CHARACTERIZATION OF MORPHOLOGY, LONGEVITY AND FUNCTIONALITY IN AN ALL-HUMAN CELL BASED TRI-CULTURE SYSTEM

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ABSTRACT

The use of primary human hepatocytes (PHHs) in preclinical pharmacological and toxicological applications has been restricted in part by the lack of a suitable culture platform that is convenient, supports a multitude of donor lots from healthy and diseased tissues, and maintains the structural and functional properties of the cells over prolonged periods of time. The sandwich culture (SC) and the co-culture systems have addressed some of these issues; however, some limitations remain. To address these limitations, an all-human Tri-Culture system has been developed that uses PHHs and primary feeder cells (FCs). Cryopreserved human feeder cells consisting of two types of stromal cells at a 1:1 ratio 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). Morphology and functionality, including albumin (Alb) and urea secretion and cytochrome P450 (CYP) activity, were evaluated over a 3-week culture period. PHHs from multiple adult donor lots in the TCS exhibited a healthy and stable morphology, including multicellular cluster formation, for up to 42 days. 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. Albumin 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) over a 3-week period compared to SC monoculture PHHs. Gene expression of *Alb, CYP1A2, CYP2B6*, and *CYP3A4* in the TCS PHHs at day 4 was increased 2-fold on average compared to the PHHs in the SC monoculture. Phase 1 and phase 2 metabolic function (midazolam 1-hydroxylation rates: 10-20 pmol/min/10^6 cells, 7-ethoxycoumarin glucuronidation rates: 300-600 pmol/min/10^6 cells) was stable after 5 days for at least 2 weeks. Overall, these results show that the Tri-Culture model represents a convenient all-human hepatic culture system that maintains both hepatocellular morphology and metabolic function over several weeks.

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

Prepare Media
1. Thaw overnight
2. Add supplements
3. Warm to 37°C

→ 30 mins. →

Plate Feeder Cells
Thaw, count, and plate FCs

→ 60 mins. →

Plate PHHs
Thaw, count, and plate PHHs

→ 2-4 hrs. →

Change Media
Daily media changes

Figure 1. Setting up the TCS.

1. Expression of ZO-1, Connexin-32, Cytokeratin 18 (CK18), Alb, CYP1A2, and Vimentin (VIM) 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.

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

RESULTS

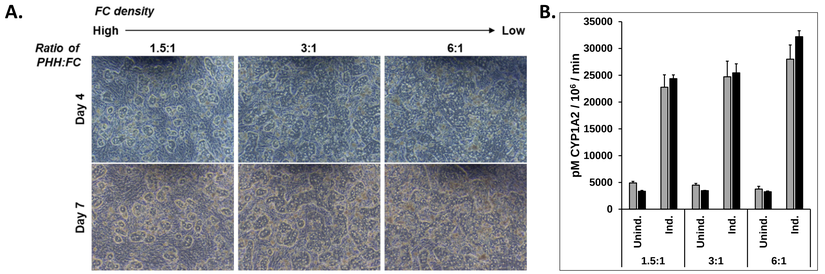


Figure 2. Optimized Ratio of PHHs:FCs in the TCS. (A) Representative images of various seeding densities (1.5:1, 3:1, and 6:1) of PHHs:FCs on days 4 and 7. (B) Induction of CYP1A2 on days 4 (grey bars) and 7 (black bars) compared to uninduced PHHs. Error bars represent standard deviation (n≥3 wells). Magnification 10X.

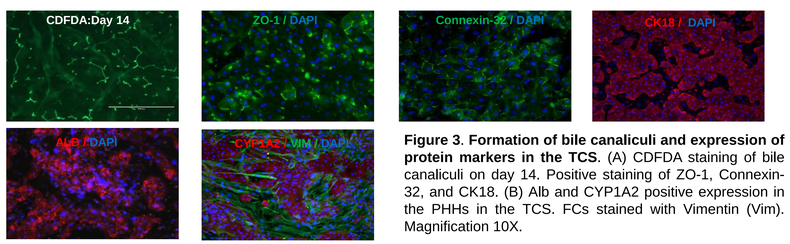


Figure 3. Formation of bile canaliculi and expression of protein markers in the TCS. (A) CDFDA staining of bile canaliculi on day 14. Positive staining of ZO-1, Connexin-32, and CK18. (B) Alb and CYP1A2 positive expression in the PHHs in the TCS. FCs stained with Vimentin (Vim). Magnification 10X.

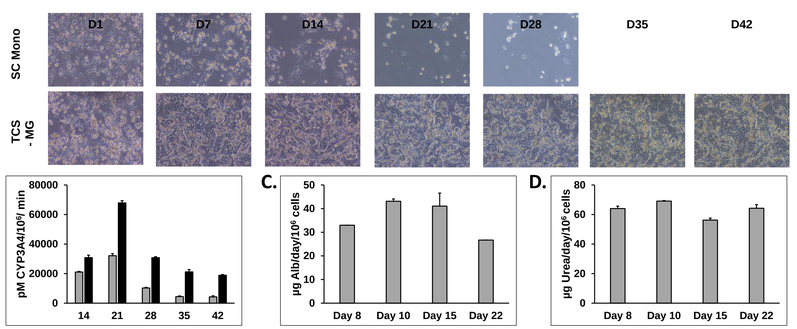


Figure 4. Stability of the TCS over time. (A) Representative images of a SC monoculture and TCS without Matrigel overlay (-MG) over a 42 day culture period (D42). (B) Induced CYP3A4 activity of PHHs in the TCS (black bars) over a 42 day culture period compared to SC monoculture (grey bars). (C) Alb and (D) urea levels in PHHs in the TCS over a 22 day culture period. Error bars represent standard deviation (n≥3 wells). Magnification 10X.

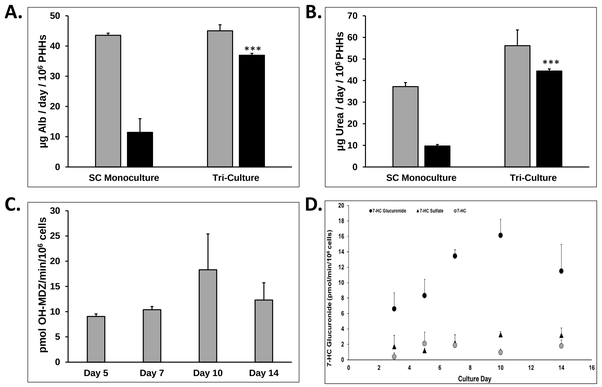


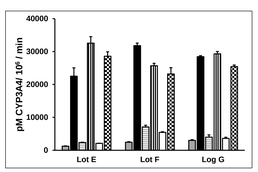
Figure 5. PHH Functionality in the TCS. (A) Alb and (B) urea in the SC monoculture and TCS on days 7 (grey bars) and 14 (black bars). (C) Phase I metabolism was determined by measuring 1'-hydroxymidazolam (OH-MDZ) on days 5, 7, 10, and 14. (D) The Phase 1 metabolite 7-hydroxycoumarin (7-HC, grey circles), and the Phase 2 metabolites, 7-hydroxycoumarin glucuronide (7-HCG, black circles) and 7-hydroxycoumarin sulfate (7-HCS, black triangles), were examined over 14 days of culture. ***$p \leq 0.001$ compared to SC monoculture on day 14.

Table 2. Contribution of FCs to OH-MDZ formation

Timept. (Day)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean± STD
5	0	0	0	NA	0
7	0	0	0	NA	0
10	0	0	0	NA	0
14	0	0	0	NA	0

A.

		Uninduced (pM CYP / 10^6 / min)	Induced (pM 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



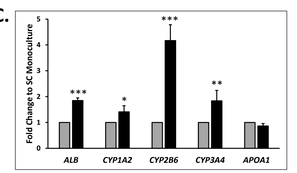


Figure 6. CYP1A2, 2B6, & 3A4 induction in the TCS. (A) Table of CYP1A2, CYP2B6, and CYP3A4 induction in the TCS compared to SC monoculture on day 4. (B) Three different donor lots (Lots E, F, and G) were cultured for 16 days in the TCS with uninduced (grey, horizontal strip, and white bars) and induced CYP3A4 (black, vertical strip, and checkered bars) enzyme activity being measured on days 4 (grey and black bars), 10 (striped bars), and 16 (white and checkered bars). (C) 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. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ 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 collagen or 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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