Cultivating liver cells on printed arrays of hepatocyte growth factor

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ABSTRACT

Growth factors are commonly present in soluble form during *in vitro* cell cultivation experiments in order to provide signals for cellular proliferation or differentiation. In contrast to these traditional experiments, we investigated solid-phase presentation of a hepatocyte growth factor (HGF), a protein important in liver development and regeneration, on microarrays of extracellular matrix (ECM) proteins. In our experiments, HGF was mixed in solution with ECM proteins (collagen (I), (IV) or laminin) and robotically printed onto silane-modified glass slides. Primary rat hepatocytes were seeded onto HGF/ECM protein microarrays and formed cellular clusters that corresponded in size to the dimensions of individual protein spots (500 μm diameter). Analysis of liver-specific products, albumin and α1-antitrypsin, revealed several fold higher levels of expression of these proteins in hepatocytes cultured on HGF/ECM microarrays compared to cells cultivated on ECM proteins alone. In addition, cultivation of hepatocytes on HGF/ECM protein spots led to spontaneous reorganization of cellular clusters from a monolayer into three-dimensional spheroids. We also investigated the effects of surface-tethered HGF on hepatocytes co-cultivated with stromal cells and observed a significantly higher level of albumin in co-cultures where hepatocytes were stimulated by HGF/ECM spots compared to co-cultures created on ECM protein islands without the growth factor. In summary, our study suggests that incorporation of HGF into ECM protein microarrays has a profound and long-lasting effect on the morphology and phenotype of primary hepatocytes. In the future, the number of growth factors printed on ECM microarrays will be expanded to enable multiplexed and combinatorial screening of inducers of cellular differentiation or proliferation.

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1. Introduction

Growth factors play an important role in regulating cellular behavior, including stimulation of proliferation [1,2], migration [3,4] and differentiation [5,6]. In the liver, production and release of growth factors are part of a complex interplay between mature and immature hepatocytes, non-parenchymal cells and recruited inflammatory cells. Hepatocyte growth factor (HGF), a mesenchyme-derived protein, has been found to play a central role in liver development and regeneration after injury. HGF is a pleiotropic morphogen that has been shown to have mitogenic, motogenic, and antiapoptotic effects [6,7]. In addition, HGF is being explored as an anti-fibrotic agent and may have applications for treatment of liver fibrosis [8,9].

Current *in vitro* cell cultivation strategies commonly rely on providing growth factors in the soluble form. These traditional approaches require significant amounts of expensive growth factors (GFs) and are not optimal for stem cell differentiation or primary cell maintenance studies where frequent media changes are required. This presents a particularly challenging problem in stem cell differentiation studies where a formulation of GFs required for stem cell lineage selection is often unknown, requiring extensive and expensive experiments involving combinations of GFs [10–13].

In contrast to *in vitro* experiments where GF molecules are present in solution, *in vivo*, GFs bind to ECM matrix proteins and are dynamically released during matrix remodeling and protease secretion by the surrounding cells [14–16]. To mimic solid-phase presentation observed *in vivo* a number of reports have described strategies for surface immobilization of GF molecules via covalent tethering [17–20]. In addition, given that *in vivo* GFs form secondary bonds with either glycosaminoglycans [2,21–24] or with matrix proteins [15,25–27], non-covalent binding represents an alternative route for surface immobilization of these molecules.

Association with ECM proteins may provide additional benefits of stabilizing GF molecules against proteolytic degradation and enhancing their activity [28].

Beyond offering a more physiological scenario, surface immobilization of GF molecules makes it possible to design strategies for high-throughput screening of cell function. Robotic microarraying, a technology originally designed for high-throughput screening of DNA hybridization events [29], is particularly amenable for printing arrays of biomolecules on cell culture surfaces. This robotic printing technology has been previously employed for high-throughput studies of cell–ECM [30], cell–biomaterial [31] and cell–small molecule interactions [32]. More recently Soen and Davis employed arrays of ECM proteins and morphogens to investigate differentiation of primary neuronal cells [33].

We have previously reported on the use of ECM protein microarrays for cultivation of hepatocytes in mono- and co-cultures [34]. These microarray-based cell cultures were complemented with methods for the analysis of gene expression and secreted product signatures within the local microenvironment [35,36]. In the present study, we describe GF presentation on printed ECM microarrays as a means to deliver stimuli to cultured hepatocytes. This study represents a step towards an integrated cell culture platform where signals are delivered and cell function is detected in a high-throughput and location-specific manner.

2. Materials and methods

2.1. Chemicals and materials

Glass slides (75×25 mm²) were obtained from VWR (West Chester, PA). (3-Acryloxypropyl)trichlorosilane was purchased from Gelest, Inc. (Morrisville, PA). Sulfuric acid, hydrogen peroxide, ethanol, collagenase, collagen from rat tail (type I), collagen IV, laminin, hepatocyte growth factor (HGF), streptavidin-conjugated Alexa 546 and protease inhibitor cocktail were obtained from Sigma–Aldrich (St. Louis, MO). Concentrated phosphate-buffered saline ($10 \times$ PBS) was purchased from Lonza (Walkersville, MD). Minimal essential medium (MEM), sodium pyruvate, nonessential amino acids, fetal bovine serum (FBS), Superscript III, RNaseOut (RNase inhibitor), dNTPs and biotinylated anti-HGF antibodies were purchased from Invitrogen (Carlsbad, CA). 384-well polypropylene microarray plates were obtained from Genetix (New Milton, Hampshire). Goat anti-rat cross-adsorbed albumin antibody, reference serum, and HGF ELISA Quantitation Kit were obtained from Bethyl Laboratories (Montgomery, TX). Goat anti-rat IgG Texas Red conjugate was purchased from Santa Cruz Biotechnologies, Inc. Formalin was purchased from Fisher (Pittsburgh, PA). Slide-A-Lyzer Mini Dialysis Units were purchased from Pierce (Rockford, IL).

2.2. Preparation of glass substrates

Glass slides were cleaned by immersion in "piranha" solution consisting of 3:1 ratio of aqueous solutions of 50% v/v of sulfuric acid and 30% w/v of hydrogen peroxide for 30 min (*caution: this mixture reacts violently with organic materials and must be handled with extreme care*). The glass slides were thoroughly rinsed with deionized water, dried under nitrogen, and kept in Class 10000 air prior to use. For silane modification, the glass slides were exposed to oxygen plasma for 5 min at 300 W (YES3, Yield Engineering Systems, Livermore, CA) and then placed for 10 min in a 2 mM solution of (3-acrylopropyl)trichlorosilane diluted in anhydrous toluene. The reaction was performed in a glove box under a nitrogen blanket to avoid exposure to atmospheric moisture. After silanization, the slides were rinsed with fresh toluene, dried under nitrogen, and cured at 100 °C for 4 h.

2.3. Printing ECM and growth factor microarrays

All ECM proteins employed for printing were dissolved in $1 \times$ PBS + 0.005% Tween-20 at 0.2 mg/mL concentration. HGF was mixed with the ECM solution to a final concentration of 500 ng/mL and allowed to bind to the ECM protein for 30 min at room temperature prior to printing. Protein microarrays were contact-printed under ambient conditions on silane-modified 75×25 mm² glass slides using a hand-held MicroCaster. The pins collected protein (0.2 mg/mL ECM + 500 ng/mL HGF in $1 \times$ PBS with 0.005% Tween) from a 382-well plate, dispensing 20–70 nL of solution onto the glass slide and forming circular spots ~500 μm in diameter. Protein arrays were kept in a refrigerator before use and were functional for at least one week.

2.4. Characterization of HGF retention on ECM microarrays

An array of ECM/HGF (6×6) was printed onto silane-modified glass slides as described before and incubated in $1 \times$ PBS at 37 °C for 2 h. HGF molecules were extracted from the silane surface using 50 μL of 4 M guanidine–HCl (pH 7.2) supplemented with 0.2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 10 mM EDTA, 0.05 M Tris and protease inhibitor cocktail. The guanidine solution was incubated with the HGF microarray for 30 min. The supernatant was buffer-exchanged with 6 M urea containing 0.05 M Tris (pH 7.4) using Slide-A-Lyzer Mini Dialysis Units with 3500 MWCO for 1 h. The concentration of HGF extracted from the printed microarray was determined according to the manufacturer's instructions using the HGF ELISA.

Immunofluorescent staining was used to determine retention of HGF on ECM microarrays after 1, 3 and 5 days in media at cell culture conditions. Protein solution containing 500 ng/mL HGF and 0.2 mg/mL collagen (I) was printed onto silanized glass slides and in cell culture media at 37 °C. At the desired time point, glass substrates were removed from media and incubated with 1 μg/ml (in $1 \times$ PBS) of anti-human HGF biotin conjugate at 37° for 2 h followed by incubation in 10 μg/ml of streptavidin, Alexa Fluor® 546 conjugate for 1 h at room temperature. Samples were washed between each staining step with $1 \times$ PBS + 0.05% Tween-20. In order to create a quantitative readout of fluorescence signal emanating from the array, the laser microarray scanner (Agilent G2565BA fluorescent scanner, Expression Analysis Facility, UC Davis Genome Center) was employed to scan the glass slides at a spot pixel resolution of 5 μm. The fluorescence intensity of each array element was determined using GenePix Pro 6.0 data analysis software (Molecular Devices, Downingtown, PA). Fluorescent intensity was converted to a percentage of the control array.

2.5. Cultivation of primary hepatocytes on HGF microarrays

Our studies employed primary rat hepatocytes. Cells were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 125–200 g, using a two-step collagenase perfusion procedure as described previously [37]. Typically, 100–200 million hepatocytes were obtained with viability >90% as determined by trypan blue exclusion. Primary hepatocytes were maintained in DMEM supplemented with epidermal growth factor (EGF), glucagon, hydrocortisone sodium succinate, recombinant human insulin, 200 units/mL penicillin, 200 μg/mL streptomycin and 10% FBS.

For cell seeding experiments, a glass slide containing printed arrays of ECM + HGF was cut to fit into a 6-well plate. Hepatocytes were seeded to form cellular arrays using protocols described earlier [34]. In brief, glass slides containing printed ECM/HGF spots were first exposed to 3 mL of rat primary hepatocytes suspended in culture medium at a concentration of 1×10^6 cells/mL. After 1 h of incubation at 37 °C, hepatocytes became localized on ECM/HGF domains, but did not attach on the surrounding silane-modified surface. The samples were then washed twice in PBS to remove unbound hepatocytes and fresh media was added to the sample well.

Murine 3T3 fibroblasts were maintained in DMEM supplemented with 10% FBS, 200 units/mL penicillin, and 200 μg/mL streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Cells were cultured until 90% confluence and then passaged. When performing co-culture experiments, hepatocytes were allowed to spread out on the protein spots overnight. The following day 3T3 fibroblasts were seeded on the sample at 0.25×10^6 cells/mL and were allowed to attach for 30 min. Unbound cells were washed away and fresh media was added as previously described. In our previous experiments [34,35] modification of glass substrates with acrylated silane was found to render these surfaces partially non-fouling. The silane layer prevented attachment of primary hepatocytes seeded first but supported adhesion of 3T3 fibroblasts that were seeded in the second step. Therefore, this micropatterning strategy led to hepatocytes residing on protein islands and fibroblasts adhering on the surrounding glass regions.
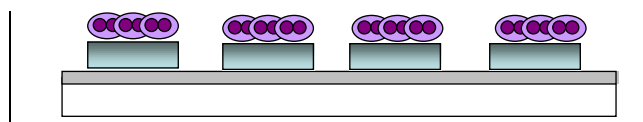
In our experiments, hepatocytes cultured on HGF/ECM microarrays were compared with cells cultured on ECM protein arrays without HGF. Another control experiment performed in parallel involved cultivation of hepatocytes on ECM protein arrays with HGF present in solution at a concentration of 10 ng/mL. Importantly, in contrast to HGF microarrays where no exchange or supplementation of GF molecules was possible, soluble GF was changed daily along with the culture media. In all three cases hepatic function was analyzed as described below.

2.6. Analysis of hepatic function

Expression of hepatic phenotype was assessed by intracellular staining of albumin, ELISA of albumin and real-time RT-PCR of albumin and α1-antitrypsin gene expression. For immunostaining, cells were fixed in 4% formalin in PBS for 20 min and then permeabilized with 0.1% Triton X-100 for 5 min. The cells were then incubated in blocking solution (1% bovine serum albumin (BSA) in 1X PBS) for 1 h at room temperature and exposed to 1:250 diluted anti-rat serum albumin antibody for 2 h at 37 °C. Finally, cells were incubated in 1:100 diluted anti-mouse IgG conjugated with Texas Red for visualization. Cells were washed between each step with 1X PBS three times for 5 min. All incubations were performed at room temperature if not specified. Stained cells were visualized and imaged using
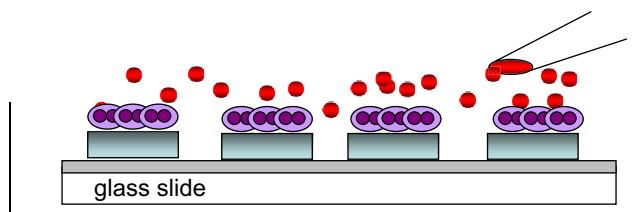
Scenario 1: daily addition of HGF to culture media

Scenario 2: no HGF, just culture media
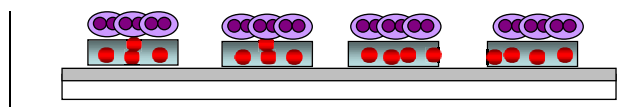
Scenario 3: solid-phase presentation of HGF

Fig. 1. Schematic representation of cell microarray experiments. Primary rat hepatocytes were cultured on protein microarrays. Three scenarios were compared: hepatocytes on ECM protein spots with HGF added in solution (scenario 1), hepatocytes on ECM spots without HGF (scenario 2) and hepatocytes on ECM spots with surface-immobilized HGF (scenario 3). Immobilization of HGF on ECM protein spots had a profound and long-lasting effect on morphology and phenotype of hepatocytes.

a confocal microscope (Zeiss LSM Pascal). Cell culture media was collected everyday and analyzed for secreted albumin content using standard protocols described previously [34]. Albumin concentration was estimated using a standard kit from Bethyl Laboratories.

For real-time RT-PCR experiments, cells were collected from microarrays using trypsin for 10 min at 37°. Extracted cells were stabilized in 100 μL of lysis buffer and stored at −20 °C. Total RNA was extracted from the cell lysates using Absolute total mRNA isolation microprep kit (Stratagene) according to the manufacturer's instructions. cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions using 12 μL of DNAse pre-treated total mRNA. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers for rat albumin, α1-antitrypsin and GAPDH genes were selected from a database http://medgen.ugent.be/rtprimerdb. Primer (Sigma Genosys) concentrations were optimized before use. SYBR Green Master Mix (1×) was used with 1 μM of forward and reverse primers in a total volume of 12 μl that also included 1 μl cDNA. All PCR reactions were done in duplicate. PCR amplification was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 10 s and 68 °C for 1 min on Mastercycler Realplex (Eppendorf). The comparative C_t value method, using housekeeping gene (GAPDH) as an internal standard, was employed to determine relative levels of albumin and α1-AT gene expression.

2.7. Characterization of cell morphology

Cell morphology was observed daily via brightfield microscopy. To document changes in cellular conformation at higher magnification, images were obtained using a Philips XL 30 scanning electron microscope (SEM) at 10 kV beam voltage and a tilt angle of 20°. In preparation for SEM characterization, the cellular micropatterns were washed in fresh media followed by 2× wash in 50% 0.2 M sodium phosphate buffer. The patterns were then fixed in 2% glutaraldehyde dissolved in 0.2 M sodium phosphate buffer for 15 min followed by 3× wash in the buffer solution. The cells were then dehydrated by incubation for 10 min in 30%, 50%, and 70% ethanol solutions. The cells were then washed three times in 95% ethanol for 5 min each and finally two times in 100% ethanol for 5 min. In a final dehydration step, ethanol was replaced with HMDS and heated to 60 °C in order to evaporate the HMDS. The dehydrated samples were coated with 6 nm layer of Au–Pd using a sputter coater (Pelco SC7, Ted Pella, Inc., Redding, CA).

3. Results and discussion

In this study, primary rat hepatocytes were cultured on printed arrays of three liver-related ECM proteins (collagen (I), (IV) and

laminin) with or without HGF. Solid-phase presentation of HGF on ECM microarrays significantly stimulated transcription and translation of liver-specific proteins in hepatocytes and induced spontaneous formation of hepatic spheroids after 5 days in culture. These data are significant as they demonstrate that one-time presentation of HGF has a pronounced and long-lasting effect on maintenance of differentiated hepatic phenotype *in vitro*.

3.1. Printing and characterization of ECM/HGF microarray

In a typical experiment, GF/ECM microarrays were fabricated using a hand-held contact arrayer printing 500 μm diameter protein spots. Prior to printing glass substrates were treated with acrylated silane. The silanization procedure may have enhanced the quality of the printed microarray spots by rendering the surface more hydrophobic, with the water contact angle increasing from near zero (after O_2 plasma treatment) to $53 \pm 2°$ after silane modification. More importantly, the silane layer did not support attachment of primary hepatocytes and could be used to localize these cells to printed islands of ECM proteins. In addition as described in the following sections of this paper, silane coating permitted fibroblast attachment and therefore allowed us to sequentially assemble micropatterned hepatocyte–fibroblast co-cultures on protein microarrays.

When creating GF microarrays, a solution of HGF (concentration 500 ng/ml) was mixed with either collagen (I) or collagen (IV) or laminin (all at 0.2 mg/ml) in a microtiter plate and then printed onto silanized glass slide. A question central to the success of the proposed strategy of solid-phase presentation of HGF was the
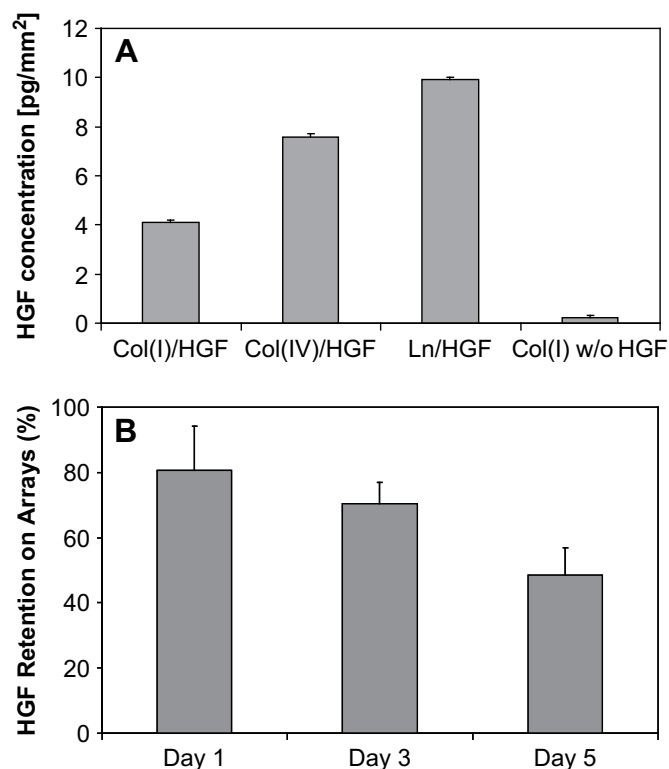

Fig. 2. Retention of HGF on ECM protein spots. HGF was mixed with one of three ECM proteins (collagen (I), (IV) or laminin) and then printed onto silane-modified glass substrates to create HGF/ECM protein spots (500 μm diameter). (A) The microarrays were then exposed to 4 M guanidine–HCl extraction buffer to remove surface-bound proteins. Presence of HGF in the extract was analyzed using ELISA. (B) HGF/col (I) microarrays were analyzed using immunofluorescent staining after incubation in culture media. For each time HGF spot replicates is $n = 20$.

retention of this morphogen on printed ECM protein spots. To answer this question we developed a protocol whereby arrays of HGF co-printed with a chosen ECM protein onto a glass substrate were immersed in $1\times$ PBS for 2 h and then removed from the surface using guanidine-based protein extraction method. HGF extracted from the surface along with carrier matrix proteins was then analyzed using ELISA. The analysis of HGF binding on microarrays of different ECM proteins showed that collagen (I) spots contained 0.8 pg of HGF/spot, whereas collagen IV and laminin contained 1.5 pg/spot, and 1.9 pg/spot respectively (Fig. 2A). These results illustrate that HGF indeed binds to all three ECM proteins. Estimating the amount of dispensed liquid per spot to be 20 nL (obtained from manufacturer) the amount of HGF present on the spot assuming 100% retention will be $\sim$10 pg/spot. Therefore, ECM protein

microarrays retained $\sim$10–20% of HGF molecules. These results are consistent with other studies that reported HGF to have moderate affinity for ECM (Kd approximately 10^{-9} mol/L) and demonstrated that 15–20% of ^{125}I-labeled HGF bound to ECM proteins that had been immobilized on polystyrene microtiter wells [15].

In addition to the amount of HGF captured on the spots immediately after printing, we investigated retention of GF molecules on the microarrays over the course of five days of incubation in cell culture media at 37 °C. The presence of HGF was revealed by incubating microarrays with biotinylated anti-HGF antibodies and fluorescently-labeled avidin. Immunofluorescence of the microarrays was quantified with a laser scanner as described by us elsewhere [36] and normalized by the fluorescence signal from the initial time point. Fig. 2B shows that the HGF signal decreased as
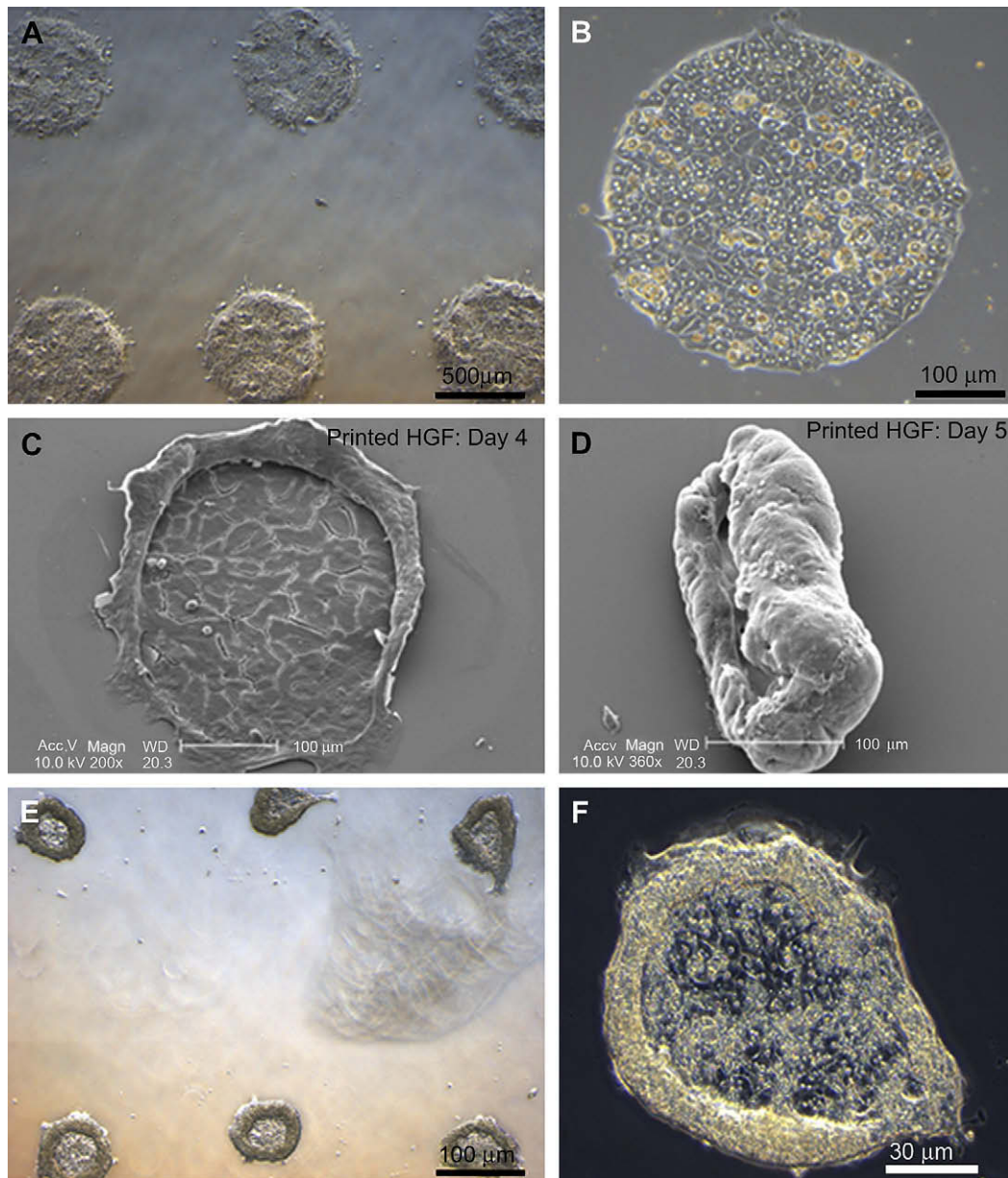

Fig. 3. Primary hepatocytes cultured on HGF/ECM microarrays. (A–B) Hepatocytes on collagen (I) spots without HGF after 5 days in culture. (C) SEM micrograph of hepatocyte cluster formed on HGF/collagen (I) spot after 4 days in culture. The edges of the spot are starting to roll up. (D) Hepatocytes cultivated on HGF/collagen for 5 days reorganized from a monolayer culture into spheroids. (E) An array of hepatocyte spheroids formed on HGF/collagen (I). During this reorganization a 500 μm diameter hepatocyte cluster compacted and rolled up into $\sim$100 μm diameter 3D spheroids. In the upper row of cell clusters one can see the boundary of 500 μm diameter cluster with a few remaining cells after 4 days in culture. (F) Brightfield image shows the presence of typical hepatocytes with large nuclei and prominent cell borders in the center of the spheroid after 4 days in culture.
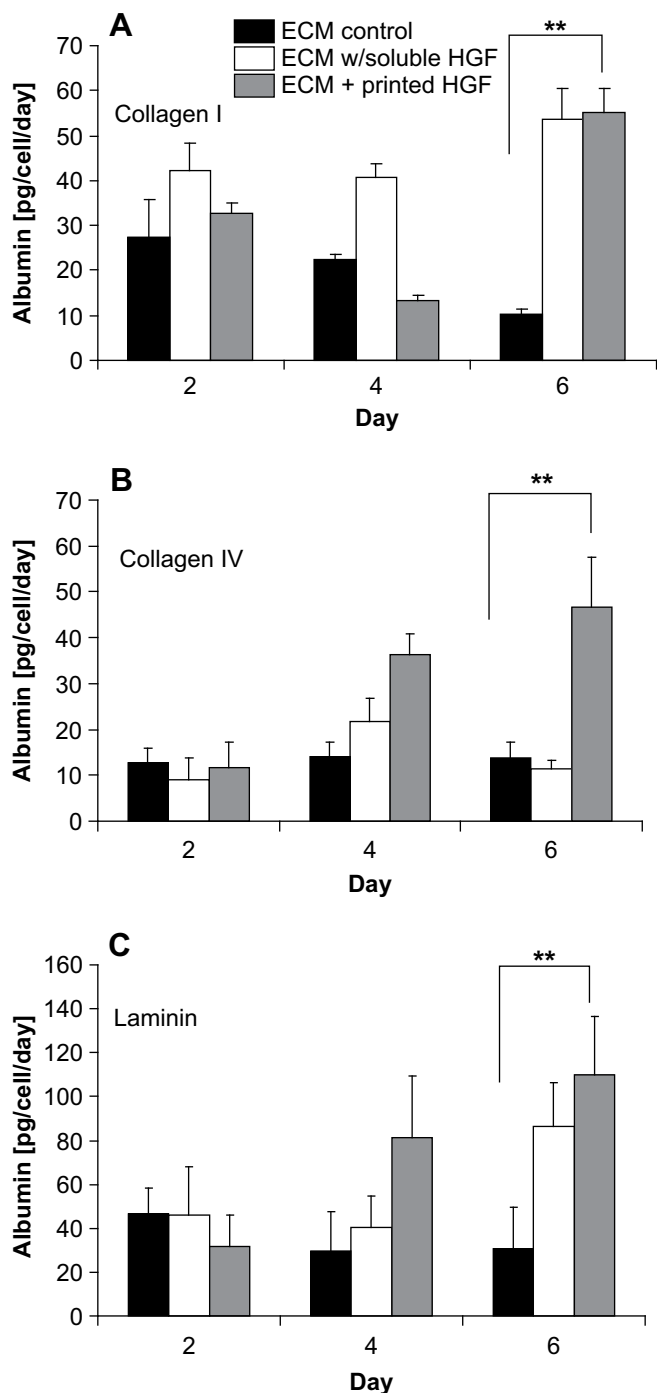
Fig. 4. Albumin synthesis in hepatocytes cultured on protein microarrays. (A) ELISA results for hepatocytes cultured on collagen (I) spots w/HGF, w/o HGF and with soluble HGF. HGF/collagen (I) spots induce significantly higher levels of albumin production compared to collagen (I) spots. ($N = 3$ samples, p-value < 0.005). (B–C) Albumin ELISA analysis of primary rat hepatocytes cultured on collagen (IV) (B) and laminin (C) suggests that solid-phase presentation of HGF significantly upregulates albumin production. ($N = 3$ samples, p-value < 0.005).

solution are added into a well of surface area of 9.6 cm^2. Assuming that all HGF molecules are adsorbed on the surface and given that 144 cell clusters are present in a well, individual cell cluster receives ~6 pg of HGF on daily basis. While this is a rough estimate, it shows that surface-bound and soluble HGF concentration seen by the cells is likely similar (same order of magnitude). In addition to interesting changes in cell morphology that were seen only in the case of surface-bound HGF, solid-phase immobilization of this morphogen was advantageous with regards to reagent conservation. When adding HGF in soluble form, 30 ng/day of reagent was used per well of a 6-well plate during a 10 day experiment, while the solid-phase presentation required one-time use of 1.44 ng per well. Therefore, surface immobilization allowed us to use nearly 200 times less of this expensive reagent.

3.2. Cultivation of hepatocytes on HGF microarrays

To test the ability of printed HGF to promote hepatic differentiation, GF/ECM spots were printed onto silane-modified glass substrates. When seeded on these glass substrates, hepatocytes selectively attached on printed arrays forming ~500 μm diameter clusters containing ~250 hepatocytes/spot. As shown in Fig. 3(A,B), cell organization on protein arrays occurred with high fidelity and minimal non-specific attachment of hepatocytes on silanized glass regions. Cell number was consistent on arrays with and without HGF (~280 cells/spot) with the exception of collagen (IV) based microarrays where 110 cells/spot were observed.

While hepatocytes cultured on ECM arrays behaved unremarkably, de-differentiating rapidly (within days), hepatocytes residing on HGF/ECM spots underwent dramatic changes in morphology, spontaneously forming three-dimensional spheroids after 4–5 days in culture. As highlighted in Fig. 3C, hepatocyte clusters started to "roll-up" after 4 days of culture on HGF/ECM spots so that by day 5 at least 75% of the clusters were in a spheroid formation (Fig. 3D). Looking at an image of an array of hepatocyte clusters (Fig. 3E) one can see the boundaries of 500 μm diameter spots occupied by hepatocytes prior to spheroid formation. Interestingly, while the edges of the spot rolled up, prototypical hepatocytes with prominent nuclei and cuboidal morphology were observed in the center of the spheroid (Fig. 3F). Organization of hepatocyte clusters into three-dimensional constructs was not observed when cells were cultured on the three ECM proteins tested (collagen (I), (IV), laminin) without HGF. Similarly, no changes in morphology of the hepatocyte spots were observed when cells cultured on ECM protein spots were supplemented with soluble HGF at 10 ng/ml concentration. Conversely, when cultured on HGF containing spots, hepatocytes formed spheroids on all three types of ECM proteins at approximately the same time – 5 days of culture. This leads us to conclude that surface-immobilized HGF provided the signals to induce changes in cell morphology. HGF is a known mitogenic factor and DNA synthesis within the hepatocyte spheroids was investigated using BrdU assay. However, DNA synthesis was not observed suggesting that HGF signalling did not induce proliferation in hepatocytes – not surprising given their terminally differentiated state. It is therefore more likely that reorganization of hepatocytes into three-dimensional constructs was connected to motogenic properties of HGF and involved cytoskeleton and cell–cell adhesion molecules. Similar effects of HGF on hepatocellular reorganization without proliferation have recently been reported by Hoshiba et al. [38]. A number of recent studies suggested that hepatocytes cultured in 3D configuration such as a spheroid are more functional compared to standard monolayer cultures [38–42]. Therefore, the organization of hepatocytes into 3D spheroids observed in Fig. 3 may also be connected to the enhancement of

a function of time in culture, however, ~50% of HGF molecules were retained on col (I) microarrays after five days in culture. These data further support the notion that HGF may be present on the surface of ECM microarrays over extended periods of time to stimulate adherent hepatocytes.

Our results illustrate that individual printed ECM spot retains ~1–2 pg of HGF. When adding HGF in soluble form, 3 ml of 10 ng/ml
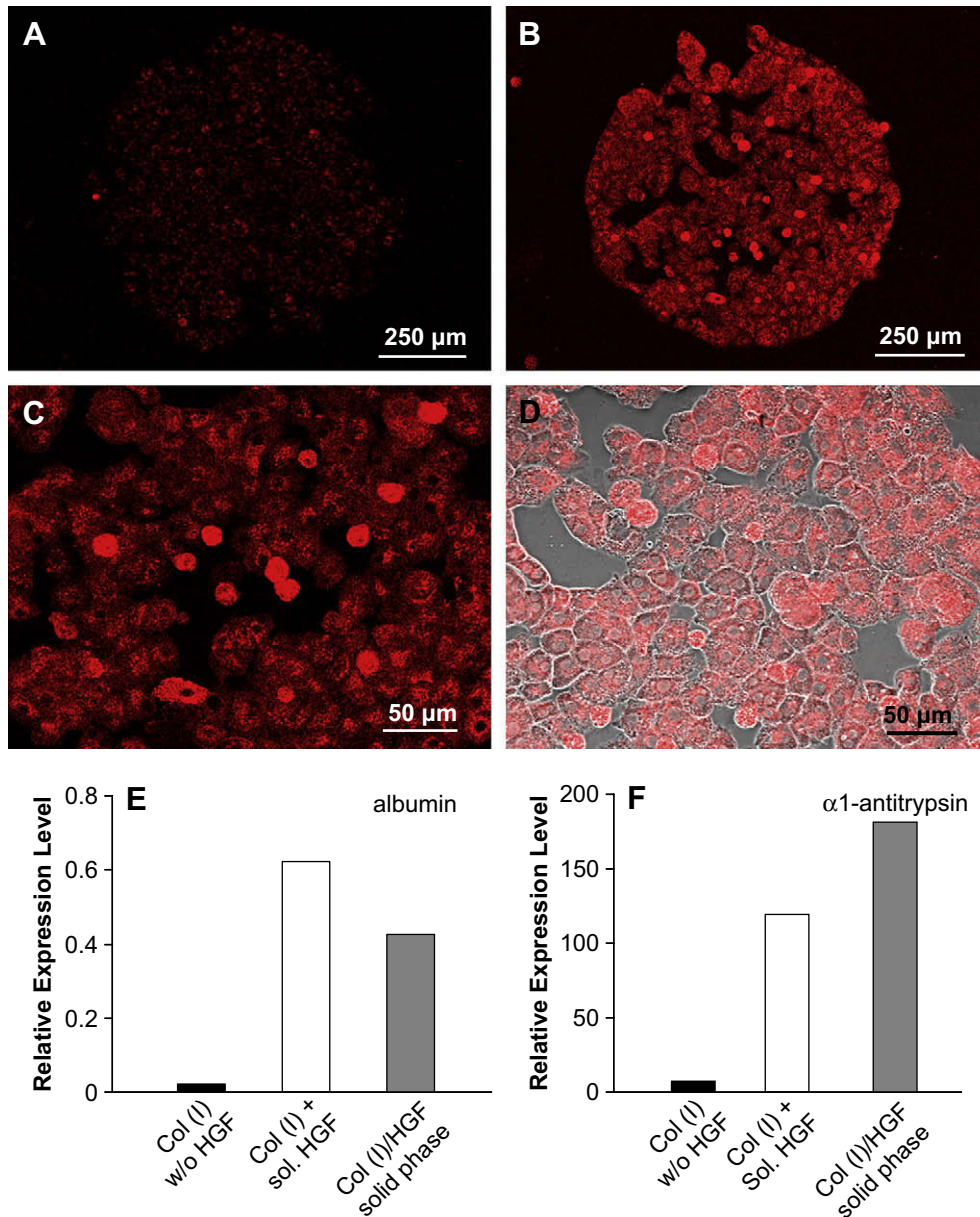
Fig. 5. Immunostaining and RT-PCR. (A) Intracellular albumin staining of hepatocytes after 4 days of cultivation on collagen (I) spots. (B) Intracellular albumin staining of hepatocytes after 4 days of cultivation on HGF/collagen (I) spots reveals significantly higher level of albumin synthesis compared to (A). (C–D) Higher magnification (63×) fluorescence (D) and merged brightfield/fluorescence image (63×) of intracellular albumin staining after 4 days of culture on HGF/collagen (I) shows localization of albumin to cytoplasm of hepatocytes. (E–F) Expression level of two liver-specific genes (albumin and α-1 antitrypsin) relative to β-actin housekeeping gene.

hepatic function induced by HGF microarrays and discussed in the following section.

It should be noted that spheroid formation comparable to that described in Fig. 3 also occurred when hepatocytes were stimulated with HGF on larger, ∼3 mm diameter, ECM spots (data not shown). However, in this latter case, spheroids were distributed on the surface randomly and not in a periodic pattern as shown in Fig. 3E.

3.2.1. Functional analysis of hepatocytes cultivated on HGF arrays

In order to assess function of hepatocytes cultured on HGF microarrays we monitored production of albumin – a liver-specific serum protein. Primary rat hepatocytes were cultured for a week on three ECM proteins – collagen I, collagen IV, and laminin – with and without HGF. An additional control performed in our studies, involved cultivation of hepatocytes on ECM microarrays with HGF added into culture media at concentration of 10 ng/ml. Thus we compared hepatic function under three scenarios: ECM microarrays alone, ECM microarrays + soluble HGF and ECM microarrays/solid-phase presented HGF (see Fig. 1 for pictorial illustration). Fig. 4 compiles the results of this study. As shown in Fig. 4A, hepatocytes cultivated on collagen (I) arrays with and without HGF produced comparable levels of albumin at the beginning of the experiment (Day 2); however, albumin production by hepatocytes on collagen (I) arrays decreased rapidly pointing to de-differentiation of these cells. This was in stark contrast to the HGF-stimulated hepatocytes that exhibited high levels of albumin synthesis (5.5-fold higher than collagen (I) controls). We also tested two other ECM proteins found in the liver – collagen IV (Fig. 4B) and laminin (Fig. 4C) – and found a similar trend in upregulation of albumin production in HGF-stimulated cells.
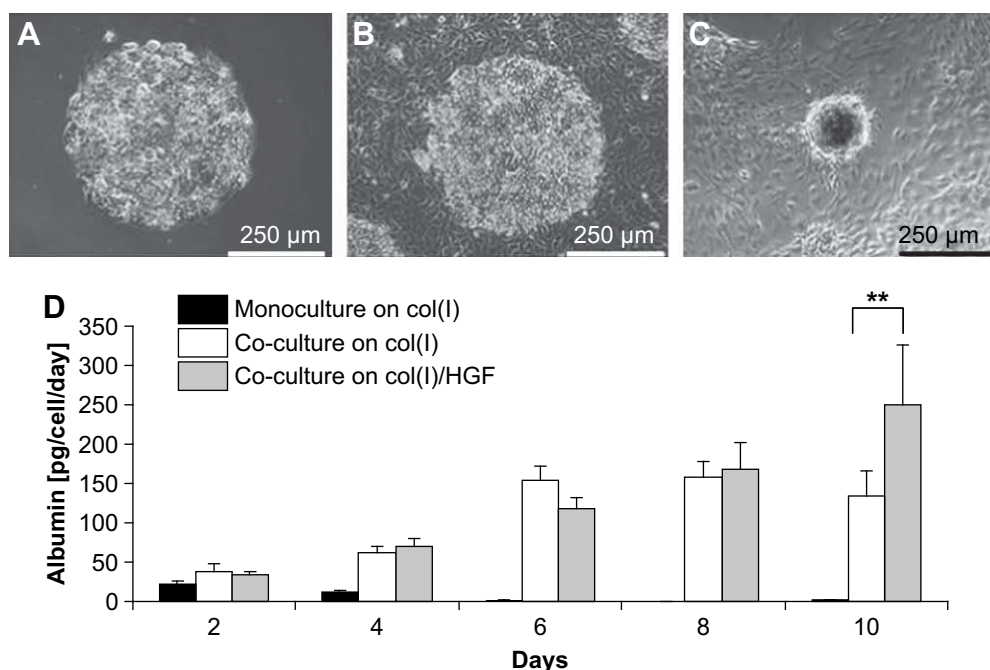
Fig. 6. Hepatocyte–fibroblast co-cultures created on HGF microarrays. (A) Monoculture; Col I control (B) Co-culture; Col I + Soluble HGF (C) Co-culture; Col I + printed HGF (D) ELISA data show enhanced maintenance in albumin secretion in ECM-bound as well as soluble HGF compared to ECM control w/o growth factor even in the presence of fibroblast co-culture.

The function of hepatocytes in contact with solid-phase presented HGF was also compared to daily additions of soluble HGF into culture media. The concentration of soluble GF was chosen based on the conditions typically used in conjunction with this GF molecule [43,44]. The direct comparison of soluble vs. solid-phase GF presentation is difficult to accomplish because it is unclear what fraction of soluble and matrix-bound GF molecules stimulates the hepatocytes in each of these two scenarios. Nevertheless, albumin ELISA results pointed to similar levels of protein synthesis in hepatocytes cultured on HGF/collagen (I) and HGF/laminin arrays compared to cells cultured on the same ECM proteins but with soluble HGF. In the case of collagen (IV) spots, a 4-fold higher albumin production was observed on solid-phase presented HGF compared to soluble HGF. In addition, we compared function of hepatocytes cultured on 500 μm and 3 mm diameter HGF/ECM spots and found hepatic albumin production to be similarly elevated in both cases (data not shown). Our results are consistent with previous studies by Bhatia and colleagues where function of hepatocytes was shown to be independent of micropattern dimension for spots larger than 500 μm in diameter [45].

Intracellular immunostaining of albumin also showed a significantly stronger signal on printed HGF/collagen I spots than on collagen I control spots (Fig. 5A,B) thereby corroborating ELISA data. A higher resolution image (63×) of albumin immunostaining shows specific cytoplasmic staining of albumin (Fig. 5C,D). The ELISA and immunostaining results were also corroborated by RT-PCR data by clearly pointing to a 20-fold upregulation in albumin gene expression in the case of printed HGF (high) and soluble HGF (Fig. 5E). RT-PCR analysis of another liver-specific protein α1-antitrypsin revealed a 23-fold higher level of expression of this gene in hepatocytes cultured on HGF/collagen (I) compared to hepatocytes on collagen (I) spots (control experiment) (see Fig. 5F).

Hepatic function results presented in this section are significant as they demonstrate that surface immobilization of HGF at the beginning of the experiment is at least as effective in maintaining differentiated hepatic phenotype as daily addition of soluble HGF into culture media. Beyond considerations of the cost of reagent, which is conserved by using HGF microarrays, our results suggest that some surface-bound HGF molecules may remain potent and active for several days under cell culture conditions, inducing lasting changes in hepatic phenotype expression.

3.2.2. Effects of printed HGF on hepatocytes in co-cultures

A number of studies have shown that co-cultivation of hepatocytes with non-parenchymal cells leads to the enhancement and maintenance of differentiated hepatic phenotype [46–48]. The co-culture format was further enhanced by Bhatia and co-workers who incorporated micropatterning strategies and showed placement of different cell types into precise locations on the same surface. In the subsequent studies, micropatterned co-cultures were used to dissect the effects of homotypic and heterotypic interactions between the hepatocytes and non-parenchymal cells [45]. In our approach, hepatocytes were seeded on the silane-modified glass substrates containing printed microarrays and became adherent exclusively on the protein islands (500 μm diameter) (Fig. 6A). The non-parenchymal cells (3T3 fibroblasts) were added in the subsequent cell seeding step, attaching on the silane-modified glass substrate around the hepatic islands (Fig. 6B). This micropatterning strategy allowed us to selectively stimulate one cell type (hepatocytes) in a co-culture format and query the effects of HGF on the hepatocytes cultured in the presence of growth factor-producing non-parenchymal cells. As illustrated in Fig. 6C, solid-phase presentation of HGF-induced formation of hepatic spheroids even in the context of a co-culture where hepatocytes residing on HGF/ECM islands (500 μm diameter) were in contact with the surrounding fibroblasts. Based on the previous reports, hepatocytes co-cultivated with 3T3 fibroblasts were expected to express higher function compared to hepatocytes cultured alone [45]. This trend was corroborated by our albumin ELISA experiments (Fig. 6D). Interestingly, hepatocyte–fibroblast co-cultures

created on HGF/collagen (I) arrays exhibited significantly higher levels (1.8-fold) of albumin production compared to co-cultures with hepatocytes sitting on collagen (I) arrays. These data are interesting as they demonstrate that solid-phase presentation of HGF had an observable and long-lasting effect even in the presence of fibroblasts – non-parenchymal cells that are likely secreting endogenous growth factors.

While there have been reports of surface-immobilized GF molecules stimulating cell function, [18,28,33] we are not aware of studies involving primary hepatocytes. These cells de-differentiate rapidly *in vitro* and require special cultivation approaches such as collagen double gel [49,50] or co-cultures [45] to rescue hepatic function and to ensure long-term maintenance. Our results are intriguing as they suggest that one-time presentation of matrix-bound HGF at the beginning of the experiment without additional supplementation is sufficient to enhance and maintain phenotype expression of primary hepatocytes after 10 days in culture. The mechanism of the phenotype induction is unclear at the moment. It has been suggested previously that GF interactions with ECM proteins may protect morphogens against proteolytic degradation [28], therefore, HGF may be stabilized by the association with matrix proteins and may remain potent and active throughout the experiment. In addition, HGF-induced organization of hepatocytes into 3D spheroids may also contribute to the enhanced phenotype expression.

4. Conclusions

The present paper investigated the use of HGF microarrays for cultivation of primary hepatocytes. Analysis of hepatic phenotype with ELISA, RT-PCR, and immunostaining techniques revealed that solid-phase presentation of HGF induced significant enhancement in synthesis and transcription of liver-specific proteins, albumin and α1-antitrypsin. In addition, hepatocytes cultured on HGF microarrays spontaneously reorganized from a monolayer to spheroid configuration – a behavior that was not observed otherwise. Solid-phase presentation of HGF was also seen to affect morphology and phenotype of hepatocytes in co-cultures with non-parenchymal cells.

Overall, the strategy of using robotic printing to create GF arrays has a number of advantages over traditional methods of cell stimulation with GF molecules. Solid-phase presentation of GF molecules associated with matrix proteins may be more physiological. It also dramatically decreases the cost of performing experiments and makes the microarray format particularly well-suited for high-throughput screening of GF–cell interactions where different combinations and/or concentrations of GF molecules may be tested in a multiplexed and combinatorial fashion.

Acknowledgments

Appendix

Figures with essential colour discrimination. Parts of Figs. 1,3 and 5 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi: 10.1016/j.biomaterials.2009.03.039.

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