Tissue Engineering

J. Cell. Mol. Med. Vol 13, No 9B, 2009 pp. 3387-3397

Functionalized self-assembling peptide hydrogel enhance maintenance of hepatocyte activity *in vitro*

Elsa Genové [a, b], Stephanie Schmitmeier [c], Ana Sala [c], Salvador Borrós [a],
Augustinus Bader [c], Linda G. Griffith [d], Carlos E. Semino [a, b, *]

[a] *Barcelona Bioengineering Center, Institut Químic de Sarrià, Universidad Ramon Llull, Barcelona, Spain*
[b] *Center for Biomedical Engineering, Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, MA, USA*
[c] *Biomedical-Biotechnological Center, Leipzig University, Leipzig, Germany*
[d] *Department of Biological Engineering and Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA*

Abstract

There is a major challenge in maintaining functional hepatocytes *in vivo* as these cells rapidly lose their metabolic properties in culture. In this work we have developed a bioengineered platform that replaces the use of the collagen I – in the traditional culture sandwich technique – by a defined extracellular matrix analogue, the self-assembling peptide hydrogel RAD16-I functionalized with biologically active motifs. Thus, after examining side by side the two culture systems we have found that in both cases hepatocytes acquired similar parenchymal morphology, presence of functional bile canaliculi structures, CYP3A2 induction by dexamethasone, urea production, secretion of proteins such as apolipoprotein (class A1, E, J), α_1-microglobulin, α_1-macroglobulin, retinol binding protein, fibronectin, α_1-inhibitor III and biotin-dependent carboxylases. Interestingly, by assessing in more detail some other hepatic markers, one of the functionalized matrix analogues – carrying the 67 kD laminin receptor ligand – enhanced the gene expression of albumin, HNF4-α, MDR2 and tyrosine aminotransferase. We conclude that the use of a synthetic culture system with designed matrix functionalization has the advantage in controlling specific cellular responses.

Keywords: extracellular matrix analogue • hepatocyte function • sandwich culture system • self-assembling peptides • collagen • liver enriched genes • real-time PCR

Introduction

The liver is a complex organ which performs many basic functions and metabolic activities including synthesis of plasma proteins, storage of glycogen, removal of toxins and metabolization of xenobiotics among others. When isolated, hepatocytes lose their surrounding microenvironement which comprises the extracellular matrix and the interaction with other hepatic cells such as sinusoidal endothelial cells, Kupffer cells, stellate cells, and resulting in a rapid loss of liver-specific functions. Many strategies have been used to obtain *in vitro* cultures which maintain these hepatocyte functions, including the use of extracellular matrices like Matrigel, collagen or liver derived basement membrane matrix [1–8] or cocultures with hepatic non-parenchymal cells [9–12]. Most recently there has been an approach towards the use of synthetic biomaterials such as hydrogels, porous membranes or the potential utility of perfused bioreactors to assess these goals [13–17].

We previously demonstrated the use of the self-assembling peptide hydrogel Puramatrix (RAD16-I, peptide sequence Ac-RADARADARADARADA-CONH$_2$, R arginine, A alanine, D aspartic acid) for certain tissue engineering applications. This synthetic and defined biomaterial is made of interweaving nanofibres 10–20 nm in diameter, which highly mimics some features of the extracellular matrix. This scaffold has previously been shown to promote the differentiation of a liver progenitor cell line (Lig-8) into hepatocyte-like spheroids [18]. In another study, it was used to entrap hippocampal neural cells [19] and, to enable osteogenic differentiation of mouse embryonic stem cells and mouse embryonic fibroblasts [20]. Moreover, we designed and characterized modified peptide scaffolds with biologically active peptide sequences in order to create instructive synthetic extracellular matrices [21].

*Correspondence to: Carlos E. SEMINO,
Center for Biomedical Engineering, NE47-383,
Biological Engineering Division, Massachusetts Institute of Technology,
500 Technology Sq., Cambridge, MA, 02139 USA.
Tel.: [1] 617-258-0249
Fax: [1] 617-258-5239
E-mail: semino@mit.edu

In this work, we have used functionalized self-assembling peptide scaffolds to build a hepatocyte culture system based on the collagen sandwich configuration, considered today as the gold standard for primary hepatocyte culture *in vitro* [5, 7] which recreates hepatic architecture. Here, we have combined RAD16-I with a modified self-assembling peptide scaffold containing the integrin-binding sequence GRGDSP (RGD = Ac-G<u>RGD</u>SP-GG-RADARADARADARADA-CONH$_2$), the 67 kD laminin receptor binding sequence YIGSR (YIG), or the heparin binding sequence present in collagen IV TAGSCLRKFSTM (TAG) in order to create a microenvironement that has features of extracellular matrix *in vivo* [21]. We have compared the maintenance of some liver specific functions of these sandwich systems to the results obtained using a similar collagen sandwich counterpart. We have analysed several liver-specific functions in terms of gene expression by means of quantitative PCR, examined albumin and urea secretion, and studied several cellular proteins of interest such as albumin, HNF4-α and biotin-dependent carboxylases. We also analysed the secreted proteome in which we detected several plasma proteins and proteases such as apolipoproteins A1, E and J, α_1-microglobulin, α_1-macroglobulin, retinol binding protein, fibronectin and α_1-inhibitor III. We have found that, hepatocytes cultured in a peptide sandwich configuration maintain similar levels of differentiation as in collagen sandwich cultures, as observed by comparable levels of the liver-specific functions studied. We have also observed that CYP3A2, one of the key Phase-I drug-metabolizing enzymes, can be induced to the same levels of that in collagen sandwiches. This peptide culture system presents the advantage of being completely synthetic and defined. This provides a solution to the problems associated with the use of a naturally derived matrix, including batch to batch deviations and the possible carryover of pathogens. Moreover, peptide sequences can be added to the matrix in a facile manner by direct solid phase synthesis, systematic variation of molecular cues in the extracellular environment and tailoring of that environment to promote specific activities.

Materials and methods

Hepatocyte isolation

Hepatocytes were obtained from male Fischer rats weighing 150–230 g by a perfusion using Liberase Blendzyme 3 solution (Roche, Indianapolis, IN, USA, 1814184). Each isolation yielded hepatocytes with viabilities 75–95% as based on trypan blue exclusion. Hepatocytes were suspended in hepatocyte growth medium (see below) and used for collagen or peptide sandwiched cultures.

Culture of primary hepatocytes in a collagen sandwich system

Collagen (Rat tail collagen, BD Biosciences, San Jose, CA, USA, 354236) substrates were prepared at a final concentration of 2 mg/ml following the manufacturer's instructions. Briefly, the collagen solution was diluted in water and PBS 10×, and neutralized with 1M NaOH. A total of 80 or 40 μl of the collagen solution were loaded into the bottom of the tissue culture insert (Millipore, Billerica, MA, USA, PICM 1250) and the collagen was allowed to gel at 37°C for 30 min. The 80 μl and 40 μl gels provided 1- and 0.5-mm-thick gels, respectively, and will be referred to by height in the text. After that time, hepatocyte culture medium (HCM, Cambrex, MD, USA, CC-3198, containing 2% fatty acid free bovine serum albumin [BSA], transferrin, insulin, recombinant human epidermal growth factor, ascorbic acid, hydrocortisone and Gentamycin/Amphotericin) was added to the system. Then, 65,000 cells/cm^2 were loaded into the tissue culture insert. Cultures were incubated at 37°C overnight. The next day, the unattached cells were removed by washing two times with sucrose 10% and another layer (80 or 40 μl) of collagen were added on top and allowed to gel in the incubator for 30 min. After that time culture medium was added to the bottom of the well and inside the insert. Cultures were maintained for 10 days in a water-jacketed incubator at 37°C and 5% CO$_2$. Culture medium was changed daily.

Culture of primary hepatocytes in a peptide sandwich system

Peptide scaffolds were prepared by diluting a 1% (w/v in water) peptide hydrogel RAD16-I solution (Puramatrix, BD Biosciences, 354250) with an equal volume of 20% sucrose (w/v in water) to achieve final peptide concentration of 0.5% (w/v in 10% sucrose). When the modified peptides were used, they were mixed in a proportion 95:5 with the prototypic peptide (RAD16-I) before dilution with the sucrose solution, vortexed and sonicated for 10 min. to ensure homogeneous mixing. The peptide solution becomes a hydrogel when it contacts a salt solution, such as a buffer or culture media [22]. Thus, 80 or 40 μl volume of peptide was loaded into the bottom of a tissue culture insert (Millipore, PICM 1250), and 200 μl of HCM was added underneath the insert membrane to initiate gel formation, giving rise to 1 and 0.5-mm-thick gels. After the peptide gel was set (10–15 min.), 200 μl of HCM was added into the insert, and the gels were allowed to equilibrate for 30 min. in the incubator at 37°C. The media were then changed and a volume of hepatocyte cell suspension was added to give a final cell density of 65,000 cells/cm^2 and cultures were incubated overnight to allow cell attachment. The next day, unattached cells were washed by rinsing them three times with sucrose 10%. Then, 80 or 40 μl of peptide were added on top, to entrap the cells in a sandwich fashion. 200 μl of media were added below the insert membrane, and the solution was allowed to gel for 30 min. After that time the peptide was equilibrated by carefully adding 20 μl of media on top, which was then left to sit for 20 min., and then the process was repeated one more time. Finally, a 50 μl volume of medium was carefully added into the insert and 650 μl were added outside of it. The medium was carefully changed daily, to avoid disturbing the upper peptide layer. Cultures were maintained for 10 days in a water-jacketed incubator at 37°C in 5% CO$_2$.

Bile canalicular-like structure staining

Transport in bile canalicular-like structure was visualized by treatment of the cultures with fluorescein diacetate (FDA). Briefly, to assess functional transportation by bile canaliculi, media containing 2.5 μg/ml of FDA (Molecular Probes, San Francisco, CA, USA) was added to 7-day peptide

sandwich cultures. Cells were incubated for 20 min. and visualized under a Nikon Epifluorescence microscope (Nikon, Boston, MA, USA).

RNA isolation and qPCR analysis

RNA of days 7 and 10 (not included) cultures was obtained by treating the cells with Trizol Reagent (Invitrogen, Carlsbad, CA, USA, 15596–026), followed by a extraction with an RNEasy kit (Qiagen, Valencia, CA, USA, 74104). RNA of freshly isolated hepatocytes was extracted and used as a control. The obtained RNA was quantified, the quality was assessed, and then samples were treated with DNAse I (Invitrogen, 18068) to remove contaminant DNA. Then, 100 or 150 ng was reverse transcribed with an Omniscript reverse transcription kit (Qiagen, 205111) using random hexamers. Finally q-PCR was carried out using QuantiTect SYBR Green PCR kit (Qiagen, 204143) in a MJ Opticon Monitor instrument as it has been previously described [23]. The primer sequences were the following: albumin forward 5′-GGTGCAGGAAGTAACAGACTTTG-3′ and reverse 5′-TAACTTGTCTCCGAAGAGAGTGTG-3′; HNF4-α forward 5′-CTGAGACTC-CACAGCCATCA-3′ and reverse 5′-CTAGATGGCTTCCTGCTTGG-3′, CYP3A2 forward 5′-GTAGTACTCTTTCCATTCCTCACCC-3′ and reverse 5′-GGTGCT-TATGCTTAGAATCCAGAC-3′; TAT forward 5′-CATCGTGGACAACATGAAGG-3′ and reverse 5′-TTGTACTTCCCCGAGTCCAG-3′; MDR2 forward 5′-CTTTGTGGTGGGGACACTCT-3′ and reverse 5′-CCAAGAAGACCAGCGA-GAAC-3′; 18s forward 5′-GCAATTATTCCCCATGAACG-3′ and reverse 5′-GGCCTCACTAAACCATCCAA-3′.

Relative gene fold enrichments were determined by the $2^{-\Delta\Delta Ct}$ method using the ribosomal unit 18s as a housekeeping gene. Samples were compared to the gene expression in freshly isolated hepatocytes. Experiments were performed in duplicate with three samples by experiment. The data represent the media value and the standard deviation of two experiments combined. Sizes of PCR products were analysed using a 2% agarose gel, and stained with ethidium bromide. ANOVA tests were performed to analyse statistical significance.

Albumin secretion

Media samples corresponding to days 3, 5, 7 and 10 hepatocyte sandwich cultures were obtained and kept at –20°C for further analysis. Albumin synthesis was measured with a specific rat albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA, E110-125). Briefly, 96-well plates were coated with a sheep anti-rat albumin antibody (1:100 in a carbonate-bicarbonate buffer pH 9.6) for an hour. After that, the wells were washed three times with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween-20 pH 8) and they were blocked for 30 min. with blocking solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween-20, 1% BSA, pH 8). Then, 100 μl of standards and samples (1:600 dilution) were incubated for 1 hr, following five washes with washing solution. Then, the wells were incubated for another hour with 100 μl of a secondary antibody HRP conjugated solution (1:50,000), and the wells were washed again five times with washing buffer. Finally, wells were incubated 15 min. with 100 μl of TMB substrate, and the reaction was stopped by adding 100 μl of H_2SO_4 2 M. Absorbances were assessed by reading at 450 nm wavelength in a microplate reader. Values were normalized using the cellular lysate total protein content value. Each experiment was performed in duplicate and the data represent the media value and the standard deviation of the two experiments combined. ANOVA tests were performed to analyse statistical significance.

Urea production

Media samples from hepatocyte sandwich cultures were collected from peptide and collagen cultures and stored at –20°C until analysis. Urea was quantitated using a urease method. Briefly, 10 μl of sample were incubated with 25 μl of urease solution (Sigma-Aldrich, St. Louis, MO, USA, US-3383) for 20 min. at room temperature. After that time, 50 μl of phenol nitroprusside (Sigma-Aldrich, P6994), 50 μl of alkaline hypochlorite (Sigma-Aldrich, A1727) and 200 μl of Milli-Q water were added to each well, and were incubated for 30 min. at room temperature. Absorbance was measured at 590 nm. Absorbance values were quantified using a calibration curve ranging from 0 to 150 mg urea/l, and values were normalized against the cell lysate total protein value. Each experiment was performed by duplicate with three samples per experiment. The data represent the media value and the standard deviation of the two experiments combined. ANOVA tests were performed to analyse statistical significance.

Total protein quantification

Hepatocyte cultures were lysed using 300 μl of RIPA buffer (Boston Bioproducts, Worcester, MA, USA, BP-115D) containing protease inhibitors (Complete Mini protease inhibitor cocktail, Roche, 1836153). Lysates were sonicated for 5 min. and the solutions were centrifuged for 10 min. at 10,000 rpm to remove cell and scaffold debris. Total protein contained in the supernatant was quantified using the BCA assay kit (Pierce Biotechnology, Rockford, IL, USA, 23225) following the manufacturer's instructions.

SDS-electrophoresis and Western blotting

Hepatocytes for each culture condition were lysed using RIPA buffer (Boston Bioproducts, BP-115D) containing a cocktail of protease inhibitors (Roche Complete mini, Indianapolis, IN, USA, 1836153), samples were sonicated and maintained at –20°C until analysed. Total protein was quantitated with a BCA Protein Assay kit (Pierce Biotechnology, 23225). Volumes containing 5 μg of protein were mixed with sample buffer (Invitrogen, NP0007) boiled for 5 min. and loaded into a 12% Bis-Tris gel electrophoresis system (Invitrogen, NP0301). Proteins were electrophoresed using MOPS buffer (Invitrogen, NP0001), and then they were transferred for 2 hrs to a polyvinyliden difluoride membrane (Invitrogen, LC2002) and used to perform Western blot. The membranes were first blocked for 3 hrs with blocking buffer (5% dry skim milk in TBST buffer). Then they were incubated independently overnight at 4°C with several antibodies diluted in the same blocking buffer: sheep anti-rat albumin-HRP conjugated antibody (1:20,000 dilution, Bethyl Laboratories, A110-134P), goat anti-HNF4-α antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-6556), goat anti-actin antibody (1:500 dilution, Santa Cruz Biotechnology, sc-1615). Each membrane was then washed three times with blocking buffer. A secondary antibody HRP conjugated was used for detection by chemiluminescence. Goat polyclonal antimouse IgG-HRP conjugated (1:1000 dilution, Santa Cruz Biotechnology, sc-2302) was used for HNF4-α and actin detection. The membranes were incubated for 6 hrs with the secondary antibody, then washed once for 20 min. with blocking buffer and twice with TBS. Western blots were developed using a chemiluminescent system (Supersignal West Pico Chemiluminiscent Substrate, Pierce Biotechnology, 34080) and protein bands visualized using an FluorChem gel imaging system (Fluorchem, Alpha Innotech, St. Leandro, CA, USA).

For biotin dependent carboxylases detection, the membranes were blocked with blocking buffer for 3 hrs and then incubated overnight at 4°C with avidin-HRP conjugated in TBS (1:2000 dilution, Peprotech, Rocky Hill, NJ, USA). Membranes were washed t times with TBS and bands were visualized by chemiluminescence as explained above.

Analysis of secreted protein by a proteomic approach

Media samples from hepatocyte sandwich cultures were electrophoresed using a 12% Bis-Tris gels and MOPS buffer. Gels were stained with coomasie blue staining solution and HCM was loaded as a control. Differential bands from samples to controls were cut and identified using a proteomic approach. Briefly, bands were desalted and digested with trypsin, and eluted using an Agilent 1100 Nanoflow HPLC system connected to a Thermo Electron LTQ Ion Trap (Waltham, MA, USA) mass spectrometer. Ions were scanned in a range molecular weight range from 500 to 3500 Da. Probability-based validation of protein identifications were performed with a modified SEQUEST algorithm to match the fragmentation patterns with the theoretical fragment patterns of tryptic peptides in the Uniprot Rat protein database. Ions were considered valid when Xcorr was 2.0 for peptides with $m/z = 1$, 2.5 for peptides with $m/z = 2$ and 3.5 for peptides with $m/z = 3$.

Cytochrome CYP3A2 induction with dexamethasone

Cells (65,000 cells/cm^2) were cultured for 5 days under standard conditions described above and medium containing 10 μM dexamethasone (DEX; MP Biomedicals, Solon, OH, USA, 190040) was added on day 6. RNA samples were taken at day 7 for analysis of the cytochrome CYP3A2 by qPCR.

Results

The use of peptide scaffolds for long-term hepatic cultures were evaluated by determining liver-specific functions of hepatocytes cultured in such systems. The results obtained were compared with those from the standard collagen sandwich model used as a control. The collagen and peptide hepatocyte sandwich cultures were prepared as shown in Fig. 1. Matrix substrates were prepared first, then hepatocytes were added, incubated overnight at 37°C, washed to remove non-adherent cells and finally another layer of gel was added to entrap the cells in a sandwich fashion. For the peptide hydrogel matrices, a 0.5% solution in sucrose 10% was gelled through contact with culture medium (see 'Materials and methods').

We first observed that the morphology and appearance of hepatocytes cultured in both collagen and peptide hydrogel sandwich systems were similar over the entire culture period (Fig. 2A). In all cases – including cultures with functionalized peptide hydrogels – hepatocytes became flattened, adopted an *in vivo*-like polygonal shape and by day 3 established extensive cell–cell con-
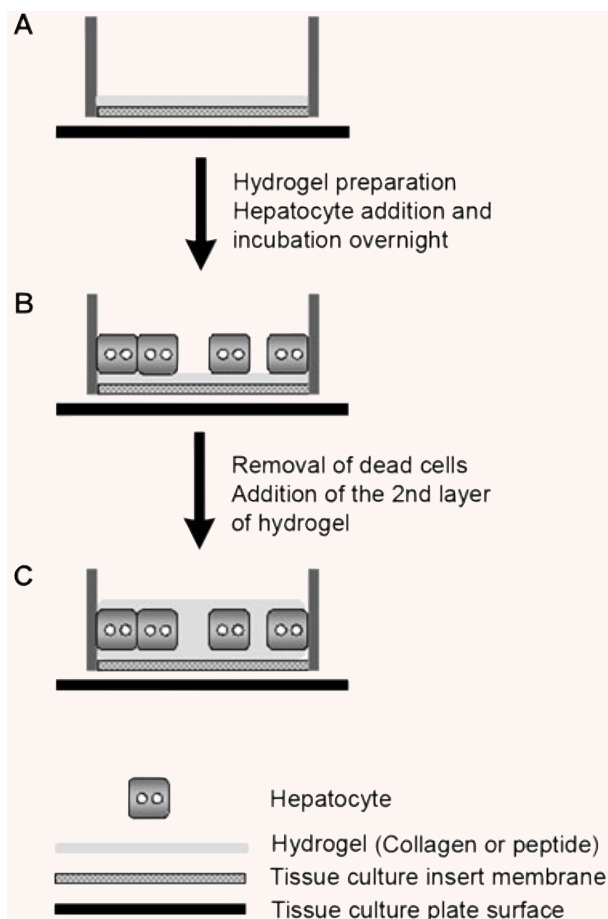

Fig. 1 Process of entrapping hepatocytes in a sandwich fashion. (**A**) First the collagen or peptide matrix is prepared as described in 'Materials and methods'. (**B**) Primary hepatocytes at a density of 65,000 cells/cm^2 are added onto the matrix. (**C**) After overnight incubation at 37°C, unattached cells are removed, and a second layer of collagen or peptide is added to the cells.

tacts with a reorganization of bile canaliculi-like structures. To demonstrate the functionality of the intercellular biliary zones, the cultures were incubated in presence of FDA. We observed that they were capable of metabolizing and transporting the dye from the cytoplasm into the canaliculi-like structures, as demonstrated by fluorescence microscopy (Fig. 2B). A similar observation has (previously) been reported for primary hepatocytes in collagen sandwich cultures [24–26].

Moreover, we wanted to assess gene expression analysis by means of quantitative PCR between freshly isolated hepatocytes and our culture systems (Fig. 3). In addition, because matrix thickness might influence nutrient and oxygen transport, and therefore hepatocyte function, we studied gene expression in cultures with 1 mm and 0.5 mm gel heights [27] (see 'Materials and methods'). As metrics of hepatocyte differentiation, we examined albumin and

Fig. 2 Hepatocyte sandwich cultures develop a compact parenchyma monolayer with visible bile canalicular-like structures. (**A**) Phase contrast microscopy images showing the morphology of primary rat hepatocytes in the (a) collagen, (b) RAD16-I, (c) YIG, (d) RGD and (e) TAG peptide sandwich cultures. (**B**) Bile canalicular-like structures in hepatocytes in the (a, e) RAD16-I, (b, f) YIG, (c, g) RAD and (d, h) TAG peptide scaffold cultures were visualized by incubating the cells with FDA (a–d) Phase contrast and (e–h) fluorescence images were taken at day 7 of culture.
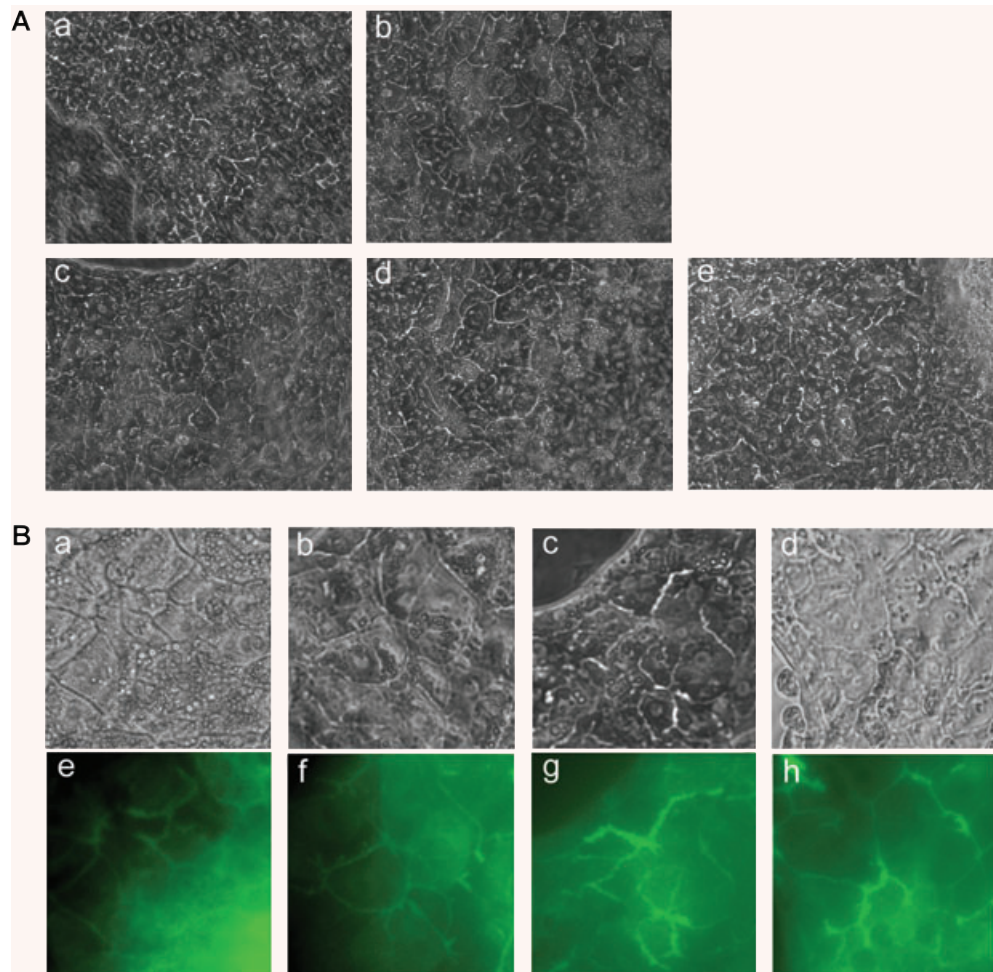
HNF4-α, one hepatic metabolic enzyme, tyrosine aminotransferase (TAT) and MDR2 as a canalicular membrane marker transporter [28] on day 7. We observed that compared with the 1 mm hydrogel cultures, the use of the 0.5 mm collagen and peptide matrices resulted in a higher expression of all the genes studied, suggesting that mass transfer through the hydrogel is affected by the gel thickness (Fig. 3). Therefore, we decided to use the 0.5-mm-thick gels for the rest of experiments and we included the modified self-assembling peptide TAG ('Materials and methods'). Despite the down-regulation of most of the genes studied in the 1 mm gels relative to freshly isolated hepatocytes (Fig. 3A), it can be observed that the gene expression profile was similar in the collagen and the peptide sandwiches. The functionalized peptide YIG-containing matrix exhibited the highest levels of gene expression for all genes studied (Fig. 3A). Nevertheless, the gene expression for MDR2 remained strongly down-regulated as compared to freshly isolated hepatocytes over the entire culture period (Fig. 3A). Looking deeper into the gene expression analysis for the 0.5 mm

gels, it can be observed that in general peptide hydrogel and collagen cultures remained mainly in the same levels of expression. Relative expression values that fell between −1 and 1 were considered the same as fresh hepatocytes. In all conditions tested it was observed that albumin and HNF4-α have already reached the expression levels of freshly isolated hepatocytes. In the case of albumin, all the peptide hydrogel cultures were significantly different than collagen. For HNF4-α significant differences were only observed for RAD16-I and YIG. In addition, MDR2 remained down-regulated in almost all conditions tested, except for YIG, which reached the relative levels of freshly isolated hepatocytes, and significantly different than collagen (Fig. 3B). However, RAD16-I and TAG presented a slight but significative up-regulation in MDR2 compared to collagen (Fig. 3B). Moreover, TAT in collagen cultures remained somehow down-regulated, but its expression in peptide hydrogel sandwich cultures was comparable to the positive control (freshly isolated hepatocytes), and significantly different than collagen cultures (Fig. 3B).
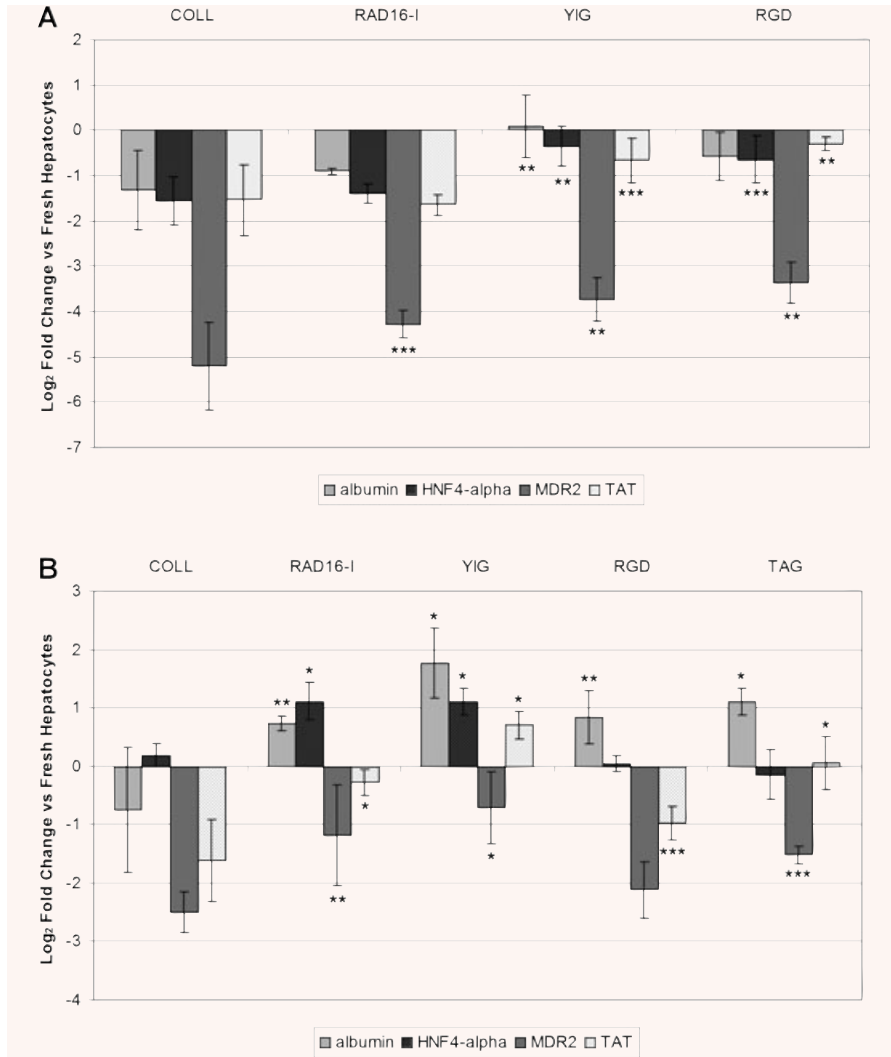
Fig. 3 Quantitative PCR analysis of liver-specific genes in hepatic cultures in 1 mm and 0.5 mm matrices. The expression of liver-specific genes in the 1 mm and 500 μm hydrogel cultures were examined by means of qPCR and were expressed as $\log_2$-fold change relative to freshly isolated hepatocytes in (**A**) 1 mm and (**B**) 0.5 mm-hydrogel systems. Expression levels were normalized to 18s RNA levels which were invariant per cell across the different culture systems. Data were obtained from four independent experiments, each of which was performed normalizing to the 18s corresponding to each hepatocyte isolation. Data are presented as mean ± S.D. (*$P < 0.001$, **$P < 0.01$, ***$P < 0.05$).

The effect of peptide hydrogels on hepatocyte-specific functionality was evaluated and compared with the collagen sandwich system by assessing cell-specific albumin and urea secretion over the entire culture period. In all culture systems, the albumin secretory capacity gradually increased between culture days 3 and 7 and remained stable thereafter (Fig. 4A). As shown by immunoblotting, albumin was expressed at a level that was similar in all hepatocyte cultures on days 7 and (Fig. 4B and C).

Furthermore, urea present in the media (indicative of amino acid catabolism) was analysed (Fig. 5). Under all culture conditions, the urea concentration slightly decreased between days 3 and 5 of culture and did not change thereafter (Fig. 5A). Similar to albumin, levels of urea production were comparable in the collagen sandwich and peptide scaffold cultures (Fig. 5A). Western blots analysis of the transcription factor HNF4-α demonstrated that it is expressed in all culture conditions (Fig. 5B). However, compared to freshly isolated hepatocytes control, an additional band of higher molecular weight was detected in all culture conditions, suggesting that the *in vitro* system might be synthesizing an additional isoform also recognized by the antibody against-HNF4-α. Additionally, we detected biotin-dependent carboxylases, which are enzymes highly expressed in liver [29–31]. In mammals, only four different biotin-dependent carboxylases have been detected so far, acetyl-CoA carboxylase (ACC, MW 270 kD), pyruvate carboxylase (PC, MW 125 kD), 3-methyl-crotonyl-CoA carboxylase (MCC, MW 75 kD) and propionyl-CoA carboxylase (PPC, MW 72 kD) [29–31]. These enzymes, are involved in the oxidation of odd-chain fatty acids, catabolism of branched amino acids such as leucine, lipogenesis and gluconeogenesis, respectively [29–31]. Using an assay, based on biotin-avidin interaction, we were able to detect three biotin-dependent carboxylases also present in freshly isolated hepatocytes (Fig. 5C). These enzymes were also present in all peptide hydrogel cultures, but interestingly, collagen sandwich cultures did not appear to express PPC (Fig. 5C).

Fig. 4 Analysis of secreted albumin. (**A**) Analysis of secreted rat albumin present in the media normalized by total protein present in the cellular lysate. Data are presented as mean ± S.D. from two different experiments. COLL: collagen. (**B** and **C**) Albumin Western blot of the cellular lysate. F.H: Freshly isolated hepatocytes.
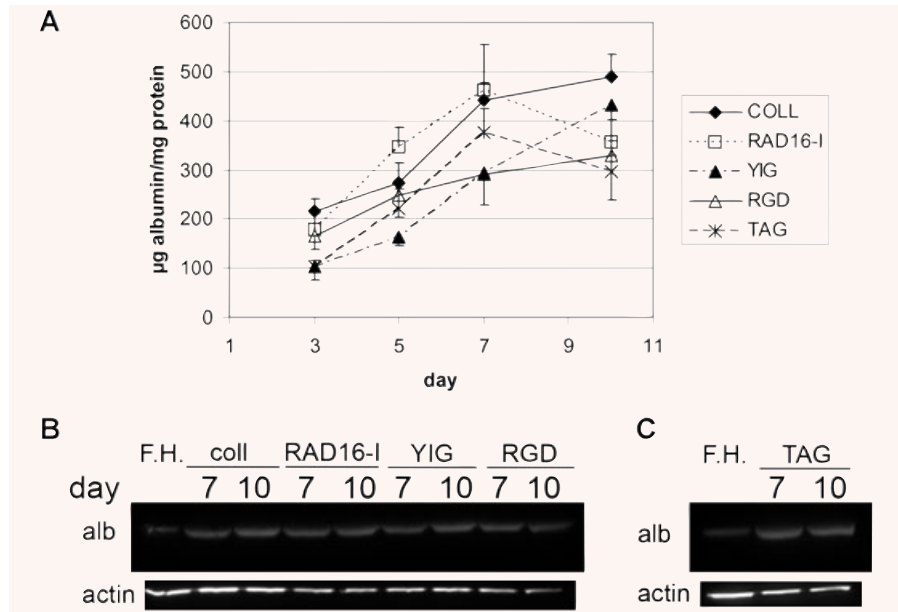

Fig. 5 Analysis of adult hepatocyte markers. (**A**) Urea present in the media at different time-points. The results are normalized against total cellular protein present in the lysates. Data are presented as mean ± S.D. from two different experiments. COLL: collagen (**B**) HNF4-α Western blots (**C**) Biotin dependent carboxylases present in the protein lysates. PC: pyruvate carboxylase, MCC: methylcrotonyl-CoA carboxylase and PCC: propionyl-CoA carboxylase. F.H.: Freshly isolated hepatocytes.
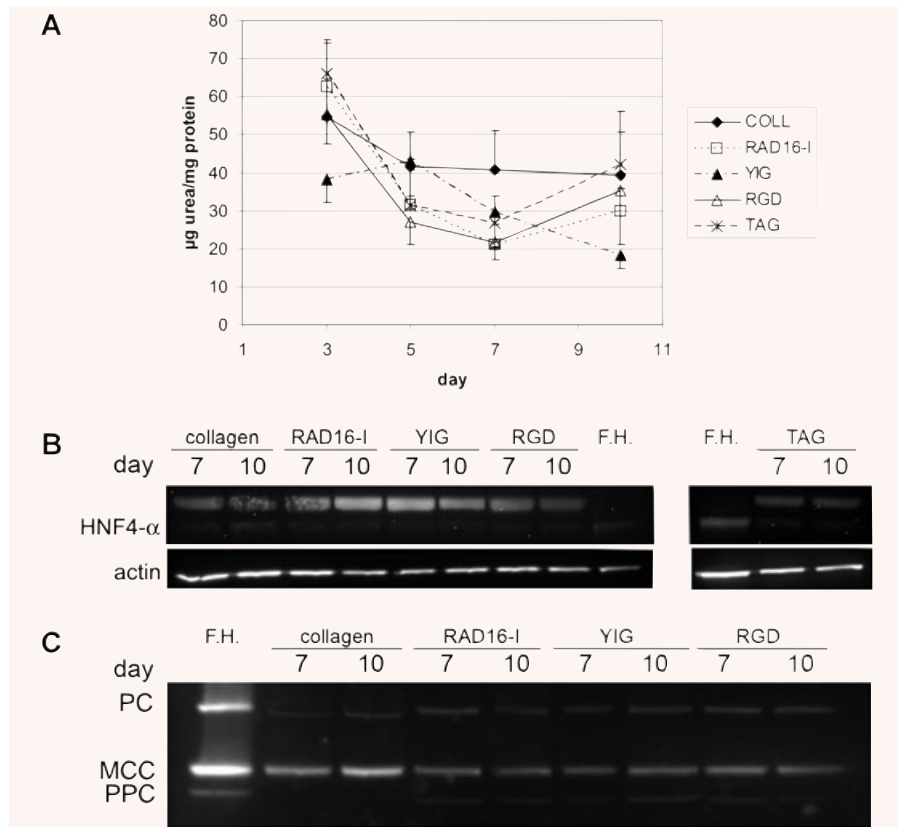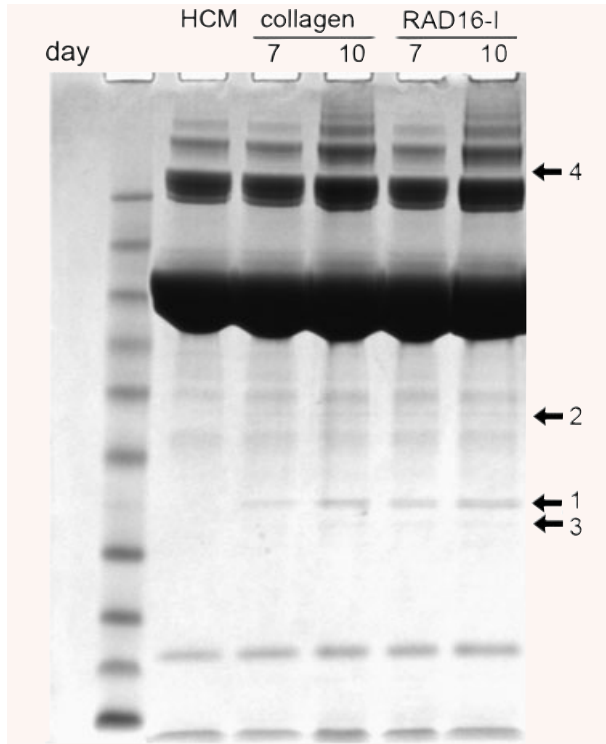
Fig. 6 Protein secretion analysis by SDS gel. SDS protein gel of media obtained at day 7 and day 10 collagen and peptide hydrogel sandwich cultures. HCM served as a control. Arrows indicate differential bands not observed in the control media. Bands were named 1–4.

Moreover, we examined and analysed the secreted proteome of hepatocytes in culture. This is a very interesting approach, since hepatocytes secrete a large amount of plasma proteins and some of them can be used as markers for diagnosing diseases [32]. Therefore, we ran coomassie blue-stained protein gels of culture media obtained at day 7 and day 10 for collagen and RAD16-I peptide hydrogel culture systems (Fig. 6). We included media as our control in one lane in order to differentiate medium containing proteins from the ones secreted into the medium. We analysed bands present in both collagen and RAD16-I ensuring that the isolated material is released by the hepatocytes. Four differential bands for both collagen and RAD16-I peptide cultures were identified and were then excised from the gels. The proteins contained in each band were tryptic digested and the remaining peptides were separated and analysed by nanoflow-HPLC MS/MS ('Materials and methods'). A brief report of the proteins obtained and their function is showed in Table 1. Several proteins were identified in both matrix systems (collagen and RAD16-I) Table 1. They are mainly plasma proteins or protease inhibitors, and their function is briefly described in Table 1 [33–49]. These findings are in concordance with a previous study using rat hepatocyte collagen sandwich cultures [32]. In some other cases, protein function is still unknown such as hypothetical protein Q4FZU2. Also, some

other proteins such as α_1-macroglobulin were found in a zone of lower molecular weight than expected, which could be due to proteolysis during the culture period (Table 1).

Inducibility of CYP3A2 by DEX using rat hepatic culture models has previously been described [6, 50, 51]. Thus, it was of interest to investigate whether hepatocytes also respond to DEX by overexpressing CYP3A2 when cultured in the peptide scaffolds. The CYP3A2 gene exhibited a high down-regulation relative to freshly isolated hepatocytes in peptide and collagen sandwich configuration at day 7 (Fig. 7A). However, when cultures were incubated for 24 hrs with DEX 10 μM the expression was up-regulated in both collagen and peptide sandwiches compared to untreated cells, indicating that hepatocyte in sandwiched cultures have the ability to respond to external inductive stimuli (Fig. 7B).

Discussion

Tissue architecture and parenchymal cell morphology reflect the functional differentiation of the liver. Hepatocytes are arranged as monolayer plates *in vivo*, an organization which allows the cells to be highly vascularized. Sandwiching hepatocytes between two layers of collagen has shown to provide the cells with an *in vivo*-like environment that enables the cells to preserve their specific functionality for several weeks [3–5]. In this work we have used synthetic extracellular matrix analogues in order to recreate the most commonly used hepatocyte culture system *in vitro*, the collagen sandwich [3–5]. We suggest that the geometrical factor in combination with the use of an artificial extracellular matrix may provide the hepatocytes a unique microenvironment which might allow them to retain important liver-specific functions. In this study, all experiments were performed in parallel to collagen sandwich system, which is considered today as the gold standard for the culture of hepatocytes *in vitro*. [3–5].

We have observed that in peptide cultures hepatocyte morphology appears to be polygonal and with a vast network of bile canalicular-like structures as it happens in collagen cultures (Fig. 2A and B). These canalicular-like structures proved to be functional by the fact that they can metabolize and transport FDA through them (Fig. 2B), as it had been previously shown for collagen sandwich cultures [24, 25]. We chose some hepatocyte markers to assess function and gene and protein expression. In terms of gene expression, the levels of relative expression of the genes studied was comparable, except for hepatocytes cultured in peptide hydrogel sandwiches containing the peptide YIG, which presented some degree of up-regulation especially for HNF4-α and albumin. Generally speaking, for all the functional experiments tested, hepatocytes in peptide sandwich cultures behaved correspondingly similar to collagen sandwich cultures. The same happened in terms of gene and protein expression, and in relation to the proteomic profile secreted to the culture medium. However, further efforts are required to improve the system to fully express liver-specific genes and to perform liver-specific functions. For

Fig. 7 DEX induction of CYP3A2 expression. (**A**) CYP3A2 gene expression relative to freshly isolated hepatocytes by quantitative PCR at different time-points. (**B**) CYP3A2 expression at day 7, after a 24 hrs induction with DEX 10 μM, COLL: collagen. Data were obtained from two independent experiments, each of which was performed normalizing to the 18s corresponding to each hepatocyte isolation. Data are presented as mean ± S.D. (*$P <$ 0.001, **$P < 0.01$, ***$P < 0.05$).
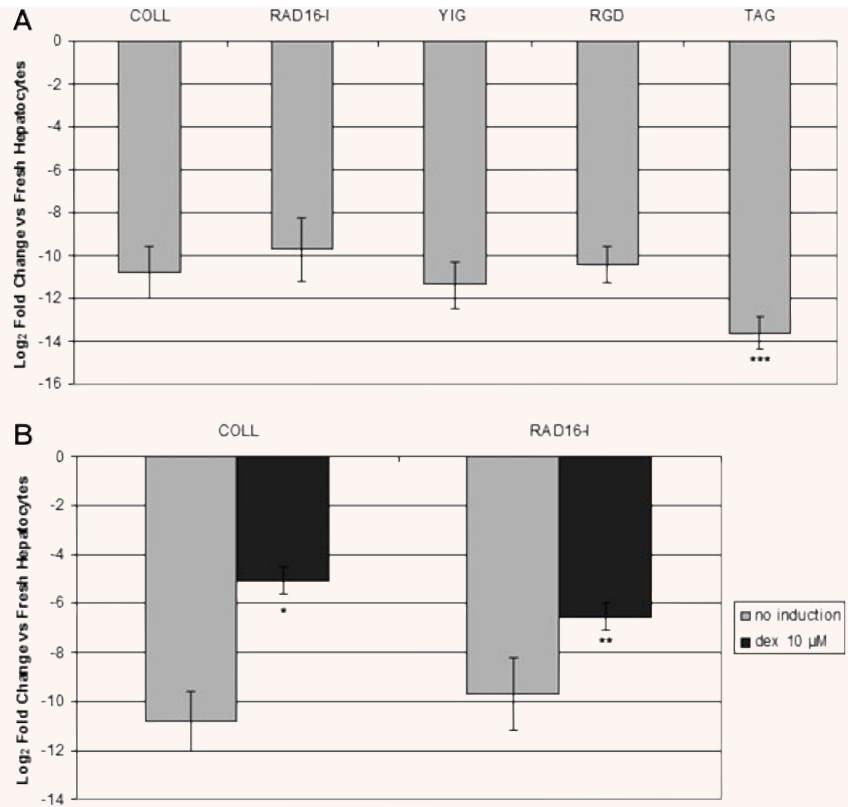
Table 1 Secreted proteins found in both collagen and RAD16-I culture medium

Protein	Band	MW	Function	References
Apolipoprotein A1 (ApoA1)	1	30 kD	Involved in reverse cholesterol transport	[33–35]
α_1-microglobulin/bikunin precursor (AMBP)	1	39 kD	a-1-microglobulin has immunoregulatory properties.Binds to IgGA Bikunin is a serine protease inhibitor	[36–38]
Apolipoprotein E (ApoE)	2	36 kD	Lipid transport. Formation of VLDL and cholymicrons	[39]
Haptoglobin	2	38.5 kD	Free hemoglobin turnover	[40, 41]
Apolipoprotein J (ApoJ, clusterin)	2	51 kD	Immune regulation, lipid transport, remodeling, apoptosis	[42–44]
Q4FZU2	2	59 kD	Hypothetical protein. Unknown	
α_1-macroglobulin*	2	167 kD	Protease inhibitor	[45]
Retinol binding protein RETBP	3	23 kD	Transports retinol from liver	[46]
Fibronectin	4	272 kD	Extracellular matrix and plasma protein	[47]
α_1 inhibitor III	4	165 kD	Protease inhibitor	[48, 49]

*Indicates proteins detected in a MW zone where they were not expected to be found.

example, it would be essential for the implementation of the system in drug screening applications, the expression of cytochromes P450 or phase I enzymes which are the responsible of the biotransformation of drugs and other xenobiotics. In particular, CYP3A2, which represents the 30% of the total cytochromes P450, metabolizes the 50% of the marketed drugs. CYP P450, especially CYP3A2, is lost rapidly after hepatocyte isolation and its expression is recovered with difficulty. However, it can be induced chemically with DEX. In collagen and peptide hydrogel sandwich systems, CYP3A2 appears to be highly down-regulated (Fig 7A), but it is induced significantly in both systems using DEX (Fig. 7B). To overcome this drawback, different strategies can be used to better recreate the hepatocyte microenvironement, such as the use of a hepatocyte non-parenchymal cell coculture or the integration of the hydrogel sandwich into a bioreactor that will allow better oxygen and mass transfer, as previously described for other systems [13–17]. Nevertheless, this peptide sandwich model represents an enormous advance through the use of a defined and synthetic extracellular matrices analogue to obtain a truly comparable system to what is considered the gold standard in long-term hepatocyte *in vitro* culture. We suggest that these promising results are an important beginning for the application of the self-assembling peptide hydrogel in hepatic reconstruction *in vitro* for many biomedical applications.

Acknowledgements

References

1. **Zhang S, Yan L, Altman M, *et al*.** Biological surface engineering: a simple system for cell pattern formation. *Biomaterials*. 1999; 20: 1213–20.

2. **Ben Ze'ev A, Robinson GS, Buchter NL, *et al*.** Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *PNAS*. 1988; 85: 2161–5.

3. **Dunn JC, Yarmush ML, Koebe HG, *et al*.** Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J*. 1989; 3: 174–7.

4. **Dunn JC, Tompkins RG, Yarmush ML.** Long-term *in vitro* function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol Progr*. 1991; 7: 237–45.

5. **Berthiaume F, Moghe PV, Toner M, *et al*.** Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. *FASEB J*. 1996; 10: 1471–84.

6. **Silva JM, Morin PE, Day SH, *et al*.** Refinement of an *in vitro* cell model for cytochrome P450 induction. *Drug Metab Dispos*. 1998; 26: 490–6.

7. **Wang Y, Liu H, Guo H, *et al*.** Primary hepatocyte culture in collagen gel mixture and collagen sandwich. *World J Gastroenterol*. 2004; 10: 699–02.

8. **Zeisberg M, Kramer K, Sindhi N, *et al*.** De-differentiation of primary human hepatocytes depends on the composition of specialized liver basement membrane. *Mol Cell Biochem*. 2006; 283: 181–9.

9. **Bhatia SN, Balis UJ, Yarmush ML, *et al*.** Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J*. 1999; 13: 1883–900.

10. **Uyama N, Shimahara Y, Kawada N, *et al*.** Regulation of cultured rat hepatocyte proliferation by stellate cells. *J Hepatol*. 2002; 36: 590–609.

11. **Kan P, Miyoshi H, Ohshima N.** Perfusion of medium with supplemented growth factors changes metabolic activities and cell morphology of hepatocyte-nonparenchymal cell coculture. *Tissue Eng*. 2004; 10: 1297–307.

12. **Auth Marcus KH, Woitaschek D, Beste M, *et al*.** Preservation of the synthetic and metabolic capacity of isolated human hepatocytes by coculture with human biliary epithelial cells. *Liver Transpl*. 2005; 11: 410–9.

13. **Powers MJ, Janigian DM, Wack KE, *et al*.** Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. *Tissue Eng*. 2002; 8: 499–513.

14. **De Bartolo L, Morelli S, Rende M, *et al*.** New modified polyetheretherketone membrane for liver cell culture in biohybrid systems: adhesion and specific functions of isolated hepatocytes. *Biomaterials*. 2004; 25: 3621–9.

15. **Sudo R, Mitaka T, Ikeda M, *et al*.** Reconstruction of 3D stacked-up structures by rat small hepatocytes on microporous membranes. *FASEB J*. 2005; 19: 1695–7.

16. **De Bartolo L, Salerno S, Morelli S, *et al*.** Long-term maintenance of human hepatocytes in oxygen-permeable membrane bioreactor. *Biomaterials*. 2006; 27: 4794–803.

17. **Schmitmeier S, Langsch A, Jasmund I, *et al*.** Development and characterization of a small-scale bioreactor based on a bioartificial hepatic culture model for predictive pharmacological *in vitro* screenings. *Biotechnol Bioeng*. 2006; 95: 1198–206.

18. **Semino CE, Merok JR, Crane GG, *et al*.** Functional differentiation of hepatocyte-like spheroid structures from putative liver progenitor cells in three-dimensional peptide scaffolds. *Differentiation*. 2003; 71: 262–70.

19. **Semino CE, Kasahara J, Hayashi Y, *et al*.** Entrapment of migrating hippocampal neural cells in three-dimensional peptide nanofiber scaffold. *Tissue Eng*. 2004; 10: 643–55.

20. **Garreta E, Genove E, Borros S, *et al*.** Osteogenic differentiation of mouse embryonic stem cells and mouse embryonic fibroblasts in a three-dimensional self-assembling peptide scaffold. *Tissue Eng*. 2006; 12: 2215–27.

21. **Genove E, Shen C, Zhang S, *et al*.** The effect of functionalized self-assembling peptide scaffolds on human aortic endothelial cell function. *Biomaterials*. 2005; 26: 3341–51.

22. **Zhang S, Holmes T, Lockshin C, *et al*.** Spontaneous assembly of a self-complementary oligopeptide to form a stable

macroscopic membrane. *PNAS*. 1993; 90: 3334–8.

23. **Sivaraman A, Leach JK, Townsend S, et al.** A microscale *in vitro* physiological model of the liver: predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab.* 2005; 6: 569–91.

24. **Barth CA, Schwarz LR.** Transcellular transport of fluorescein in hepatocyte monolayers: evidence for functional polarity of cells in culture. *PNAS*.1982; 79: 4985–7.

25. **LeCluyse EL, Audus KL, Hochman JH.** Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. *Am J Physiol.* 1994; 266: 1764–74.

26. **Hoffmaster KA, Turncliff RZ, LeCluyse EL, et al.** P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes: relevance to the hepatobiliary disposition of a model opioid peptide. *Pharm Res.* 2004; 21: 1294–302.

27. **Li RH, Altreuter DH, Gentile FT.** Transport characterization of hydrogel matrixes for cell encapsulation. *Biotechnol Bioeng.* 1996; 50: 365–73.

28. **Muller M, Jansen PL.** Molecular aspects of hepatobiliary transport. *Am J Physiol.* 1997; 272: 1285–303.

29. **Jitrapakdee S, Wallace JC.** Structure, function and regulation of pyruvate carboxylase. *Biochem J.* 1999; 340: 1–16.

30. **Baumgartner MR, Almashanu S, Suormala T, et al.** The molecular basis of human 3-methylcrotonyl-CoA carboxylase deficiency. *J Clin Invest.* 2001; 107: 495–504.

31. **Jiang H, Rao KS, Yee VC, et al.** Characterization of four variant forms of human propionyl-coa carboxylase expressed in escherichia coli. *J Biol Chem.* 2005; 280: 27719–27.

32. **Farkas D, Bhat VB, Mandapati S, et al.** Characterization of the secreted proteome of rat hepatocytes cultured in collagen sandwiches. *Chem Res Toxicol.* 2005; 18: 1132–9.

33. **Frank PG, Marcel YL.** Apolipoprotein A-I: structure-function relationships. *J Lipid Res.* 2000; 41: 853–72.

34. **Ajees AA, Anantharamaiah GM, Mishra VK, et al.** Crystal structure of human apolipoprotein A-I: insights into its protective effect against cardiovascular diseases. *PNAS*. 2006; 103: 2126–31.

35. **Barter PJ, Rye KA.** The rationale for using apoA-I as a clinical marker of cardiovascular risk. *J Intern Med.* 2006; 259: 447–54.

36. **Falkenberg C, Grubb A, Aakerstroem B.** Isolation of rat serum a1-microglobulin. Identification of a complex with a1-inhibitor-3, a rat a2-macroglobulin homolog. *J Biol Chem.* 1990; 265: 16150–7.

37. **Rouet P, Raguenez G, Ruminy P, et al.** An array of binding sites for hepatocyte nuclear factor 4 of high and low affinities modulates the liver-specific enhancer for the human a1-microglobulin/bikunin precursor. *Biochem J.* 1998; 334: 577–84.

38. **Logdberg LE, Akerstrom B, Badve S.** Tissue distribution of the lipocalin alpha-1 microglobulin in the developing human fetus. *J Histochem Cytochem.* 2000; 48: 1545–52.

39. **Eichner JE, Terence DS, Perveen G, et al.** Apolipoprotein E polymorphism and cardiovascular disease: a HuGE review. *Am J Epidemiol.* 2002; 155: 487–95.

40. **Alper CA, Peters JH, Birch AG, et al.** Haptoglobin synthesis. I. *In vivo* studies of the production of haptoglobin, fibrinogen, and g-globulin by the canine liver. *J Clin Invest.* 1965; 44: 574–81.

41. **Ship NJ, Toprak A, Lai RP, et al.** Binding of acellular, native and cross-linked human hemoglobins to haptoglobin: enhanced distribution and clearance in the rat. *Am J Physiol.* 2005; 288: 1301–9.

42. **Kapron JT, Hilliard GM, Lakins JN, et al.** Identification and characterization of glycosylation sites in human serum clusterin. *Prot Sci.* 1997; 6: 2120–33.

43. **Min BH, Jeong SY, Kang SW, et al.** Transient expression of clusterin (sulfated glycoprotein-2) during development of rat pancreas. *J Endocrinol.* 1998; 158: 43–52.

44. **Calero M, Tokuda T, Rostagno A, et al.** Functional and structural properties of lipid-associated apolipoprotein J (clusterin). *Biochem J.* 1999; 344: 375–83.

45. **Tsuji A, Akamatsu T, Nagamune H, et al.** Identification of targeting proteinase for rat a1-macroglobulin *in vivo*. Mast-cell tryptase is a major component of the a1-macroglobulin-proteinase complex endocytosed into rat liver lysosomes. *Biochem J.* 1994; 298: 79–85.

46. **Soprano DR, Soprano KJ, Goodman DS.** Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J Lipid Res.* 1986; 27: 166–71.

47. **Tamkun JW, Hynes RO.** Plasma fibronectin is synthesized and secreted by hepatocytes. *J Biol Chem.* 1983; 258: 4641–7.

48. **Braciak TA, Northemann W, Hudson GO, et al.** Sequence and acute phase regulation of rat alpha 1-inhibitor III messenger RNA. *J Biol Chem.* 1988; 263: 3999–4012.

49. **Abraham LJ, Bradshaw AD, Shiels BR, et al.** Hepatic transcription of the acute-phase a1-inhibitor III gene is controlled by a novel combination of cis-acting regulatory elements. *Mol Cell Biol.* 1990; 10: 3483–91.

50. **Turncliff RZ, Meier PJ, Brouwer KL.** Effect of dexamethasone treatment on the expression and function of transport proteins in sandwich-cultured rat hepatocytes. *Drug Metab Dispos.* 2004; 32: 834–9.

51. **Abu-Absi SF, Hu WS, Hansen LK.** Dexamethasone effects on rat hepatocyte spheroid formation and function. *Tissue Eng.* 2005; 11: 415–26.