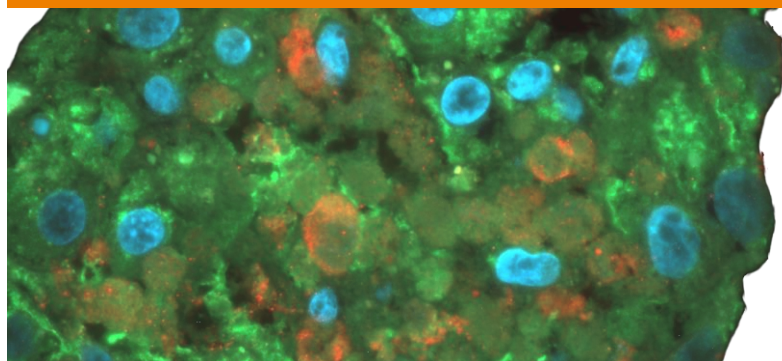


## Generation of HepaRG Spheroids in the CERO A 3D model for investigation of Hepatitis B Virus infections



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### Introduction

Research on Hepatitis B Virus (HBV) is hampered by the fact, that no mouse models exist for HBV infections. *In vitro* models more closely mimicking the *in vivo* structure and featuring several gradients like nutrients and oxygen are required.

HepaRG is a cell line derived from a Hepatitis C Virus positive patient upon liver resection. This cell line is kept in a proliferative state, but can be differentiated into hepatocyte-like cells (HLCs) and cholangiocyte-like cells (CLCs) by the addition of 1.8% DMSO into the medium. HLCs share a variety of similarities with primary human hepatocytes (PHHs) which are considered to be the “gold standard” of cell culture model for the liver, drug screenings, metabolism and viral infection studies. The HepaRGs show low batch-to-batch variations, good proliferation and have a stable metabolism, once differentiated.

Standard 2D culture of HepaRG is not supportive of the key liver features such as oxygen gradient along the liver’s small building blocks - liver sinusoids. We therefore aimed to generate a 3D model of HepaRG to investigate the dynamics of an HBV infection and also of antiviral therapy along an oxygen gradient. Using the CERO, we found HepaRG spheroids to be viable in long term culture over 80 days - highly expressing albumin, a hepatocyte marker. This indicates good differentiation efficiency into HCLs even without DMSO in the liquid spheroid culture.

### Methods

#### HepaRG cells and cultivation

HepaRG cells were obtained with an MTA from the Cancer Research Center of Lyon (CRCL), INSERM 1052. Cells were cultured in William’s Medium E + GlutaMAX (Gibco), supplemented with 10% FetalClone II (HyClone), 1% Penicillin/Streptomycin (Gibco), 17.12 µg/mL Hydrocortisone (Pfizer) and 0,023 I.E./mL (Sanofi). Cells were split once per week 1:6 and cultivated at 37°C with 5% CO<sub>2</sub>. HepaRG spheroids were cultured in the same medium, but with only 5% FCS.

#### Spheroid generation

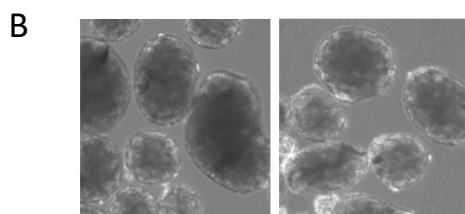
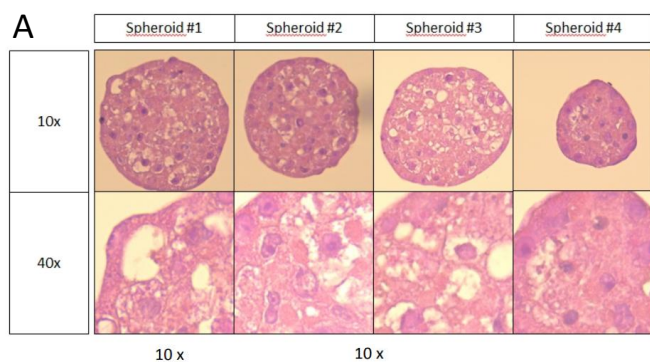
Cells were detached with Trypsin/EDTA solution, counted and pelleted. Then, cells were resuspended in fresh medium, supplemented with 10 µM Y-27632 (Cell Signalling Technologies) and 0.04% polyvinyl alcohol (Sigma Aldrich), and seeded into Aggrewell 800 24-well plates (Stem Cell Technologies) at 15000 cells/spheroid. Plates were pre-treated and cells were collected in the microwells according to the manufacturer. Medium was then replaced without Y-27632 after 24 hours, and medium was changed for 4 more days every 24 hours. 5 days after inoculation of a cell suspension into the Aggrewell plates, compact spheroids were visible, and up to 4 wells, i.e. up to 1200 spheroids, were resuspended in 45 mL medium supplemented with 0.01% CERO Solution 1 and inoculated into one CEROTube.

### Cultivation of spheroids

Spheroids were cultured always in the presence of 0.01% CERO Solution 1, which was shown by us to neither influence size, compactness nor fission/fusion ratio of spheroids. Settings in the CERO were: Rotation period 1'', Rotation pause none, Agitation period 2', Agitation pause none, Rotation speed 80 rpm, Protocol duration infinite. Medium was exchanged once per week. To do so, spheroids were transferred from the CEROTube into a 10 cm cell culture dish, and approx. 30 mL of the medium was removed with a vacuum pump through a 100 µM cell strainer. The process was repeated with 30 mL medium. Finally, the medium in the dish was filled up to 45 mL again and transferred into a CEROTube. By this procedure, extracellular matrix and cell debris, as well as small spheroids generated by fission of bigger spheroids were efficiently removed.

### Histological analysis

For histological analysis, spheroids were recovered from the CEROTube and the 45 mL was split into four 50 mL tubes, which were filled up to 50 mL with phosphate buffered saline (PBS, Gibco). Spheroids were allowed to settle by gravity, then 40 mL of the supernatant was removed and spheroids were pooled in one 50 mL tube, which was filled up to 50 mL with PBS. Spheroids were again allowed to settle by gravity flow and were washed once more with PBS. Spheroids were fixed overnight with 4% Paraformaldehyde (Carl Roth), and then dehydrated on the following day by incubation in 70%, 80%, 90% and 100% ethanol, 1 hour each, followed by a 1 hour incubation step in xylol. Spheroids were then embedded in paraffin, which was exchanged three times. 2 µM sections of spheroids were either prepared for immunohistochemistry or immunofluorescence.



**Fig. 1: Spheroids from HepaRG after 6 weeks in culture.**

**A:** H&E staining of FFPE Spheroids from HepaRG  
**B:** Phase contrast from Spheroids from HepaRG right before fixation

### Results

#### Viable Spheroids even after 80 Days

HepaRG Spheroids cells stayed viable for a prolonged time of >80 days, which was unachievable in stationary cultures, in which spheroids started to degrade 7 days after seeding and <5% viable spheroids were observed 20 days after seeding.

#### Differentiation into a HLC

In addition, spheroids stay quite similar in size over the time of cultivation, which is likely because of the low proliferation rate which was visualized by Ki67 staining of sections. Importantly, spheroids show strong expression of albumin (fig. 2), a marker for successful differentiation into a HLC.

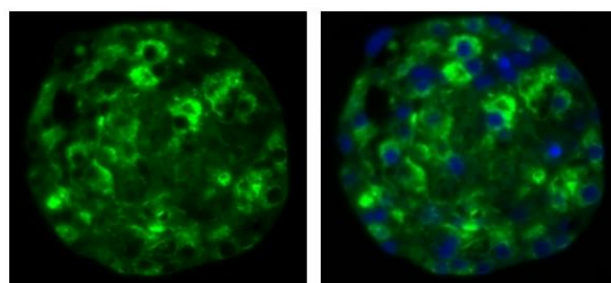
### Conclusion

#### Long-term culture for HBV Infections

We expect this 3D cultivation system to be an extremely important tool for HBV research using the HepaRG cell line, as HBV infections take 7-10 days to be self-sustaining in culture. After this time, 3D cultures of HepaRG would already start to degrade as a consequence of cell death, if they are kept in a stationary culture.

#### HBV Infection spreading a 3D Structure

In addition, expression of albumin is a good indication for a hepatic differentiation state of the cells, which rises the hope that spheroids can really be infected with HBV and that the infection could potentially spread through the 3D structure.



**Fig. 2: Spheroids from HepaRG after 6 weeks in culture.** Immunofluorescence staining of Spheroids from HepaRG for Albumin (green), nuclei counterstained in blue with DAPI

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