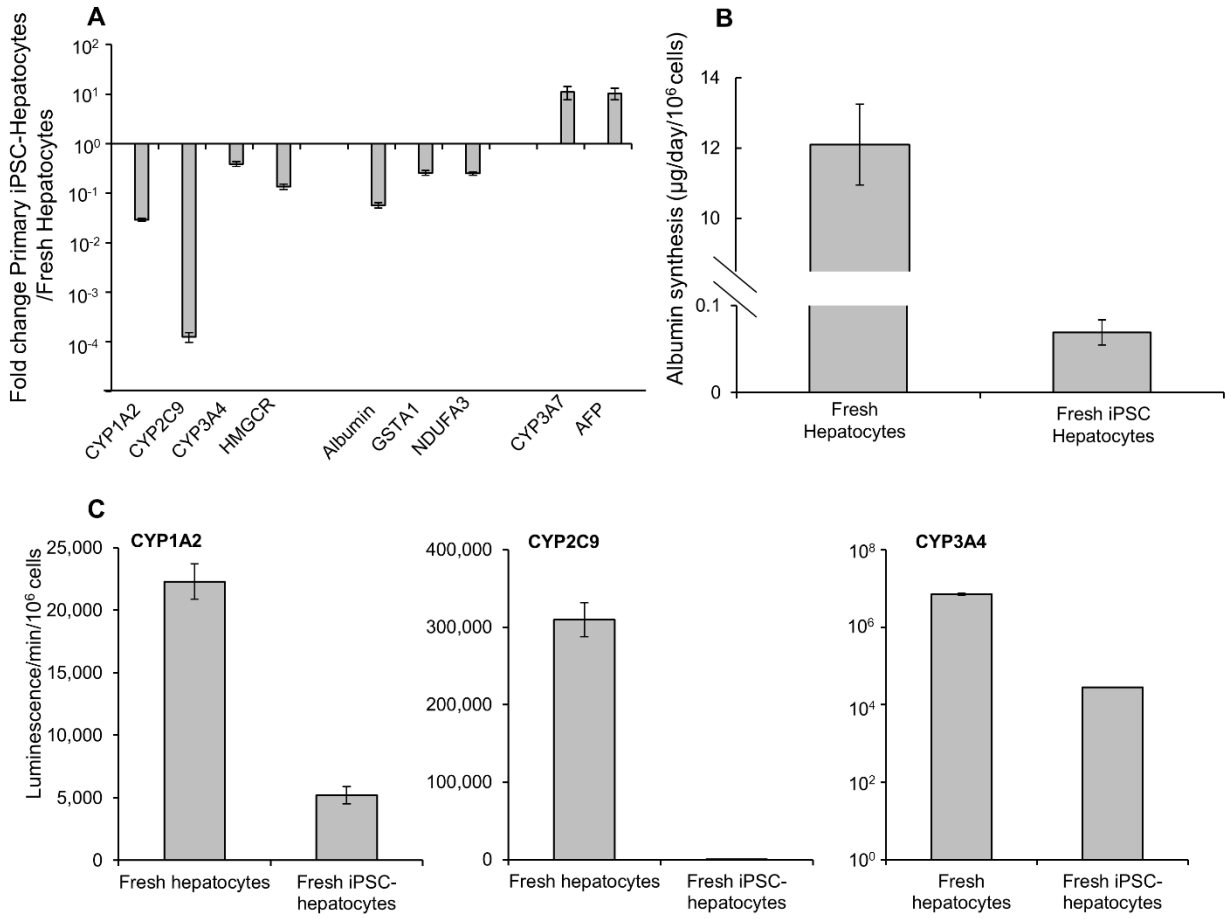
Supplemental Fig. 1:



Supplemental Fig. 1. Preparation and characterization of liver ECM. (A) Liver decellularization was carried out by sequential perfusion steps using reagent mixtures depicted in the figure; (B) growth factor content of native and decellularized liver, PLLA-collagen bioscaffold (#), and matrigel-collagen coating (‡) used for the sandwich control (n=4 for each group); (C) Western blot depicting content of ECM proteins within each scaffold environment; (D) H&E staining of native, untreated rat liver and decellularized liver ECM reveals complete

lack of cells after decellularization; anti-fibronectin and anti-laminin staining of the native rat liver and the decellularized liver ECM demonstrates that fibronectin and laminin are strongly expressed around vasculature remnants and Glisson's capsule (scale bar: 50μm) and SEM images of native rat liver and decellularized rat liver ECM depicting a porous ECM scaffold (* $p<0.05$, ** $p<0.01$).

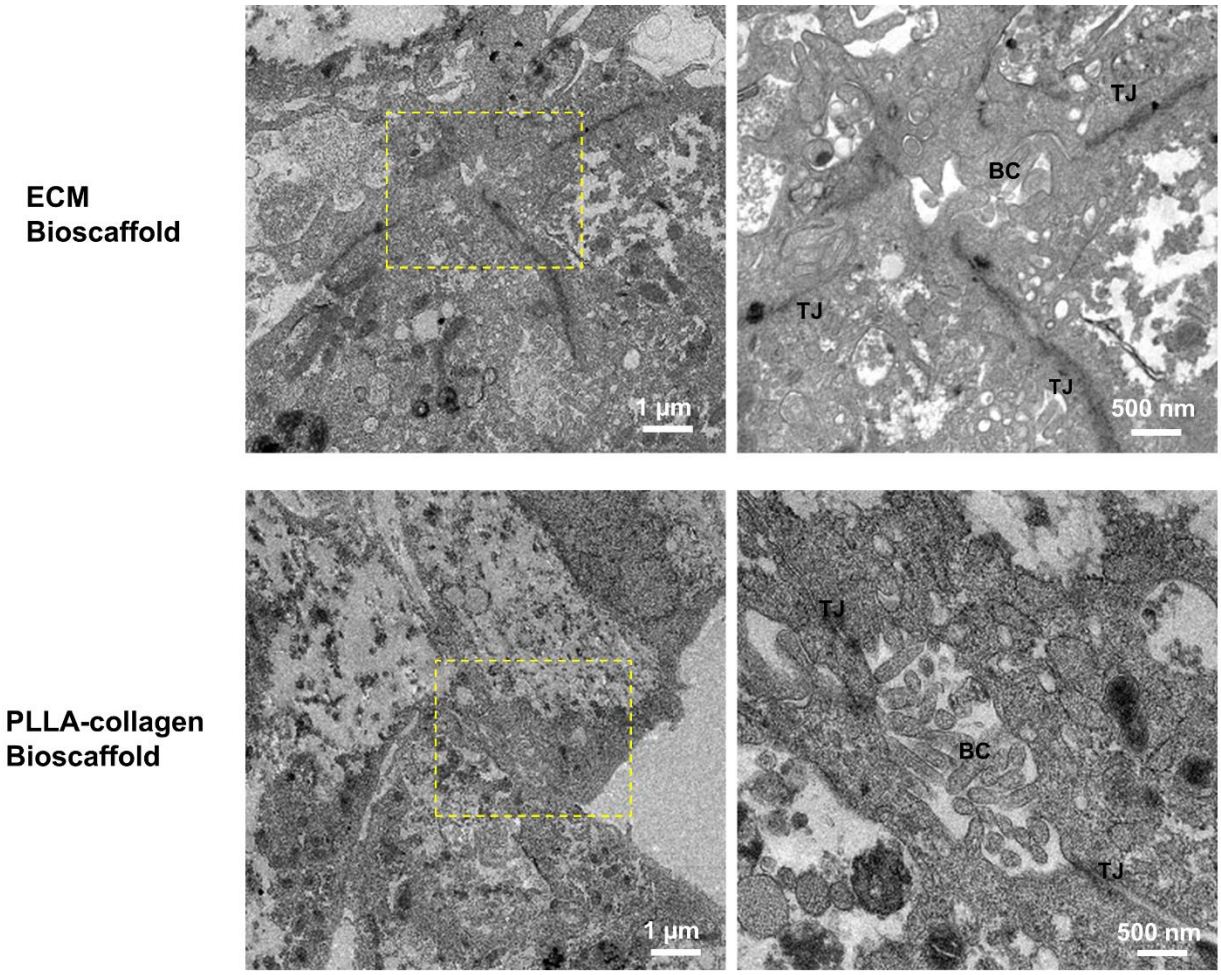
Supplemental Fig. 2:



Supplemental Fig. 2. Baseline characterization of iPSC-hepatocytes: (A) Baseline characterization of fresh iPSC-hepatocytes showing expression of synthetic and metabolic genes that are decreased relative to fresh primary hepatocytes; (B) Albumin synthesis by fresh primary hepatocytes at day 0 compared to iPSC-hepatocytes at day 0; and (C) Decreased

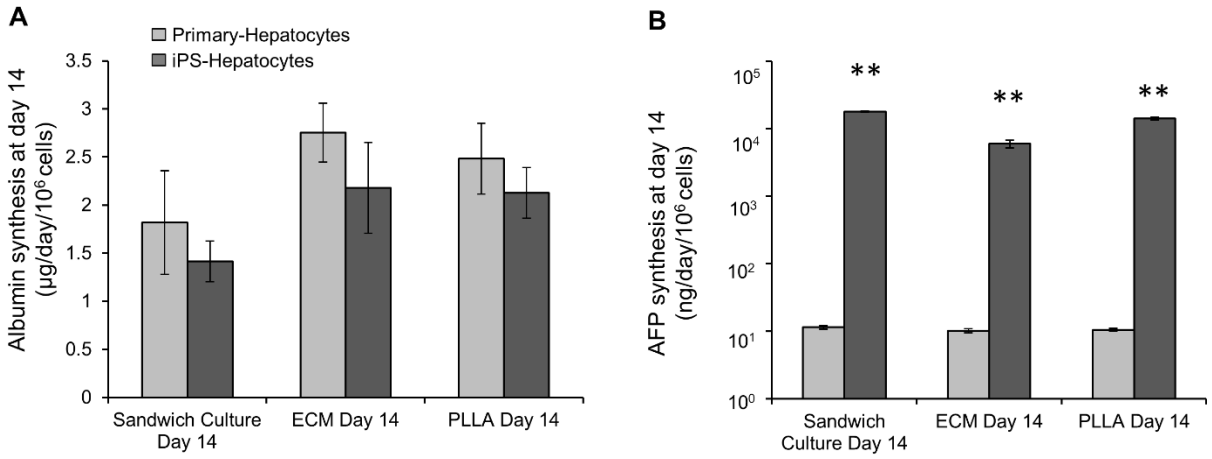
baseline activity of P450 isotypes (CYP1A2, CYP2C9 and CYP3A4) in day 0 iPSC-hepatocytes compared to day 0 fresh primary hepatocytes (n=4 for each group).

Supplemental Fig. 3:



Supplemental Fig. 3. iPSC-hepatocytes develop cell-cell contacts and biliary canaliculi within 3D scaffolds: Low magnification TEM of several adjacent iPSC-hepatocytes in ECM or PLLA-collagen scaffolds at day 7 (left). High magnification of yellow dashed box shows biliary canaliculi-like structures (right) flanked by tight junctions (LG = Lipid globule, BC= biliary canaliculi-like structure, TJ = tight junction, M = mitochondria).

Supplemental Fig. 4:



Supplemental Fig. 4. Comparison of albumin and AFP expression in iPSC-hepatocytes and primary human hepatocytes: (A) Albumin and (B) AFP biosynthesis in iPSC-hepatocytes relative to cryo-preserved primary hepatocytes after 14 days of culture in sandwich control culture or ECM or PLLA-collagen scaffolds (** $p<0.01$, $n=4$ for each group).

Primers used in Quantitative RT-PCR:

Primer sequences design (5'-3') are:

Cyclophilin: F(forward)-CACAGGAGGAAAGAGCATCTAC

R(reverse)-CACAGACGGTCACTCAAAGAA;

HMGCR: F-CCTGTTGGAGTGGCAGGACCC

R-TGGTGCTGGCCACAAGACAACC;

ALB: F-AGCATGGGCAGTAGCTCGCCT

R-AGGTCCGCCCTGTCATCAGCA;

CYP2C9: F-GGATTTGCCTCTGTGCCGCC

R-GCAGCCAGGCCATCTGCTCTT;

CYP1A2: F-TCCCAGTCTGTTCCCTTCTCGGC

	R-TTGACAGTGCCAGGTGCGGG;
GSTA1	F-GTTTCTACAGCCTGGCAGCCCA
	R-AGTTCTTGGCCTCCATGACTGC;
NDUFA3:	F-CGTGTCCTTCGTCGTCGGGG
	R-CTGGGCACGTCGGGCATGTT;
AFP:	F-GCTGACCTGGCTACCATATTT
	R-GGGATGCCTTCTTGCTATCTC;
CYP3A7:	F-CTGAGAAGTTCCTCCCTGAAAG
	R-GCACGTACAGAATCCCTGATTA;
CYP3A4:	F-CTGCTTCTCACGGGACTATTT
	R-CCTCCCAAACTGCTAGGATTAC.

Supplemental References:

1. Soto-Gutierrez A, Zhang L, Medberry C et al. A whole-organ regenerative medicine approach for liver replacement. **Tissue Engineering Part C, Methods**. 2011;17:677-686.