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# Differentiation of liver progenitor cell line to functional organotypic cultures in 3D nanofibrillar cellulose and hyaluronan-gelatin hydrogels

Melina M. Malinen [a], Liisa K. Kanninen [a], Anne Corlu [b], Helena M. Isoniemi [c], Yan-Ru Lou [a], Marjo L. Yliperttula [a], Arto O. Urtti [a,d,*]

[a] Centre for Drug Research, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.O. Box 56, FI-00014 Helsinki, Finland
[b] Inserm UMR991, Liver Metabolism and Cancer, Université de Rennes 1, F-35043 Rennes, France
[c] HUS, Transplantation and Liver Surgery Clinic, P.O. Box 340, FI-00029 HUS, Finland
[d] School of Pharmacy, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland

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ABSTRACT

Physiologically relevant hepatic cell culture models must be based on three-dimensional (3D) culture of human cells. However, liver cells are generally cultured in two-dimensional (2D) format that deviates from the normal *in vivo* morphology. We generated 3D culture environment for HepaRG liver progenitor cells using wood-derived nanofibrillar cellulose (NFC) and hyaluronan-gelatin (HG) hydrogels. Culture of undifferentiated HepaRG cells in NFC and HG hydrogels induced formation of 3D multicellular spheroids with apicobasal polarity and functional bile canaliculi-like structures, structural hallmarks of the liver tissue. Furthermore, hepatobiliary drug transporters, MRP2 and MDR1, were localized on the canalicular membranes of the spheroids and vectorial transport of fluorescent probes towards the biliary compartment was demonstrated. Cell culture in 3D hydrogel supported the mRNA expression of hepatocyte markers (albumin and CYP3A4), and metabolic activity of CYP3A4 in the HepaRG cell cultures. On the contrary, the 3D hydrogel cultures with pre-differentiated HepaRG cells showed decreasing expression of albumin and CYP3A4 transcripts as well as CYP3A4 activity. It is concluded that NFC and HG hydrogels expedite the hepatic differentiation of HepaRG liver progenitor cells better than the standard 2D culture environment. This was shown as improved cell morphology, expression and localization of hepatic markers, metabolic activity and vectorial transport. The NFC and HG hydrogels are promising materials for hepatic cell culture and tissue engineering.



## 1. Introduction

Pharmaceutical industry, regulatory authorities, and academic investigators need liver cell cultures to predict and estimate metabolism, excretion and toxicity of drugs and other chemicals in the human liver. Due to the inter-species differences animals and animal cells lead frequently to misleading, and sometimes hazardous, estimates of pharmacokinetics and toxicity in humans. Therefore, Food and Drug Administration of the United States has emphasized the need for improved preclinical cell models for drug development in their Critical Path Initiative.

Human liver microsomes are used to study xenobiotic metabolism, but the microsomes do not have drug transporters or transcription machinery. This limits seriously their usefulness in pharmacokinetics and toxicology. Primary human hepatocyte cultures in 2D are the gold standard in *in vitro* evaluation of hepatic

*Abbreviations:* 2D, two-dimensional; 3D, three-dimensional; ABC transporter, ATP-binding cassette transporter; ALB, Albumin; Calcein-AM, calcein-acetoxymethyl ester; CycloG, cyclophilin G (peptidylprolyl isomerase G); CYP, cytochrome P450; DMSO, dimethyl sulfoxide; dPBS, Dulbecco's modified phosphate salt buffer; ECM, extracellular matrix; F-actin, filamentous actin; HBSS, Hank's balanced salt solution; HG, hyaluronan-gelatin hydrogel (Extracel™); HNF4A, hepatic nuclear factor 4A; MDR1, multidrug resistance protein 1 (ABCB1, P-glycoprotein); MRP, multidrug resistance-associated protein; MRP2, ATP-binding cassette sub-family C member 2 (ABCC2); NFC, nanofibrillar cellulose (Growdex™); PBS, phosphate salt buffer; RT, room temperature; RT-PCR, real time polymerase chain reaction.

* Corresponding author. Centre for Drug Research, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.O. Box 56, FI-00014 Helsinki, Finland. Tel.: +358 919159636; fax: +358 919159725.
*E-mail address:* arto.urtti@helsinki.fi (A.O. Urtti).

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**Table 1**
HepaRG cultures were performed in distinct culture dishes depending on the end-point analysis.

| | Standard tissue culture treated 96-well plates | Ultra low Attachment 96-well plates (Corning Inc.) | Lab-tek chambered#1.0 borosilicate coverglass system (Thermo Scientific) | Lab-tek chamber slide system with Permanox® plastic (Thermo Scientific) | μ-plate 96-well plates (ibidi GmbH) |
|---|---|---|---|---|---|
| alamarBlue | 2D | 3D | | | |
| CYP activity | 2D | 3D | | | |
| PCR | 2D | 3D | | | |
| gDNA | 2D | 3D | | | |
| Live/dead | | | 2D & 3D | | |
| Transporter activity | | | 2D & 3D | | 2D & 3D |
| Immuno-histochemistry | | | | 2D & 3D | |

metabolism and toxicity. However, the availability of human primary hepatocytes is limited, they show substantial functional variability and restricted lifespan, and their drug transporter activity is low unless they are 'sandwich-cultured' [1,2]. Immortal human liver cell lines, such as HepG2 and HepaRG are widely used in *in vitro* studies [3,4]. Compared to HepG2 cells, HepaRG liver progenitor cells generate improved hepatic phenotype in culture and this continuous cell line has been successfully applied in the evaluation of chemicals and drug candidates [4–8]. However, 2D format of the current HepaRG cultures clearly deviates from the vectorial 3D morphology of the hepatocytes in the liver. Overall, the existing liver cell models are not satisfactory and more representative cell models are needed for biological research and drug and chemical testing.

Cellular phenotype can be tuned with the culture environment, particularly with extracellular matrix mimicking biomaterials. Preferably the biomaterials should provide fibrillar structures, extracellular matrix mimicking stiffness and hydrous environment with unrestricted permeation of nutrients and endogenous factors [9–13]. We investigated hydrogels of native wood-derived nanofibrillar cellulose (NFC) and hyaluronan-gelatin (HG) as supporting materials in the 3D culture of HepaRG liver progenitor cells.

## 2. Materials and methods

### 2.1. Biomaterials

Growdex™ nanofibrillar cellulose (NFC) hydrogel was obtained from UPM Corporation, Finland. The preparation and properties of NFC hydrogel have been described in detail earlier [12]. The NFC concentration of the hydrogel was 1.7 wt% and the product was sterile. Due to the used raw material, the NFC hydrogel contains also substantial amounts of hemicellulose, mainly xylene (25%), which generates a slightly anionic surface charge (−2 mV) on the fibrils. Extracel® (HG) hydrogel is based on thiol-modified hyaluronan, thiol-modified gelatin and crosslinker (polyethylene glycol diacrylate) (PEGDA) [21,22]. This material was obtained from Glycosan Biosystems, USA.

### 2.2. Human liver tissue

Human liver tissue was obtained from harvested organs for liver transplantation in the Transplantation and Liver Surgery Clinic (Helsinki, Finland). Donor livers were from brain dead male (age 41) and female (ages of 58, 13 and five) subjects with beating heart, normal liver function, negative hepatitis serology, and non-pathologic liver histology. The livers were flushed *in situ* with University of Wisconsin solution and kept at +4 °C until resection. Liver segments that were redundant for liver transplantations were sliced and stored at −70 °C in TRI reagent (Sigma–Aldrich) or at −20 °C in RNAlater (Qiagen). RNA was extracted (see paragraph for real time polymerase chain reaction). The research was authorized by National Supervisory Authority for Welfare and Health and by the Hospital District of Helsinki and Uusimaa Ethics Committee Department of Surgery.

### 2.3. HepaRG cell line

HepaRG cells have been derived from a liver tumor of a female patient who suffered from hepatitis C virus and hepatocarcinoma [23]. HepaRG cells are capable of differentiating into biliary-like epithelial cells (cholangiocyte-like cells) and hepatocyte-like cells. At low culture density, the cells express markers of early liver progenitors, and at confluence the cells will be committed to hepatocyte-like differentiation [24]. The differentiation into mature hepatocyte-like cells is potentialized by dimethyl sulfoxide (DMSO) treatment. Subcultivation at low-density induces dedifferentiation of differentiated cells into early liver progenitors.

### 2.4. Cell cultures

The 2D HepaRG cultures were used as a benchmark to compare with the 3D hydrogel cell cultures. HepaRG cells were cultured in 2D format as described previously [23,24]. Shortly, HepaRG cells were plated either at progenitor state to study the differentiation process or at differentiated state (obtained with DMSO treatment) to study the maintenance of the differentiation. Undifferentiated progenitor cells were seeded at low-density ($2.6 \times 10^4$ cells/cm$^2$) and differentiated at high-density ($45 \times 10^4$ cells/cm$^2$). The cell cultures were placed to the different dishes depending on the end-point analyses (Table 1). The medium volume was set to be 400 μl/cm$^2$ in low-density cultures and 600 μl/cm$^2$ in high-density cultures, hereby representing the equal total volumes with 3D hydrogel cultures.

The HepaRG cells were embedded in 3D NFC and HG hydrogels. Undifferentiated cells were seeded at low-density (one million cells/ml of hydrogel) and differentiated at high-density (nine million cells/ml of hydrogel). NFC hydrogel-based 3D cell cultures were prepared by mixing the HepaRG cell suspension with 1.7 wt% NCF hydrogel to achieve 1 wt% hydrogels with desired cell density. The mixing was performed by pipetting up and down with low-retention pipette tips (TipOne®, Starlab Group). HG hydrogel-based 3D cell cultures were prepared according to the manufacturer's instructions (Glycosan Biosystems). Briefly, the cells were mixed with HG solutions followed by the gelification at 37 °C in an atmosphere containing 5% $CO_2$. The cell cultures were placed to the different dishes depending on the endpoints analysis (Table 1). The volume of the hydrogel cell cultures was set to be 200 μl/cm$^2$ in each dish. The medium volume (on the top of the hydrogel) was set to be 200 μl/cm$^2$ for low-density cultures and 400 μl/cm$^2$ for high-density cultures, hereby representing the equal total volumes with 2D cultures.

The low-density cultures were maintained with the standard HepaRG growth medium and the high-density cultures with HepaRG differentiation medium supplemented with DMSO [23]. The equal volume of the medium was renewed daily both from the 2D and 3D hydrogel cultures (2/3 of the medium volume in the hydrogel cultures). The cell cultures were maintained at 37 °C in an atmosphere containing 5% $CO_2$.

### 2.5. Microscope analysis of cell growth

Cell cultures were monitored by phase contrast microscopy (Leica DM IL LED) and images were taken over the time. The average diameter of spheroids was defined from the phase contrast images with LAS EZ software (Leica Microsystems) using the distance line facility. Minimum of 10 spheroids were measured to attain the average size.

### 2.6. Cell viability

The mitochondrial metabolic activity of the cells was determined using oxidation-reduction indicator, resazurin (alamarBlue® Cell Viability Reagent, Invitrogen), 1/10 of the co-volume of medium and hydrogel. To ensure the mixing of the indicator in hydrogels the culture plates were gently shaken (150 rpm) for 10 min both in the beginning and the end of the incubation (Heidolph incubator 1000 equipped with Titramax 1000 shaker). After exposure of 3 h to resazurin at 37 °C in 5% $CO_2$, 50 μl of medium was transferred from each culture well to another 96-well plate and the fluorescent metabolite of resazurin (resorufin) was recorded with a plate reader (Varioskan Flash, Thermo Fisher) using excitation at 560 nm and emission at 590 nm. Three independent experiments of both low-density and high-density cultures were carried out. The mitochondrial metabolic activity of the cells was examined within the same wells as CYP3A4 activity and RT-PCR.

Cell viability was analyzed using LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes™). In this case, 0.5 μM calcein-AM and 2 μM ethidium homodimer-1 were added to the cultured cells in Hank's balanced salt solution (HBSS). The conversion of

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