

RESEARCH ARTICLE

Methylcellulose as a scaffold in the culture of liver-organoids for the potential of treating acute liver failure

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Much progress has been made in understanding the development of human organs through advanced cellular and molecular techniques. Acute liver failure (ALF) in children is a life-threatening condition that relies on liver or hepatocyte transplantation. The translation of novel regenerative medicine strategies for the treatment of ALF is however somewhat limited. Here, we show that *in vitro* liver-organoids derived from human umbilical cord derived mesenchymal stem cells and human cadaveric donor-derived hepatocytes, cultured in a clinically appropriate manner, exhibit liver function. We obtained organoids that varied in size and morphology which produced albumin, and detoxified ammonium chloride into urea. Immunohistochemistry of these organoids revealed hepatocyte specific, non-parenchymal markers and histological organisation. Our *in vitro* findings indicate that these organoids may be a useful bridge in ALF while awaiting liver recovery or transplant. The organoid culture system we have established here is also well suited to drug screening and disease modeling.

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INTRODUCTION

There have been significant conceptual and practical advances with regards to regenerative medicine for the treatment of disease. This has ranged from the classic use of embryonic stem cells, their culture and ability to differentiate into practically any cells of the body, to reprogramming of fully differentiated cells into an embryonic state followed by differentiation to a chosen cell type, which is now mainstream research [1]. These now significantly impact not only on treating disease but the potential for modelling disease in culture dishes that was previously not possible. The development of liver has been of prominent interest, as liver disease is the fifth biggest cause of deaths in the UK.

Acute liver failure (ALF) in children is a life-threatening condition. The prognosis of ALF is generally poor and medical intervention relies on a liver [2,3] or hepatocyte [4,5] transplantation if the native liver is unable to recover. The major limitation of the technique today is the availability of donor organs. Many patients who might benefit from a transplant die before a suitable organ can be found. Whilst much progress has been made in the study and understanding of the biology of liver [6], there have been limited clinical advancements in the treatment of ALF utilizing novel regenerative techniques, such as the use of embryonic stem cell techniques to induced pluripotent stem cell-derived hepatocytes. Human biocompatible scaffolds to grow liver type/functional cells is ever increasing. The main challenges in translating advances in basic science of cell therapy into

the clinic has been determining the best route of delivery, the rapid elimination of transplanted cells by the recipient, poor engraftment and proliferation of transplanted cells within the liver [7].

Stem cell-based technologies have been extensively researched *in vitro* and *in vivo*. Mesenchymal stem/stromal cells (MSCs) are a population of immature cells that can give rise to more mature cell types such as adipogenic, osteogenic and chondrogenic cells. These cells can be easily cultured from adult bone marrow and full-term umbilical cord (blood or the Wharton's jelly). MSCs have also been isolated from muscle connective tissue, adipose tissue and in some circumstances peripheral blood. Culture protocols usually include direct plastic adherence or enrichment by CD marker selection. The well-established characteristics of MSCs, have been the basis of the extensive use in clinical applications. MSCs have been used in the treatment of liver disease with varying results [8].

Tissue engineering strategies for regenerative medicine are yet another avenue of great advancement. Novel scaffolds-engineered [9], naturally occurring [10] or synthetic material [11] are under extensive scrutiny. There is an unmet need to use novel organoid-based strategies that is suitable for transplantation in ALF until the patient receives a liver transplant or the native liver recovers. As such, we proposed to exploit novel organoid-strategies as a means for clinical application. Here we hypothesized that using methylcellulose as a scaffold to obtain liver-organoids maybe a useful approach towards clinical use.

RESULTS

Establishment of organoids in matrigel

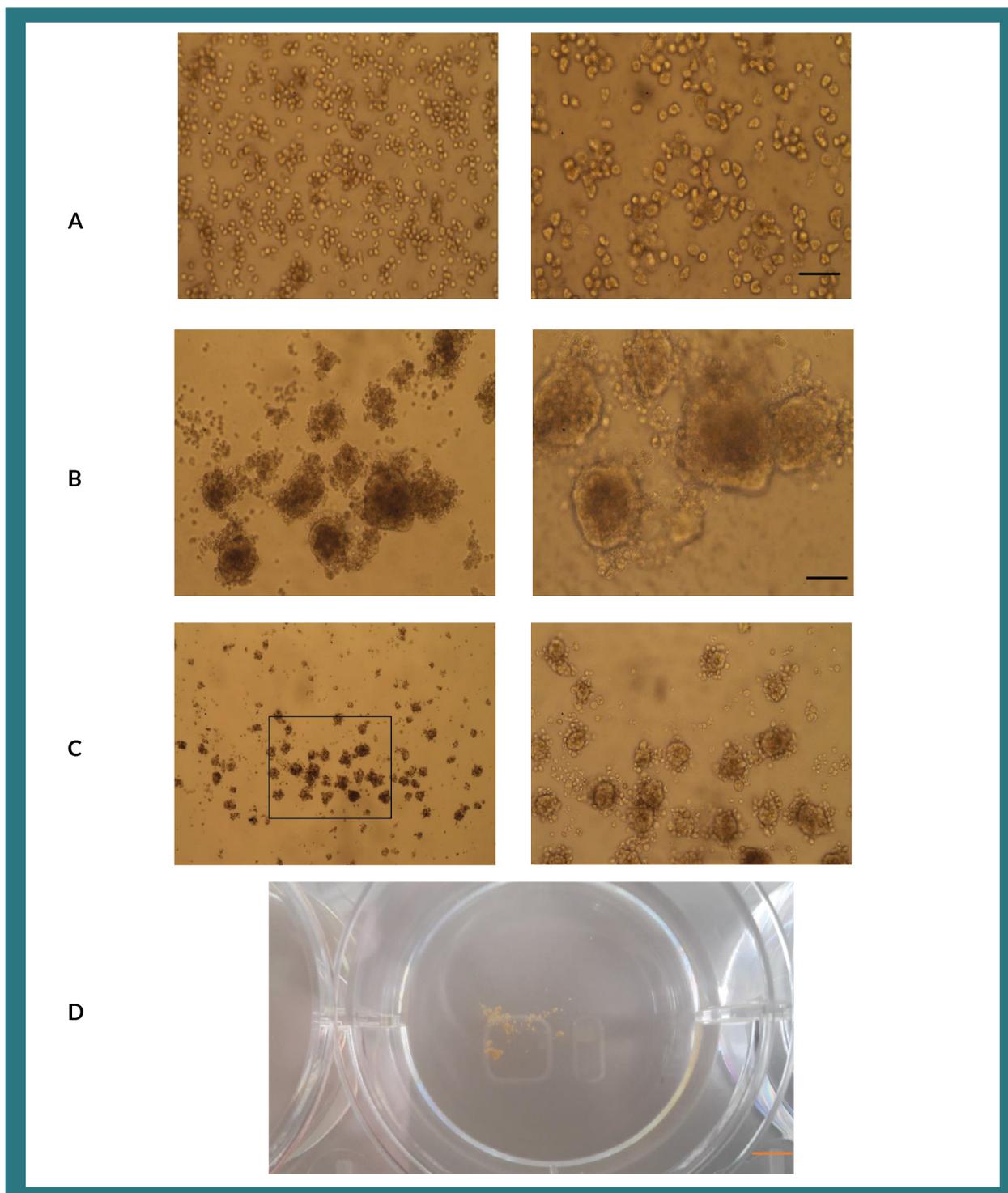
Our initial strategy to develop liver organoids, stems from the seminal research of Takebe and colleagues [12]. Whilst Takebe *et al.* used human induced pluripotent stem cell (hiPSC) derived hepatocytes, MSCs and human umbilical vein endothelial cells (HUVECS) cultured in matrigel with a host of factors to obtain organoids, we used adult hepatocytes from cadaveric donors and MSCs cultured in serum free matrigel. Organoids were analyzed after 14 days in culture (Figure S1A). Confocal imaging of these organoids revealed strong expression of hepatocyte markers (Figure S1B). Our rationale for the ratio of cells to be cultured was based on our previous experience of a 2-D co-culture system of hepatocytes and MSCs, which resulted in improved hepatocyte function [13]. We tested four different ratios of hepatocytes cultured with MSCs. Albumin production and ability to metabolise ammonium chloride to urea were used as functional readouts of the resulting organoids after two weeks in culture. Hepatocytes and MSC cultured in a two to one ratio respectively, gave rise to the highest albumin production (Figure S2A). Interestingly, the urea production was the highest when using a one to one ratio (Figure S2B). When using a two to one ratio of cultured cells, these organoids produced albumin over a period of 2 weeks, peaking from day 10 onwards (Figure S3A). These organoids were also able to metabolise ammonium chloride to produce urea from day 4 peaking at day 14 as well (Figure S3B).

Establishing organoids using methylcellulose as a scaffold

Matrigel whilst proving to be an excellent scaffold for studying and understanding basic biology of organ development, its clinical application is somewhat restricted as it is a gelatinous substance derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. Therefore, we hypothesized that substituting matrigel with methylcellulose as a scaffold for the growth of organoids might be a useful approach to using organoids for future clinical application (Figure S4). Having studied the composition of growth factors present in matrigel (Corning), we tested the effects of transforming growth factor- β (TGF- β), hepatocyte growth factor (HGF) and epidermal growth factor (EGF); individually and in combination, in methylcellulose for the ability to produce organoids. A two-part hepatocyte to one-part MSC ratio were maintained and cultured in the presence of methylcellulose and growth factors in non-tissue culture 6-well plates. When we cultured MSC and hepatocytes with a single cytokine only, no organoids formed (Figure 1A) 14 days into culture. However, the combination of TGF- β and EGF growth factor gave rise to organoids of varying sizes (50–600 μ m) (Figure 1B). Surprisingly, the addition of all three growth factors in culture yielded organoids that weren't as big in comparison to organoids from TGF- β and EGF growth factor combination (Figure 1C). The combination of TGF- β and EGF growth factor (Figure 1B) cultured organoids were macroscopic after 14 days in culture, varying in shapes and sizes (Figure 1D).

► **FIGURE 1**

Morphology of organoids grown in methylcellulose.



(A) Left hand panel: hepatocytes and MSCs cultured in a 2:1 ratio respectively in EGF only; right hand panel: as in left hand panel but at higher magnification. No organoid formation was observed after 14 days in culture. Scale bar = 100 micron. (B) Left hand panel: organoids obtained from hepatocytes and MSCs cultured in a 2:1 ratio respectively in EGF and TGF- β ; right hand panel: as in left hand panel but at higher magnification. Organoids formation was observed after 14 days from culture initiation. Scale bar = 100 micron. (C) Left hand panel: organoids obtained from hepatocytes and MSCs cultured in a 2:1 ratio respectively in EGF, HGF and TGF- β ; right hand panel: as in left hand panel but at higher magnification. Organoids formation was observed after 14 days from culture initiation, however morphologically not the same as in B. Scale bar = 100 micron. (D) Organoids grown in non-tissue culture treated 6-well plates. After 2 weeks in culture many were macroscopic and heterogenous in shape. Scale bar = 200 micron.

Liver function activities of organoids cultured in methylcellulose

We next tested the ability of organoids to produce albumin and detoxify ammonium chloride, in optimally cultured in the conditions. We compared these conditions, to that of organoids obtained using matrigel as a reference point. Albumin production was highest from organoids derived from cultures containing TGF- β and EGF (Figure 2A). Again, surprisingly, organoids derived from the culture in all three growth factors, yielded the least amount albumin production. This pattern of albumin production co-related with the morphology of organoids obtained as above. When organoids were assayed for the ability to detoxify ammonium chloride to urea, a similar pattern was observed. Urea production was highest from organoids derived from the culture containing TGF- β and EGF in comparison to the organoids derived from all three growth factors (Figure 2B). A similar co-relation of function to morphology was observed.

Histological & biochemical properties of organoids derived from methylcellulose

Having established the functionality of these organoids, we then analysed the histology of these optimally cultured organoids. These organoids expressed hepatocyte specific marker, OCH1E5 and; biliary marker, cytokeratin 7 (Figure 3B) and Control H&E section (Figure 3A) indicating that cells of both hepatocyte and biliary lineages were present in the organoids. These organoids also expressed Cytokeratin 19 (Figure 3B, left and middle

panels). Furthermore, confocal, microscopy also revealed CD31 expression in these organoids suggesting endothelial cell formation (Figure 3C).

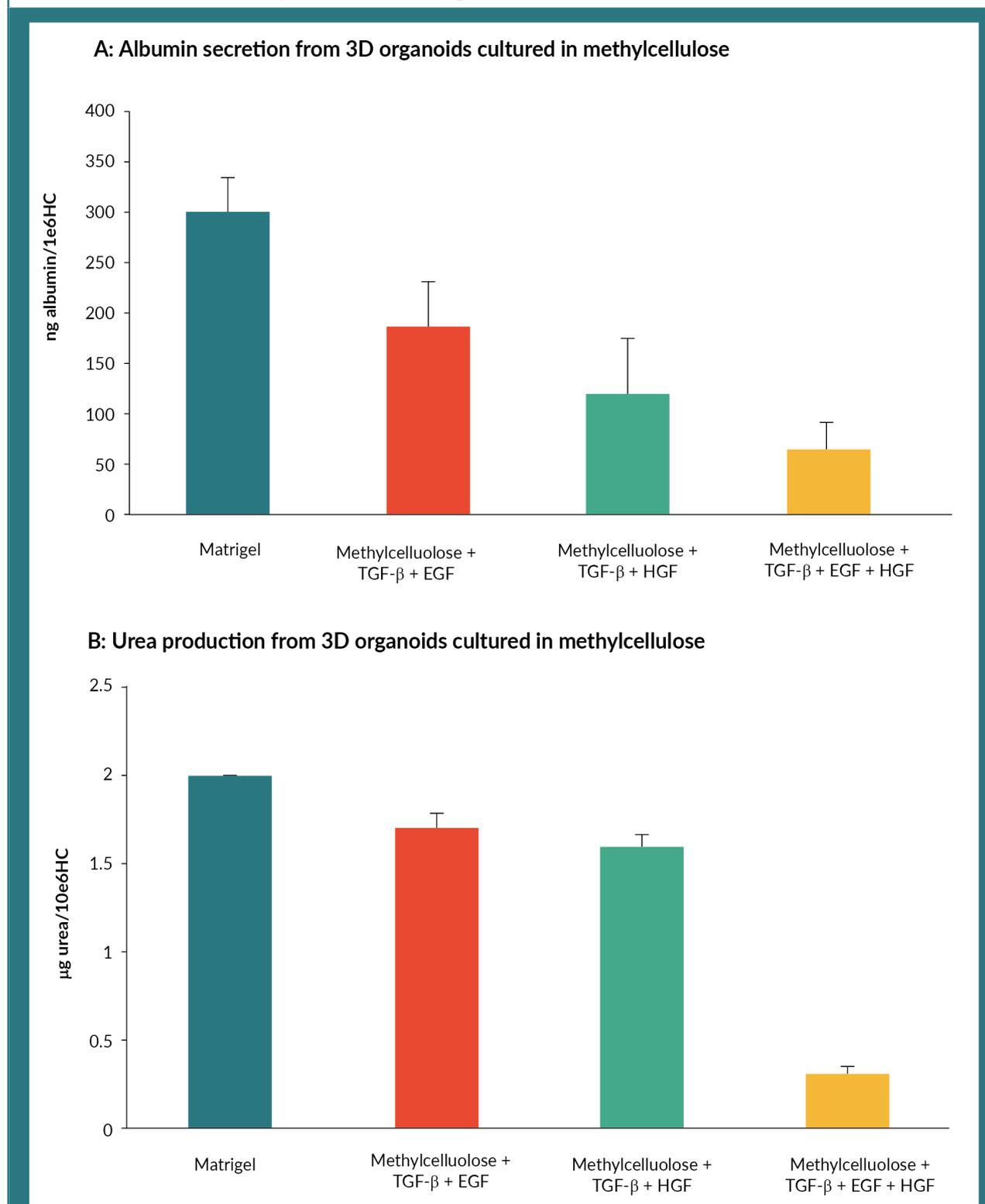
Finally, we assessed the ability of organoids to conjugate bilirubin. Albeit relatively lower levels compared to the HepG-2 cell line, these organoids could conjugate bilirubin (Figure 4A). Strikingly, we found the expression of MRP-2 in these organoids, suggesting an attempt at organisation of the hepatocytes into a lobule (Figure 4B, middle panel) and compellingly, bile staining as shown in a Fouchet stain (Figure 4C).

DISCUSSION

In this study, we show that methylcellulose, an inert semi-solid media, could be utilized as a scaffold to establish liver-organoids that resemble liver structure and function. The results we obtained here are comparable to that of using matrigel as a scaffold. There was also a good correlation of the morphology of organoids obtained to functional activity. Albeit the lowered functional activities (albumin and urea production) using methylcellulose as opposed to matrigel, the suitability of using methylcellulose towards clinical grade expansion of organoids is highly compelling. Further optimization and scaling up of the process is underway. Methylcellulose is a synthetic chemical product derived from cellulose. Cellulose is heated with sodium hydroxide and the resulting substitution of hydroxyl residues on cellulose are replaced with methoxide [14]. Methylcellulose is used in a wide array of applications

► **FIGURE 2**

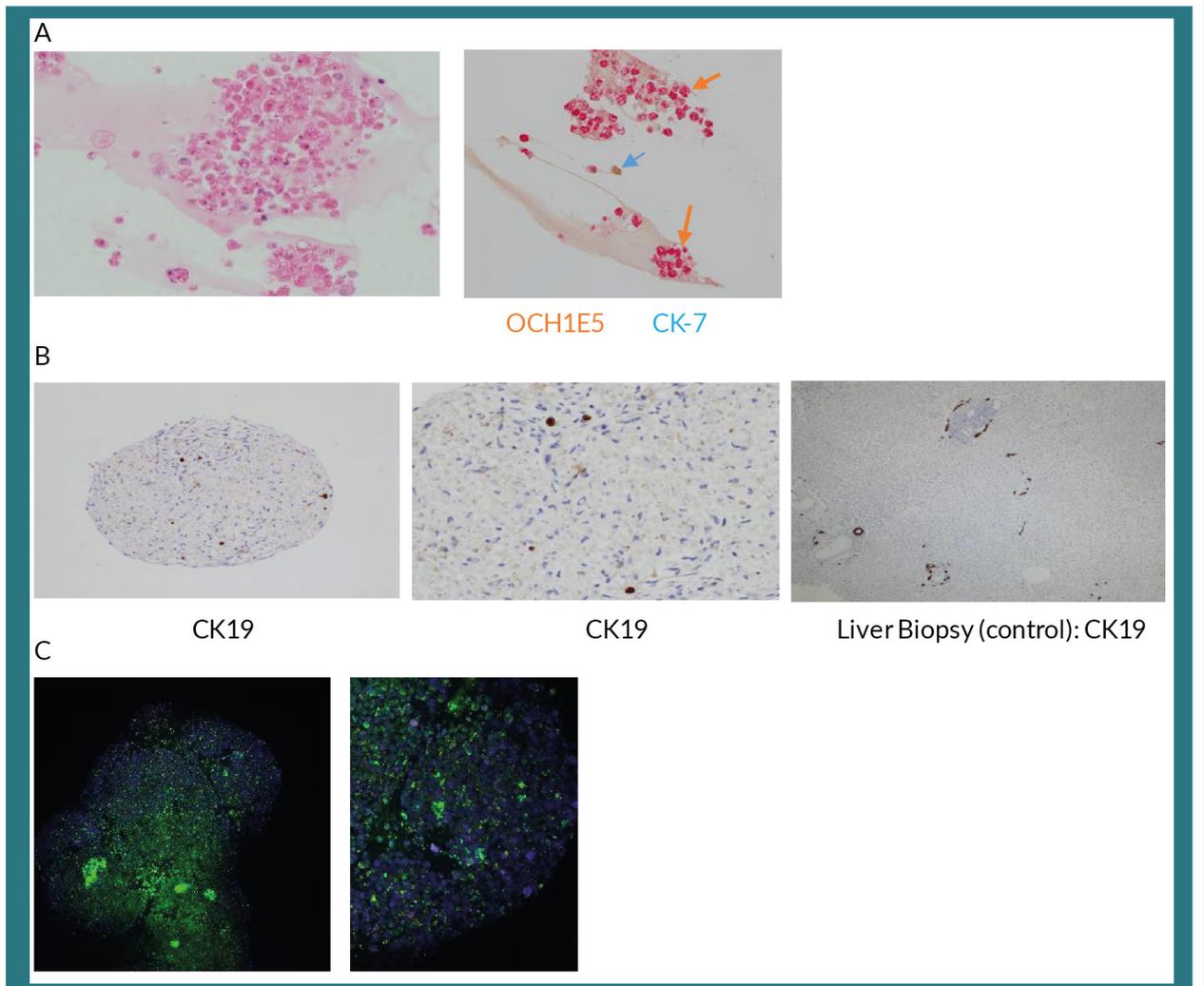
Albumin secretion and urea production from organoids.



Albumin secretion from organoids was measured by ELISA method. **(A)** Albumin production from organoids cultured in Matrigel (as a base line) was compared to that which was cultured in methylcellulose. The combination of TGF- β and EGF growth factors in methylcellulose yielded the highest amounts of albumin production compared to other cytokine combinations in methylcellulose (n = 3 independent experiments, each performed in triplicate). **(B)** Urea production of similar organoids were measured by colorimetric means. Here too the combination of TGF- β and EGF growth factors yielded the highest amounts of urea production compared to other cytokine combinations in methylcellulose (n = 3 independent experiments, each performed in triplicate).

► FIGURE 3

Immunohistochemical profile of organoids.



Immunohistochemistry was performed to characterize organoids. (A) Organoids stained by H&E (left hand panel), and organoids expressing hepatocyte (Orange arrow) and biliary marker (blue arrow)(right hand panel). (B) Organoids expressing CK-19 (left and middle panel) and control H&E stained sections of a liver biopsy (right hand panel). (C) Organoids expressing CD31 (green) and nuclear stained DAPI (blue).

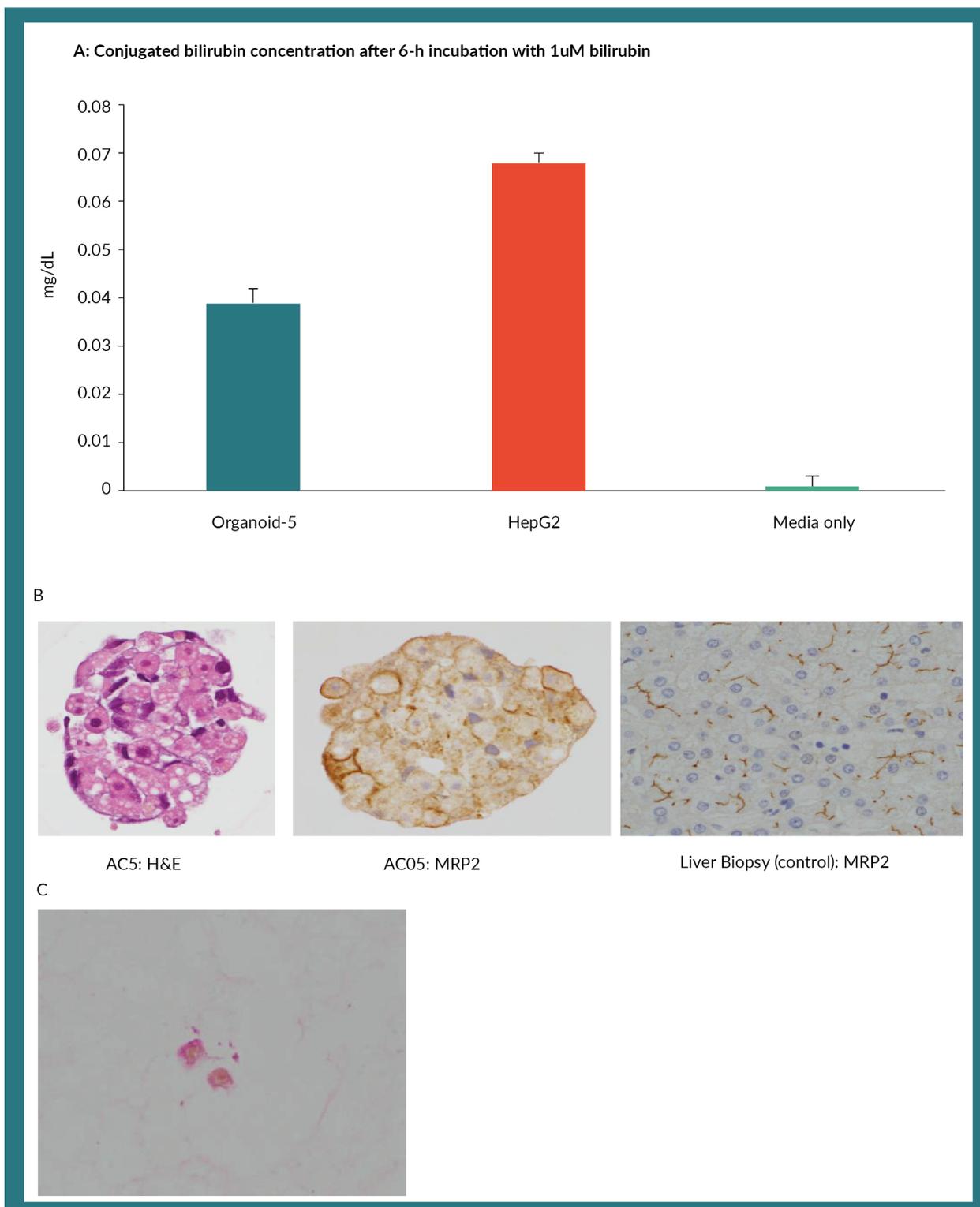
in industry and in the clinic. It is a well-known agent for the treatment of constipation [15] and its derivatives can be used as artificial tears [16,17] or saliva. In the laboratory, methylcellulose has long been used as a scaffold for the development of colony forming cells from haematopoietic stem/progenitor cells and is a good support for studying stem cell proliferation and differentiation [18–20]. As such, it should be relatively easy to implement a

clinical grade strategy using methylcellulose as a scaffold to obtain liver-organoids.

A further advantage to using methylcellulose as a scaffold is the flexibility to assess the effects of different growth factors, tailor-made chemicals or synthetic combinations on organoid formation. We found that the use of EGF and TGF- β together were crucial in establishing organoids. EGF is a member of the tyrosine kinase

► **FIGURE 4**

Organoids exhibiting biliary function.



(A) Organoids were able to conjugate bile as in control cells, as measured biochemically. (B) Immunohistochemistry confirmed this as organoids stained positively for MRP-2 antibody (middle panel), H&E left panel and control tissue (right panel) thus suggesting structure formation. (C) Fouchet staining of organoids showing bile stain, indicating the presence of conjugated bile.

receptor family [21] and is important in the upregulation of MAP kinase signalling pathway leading to DNA synthesis and cell proliferation [22]. TGF- β on the other hand is a growth factor known to induce apoptosis in lymphocytes and hepatocytes in mice [23]. It is also thought to stop proliferation and induce differentiation in stem cells [24]. It is intriguing that the synergistic effect of EGF and TGF- β in our experiments was necessary for the formation of organoids, to which the exact mechanism of organoid development remains to be determined. The simplicity of this system means that it can also be scaled up for drug screening and cell signalling experiments as well.

We and others have previously shown that hepatocytes isolated from a donor liver, can be used to treat children with ALF effectively [25–27]. Further improvements to hepatocyte transplantation has been the method of encapsulating hepatocytes with alginate beads [28,29]. This encapsulating method eliminates the need for immunosuppressing drugs that are routinely used in liver or non-encapsulated hepatocyte transplantation. Such alginate encapsulated human hepatocyte transplants in humans although not necessarily curative, have shown to be useful as a bridge to native liver recovery or until a suitable donor liver is found [Anil Dhawan, Personal Communication/Unpublished Data].

Recently, bio-fabrication techniques have been developed using 3D plotting with methylcellulose and alginate. MSCs cultured in these 3D scaffolds retained viability and differentiation properties [30,31]. Taken together, it is highly conceivable to be able to establish organoids

in methylcellulose, as done here and then encapsulating the resulting organoids in alginate-methylcellulose 3D scaffolds [32]. Such a strategy should be highly applicable in the treatment of ALF.

METHODS

Hepatocyte & MSC isolation

Primary hepatocyte isolation and research activities were ethically approved via the National Research Ethics Service (King's College Hospital local research ethics committee; LREC 01-016). Organs were donated through the National Health Service Blood and Transplant (NHSBT) and offered for hepatocyte transplantation and/or research following decline for solid organ transplantation. Hepatocytes were derived from donor as previously described [33]. All hepatocyte isolation procedures were performed to Good Manufacturing Practises (GMP) standards (Cell Therapy Unit, King's College Hospital) and governed by the Human Tissue Authority for the storage, clinical and research use of these cells. Briefly, surgically obtained split liver lobes were weighed and cannulated [26]. This was followed by an in-house modified collagenase perfusion step, followed by a perfusion to inactivate the collagenase. Perfused liver lobes were then macerated using scissors and forceps and filtered through gauze until clumps had reduced. Hepatocytes were obtained by centrifugation at 50g, where the hepatocyte fraction was collected from the cell pellet. Cell number and viability was determined by trypan blue exclusion. Cells were frozen in cryovials (research use) and cryobags (clinical use), using a controlled rate freezer

and stored at -180°C vapour phase nitrogen freezers.

MSCs were isolated from Wharton's jelly (WJ) of umbilical cords obtained following caesarean section deliveries at King's College Hospital. These umbilical cords were procured via the Anthony Nolan Trust, according to King's College Hospital Institutional guidelines. MSCs were isolated at research laboratory grade standards. Briefly, cords were collected in PBS containing $40\mu\text{g}/\text{ml}$ of gentamicin [34]. Cords were cut into sections to expose the WJ, which was minced into fine pieces ($1\text{--}3\text{ mm}^2$). The explants were placed in sterile petri dishes and immersed with MSC culture medium, consisting of MEM α (Life Technologies), 5% Stemulate (Cook Regentec), $40\mu\text{g}/\text{ml}$ of gentamicin and allowed to adhere for up to 5 minutes. Cord samples were then incubated at 37°C , 5% CO_2 in atmosphere and half medium replenished every 3–6 days. Gentamicin was used in the MSC medium for the first 1–2 weeks of culture and then replaced by penicillin/streptomycin ($100\text{ units}/\text{ml}$ and $100\mu\text{g}/\text{ml}$, respectively). Once cell cultures were established, they were expanded, quality controlled and cryopreserved. Cells were positive for MSC markers (i.e., CD73, CD90 and CD105; expression higher than 75%) and negative for hematopoietic markers (i.e., CD14, CD34 and CD45; expression lower than 2%) by flow cytometry.

Organoid formation

The initial establishment of liver organoids was adapted from Takebe *et al.* Briefly, hepatocytes and MSC

were thawed and cultured in round bottom non-tissue culture treated 96 well plates. The culture media consisted of DMEM/F12 high glucose (Gibco) as the basal media supplemented with N2 (Gibco) and B27 (Gibco) supplements, Penicillin and Streptomycin and L-glutamine. Matrigel (Corning) was added to a final concentration of 1%. The initial ratio of hepatocytes to MSC was 1:1 and 10^5 cells in $200\mu\text{l}$ total volume used. Plates were incubated at 37°C , 5% CO_2 in atmosphere. Different cell ratios were investigated. Plates were cultured for 2 weeks and monitored daily for the growth of organoids.

Having established the system in Matrigel, methylcellulose (Bio-Techne) was substituted as a scaffold. We also scaled up the process into non-tissue culture treated six well plates, omitting the use of Penicillin and Streptomycin. Methylcellulose was added to a similar culture media as above to a final concentration of 1.2%. $10\text{ng}/\text{ml}$ final concentration of TGF- β , HGF and EGF (all from BioTechne) was used in cultures, either individually and/or in combination. Plates were incubated at 37°C in 5% CO_2 in atmosphere. Plates were cultured for 2 weeks and monitored daily for the growth of organoids.

Albumin assay

Albumin secretion from organoids were measured by a standard ELISA protocol as previously described [13]. Briefly, organoids were transferred to fresh plates and incubated with media (less the scaffold) for 8 to 18 hours, and media was then removed and stored at -20°C until the assay.

Urea assay

Urea assay was performed according to previously established methods [13]. Again, organoids were transferred to new plates and incubated with media (less scaffold) in the presence of 4mM Ammonium Chloride. Urea production in the media was measured 6 hours later using QuantiChrome Urea Assay Kit (BioAssay Systems, Cambridge, UK).

Bile conjugation assay

Total and conjugated bile was determined by using a bilirubin assay kit from Sigma. One μM unconjugated bilirubin was used to spike organoids. This assay is based on the Jendrassik-Grof method which utilizes the reaction of bilirubin with diazotized sulfonic acid resulting in a colorimetric product measured at 530 nm, proportionate to the bilirubin present in the sample.

Histology

Developing and established organoids were fixed in 4% paraformaldehyde. Fixed organoids were embedded in 2.5% agarose. The set agarose-organoid material was then routinely processed. Five-micron thin paraffin embedded sections were placed on poly-lysine coated slides. Standard immunohistochemistry protocols were employed. Details of antibodies used in Table S1. All immune-stained sections were counter stained with Haematoxylin and permanently mounted.

Confocal microscopy was performed on live organoids mildly permeabilised with 0.01% triton-x

in PBS. Organoids were then stained with respective antibodies (Table S1), washed twice in PBS and mounted onto glass slides. Confocal microscopy was performed on a Leica SP5 microscope.

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AUTHOR CONTRIBUTIONS

Anil Chandrashekran, Anil Dhawan conceived and designed experiments; Anil Chandrashekran, Tharindu Premachandra, Chris Starling performed experiments; Anil Chandrashekran, Maesha Deheragoda, Anil Dhawan analyzed and interpreted data; Sharon Lehec, Ragai R Mitry, Celine Filippi, Valeria Iansante, Anil Chandrashekran for GMP hepatocyte isolation; Valeria Iansante responsible for MSC isolation; SB, Emer Fitzpatrick obtained and provided clinical samples; Ragai R Mitry, Celine Filippi, David Hay provided useful discussion, insight and support. Anil Chandrashekran, Anil Dhawan wrote the paper.



FINANCIAL & COMPETING INTERESTS DISCLOSURE

The authors have no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject

matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

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AFFILIATIONS

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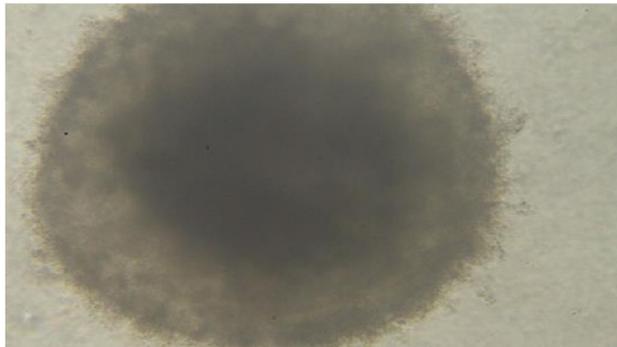
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Supplementary figure 1

A



B

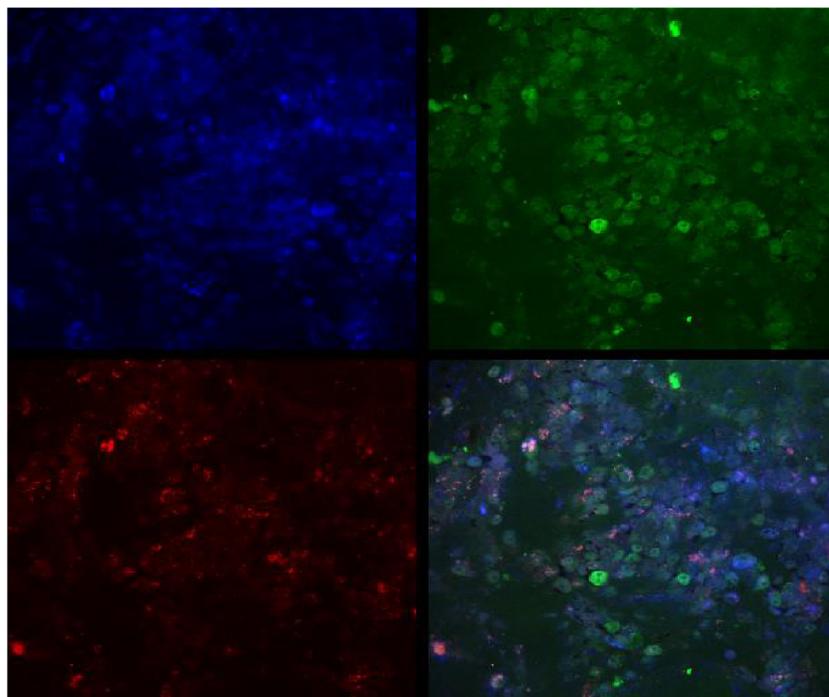


Figure S1 Organoids cultured in matrigel

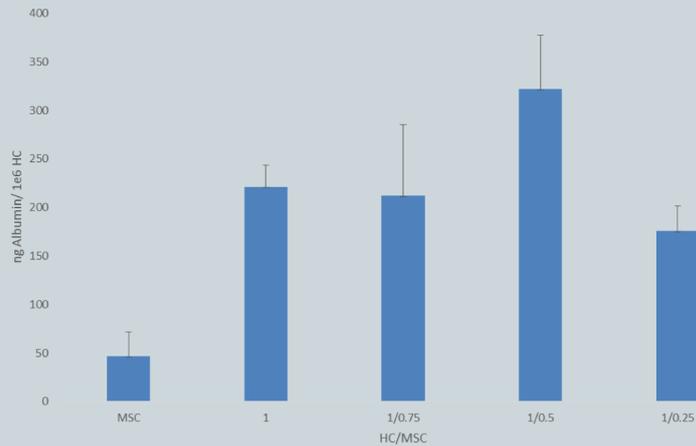
A. Morphology of organoids from a 96-well plate

B. Confocal imaging of hepatocyte (top right panel, in green) and CD105 marker (bottom left panel-in red and merged image (bottom right panel). Dapi stain of nuclei (Top left panel-in blue)

Supplementary figure 2

Albumin secretion from HC-MSC derived Organoids

A



Urea production from HC-MSC derived Organoids

B

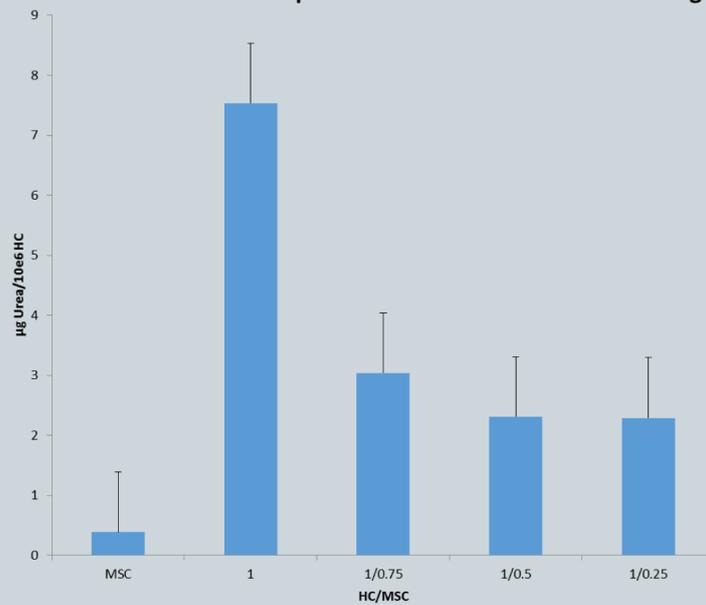


Figure S2 Cell ratio analysis for organoid development

A. Albumin ELISA demonstrating that a 2:1 hepatocyte: MSC ratio gave rise to the highest expression

B. Urea production was highest using a 1:1 hepatocyte: MSC ratio

Supplementary figure 3

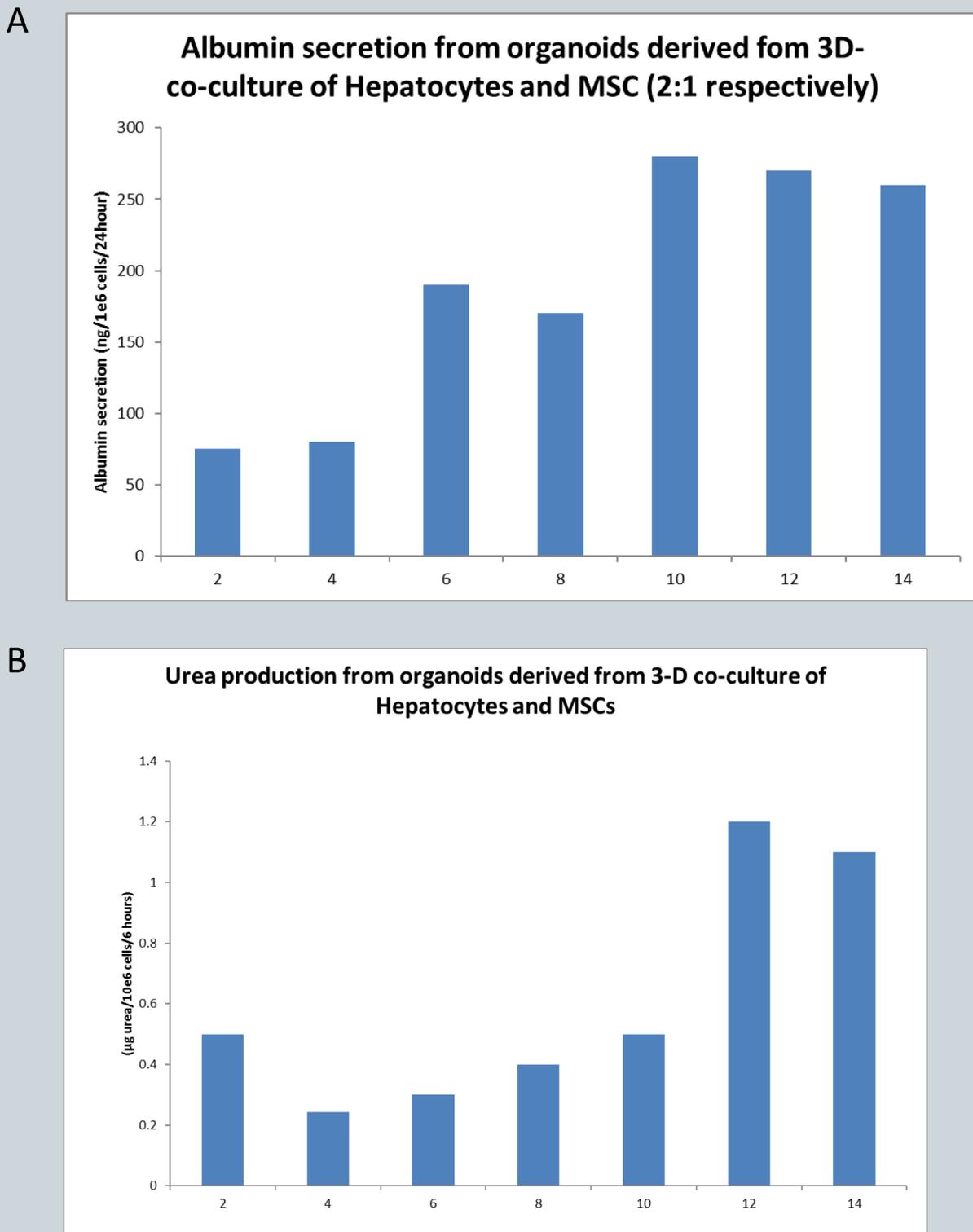


Figure S3 Time function of organoids

A. Albumin production was detected from day two in culture and peaking at day 10

B. Urea production was like albumin production except that a slightly higher level was noted on day 2. Urea production peaked at day 12

Supplementary figure 4

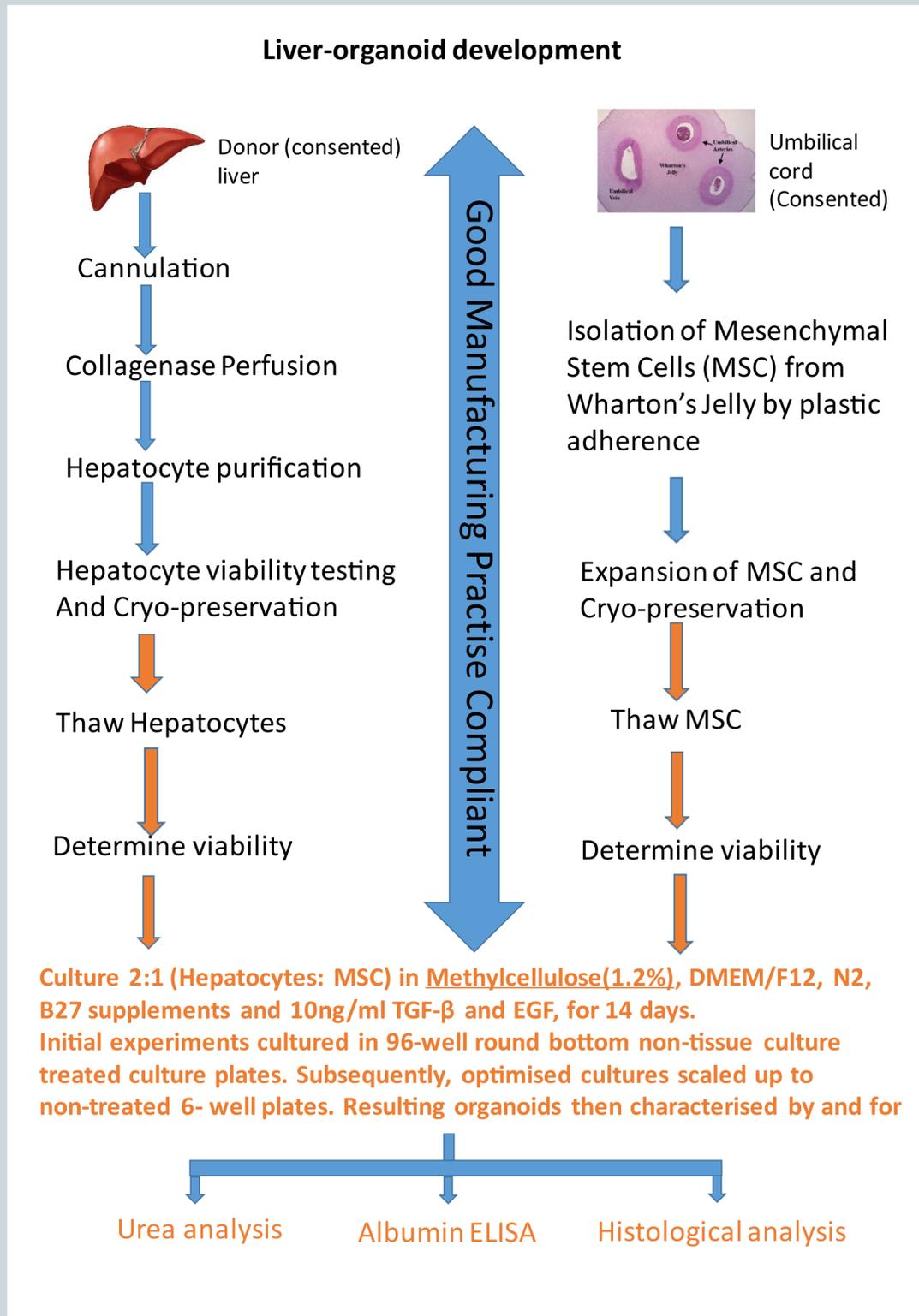


Figure S4 Schematic production for developing organoids suitable for transplantation

Antibody	Supplier	Catalogue #	Pretreatment	Dilution	Bond Protocol	Control	Result
cMOAT (F)	Abcam	Ab3373-250	ER2	1:40	F	Normal liver	Canaliculi +ve
CK 7	Dako	M7018	ER1	1:200	F	Normal liver	Bile ducts +ve
OCH1E5	Dako	M7158	ER1	1:100	F	Normal liver	Hepatocytes +ve
Cytokeratin 19	Abcam	ab52625	-	1:200	-	Normal liver	Bile ducts +ve
CD31	Abcam	ab28364	-	1:50	-	-	Endothelial cells +ve
CD105-PE	Biolegend	323205	-	1:50	-	MSC-P1	MSC +ve