Long-Term Culture of Human Liver Tissue with Advanced Hepatic Functions

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Supplementary Figure Legends

Supplementary Figure 1. ICC scaffold demonstrates uniform porosity and porous structure across z-axis.

Supplementary Figure 2. Surface functionalization of ICC scaffolds with type I collagen.

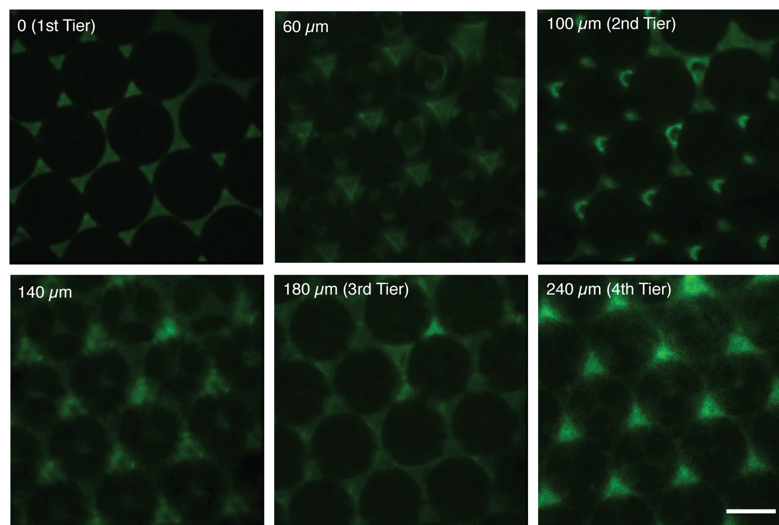
Supplementary Figure 3. HepG2 cells do not exhibit significant CYP activity compared to primary liver cells, while FTLC in Col I-ICC scaffolds maintain drug metabolizing activity for months.

Supplementary Figure 4. Col-ICC facilitates the maturation of adult liver metabolic activities.

Supplementary Figure 5. Infection of ICC cultures with cell culture-produced HCVcc genotype 2a. The engineered liver tissue supports the full life cycle of J6/JFH1 HCV genotype 2a (HCVcc) infection.

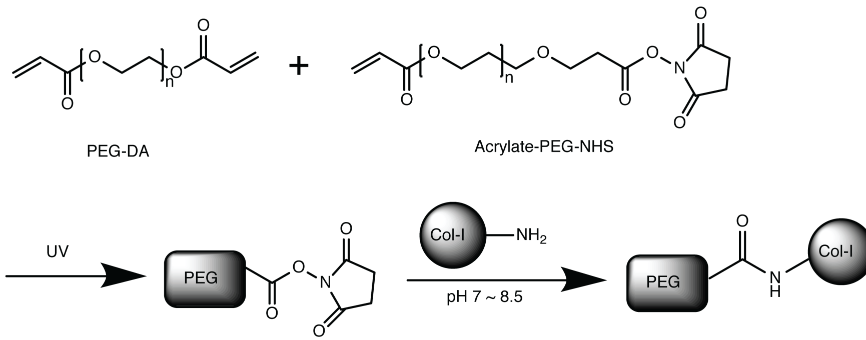
Supplementary Table 1. Comparison of the presently described ICC system with current state-of-the-art *in vitro* liver models based on primary human cells.

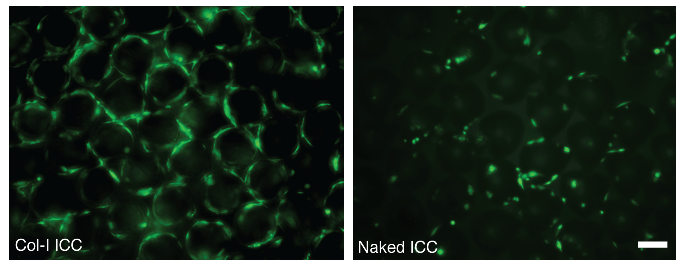
Supplementary Figure 1. ICC scaffold demonstrates uniform porosity and porous structure across the z-axis. Fluorescein-conjugated-PEG was incorporated into ICC to enable confocal microscopic imaging. Uniform pore size, 3D hexagonal arrangement and high-interconnected windows were observed across 4 tiers of ICCs that span across 240 μm in z-axis.



Supplementary Figure 2. Surface functionalization of ICC scaffolds with type I collagen. (**A**) 10 wt.% of N-hydroxysuccinimide (Acrylate-PEG-NHS) was incorporated into diacrylate-PEG (PEG-DA) to provide amine-reactive ligand for type I collagen (Col) conjugation. (**B**) HUVECs transduced with lentiviral EGFP reporter were seeded into Col-I ICC and reached confluence in 1 day post-seeding, whereas minimal cell attachment was observed on Naked ICC (scale bar, 100 μm). (**C**) EGFP-HUVECs established a confluent morphology across multiple tiers in ICCs 1 day post-seeding, as demonstrated by confocal microscopy (scale bar, 100 μm). Statistical significance was analyzed by one-way ANOVA with Tukey's post test: ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, N.D. none detected.

A





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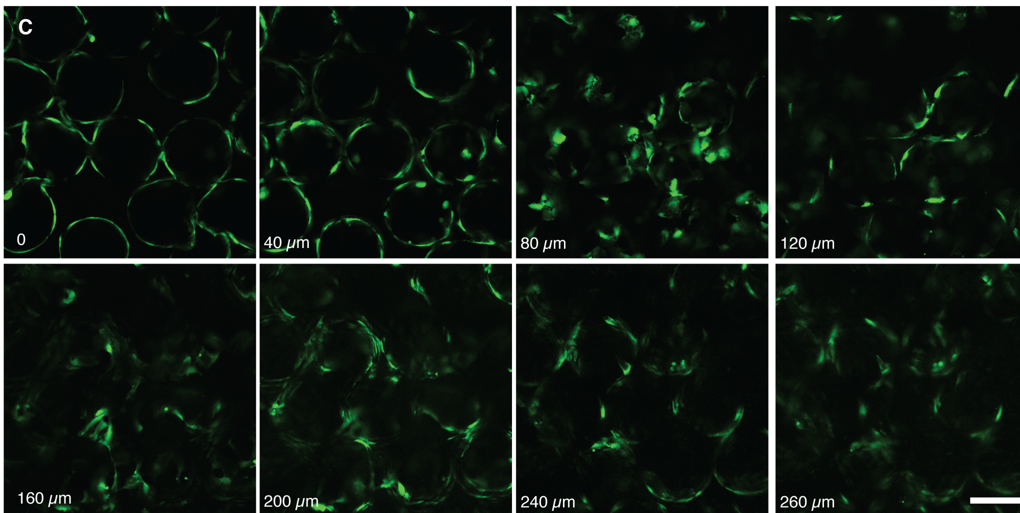
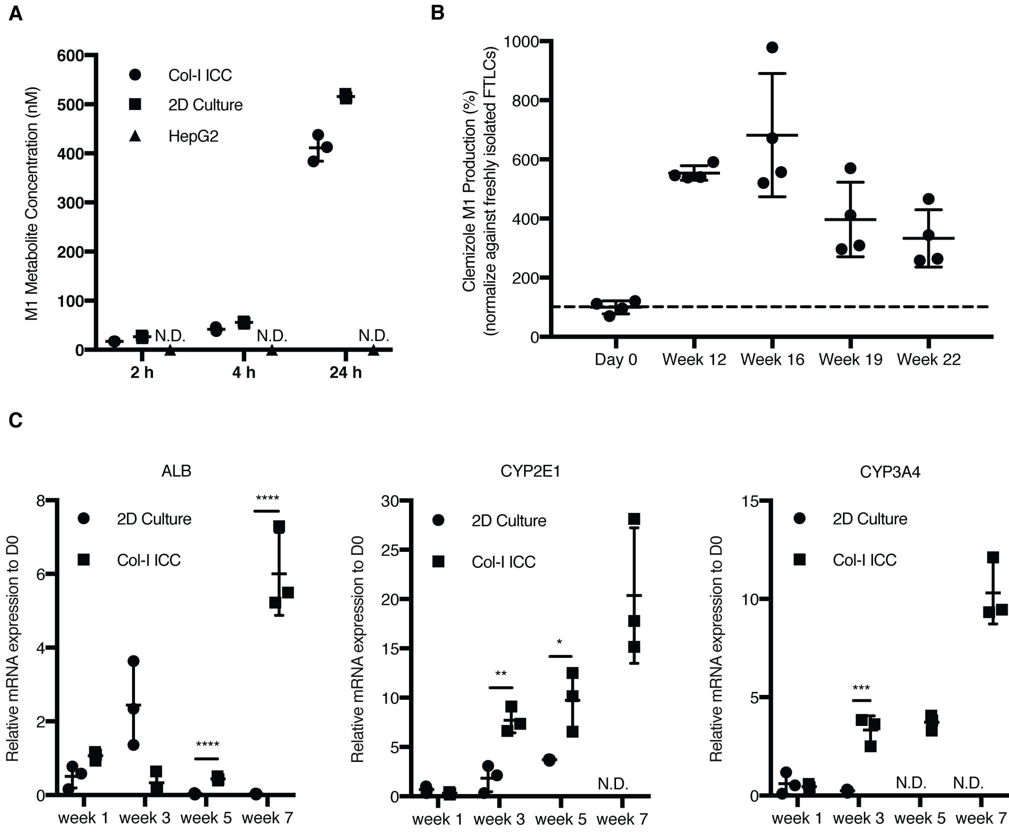
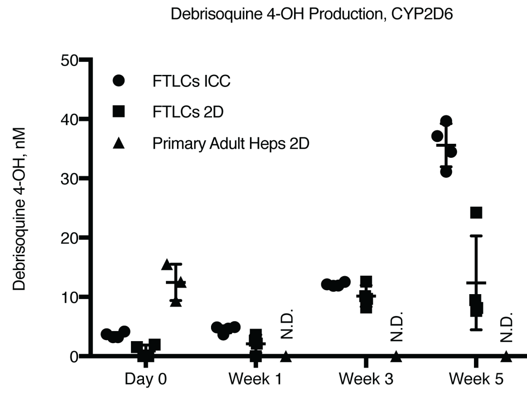


Supplementary Figure 3. HepG2 cells do not exhibit significant CYP activity compared to primary liver cells, while FTLC in ICC-Col I scaffolds maintain drug metabolizing activity for months. (**A**) Freshly isolated FTLCs in different platforms and HepG2 on 2D tissue culture plates were treated with Clemizole. Supernatants were collected at 2, 4 and 24 hours to monitor the production of M1 metabolites, n=3. (**B**) Long-term preservation of metabolic function was assessed by the production of clemizole M1 metabolite. FTLCs in Col-I ICC were treated with Clemizole for overnight at day 0, 85 and 112, 133 and 148 post-seeding, and assayed for M1 metabolite with LC/MS. Results were normalized against freshly isolated FTLC (day 0 value), n=4. (**C**) Long term expression of hepatocyte markers in the ICC cultures. RT-qPCR assays for hepatocyte-specific markers (ALB, CYP2E1, CYP3A4) performed up to 7 weeks post seeding in ICC vs. standard 2D cultures. Statistical significance was analyzed by unpaired t test two-tailed: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0005$, N.D. none detected.


A
M1 Metabolite Concentration (nM)
600
500
400
300
200
100
0
Col-I ICC
2D Culture
HepG2
N.D.
2 h
4 h
24 h
B
Clemizole M1 Production (%)
(normalize against freshly isolated FTLCs)
1000
800
600
400
200
0
Day 0
Week 12
Week 16
Week 19
Week 22
C
ALB
CYP2E1
CYP3A4
Relative mRNA expression to D0
2D Culture
Col-I ICC
****
**
*
***
N.D.
week 1
week 3
week 5
week 7

Supplementary Figure 4. Col-I ICC facilitates the maturation of adult liver metabolic activities. Cultures of FTLCs in Col-I ICC, FTLCs in standard 2D culture, or cryopreserved primary adult human hepatocytes in standard 2D culture were treated overnight with debrisoquine, s-mephenytion and clemizole at the indicated time points. The production of these compounds' major metabolites was then measured. (**A**) debrisoquine 4-OH metabolite generated from debrisoquine by CYP2D6 metabolism, n=4. (**B**) s-mephenytoin 4-OH metabolite generated from mephenytoin by CYP2C19 metabolism, n=4. (**C**) clemizole M1 metabolite generated from clemizole by CYP3A4 metabolism, n=3. N.D. none detected.

A



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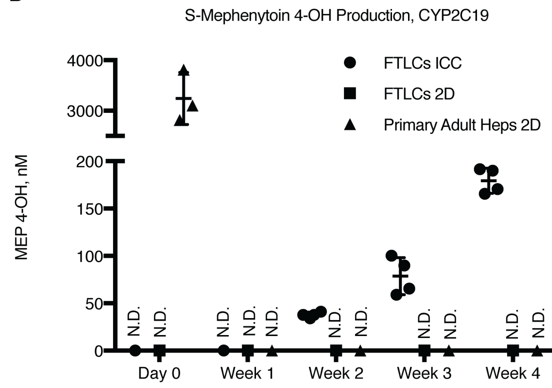

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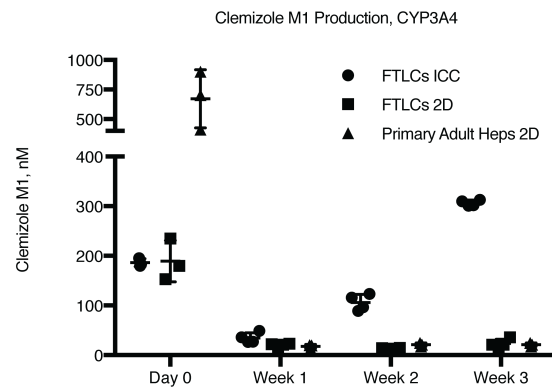
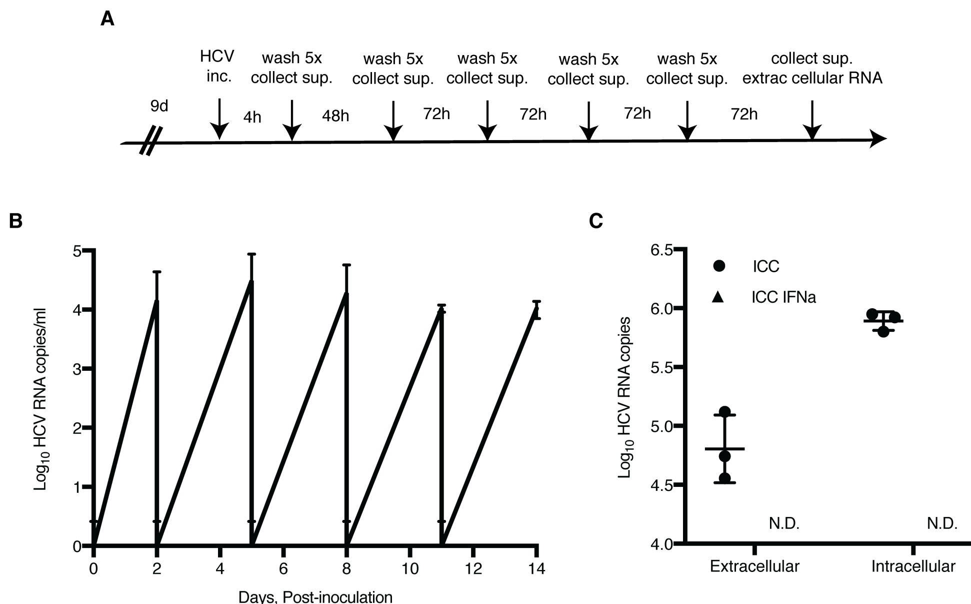


Supplementary Figure 5. Infection of ICC cultures with cell culture produced HCVcc genotype 2a. Human engineered liver supports full life cycle of J6/JFH1 HCV genotype 2a (HCVcc) infection. **(A)** Experimental set up for infection with HCVcc. FTLCs in ICC were inoculated with HCVcc at Day 9 post-seeding. At 4 h post-inoculation, virus was removed, cultures were washed five times with PBS and fresh medium was added; media samples were collected pre- and post-wash at the interval of 2 or 3 days. At Day 14 post-inoculation, cultures were terminated and intracellular RNA was extracted for analysis. **(B)** RT-qPCR HCV RNA results collected from the pre- and post-wash of HCVcc inoculated cultures demonstrate persistent HCV production in FTLCs Col-I ICC. **(C)** Parallel sets of cultures were treated with vehicle control or 50 unit/ml of interferon alpha (IFNa) from Day 1 post-inoculation. At Day 14 post-inoculation, IFNa treatment eradicated detectable HCV in both extra- and intracellular samples, n=3. N.D. none detected.



Supplementary Table 1. Comparison of the presently described ICC system with current state-of-the-art *in vitro* liver models based on primary human cells. An abundance of *in vitro* liver model systems have been reported in recent decades. However, many of such systems use liver cancer cell lines or immortalized hepatic cell lines, which often have lost key markers of their former differentiated state. As such, the gold standard for recapitulating advanced hepatic functions in culture systems remains human primary hepatocytes. In this table, we compare the ICC system with other liver cell cultures that only use human primary cells. To our knowledge, there are about 20 such reports. In particular, we compared the long-term performance *in vitro*, *e.g.*, how long the culture systems can support a hepatic phenotype (e.g. albumin production, liver-specific protein expression, etc.); how long they can maintain drug metabolism capability; and whether they can support HCV infection, and whether the infection is mediated by cell culture-derived HCV (HCVcc) or natural patient inoculums of HCV (in general, the use of HCV patient sera is considered more physiologically relevant than using HCVcc). Based on these specifications, the ICC system demonstrates superior features to the other systems in nearly all performance criteria. The ICC system has the potential to serve as a scalable platform for studies of long-term human-specific drug metabolism and toxicities, as well as to provide a highly capable liver model that simultaneously supports virus infection and testing of antiviral drugs.

Platform	Cell source	Hepatic phenotype	Drug metabolism	HCV infection	Reference
ECM-functionalized 3D ICC scaffold	Fetal total liver cells (16-22 w)	> 5 months	> 5 months	HCV patient sera	This study
Micropatterning co-culture	Adult hepatocyte, iPS	2-6 weeks	6 weeks	HCVcc	[1-3]
Collagen sandwich	Adult hepatocyte	2 weeks	2 weeks	N.A.	[4]
Hyaluronic acid with liver ECM extract	Adult hepatocyte	4 weeks	4 weeks	N.A.	[5]
Spheroid in bioreactor	Adult hepatocyte	3-4 weeks	4 weeks	N.A.	[6]
Microfluidic chip with endothelial-like barrier	Adult hepatocyte	7 days	< 7 days	N.A.	[7]
Decellularized organ	Fetal liver cell (17-21 w)	7 days	N.A.	N.A.	[8]
Cell sheet	Adult hepatocyte	3 days	3 days	N.A.	[9]
Nanopillar plate	iPS/ES	5 weeks	5 weeks	N.A.	[10]
Perfusion-based microfluidic chip	Adult hepatocyte	7 days	N.A.	N.A.	[11]
HepaChip[R]	Adult hepatocyte	3 days	3 days	N.A.	[12]
2D collagen matrix	Adult hepatocyte	3 weeks	N.A.	HCV patient sera	[13]
Alginate microencapsulation	Adult hepatocyte	7 days	3 days	N.A.	[14]

Hepatosphere	Adult hepatocyte	20 days	N.A.	N.A.	[15]
Collagen-I plate	Fetal liver cell (16-24 w)	4 weeks	N.A.	HCVcc	[16]
Perfusion bioreactor	Fetal liver cell (17-20 w)	10 days	N.A.	N.A.	[17]
Conditioned 2D culture	Fetal hepatocyte (8-17 w)	> 4 months	N.A.	HCV patient sera	[18,19]

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