AN ALL-HUMAN CELL BASED TRI-CULTURE SYSTEM DISTINGUISHED THROUGH MORPHOLOGY, LONGEVITY, AND FUNCTIONALITY

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Abstract

ABSTRACT

Several major limitations exist for the use of primary human hepatocytes (PHHs) in preclinical pharmacological and toxicological applications including maintenance of structural and functional properties of cells over prolonged periods of time, support of a multitude of lots from healthy and diseased tissues, and lack of a suitable culture platform that is convenient. Although the sandwich culture and co-culture systems have addressed some of these limitations, further advancement is needed. An all-human tri-culture system has been developed to address these limitations using primary human feeder cells (FCs) and PHHs.

Cryopreserved human feeder cells (FCs) consisting of two types of stromal cells were thawed and seeded onto a 24-well collagen coated plate. Cryopreserved adult PHHs were then thawed and plated onto the FCs, creating a Tri-Culture System (TCS). Albumin (Alb) and urea levels were measured through ELISA and a colorimetric assay. Cytochrome P450 (CYP) and phase 2 conjugation activities were determined by LC-MS/MS and P450 Glo assays. RT-PCR and IFC was performed for gene and protein expression, respectively. CDFDA staining was used for bile canaliculi functional activity.

A healthy and stable hepatocellular morphology and cytoarchitecture, including trabecular cluster formation, was maintained in PHHs from multiple adult donor lots for up to 42 days without an ECM overlay. Extensive anastomosing networks of bile canaliculi with tight and gap junctions were established during the initial 5 days of culture and remained stable throughout the remainder of the culture period. Physiological levels of Alb and urea secretion were measured over 21 days of culture period. Both Alb and urea production levels were significantly higher in the TCS (30-40 µg/day/10^6 cells and 55-70 µg/day/10^6 cells, respectively) compared to monoculture PHHs. Phase 1 and phase 2 metabolic functions (midazolam 1-hydroxylation rates: 10-20 pmol/min/10^6 cells, 7-ethoxycoumarin glucuronidation rates: 300-600 pmol/min/10^6 cells) were stable after 5 days for at least 2 weeks. Gene expression of *Alb*, *CYP1A2*, *CYP2B6*, and *CYP3A4* in the TCS PHHs at day 4 was 2-fold higher on average compared to the PHHs in the monoculture.

PHHs maintained in the Tri-Culture System exhibited enhanced cellular morphology, polarity, and functionality for > 2 weeks. This novel culture platform represents a convenient all-human hepatic culture system for toxicological and pharmacological testing applications.

INTRODUCTION

Existing limitations for finding suitable culture models or systems that mimic the *in vivo* liver microenvironment include the ability of the hepatocytes to maintain their morphology and functionality over prolonged periods of time.[1] Animal models can be used, but there are marked species differences in hepatic metabolic and receptor-signaling pathways.[2] Mono- and sandwich culture model systems also have corresponding serious limitations including the short- to mid-term loss of cell polarity and metabolic functions.[3] Co-culture and tri-culture models that currently exist combine hepatocytes with one or more different type of FCs. However, these systems use non-human FCs, primarily mouse or rat origin.[4] In this newly developed TCS, two different types of frozen primary human FCs and PHHs are used. The kit component of cryopreserved cells is convenient and has an ease of use for the researcher that allows control over the experimental work flow in a standard plate format. It can be set up in a matter of hours and tested in multiple applications that require a sustained hepatocyte polarity and phenotype.

MATERIALS & METHODS

Table 1. Donor information for PHH lots.

Donor ID	Sex	Age (y/o)	Ethnicity	BMI
A	F	31	Caucasian	37
B	M	54	Caucasian	36
C	F	28	Caucasian	26
D	F	27	Caucasian	31
E	M	23	African American	34
F	F	41	Caucasian	30
G	M	42	African American	31
H	F	48	Caucasian	24
I	M	56	Caucasian	29
J	M	40	Caucasian	28
K	F	48	Caucasian	24

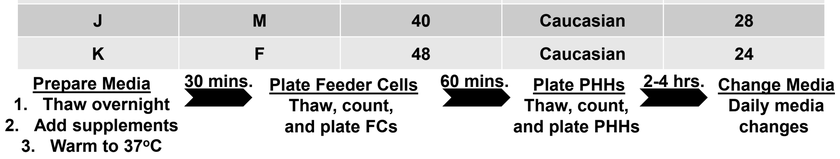

Figure 1. Setting up the TCS.

1. Expression of ZO-1, Connexin-32(CX-32), Cytokeratin 18 (CK18), and Alb were assessed by immunocytochemistry with antibodies from Thermo Fisher and Abcam respectively. 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain was used to stain nuclei. 5-(and-6)-Carboxy-2',7'-Dichlorofluorescein Diacetate (5µM) (CDFDA, Thermo Fisher) was used for bile canaliculi staining.
2. Cells were lysed in RLT buffer (Qiagen). Total RNA was isolated using the RNeasy Mini Kit (Qiagen). cDNA was prepped using the QuantiNova SYBR Green RT-PCR kit (Qiagen). PCR amplification was done on a StepOnePlus RT PCR System (Applied Biosystems) and analyzed with StepOne Software. Data was normalized to GAPDH and analyzed using the $2^{-\Delta\Delta C}{}_T$ method.
3. Supernatant was collected for Alb and urea detection. Alb was measured using an ELISA kit (Abcam), and a colorimetric assay (StanBio Labs) was used to measure urea synthesis.
4. CYP1A2, CYP2B6, and CYP3A4 activity were measured in PHHs after being induced for 48hrs with Omeprazole (100µM), CITCO (100nM), and Rifampicin (25µM) using a P450-Glo Assay (Promega).
5. Phase I metabolism was determined by LC MS/MS using Midazolam (15µM) as a probe. Samples were analyzed for concentrations of 1'-hydroxymidazolam (OH-MDZ).
6. Phase 2 metabolism was determined by LC MS/MS. Cells were exposed to 100µM 7-ethoxycoumarin (7-EC) from Sigma. Samples were collected following a 30(60)-minute incubation period and analyzed for production of 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin glucuronide (7-HCG) and 7-hydroxycoumarin sulfate (7-HCS), by LC-MS/MS.

RESULTS

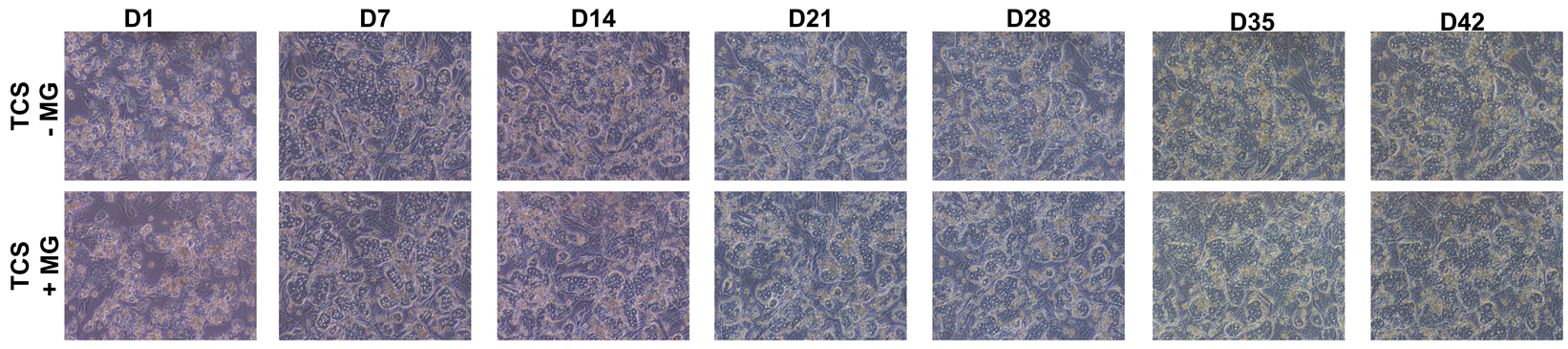

Figure 1. Morphology of the TCS over time. (A) Representative images of the TCS without Matrigel overlay (-MG) and with a Matrigel overlay (+) over a 42 day culture period (D42). Magnification 10X.

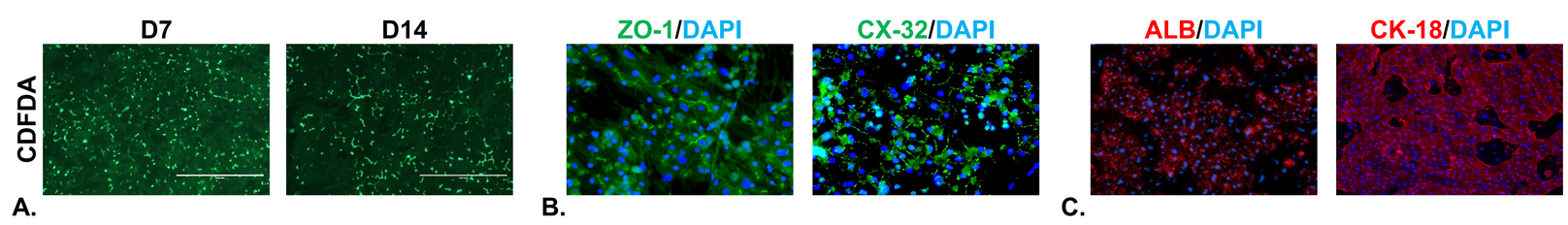

Figure 2. Formation of bile canaliculi and expression of protein markers in the TCS. (A) CDFDA staining of bile canaliculi on days 7 and 14. (B) Positive staining of ZO-1 and Connexin-32. (C) Alb and CK-18 positive expression in the PHHs in the TCS. Magnification 10X.

Table 2. Levels of Albumin and Urea (µg / 10^6 cells/day)

Timept. (Day)	Albumin	Urea
8	33.0 ± 0.02	64.0 ± 1.7
10	43.1 ± 0.98	69.1 ± 0.39
15	41.1 ± 5.5	56.3 ± 1.3
22	26.7 ± 0.05	64.3 ± 2.5

A.

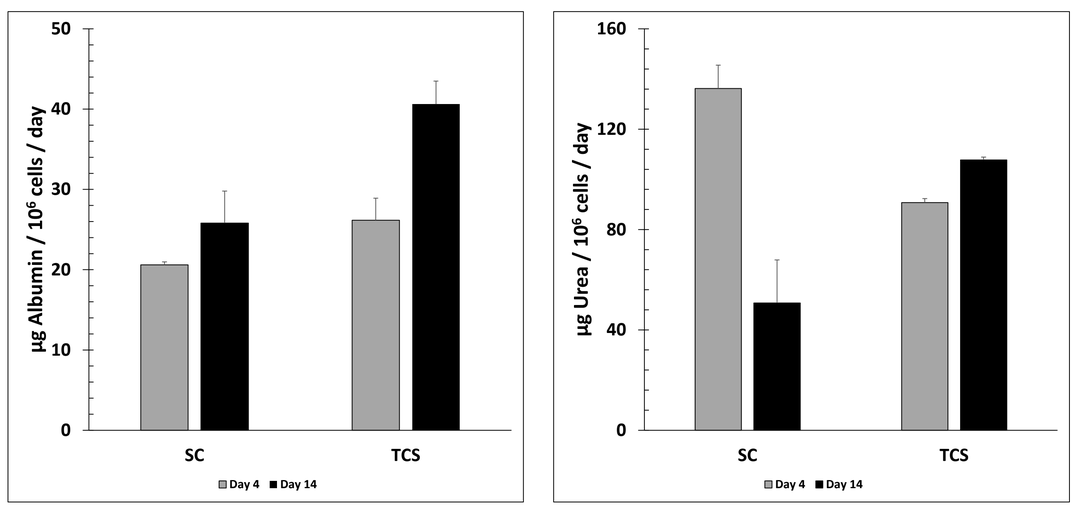

Figure 3. Stability of the TCS over time. (A) Alb and urea levels in PHHs in the TCS over a 22 day culture period. (B) Production of Alb and urea in the SC monoculture compared to the TCS on days 7 (grey bars) and 14 (black bars). Error bars represent standard deviation (n≥3 wells).

Rate of OH-MDZ formation

Timept. (Day)	OH-MDZ pmol / min / 10^6 cells
5	9.06 ± 0.5
7	10.4 ± 0.6
10	18.3 ± 7.1
14	12.3 ± 3.4

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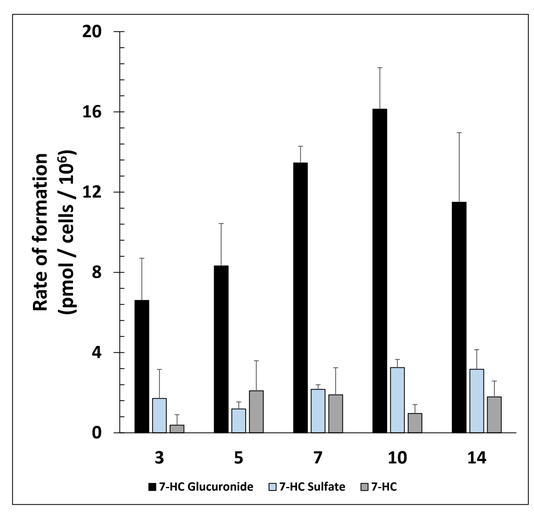

Figure 4. Metabolic Functionality in the TCS. (A) Phase I metabolism was determined by measuring 1'-hydroxymidazolam (OH-MDZ) on days 5, 7, 10, and 14. (B) The Phase 1 metabolite 7-hydroxycoumarin (7-HC, grey bars), and the Phase 2 metabolites, 7-hydroxycoumarin glucuronide (7-HCG, black bars) and 7-hydroxycoumarin sulfate (7-HCS, blue bars), were examined over 14 days of culture.

		Uninduced (pmoles CYP /10^6 /min)	Induced (pmoles CYP / 10^6 / min)
1A2	*SC Mono*	1,551 ± 643	8,970 ± 1290
	TCS	7,400 ± 781	28,922 ± 3,483
2B6	*SC Mono*	88.6 ± 107	205 ± 133
	TCS	292 ± 138	1,171 ± 253
3A4	*SC Mono*	1,441 ± 352	26,556 ± 3,067
	TCS	982 ± 358	25,300 ± 3,822

A.

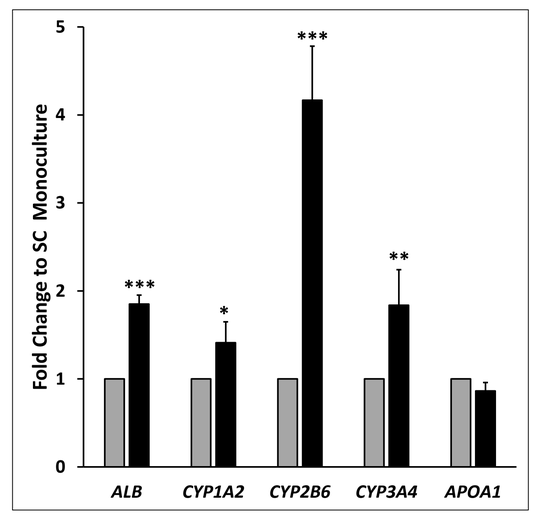

Figure 5. CYP1A2, 2B6, & 3A4 induction in the TCS. (A) CYP1A2, CYP2B6, and CYP3A4 induction in the TCS compared to SC monoculture on day 4. (B) Gene expression in the TCS (black bars) of *Alb, CYP1A2, CYP2B6, CYP3A4,* and *Apolipoprotein 1 (APOA1)* compared to expression in the SC monoculture PHHs (grey bars) on day 4. *p≤0.05, **p≤0.01, ***p≤0.001 to SC monoculture. Error bars represent standard deviation (n≥3 wells).

CONCLUSIONS

- PHHs in the TCS are able to maintain hepatocyte morphology for 42 days *in vitro* with no Matrigel overlay.
- Extensive anastomosing networks of bile canaliculi with tight and gap junctions were formed by PHHs in the TCS.
- PHHs in the TCS remained functionally stable for over 22 days when Alb and urea levels were determined.
- There was significantly higher Alb, urea, and CYP activity in PHHs in the TCS compared to PHHs in the SC monoculture.
- CYP- and Uridine 5'-diphospho-glucuronosyltransferase (UGT)-based enzyme activity and nuclear receptors were also stable during this time.
- The TCS represents a convenient, stable, and reproducible platform for pharmacological and toxicological applications in drug development and risk assessment.

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