

Abstract

The discovery and development of anti-fibrotic therapies remains heavily reliant on animal testing. There is an urgent need to develop robust and relevant *in vitro* models to support the identification and preclinical evaluation of potential new anti-fibrotic drugs¹. To this end, we have developed and characterised a novel human 3D liver co-culture model using our proprietary ORGANDOT™ platform. These liver ORGANDOT cultures (human hepatocytes, Kupffer cells and stellates) not only maintain viability and functionality for up to four weeks in culture, but can also be treated with TGFβ1 to induce a fibrotic phenotype. The TGFβ1-treated ORGANDOT cultures show a decrease in hepatocyte function and a concomitant increase in fibrogenic gene expression (COL1A1, ACTA2, SPP1, TIMP2), hyaluronic acid secretion, and collagen deposition (collagen I immunostaining). Furthermore, co-administration of an ALK5 inhibitor was able to completely prevent these fibrotic changes and rescue the functionality of the cultures. These data demonstrate the potential utility of this *in vitro* model for testing the efficacy of new anti-fibrotic drug candidates or for research into the mechanism of stellate cell activation and fibrosis induction.

Methods

Isolation of Human Hepatic Stellate Cells

Macroscopically normal resected human liver tissue was used for the isolation of stellate cells. All samples were provided with informed consent. Stellate cells were isolated in-house according to established protocols. In brief, the tissue was minced and then digested by incubating with 0.03% pronase and 0.05% collagenase B (Roche) for 60 minutes at 37°C in a shaking incubator at ~100rpm. The crude cell suspension was then filtered, washed and the stellate cells purified using a discontinuous OptiPrep™ gradient (Sigma). The freshly isolated cells were then expanded in culture in DMEM-high glucose supplemented with 16% FBS, 4mM L-glutamine and 1% penicillin-streptomycin (complete DMEM) and cryopreserved stocks created. Before each ORGANDOT experiment, stellate cells (passage 1) were revived and cultured in complete DMEM for ~7 days.

Creation of Liver ORGANDOT Cultures

Cryopreserved human hepatocytes were obtained from Triangle Research Laboratories (TRL). Cryopreserved human Kupffer cells were obtained from Life Technologies. On the day of ORGANDOT creation, hepatocytes and Kupffers were thawed according to manufacturers' protocols. Hepatocytes were pelleted at 100 x g for 8 minutes and resuspended in TRL's hepatocyte maintenance medium (MM250). Kupffer cells were pelleted at 500 x g for 5 minutes and resuspended in Life Technologies' Kupffer thawing medium (Advanced DMEM supplemented with FBS and Cocktail A). Stellate cells were harvested with TrypLE (Gibco), pelleted at 400 x g for 5 minutes and resuspended in complete DMEM. The three cell suspensions were then combined and ORGANDOT cultures created by spotting 5μL (50K hepatocytes, 3K stellates and 1.5K Kupffers) onto 12mm MilliCell CM inserts (PICM01250) with 250μL hepatocyte maintenance medium added basolaterally. ORGANDOT cultures were maintained at 37°C/ 5%CO₂ with renewal of the basolateral medium every weekday.

The ORGANDOT platform



- Primary cells
- Transfer to a semi-permeable membrane and culture at air liquid interface
- Re-aggregation of the cells over time in culture
- 3D microtissues recapitulate original tissue architecture and functionality

Image of a Liver ORGANDOT



Compound treatment

ORGANDOT cultures were maintained for 3 days before being treated with 0.5ng/mL Transforming Growth Factor-β1 (TGFβ1) (R&D Systems, 240-B) for 4 days (daily renewal of medium containing TGFβ1). For inhibitor experiments, cultures were pre-incubated with SB525334 (Tocris 3211) for 30 minutes prior to exposure to TGFβ1. For Kupffer activation experiments, ORGANDOT cultures were treated with 10μg/mL lipopolysaccharide (LPS, Sigma L4516) for 24 hours.

Cytochrome P450 assay

CYP3A4 activity of the ORGANDOT cultures was measured using Promega's P450-Glo™ CYP3A4 assay (V9001). Basolateral culture medium was replaced with fresh medium containing 3μM Luciferin-IPA (250μL basolaterally and 100μL apically) and the cultures incubated for 60 minutes at 37°C. At the end of the incubation, 100μL of medium was transferred to a luminometer plate and an equal volume of detection reagent added. The plate was left to incubate at room temperature for 20 minutes and then the luminescent signal was read on a Wallac Victor² plate reader. The ORGANDOT cultures were retained for analysis of ATP content.

ATP assay

Cellular ATP was quantified using Promega's CellTiter-Glo® 3D Cell Viability Assay. The basolateral medium was removed from each culture and 100μL of CellTiter-Glo reagent added apically. The culture plates were then shaken for 10 minutes to induce cell lysis and an equal volume of medium added per culture. Each sample was briefly pipette mixed and 100μL transferred to a luminometer plate. The plate was left to incubate at room temperature for 10 minutes and then the luminescent signal was read on a Wallac Victor² plate reader.

ELISAs

Conditioned supernatants were assayed for Albumin, IL-6 and Hyaluronic acid (Hyaluronan) using ELISA kits from Abcam (albumin ELISA, ab108788) and R&D Systems (Hyaluronan and IL-6 Duoset ELISAs, DY3614 and DY206). All ELISAs were performed as described in the manufacturers' protocols.

RNA Extraction and qRT-PCR

ORGANDOT cultures were lysed with Promega's RLA lysis buffer and then stored at -80°C pending RNA extraction. Total RNA was then extracted using Promega's SV96 Total RNA Isolation System (Z3500), converted to cDNA, then the expression of ACTA2, COL1A1, SPP1 (OPN) and TIMP2 measured by qRT-PCR. BioIVT's global standard curve was used to interpolate transcript copy number from C_T values². Copy number of target genes was normalised to GAPDH.

Immunostaining

FFPE sections (5μm) of ORGANDOT cultures were used. The sections were deparaffinised, antigen retrieved and rehydrated in a Dako PT Link apparatus at 95°C for 20 minutes using pH6.1 (collagen I) or pH9 (SMA) Flex+ 3-in-1 antigen retrieval buffer. Immunostaining was performed on a Dako Autostainer Plus using the EnVision™ FLEX visualization system. The sections were rinsed with Flex Wash buffer and then blocked for 5 minutes with EnVision™ peroxidase block. The sections were rinsed and blocked for 10 minutes with Dako serum-free protein block. The blocking solution was air-removed. Anti-collagen I antibody (abcam, ab88147) at 5μg/mL and anti-SMA antibody (Dako IR611) at 25% (1 in 4 dilution with Flex antibody diluent) were added to the sections. Sections were incubated with the primary antibodies for 30 minutes. The sections were rinsed twice before being stained with EnVision™ FLEX/HRP for 20 minutes. The sections were further rinsed twice before being stained with DAB for 10 minutes. The sections were then counterstained with hematoxylin, dehydrated in an ascending series of ethanol solutions (90-100%), cleared in xylene and cover-slipped under DePeX.

Results

Figure 1

Maintenance of viability and functionality of liver ORGANDOT co-cultures

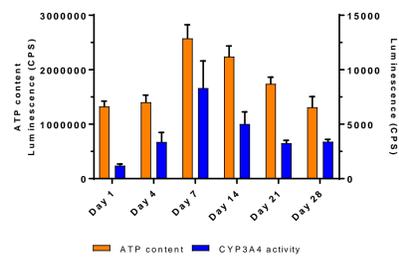


Figure 1 Liver ORGANDOT co-cultures (hepatocytes, Kupffers, and stellates) were maintained in hepatocyte maintenance medium. ATP content and CYP3A4 activity were measured on day 1, day 4, day 7, day 14, day 21 and day 28 as described in the Methods section.

Figure 2

Kupffer cell functionality is maintained in liver ORGANDOT co-cultures

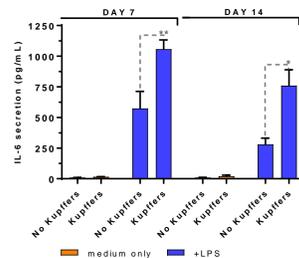


Figure 2 ORGANDOT cultures of hepatocytes and stellates (No Kupffers) or hepatocytes, Kupffers and stellates (Kupffers) were treated with LPS (10μg/mL) for 24 hours. Supernatants were retained for measurement of IL-6 secretion. Cultures containing Kupffer cells showed significantly higher levels of IL-6 secretion. ** = p<0.01 and * = p<0.05

Figure 3

TGFβ1-treated liver ORGANDOT co-cultures show reduced functionality and an increase in hyaluronic acid secretion that is prevented by the ALK5 inhibitor SB525334

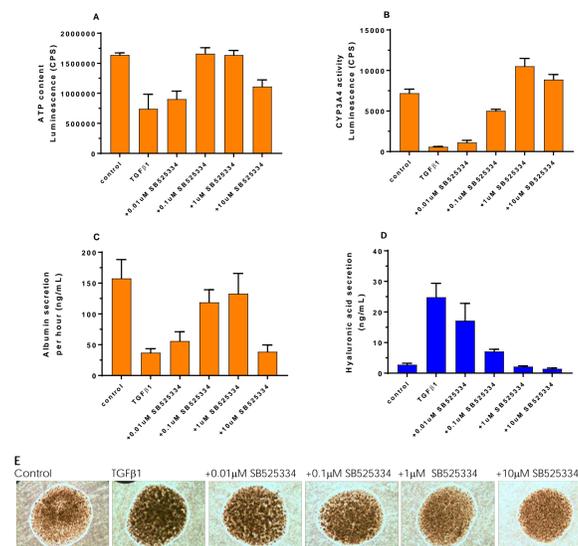


Figure 3 Liver ORGANDOT co-cultures (hepatocytes, Kupffers, and stellates) were maintained in culture for 3 days and then were treated with 0.5ng/mL TGFβ1 ± SB525334 (0.01μM, 0.1μM, 1μM or 10μM) for 4 consecutive days. On day 7, cultures were assayed for ATP content (A), CYP3A4 activity (B), and supernatants retained for analysis of albumin secretion (C) and hyaluronic acid secretion (D) by ELISA. Panel E shows representative phase contrast images captured at 12.5x magnification. TGFβ1-treated cultures showed reduced viability (ATP content) and functionality (CYP3A4 activity and albumin secretion) and an increase in hyaluronic acid secretion. Co-administration of SB525334 prevented the detrimental effect of TGFβ1 on the functionality of the cultures and reduced hyaluronic acid secretion to the same level as untreated controls.

Figure 4

TGFβ1-treated liver ORGANDOT co-cultures show an increase in fibrogenic gene expression that is prevented by the ALK5 inhibitor SB525334

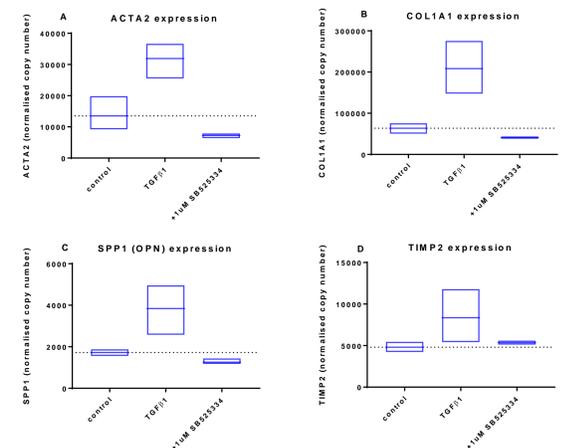


Figure 4 Liver ORGANDOT co-cultures (hepatocytes, Kupffers, and stellates) were maintained in culture for 3 days and then were treated with 0.5ng/mL TGFβ1 ± 1μM SB525334 for 4 consecutive days. On day 7, cultures were lysed and RNA extracted as described in the Methods section. Expression of ACTA2 (A), COL1A1 (B), SPP1 (C) and TIMP2 (D) were then measured by qRT-PCR. Copy number of target genes has been normalised to GAPDH. Data are displayed as min-max values with a line at the mean. TGFβ1-treated cultures showed an increase in the expression of all 4 genes, that was prevented by co-administration of SB525334.

Figure 5

TGFβ1-treated liver ORGANDOT co-cultures show an increase in collagen I deposition that is prevented by the ALK5 inhibitor SB525334

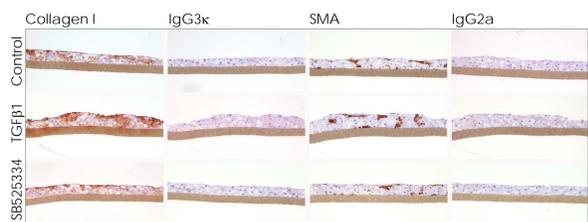


Figure 5 Liver ORGANDOT co-cultures (hepatocytes, Kupffers, and stellates) were maintained in culture for 3 days and then were treated with 0.5ng/mL TGFβ1 ± 1μM SB525334 for 4 consecutive days. On day 7, cultures were fixed in 10% NBF before being embedded in liquid paraffin. Sections were then stained with an anti-collagen I antibody, an anti-SMA antibody, or appropriate isotype controls, as described in the Methods section. Representative images were captured at 100x magnification. TGFβ1-treated cultures showed an increase in collagen I and SMA immunoreactivity that was prevented by co-administration of SB525334.

Conclusions

- Liver ORGANDOT co-cultures (human hepatocytes, Kupffers and stellates) maintain viability and functionality for up to 28 days in culture.
- Exposure to TGFβ1, a central regulator in liver fibrosis, induced a fibrotic phenotype with an increase in fibrogenic gene expression, hyaluronic acid secretion, and collagen I deposition.
- Co-administration of the ALK5 inhibitor, SB525334, was able to prevent these fibrotic changes and rescue the functionality of the ORGANDOT cultures.
- These data demonstrate the potential utility of this novel 3D *in vitro* model for evaluating new anti-fibrotic drugs or for studying the mechanism of fibrosis induction and hepatic stellate cell activation.
- This data also extend the use of the ORGANDOT platform to additional diseased models, which now include diabetes, oncology, and as demonstrated here, liver fibrosis.

References

1. Van Grunsven LA (2017) 3D *in vitro* models of liver fibrosis. *Advanced Drug Delivery Reviews* 121: 133 – 46.
2. Barber RD, Harmer DW, Coleman RA, Clark BJ (2005) GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* 21: 389-395.