Lonza
Specialized Modalities

Primary human hepatocyte 3D spheroids co-cultured with non-parenchymal cells

High throughput meets physiological relevance

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Introduction

Industry guidance provided by the FDA Modernization Act 2.0 has led to a shift away from animal-based models and towards the generation and application of physiologically-relevant, xeno-free culture models of liver disease and toxicology. 3D primary human hepatocyte (PHH) cell culture models generally provide greater physiological relevance than 2D PHH models and therefore provide more relevant data for applications such as ADME-toxicology, drug development, physiology, and human health.[1-3] The addition of non-parenchymal cells (NPCs) further increases both the complexity as well as the relevance of *in vitro* 3D PHH cell culture models, representing the complex interactions that underlie hepatic function *in vivo*.[4,5] Here, we present a 3D co-culture model using Lonza PHH spheroids and Kupffer cells (KCs), stellate cells (SCs), and liver endothelial cells (LECs) optimized for high-throughput applications. We found that Lonza HCM™ Medium produced co-cultures that maintain both PHH spheroid and NPC health and functionality through 21 days. Furthermore, we found that this protocol allows for greatly increased donor variety over 3D spheroid PHH monocultures, as hepatocytes from donors that were unable to form spheroids in monoculture do form spheroids in co-culture with NPCs. The resulting protocol provides a user-friendly, physiologically-relevant model system for a wide variety of high-throughput applications.

Methods

Materials used to create the optimized 3D spheroid PHH-NPC co-culture model can be found in Table 1. Combinations of hepatocyte and NPC donors used in this study can be found in Table 2. 3D spheroid PHH-NPC co-cultures were prepared according to protocol instructions published on the Lonza website.[6] Briefly, we first prepared a Plating Medium that consisted of Lonza Hepatocyte Culture Medium (HCM™) with the addition of 20% FBS and

Product Information:

Vendor	Catalog No.	Description
Lonza	HUCPG	Cryopreserved Primary Human Hepatocytes, Plateable (Verified for Spheroids)*, ≥ 5 million cells
Lonza	HUCPI	Cryopreserved Primary Human Hepatocytes, Plateable, DDI Qualified. (Verified for Spheroids)*, ≥ 5 million cells
Lonza	HLKC-500K	Cryopreserved Human Kupffer Cells (KC), ≥ 500,000 cells
Lonza	HUCLS-200K	Cryopreserved Human Stellate Cells (SC), ≥ 200,000 cells
Lonza	HLECP1	Cryopreserved Human Liver-derived Endothelial Cells (LEC), Passage 1, ≥ 1 million cells
Lonza	MCHT50	Human Hepatocyte Thawing Media, 50 mL
Lonza	CC-3199	HBM Basal Medium, 500 mL
Lonza	CC-4182	HCM™ SingleQuots® Supplements
Lonza	CC-3198	HCM™ Hepatocyte Culture Medium BulletKit®
Lonza	BEBP17-737E	1M HEPES Buffer, 100 mL
Corning®	CLS7007	96-well Ultra-Low Attachment Plates
Any		FBS
Any		Trypan Blue 0.4%

Table 1.
Materials used by Lonza to create the 3D spheroid PHH-NPC co-culture. *Verified for Spheroids: Lonza routinely screens plateable hepatocyte lots for spheroid formation potential. Required for 3D spheroid PHH monocultures. To learn which lots are characterized for spheroid formation, contact Scientific Support at scientific.support@lonza.com or scientific.support.eu@lonza.com.

Hepatocyte Part #	Lot#	Gender	Race	Age	BMI	Long-term Plateable	96-well Plateable	Spheroid Qualified*
HUCPG	HUM212641	Female	Caucasian	53	25	No	No	No
HUCPI	HUM211621	Male	Caucasian	33	26.6	No	Yes	No
HUCPI	HUM200711	Male	Caucasian	49	19.7	Yes	No	No
HUCPI	HUM222051	Male	Caucasian	30	30.2	Yes	Yes	Yes
HUCPG	HUM222621	Female	Caucasian	49	24.7	No	Yes	Yes
HUCPI	HUM221971	Female	Caucasian	61	20.7	Yes	Yes	Yes

Table 2.
Hepatocyte donor information for hepatocytes used in this study. * Indicates whether or not hepatocytes from this donor were qualified to form spheroids in monoculture.

25 mM HEPES to encourage spheroid formation. Next, Lonza hepatocytes (part no. HUCPI or HUCPG) were thawed and transferred to Lonza Hepatocyte Thawing Medium, while the corresponding KCs, SCs, and LECs were thawed and transferred to their own respective 15 mL conical tube with 10 mL of Plating Medium. All tubes were centrifuged, aspirated of supernatant, and then cells were resuspended in Plating Medium, counted using 0.4% trypan blue and a hemocytometer, and adjusted to final concentrations of 1 x 10^6 cells/mL for hepatocytes and 5 x 10^5 cells/mL for each NPC. An aliquot of each cell type was then transferred to a new 15 mL tube (195 µL hepatocytes, 59 µL KCs, 49 µL SCs, and 87 µL LECs) to give a final ratio of 1 hepatocyte : 0.30 KC : 0.25 SC : 0.45 LEC.[4] The resulting cell mixture was then diluted to 13 mL with Plating Medium (final density of 22,500 cells/mL) and seeded into a 96-well ultra-low attachment plate at 100 µL per well. Plates were incubated in a humidified incubator at 37°C, 5% CO_2 and left undisturbed until 5 days. 50% medium changes were performed on days 5 - 7 using fresh, pre-warmed HCM™. Spheroids formed by day 7. After day 7, 50% medium changes were performed every 2 – 3 days through day 21.

Hepatocyte spheroid morphology was assessed visually by phase-contrast microscopy. Hepatocyte health/functionality was quantitatively assessed by measuring albumin release using an ELISA on days 10, 14 and 21. NPC health and functionality was assessed via ELISA assays for cytokine release after 24 hours of LPS stimulation (1 µg/mL LPS in HCM™) on days 10, 14, and 21. All ELISAs measured supernatant collected from medium changes on those days, which was frozen until analysis. Cytokines evaluated included TNF-α (KCs), TIMP-1 (SCs), and CCL2 (LECs).

Results

Using the optimized conditions for cell culture medium and cell density described in the Methods, we found that spheroid morphology was well supported by Lonza's HCM™ medium (Figure 1), with smooth, well-defined borders and a lack of cellular detritus in the background. Normally, Lonza verifies lots of hepatocytes as spheroid forming or non-spheroid forming during the production process (Figure 1A, B). Interestingly, we were able to promote spheroid formation in hepatocytes that had been classified as non-spheroid forming by co-culturing them with

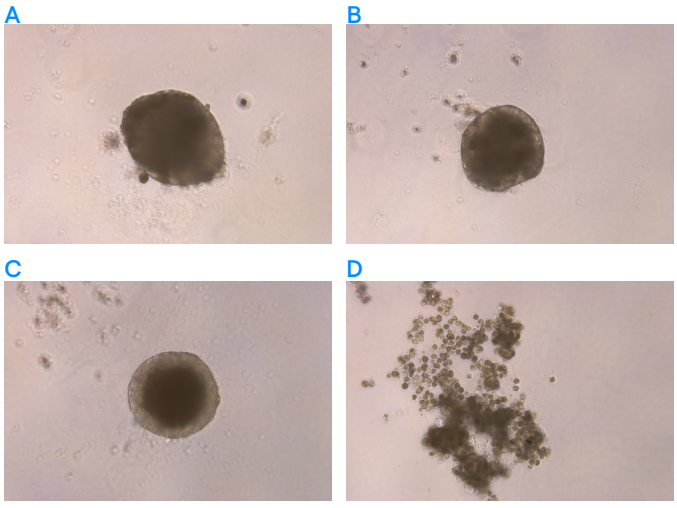

Figure 1.
Repesentative spheroids from two of the six donors used in this study. The donor shown in panels A and B was a spheroid-qualified lot and could form spheroids in co-culture (A) or monoculture (B). The donor shown in panels C and D) formed spheroids when in co-culture (C) but not in monoculture (D).

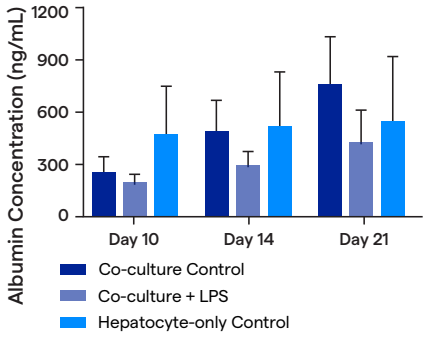

Figure 2.
Albumin release by 3D spheroid PHHs in co-culture with and without LPS stimulation and in monoculture.

NPCs (Table 2, Figure 1C), though this is only an initial observation and requires further testing. Notably, these non-spheroid-qualified hepatocytes failed to form spheroids in monoculture (Figure 1D). In general, co-culturing PHHs with NPCs greatly increased the performance of PHHs spheroids in culture, as all donors formed healthy spheroids in the 96-well format for the entire 21-day culture, regardless of whether or not they could do any of the above in monoculture (Table 2). Additionally, albumin concentrations were similar between co-culture and monoculture, and were lowest in the co-culture stimulated with LPS (Figure 2). This further indicated a positive

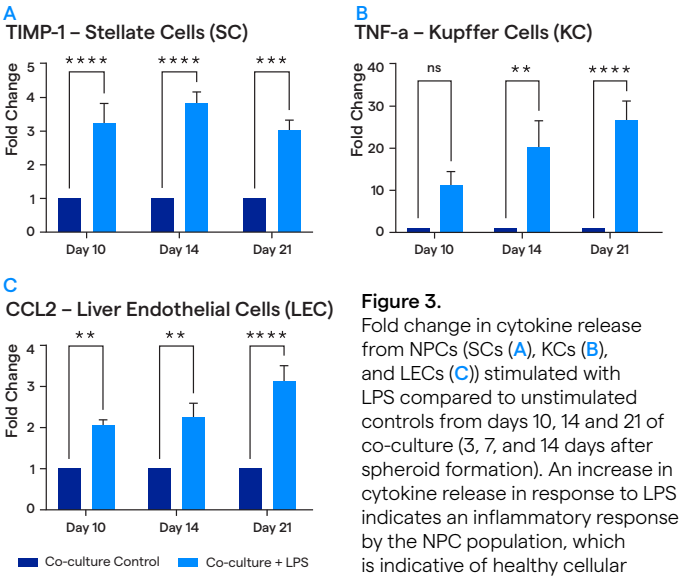

Figure 3.
Fold change in cytokine release from NPCs (SCs (A), KCs (B), and LECs (C)) stimulated with LPS compared to unstimulated controls from days 10, 14 and 21 of co-culture (3, 7, and 14 days after spheroid formation). An increase in cytokine release in response to LPS indicates an inflammatory response by the NPC population, which is indicative of healthy cellular function.[5]

physiological response by PHH spheroids to co-culturing with NPCs as well as the negative physiological response to LPS-induced inflammation expected by a healthy 3D spheroid PHH-NPC co-culture.[7]

Cytokine release for KCs, SCs, and LECs over the course of the co-culture remained significantly higher for LPS-stimulated co-cultures compared to unstimulated controls (Figure 3). Combined with the decrease in albumin levels in co-cultures stimulated with LPS (Figure 2), this indicates that the NPC fraction of the co-culture functioned as expected, with the entire co-culture exhibiting an appropriate inflammatory response upon stimulation with LPS.

Conclusions

Our study demonstrates a physiologically-relevant 3D spheroid PHH-NPC co-culture model that maintains healthy morphology and physiology through 21 total days of culture (14 days post-spheroid formation) and behaves as expected when stimulated with an inflammatory compound. More importantly, our study reveals an emergent property of hepatocyte-NPC co-cultures in a 3D format: that the addition of NPCs (a closer approximation to the contextual physiology found *in vivo* than hepatocytes in monoculture) may promote long-term spheroid formation with consistent functionality in a 96-well format by hepatocytes that cannot do so in monoculture. If true, there is a far greater donor pool available for 3D spheroid PHH-NPC co-cultures than there is for 3D spheroid PHHs alone, further enhancing our model's ability to produce data relevant to the general population obtained from drug discovery and toxicological assays. Our 3D spheroid PHH-NPC co-culture can be used in a wide variety of high-throughput applications such as ADME-toxicology, drug-induced liver injury assay panels, and safety screening assays, where obtaining physiologically-relevant data on the inflammatory cross-talk between hepatocytes and NPCs is of the highest importance.

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CD-DS051 06/25

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