Designing a Hepatocellular Microenvironment with Protein Microarraying and Poly(ethylene glycol) Photolithography

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In this study, robotic protein printing was employed as a method for designing a cellular microenvironment. Protein printing proved to be an effective strategy for creating micropatterned co-cultures of primary rat hepatocytes and 3T3 fibroblasts. Collagen spots (ca. 170 μm in diameter) were printed onto amino-silane- and glutaraldehyde-modified glass slides. Groups of 15−20 hepatocytes attached to collagen regions in a highly selective manner forming cell clusters corresponding in size to the printed collagen domains. Fibroblasts, seeded onto the same surface, adhered and spread around arrays of hepatocyte islands creating a heterotypic environment. The co-cultured hepatocytes produced and maintained high levels of liver-specific biomarkers, albumin and urea, over the course of 2 weeks. In addition, protein printing was combined with poly(ethylene glycol) photolithography to define intercellular contacts within the clusters of hepatocytes residing on individual collagen islands. Glass slides, treated with 3-acryloxypropyl trichlorosilane and imprinted with 170 μm diameter collagen spots, were micropatterned with a high-density array of 30 μm × 30 μm poly(ethylene glycol) (PEG) wells. As a result, discrete groups of ca. 9 PEG microwells became functionalized with the cell-adhesive ligand. When exposed to micropatterned surfaces, hepatocytes interacted exclusively with collagen-modified regions, attaching and becoming confined at a single-cell level within the hydrogel wells. Micropatterning strategies proposed here will lead to greater insights into hepatocellular behavior and will benefit the fields of hepatic tissue engineering and liver biology.

Introduction

The microenvironment is a defining factor in a wide range of cellular processes including proliferation, differentiation, and expression of phenotype-specific functions.[1,2] The complex cellular microenvironment is comprised of simpler components including cellular interactions with matrix proteins, neighboring cells, and soluble factors. In tissue engineering, design of the cellular microenvironment is necessary for in vitro maintenance of well-differentiated primary cells; hence, it is an important component in the development of bioartificial organs. Specifically, in hepatic tissue engineering, cell−cell communications and extracellular matrix (ECM) composition have been identified as important modulators of hepatocyte function.[3−5] Cultivating primary hepatocytes on collagen in a random co-culture with nonparenchymal cells (e.g., fibroblasts) has been shown to preserve liver-specific hepatic function for several weeks.[6,7]

Micropatterning and surface engineering techniques are emerging as important tools for dissecting the effects of microenvironment on cellular behavior by orchestrating cell−surface, cell−cell, and cell−solution interactions in a controlled manner.[8−12] In our laboratory, traditional photolithography was recently employed to create hepatocyte−fibroblast co-cultures[13] and to rationally analyze hepatic phenotype expression as a function of heterotypic and homotypic cell−cell contacts within the co-cultures.[14,15] More recently, poly(ethylene glycol) (PEG) photolithography[16] was proposed as a method for controlling interactions of individual primary hepatocytes with neighboring cells.[17] PEG-diacrylate was micropatterned on glass to manufacture arrays of wells composed of cell/protein resistant PEG walls and glass attachment pads. Modifying attachment pads with collagen (type 1) enabled adhesion and confinement of individual primary hepatocytes inside the PEG wells.[17]

Despite their obvious utility, strategies for creating cellular micropatterns often have shortcomings. For

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example, photolithography-based protein patterning, employed on our previous studies of the hepatocyte−fibroblast co-cultures, is a complex multistep process that exposes surfaces to photoresists and organic solvents. This technology also imposes strict limitations on the chemical properties and the geometry of the substrate. Moreover, photolithography and most other micropatterning strategies are poorly suited for placing multiple biomolecules on the same surface.

Robotic microarraying, on the other hand, is a micropatterning technique that is specifically designed to create surfaces with complex, spatially resolved physicochemical properties. In addition, microarraying requires minimal surface manipulation and may be employed to modify substrata with diverse geometries and chemical compositions. While originally designed for massively parallel screening of DNA hybridization events,[18] robotic microarraying technology has recently been adapted for printing proteins and is being employed widely in the emerging field of proteomics.[19,20] Thus far, protein arrays have been used extensively for biomolecular screening (e.g., antibody−cytokine interactions);[19−24] however, relatively few reports have utilized protein microarraying as a tool for designing specific cell−cell and cell−surface interactions.[25−27]

In the present study, robotic protein printing was employed to enhance our ability to design the hepatocellular microenvironment. To highlight the flexibility of this technology, three ECM proteins, collagen (type 1), laminin, and fibronectin, were printed in predefined locations on the same surface. Protein microarraying also proved a simple and effective strategy for micropatterning of heterotypic co-cultures of primary rat hepatocytes and murine 3T3 fibroblasts. Collagen was robotically printed to define hepatocyte-adhesive regions on glass. When exposed to patterned surfaces, primary hepatocytes selectively adhered to collagen domains, forming cell arrays with 15−20 cells per array element. Seeding murine 3T3 fibroblasts onto the surface containing hepatocyte arrays resulted in formation of a heterotypic co-culture. Hepatocyte-specific function was evaluated by measuring albumin and urea levels in the cell culture medium and was found to be stable over the course of 2 weeks. In addition, robotic protein printing was combined with PEG photolithography to precisely define cell−cell interactions within the clusters of hepatocytes residing on printed collagen domains. Glass slides, treated with 3-acryloxypropyl trichlorosilane and imprinted with 170 μm diameter collagen spots, were micropatterned with high-density arrays of 30 μm × 30 μm PEG hydrogel wells. As a result, discrete groups of ca. 9 wells within the array of PEG microwells were functionalized with collagen. When seeded onto micropatterned surfaces, hepatocytes interacted exclusively with groups of collagen-modified PEG wells, so that individual cells adhered and became confined in 900 μm^2 glass attachment pads of the hydrogel wells.

Experimental Section

Materials. Poly(ethylene glycol) diacrylate (PEG-DA) (MW = 575), 2,2′- dimethoxy-2-phenyl-acetophenone (DMPA), anhydrous toluene, desiccator, glovebag, ethanol, bovine serum albumin (BSA), 3-[(2-aminoethyl)amino] propyl trimethoxysilane, epidermal growth factor, and urea assay kits were obtained from Sigma-Aldrich Chemical Co. 3-Acryloxypropyl trichlorosilane was purchased from Gelest, Inc. Sulfuric acid and hydrogen peroxide were obtained from J.T. Baker. Glass slides (75 × 25 mm), tissue culture flasks, and serological pipets were obtained from Fisher Scientific. Phosphate-buffered saline (PBS) 1×, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin, streptomycin, trypan blue, trypsin/EDTA, fetal bovine serum (FBS), and bovine calf serum (BCS) were purchased from Invitrogen Life Technologies. Glucagon and insulin were obtained from Eli-Lilly, and hydrocortisone was obtained from Pharmacia and Upjohn. Fluorescein isothiocyanate (FITC) conjugated collagen (type I from bovine skin) was obtained from Molecular Probes. Fibronectin and laminin were purchased from Biomedical Technologies Inc. Cyanine (Cy)-3 and -5 fluorescent dyes were bought from Amersham Biosciences. Murine 3T3 fibroblasts were purchased from American Type Culture Collection. Primary hepatocytes were isolated from female Lewis rats (Charles River Laboratories). Rat albumin and anti-rat albumin were purchased from Cappel Laboratories.

Printing Protein Microarrays. Glass slides were cleaned by immersion in "piranha" solution consisting of a 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*). Next, the glass slides were thoroughly rinsed with deionized (DI) water and dried under nitrogen. The glass slides were then placed for 30 min in a solution containing 3-[(2-aminoethyl)-amino] propyl trimethoxysilane (2% v/v) diluted in 95% ethanol, rinsed thoroughly with DI water, and cured at 80 °C for 15 min. Amino-functionalized glass slides were stored at 4 °C until further use. Prior to printing protein arrays, the silane-modified glass slides were exposed for 30 min to a glutaraldehyde solution (1% w/v) diluted in PBS (pH 7.4), rinsed with DI water, and dried under nitrogen. For micropatterning of multiple proteins, laminin-Cy5 and fibronectin-Cy3 were conjugated in accordance with instructions from Amersham Biosciences. All proteins employed for printing were dissolved in 1× PBS at 0.1 mg/mL concentration. Protein microarrays were contact-printed under ambient conditions on modified glass substrata using a GMS 417 Arrayer (Genetic MicroSystems, Inc.) with Pin-and-Ring liquid dispensing technology. A computer-controlled robotic microarrayer collected fluorescently labeled protein (0.1 mg/mL in PBS) from a 96-well plate and dispensed 1−2 nL of protein solution on the aldehyde-derivatized glass slides forming circular spots ca. 170 μm in diameter with 375 μm center-to-center spacing. Multiprotein arrays were typically printed in 6 × 6 or 10 × 10 format. For the hepatocyte−fibroblast co-culture experiments, collagen arrays containing 4800 spots were robotically printed on 75 × 25 mm glass slides. Glass slides containing protein arrays were rinsed with 1× PBS immediately after a printing run. The quality of printed arrays was assessed using a GMS 418 scanner (Genetic MicroSystems, Inc.) Fluorescently labeled collagen was imaged using a Nikon Eclipse TE2000 inverted microsope with an attached SPOT digital camera (Diagnostic Instruments Inc.). Characterization of arrays of multiple fluorescently labeled proteins was accomplished with a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss Inc.). In these composite images, collagen-FITC and fibronectin-Cy3 appeared green and red, respectively, while laminin-Cy5 conjugate was arbitrarily assigned blue color. Collagen-modified glass slides were stored at 4 °C in air for up to 4 weeks without apparent detriment to cell patterning or function.

Combining Protein Printing and PEG Micropatterning. Figure 1B illustrates the process flow for combining PEG hydrogel micropatterning and protein printing. As a first step in this surface micropatterning procedure, glass slides were treated with acrylated trichlorosilane according to the procedure described previously.[17] Robotic printing was then employed to create arrays of collagen spots on silane-modified glass slides. Printed collagen islands were ca. 170 μm in diameter with 375 μm center-to-

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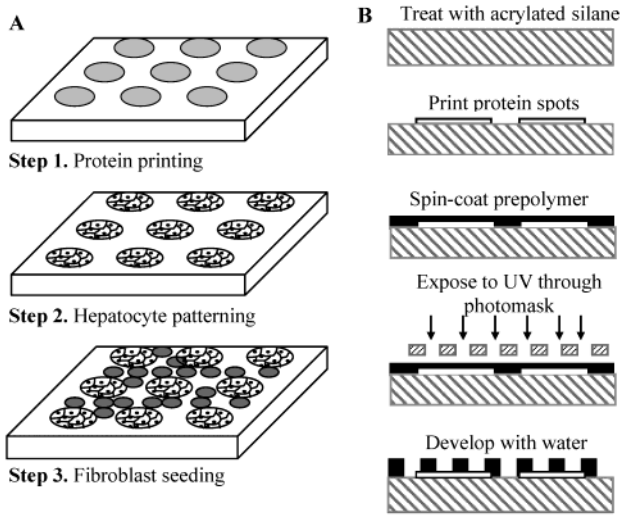

Figure 1. (A) Schematic of the cellular micropatterning process. Step 1: Collagen arrays are robotically printed on amino-silane- and glutaraldehyde-modified glass slides. Step 2: The surfaces are treated with BSA. Hepatocytes are patterned on collagen microarrays and incubated for 24 h. Step 3: The micropatterned co-culture is created by seeding fibroblasts on the same surface. The fibroblast population proliferates, occupying interhepatic space, stabilizing and enhancing liver-specific hepatocyte function. (B) Photolithographic patterning of PEG microwells on the glass substrates imprinted with protein arrays.

center spacing. In contrast to the protein printing procedure outlined in the previous section where protein was chemisorbed onto amino-silane- and glutaraldehyde-modified glass substrates, collagen immobilization on acrylated silane-derivatized glass slides was exclusively due to physiadsorption. After robotic printing, glass slides contained discrete protein regions formed on the substrate modified with the acrylated silane, a coupling agent necessary to anchor PEG hydrogel microstructures to the surface. Glass slides imprinted with protein arrays were then micropatterned with PEG in accordance with the process described before.[17] Briefly, a precursor solution of PEG-DA (MW = 575) with 1% w/v photoinitiator (DMPA) was spun at 1000 rpm for 6 s onto the protein-patterned glass slides using a spin-coater (Machine World Inc.). The uniform layer of PEG-DA precursor solution on glass was then exposed through a chrome/sodalime photomask (Advance Reproductions) to 365 nm, 15 mW/cm^2 UV light from a Q 2001 mask aligner (Quintel Co.). Exposure times ranged from 1 to 2 s. Regions of PEG-DA exposed to UV underwent free-radical polymerization and became cross-linked, while unexposed regions were dissolved in DI water after 5 min of development. The resultant micropatterns consisted of individual wells with 30 μm × 30 μm PEG hydrogel sidewalls and glass adhesion pads. PEG walls were 20 μm in width. The height of the hydrogel microstructures varied from 1 to 3 μm as measured with a Dektak^3 surface profiler (Veeco Instruments). Individual wells formed 80 × 80 microwell arrays with discrete groups of ca. 9 wells containing collagen-coated adhesion sites. A Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss Inc.) was employed to collect composite, brightfield and fluorescence images of PEG micropatterns superimposed over collagen-FITC arrays.

Cell Culture and Patterning. Rat hepatocytes were isolated and purified according to established protocols.[28] Typically 200−300 million hepatocytes were isolated from a single liver with less than 5% contamination from nonparenchymal cells. Hepatocytes were maintained at 37 °C in a humidified 10% CO_2 atmosphere in DMEM supplemented with 10% heat-inactivated FBS, 200 U/mL penicillin, 200 μg/mL streptomycin, 7.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor, 14 ng/mL glucagon, and 0.5 U/mL insulin. Murine 3T3 fibroblasts were maintained at 37 °C in a humidified 10% CO_2 atmosphere in DMEM supplemented with 10% BCS, 200 U/mL penicillin, and 200 μg/mL streptomycin. Cells were cultured until ca. 90% confluency and passaged.

Creating Micropatterned Co-cultures. A schematic showing the sequence of steps to obtain micropatterned co-cultures of hepatocytes and fibroblasts is shown in Figure 1A. Prior to cell culture, the glass slides were placed in a sterilized custom-made silicone mold to ensure a tight seal and efficient cell patterning. The collagen-modified glass slides were treated with a 1% BSA solution (diluted in 1× PBS) to prevent nonspecific cell adhesion and subsequently sterilized in ethanol. To demonstrate spatial distribution of BSA on the glass surface, collagen-patterned slides were exposed to BSA-Cy3 conjugate (1 mg/mL in 1× PBS) for 30 min, rinsed with DI water, and imaged using fluorescence microscopy.

Hepatocyte seeding was accomplished in a two-step process (Figure 1A); the collagen-patterned slides were first exposed to 3 mL of cell suspension in hepatocyte culture medium at a concentration of 1 × 10^6 cells/mL. At the end of 1 h, the medium containing unattached cells was aspirated and fresh cell suspension at the concentration given above was added again, aspirated after 1 h, and replaced with hepatocyte culture medium. To introduce the second cell type, glass slides containing surface-bound arrays of hepatocytes were exposed to a 3 mL cell suspension of fibroblasts (2.5 × 10^5 cells/mL) in fibroblast culture medium. After 2 h of incubation at 37 °C, the culture medium was aspirated and replaced with hepatocyte culture medium. Hepatocyte−fibroblast co-cultures were maintained in hepatocyte culture medium for up to 2 weeks in a humidified 10% CO_2 atmosphere at 37 °C.

Patterning Hepatocytes on Protein-Imprinted and PEG-Modified Surfaces. Glass slides, containing printed protein arrays and PEG micropatterns, were cut into 25 mm × 25 mm pieces and placed into 35 mm diameter Petri dishes. Prior to hepatocyte seeding, glass samples were sterilized by exposure to 70% ethanol for 1 h and rinsed in DI water.

Hepatocyte seeding was performed immediately after the isolation to ensure cell viability. Micropatterned glass substrates were placed in 35 mm diameter Petri dishes and exposed to 2 mL of a 5 × 10^5 cells/mL hepatocyte suspension. After 1 h of incubation at 37 °C, hepatocyte media and unattached cells were aspirated and replaced with 2 mL of fresh media. Patterned hepatocytes were incubated for 24 h at 37 °C and fixed for imaging by immersion in a 1% v/v solution of glutaraldehyde (diluted in 1× PBS) for 30 min.

Brightfield and phase contrast microscopy of the cellular micropatterns was obtained using Nikon Eclipse TE2000 and Zeiss LSM 5 Pascal Confocal instruments (Carl Zeiss Inc.). High-resolution images of the primary hepatocytes residing on micropatterned surfaces were obtained using a JSM 5600LV scanning electron microscope (SEM) (JEOL Inc.) operating at 10 mV accelerating voltage. To avoid charging effects, substrates were sputter-coated with gold−palladium to a thickness of 10 nm prior to SEM experiments.

Analysis of Urea Production and Albumin Synthesis. Hepatocyte culture medium collected every 24 h was analyzed for urea and albumin content. Urea and albumin concentrations were determined using a standard kit from Sigma Diagnostics and by enzyme-linked immunosorbent assay (ELISA), respectively.[28] The sample size in our studies was $n = 4$.

Results and Discussion

In the present study, robotic protein microarraying was investigated as a strategy for designing the hepatocellular microenvironment. Collagen microarraying was employed to define hepatocyte-specific adhesion domains on glass in order to micropattern co-cultures of primary rat hepatocytes and 3T3 fibroblasts. Furthermore, the combination of robotic protein printing and PEG photolithography allowed simultaneous control of individual hepatocyte interactions with surface-bound ligands and neighboring cells. The micropatterning strategies proposed

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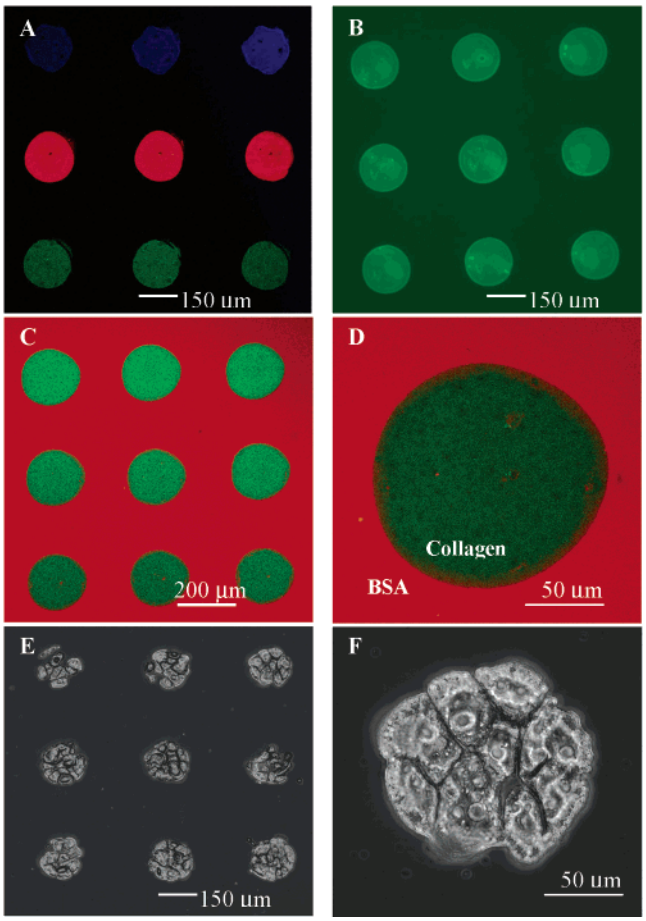

Figure 2. Protein printing and hepatocyte micropatterning. In all cases, the individual dimensions of protein spots are ca. 170 μm with 375 μm center-to-center distance. (A) Confocal image of a 3 × 3 protein array containing collagen-FITC (green), fibronectin-Cy3 (red), and laminin-Cy5 (blue). (B) Array of FITC-conjugated collagen spots (×100). (C) Collagen-FITC array after adsorption of BSA-Cy3 (red background) (×100). (D) Higher magnification view of a collagen spot with BSA-Cy3 background (×400). (E) Brightfield image of a hepatocyte pattern formed on a collagen microarray (×100). (F) Higher magnification brightfield image of an individual hepatocyte island (×400).

here will lead to better understanding of hepatocellular behavior, thus benefiting the fields of hepatic tissue engineering and liver biology.

Protein Printing and Hepatocyte Micropatterning. Although numerous strategies for surface patterning of biomolecules have been proposed,[29] these techniques either require complex, multistep surface modification processes (e.g., photolithography) or employ principles of molecular self-assembly on specific surfaces (e.g., microcontact printing on gold). In contrast, robotic microarraying is a "direct write" technology that enables patterning biomolecules in a high-density array format on a variety of substrata with minimal surface modification. In our studies, simple functionalization of glass slides with amino-silanes followed by glutaraldehyde treatment provided the protein-reactive surfaces suitable for creating the protein micropatterns presented in Figure 2A−D. In other experiments described in this paper, collagen spots were simply physiadsorbed onto glass surfaces modified with acrylated chlorosilane.

Beyond its simplicity, microarraying technology offers a unique possibility for patterning of multiple biomolecules on the same surface. Figure 2A shows three ECM proteins, collagen-FITC, fibronectin-Cy3, and laminin-Cy5, printed in array format on the same surfaces. Individual array elements in this figure are ca. 170 μm in diameter with a center-to-center distance of 375 μm. Decorating surfaces with matrix proteins is often necessary in order to induce attachment of anchorage-dependent cells. Primary hepatocytes, for example, have been shown to selectively adhere to collagen micropatterns.[13] In the present study, robotic microarraying was used to print hepatocyte-specific collagen domains on glass (see Figure 2B). Surfaces containing collagen arrays were then exposed to BSA (see Figure 2C,D) to ensure minimal nonspecific hepatocyte attachment. To visualize its distribution on the collagen-patterned surface, BSA was conjugated to a Cy-3 fluorophore. As seen from Figure 2C,D, BSA-Cy3 adsorbed uniformly around collagen spots, creating a well-defined interface between hepatocyte-adhesive and nonadhesive regions on glass. The hepatocytes adhered and spread in a highly selective manner forming circular arrays corresponding in size to the underlying collagen domains (Figure 2E,F). The morphology of hepatocytes was dependent on their individual location within the collagen-rich domains. Hepatocytes that adhered to the periphery of the collagen domains aligned themselves along the collagen−BSA interface and appeared elongated, while the cells located in the center of each collagen island spread and exhibited a normal cuboidal shape.

Creating Micropatterned Co-Cultures on Printed Collagen Arrays. The ability to elicit long-term hepatocyte-specific function in vitro is essential in hepatic tissue engineering for development of bioartificial liver devices. Co-culturing hepatocytes with nonparenchymal cells (e.g., fibroblasts) is one of the proven methods to attain this long-term stability.[6,7] Controlled micropatterning of two cell types on the same surface, described in Figure 1A, is based upon the preferential adhesion of hepatocytes to collagen domains. As discussed in the previous section, hepatocytes preferentially assembled onto collagen domains imprinted on glass. Cellular organization was reproducible on a large scale, as seen from Figure 3A, where a 10 × 8 cellular array is shown immediately after hepatocyte seeding. BSA treatment of regions not containing arrayed protein provided an excellent barrier to nonspecific hepatocyte attachment. However, BSA-coated regions supported fibroblast adhesion and proliferation. Figure 3B shows fibroblasts dispersed around hepatocyte arrays 24 h after seeding. By day 5, fibroblasts proliferate to occupy most of the interhepatic space while nonproliferating hepatocytes maintained their original location as seen in Figure 3C. Figure 3C also shows that the shape of the hepatocyte arrays in the co-culture changes over time as the cells try to bridge the distance between adjacent domains in an effort to establish hepatocyte−hepatocyte contacts. Pattern deformation continued until adjacent hepatocyte islands became connected by day 10 in culture.

Despite pattern reorganization, the initial hepatocellular microenvironment in the micropatterned co-cultures played a significant role in induction and maintenance of hepatocyte function. Similar pattern reconfiguration was previously observed by Bhatia et al. who noted that hepatocyte islands of less than 490 μm in diameter reorganized into cordlike structures after several days in culture.[15] However, these patterns elicited higher levels of production of hepatic function when compared to larger diameter islands or random co-cultures.[15,30] The efficacy

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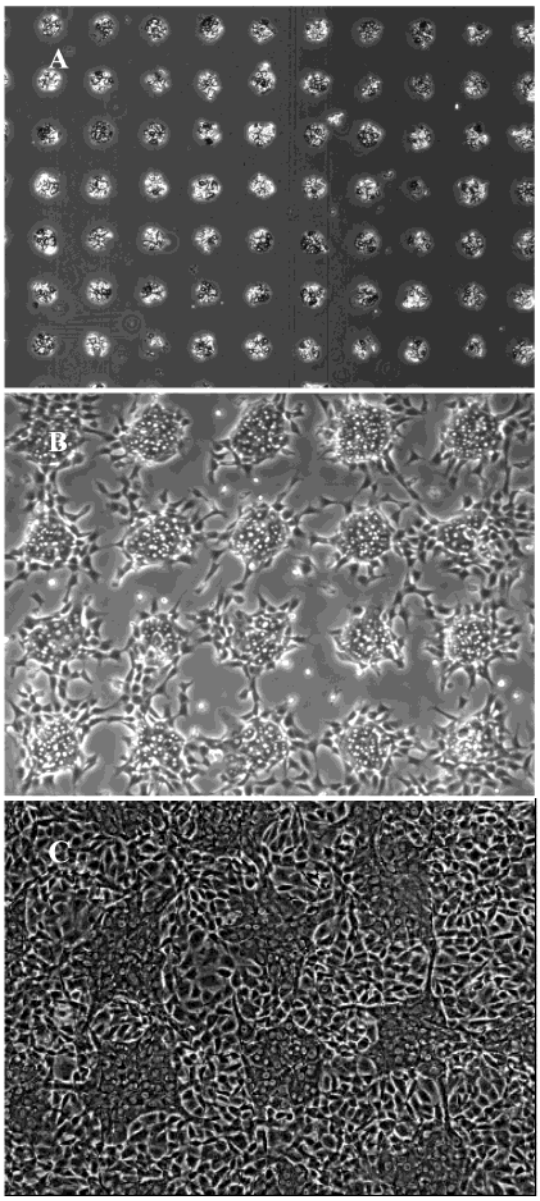

Figure 3. Micropatterned hepatocyte and fibroblast co-culture. (A) 10 × 8 array of hepatocytes prior to fibroblast seeding (×40). (B) Co-culture of hepatocytes and fibroblasts 24 h after fibroblast seeding (×100). (C) Co-culture after 5 days of incubation. Hepatocyte islands losing their circular shape and starting to form interisland contacts (×150).

of the smaller diameter hepatocyte islands in inducing hepatic function was attributed to the increase in the heterotypic hepatocyte−fibroblast interface.

Analysis of Hepatocyte-Specific Function. Albumin secretion and urea synthesis are key markers that reflect the stability and function of hepatocyte cultures in vitro. Since both markers are hepatocyte-specific and are not expressed by 3T3 fibroblasts,[31] they provide information pertinent to hepatic phenotype expression. Data presented in Figure 4 demonstrate stability of albumin synthesis and urea production over the course of 2 weeks. The albumin response (Figure 4A) followed the previously reported pattern, with the rate of protein synthesis increasing until reaching a plateau by day 8.[15] The induction and enhancement of hepatocyte function due to the addition of fibroblasts are clearly observed by comparing albumin synthesis in "control" samples that contain hepatocytes alone to hepatocytes co-cultured with fibroblasts. The observed values for albumin synthesis 24 h after seeding are 0.8×10^{-4} μg/cell and 0.85×10^{-4} μg/cell for co-culture and control samples, respectively. By day 8 in culture, the co-cultivated hepatocytes exhibited a significant increase in albumin production, ca. 10-fold higher with albumin values of 5.57×10^{-4} μg/cell/24 h and 0.55×10^{-4} μg/cell/24 h for co-culture and control samples, respectively. Similar trends were observed in the case of urea production (Figure 4B). For example, by day 8 hepatocyte and fibroblast co-cultures produced ca. 9-fold higher urea (23.6×10^{-4} μg/cell/24 h) than the control samples (2.7×10^{-4} μg/cell/24 h). In general, our data compare favorably with prior observations of the hepatocyte behavior in micropatterned co-cultures where the rate of albumin synthesis was reported to be 3.6×10^{-4} μg/cell/24 h for a fibroblast/hepatocyte ratio of 0.5.[14]

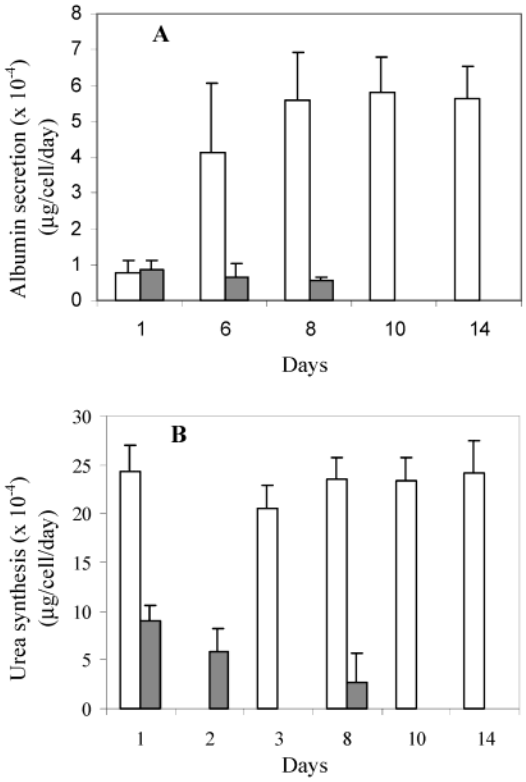

Figure 4. Hepatocyte-specific function in micropatterned co-cultures represented by albumin and urea synthesis. Dark bars represent controls (hepatocytes only), and open bars represent co-cultures. All results are based on ~10^5 hepatocytes. (A) Albumin secretion analyzed by ELISA. (B) Urea synthesis analyzed by colorimetric assay.

As discussed in the previous section, the diameter of the collagen islands is an important modulator of the hepatocyte function in the micropatterned co-cultures. Bhatia et al. reported significantly higher levels of albumin and urea production in hepatocytes co-cultured on smaller-diameter collagen islands (36, 100, and 490 μm) compared to larger-diameter collagen spots (6800 and 17 800 μm).[14,15] Thus, the 170 μm diameter collagen spots utilized for creating micropatterned co-cultures in this study are within the range of "optimal" collagen island dimensions. One should note that the robotic printer employed here is designed to dispense a constant volume (ca. 1 nL) of protein solution upon contact with the substrate, thus making it difficult to vary dimensions of individual protein spots. However, microarrayers employing noncontact printing (e.g., ink-jet printing) are commercially available

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and may be used to modulate protein spot dimensions by dispensing picoliter to nanoliter volumes of protein solutions.[32] Therefore, it may be possible to print protein islands as small as 30−50 μm in diameter.

Combining Robotic Protein Printing and PEG Photolithography. Robotic protein printing is an excellent method for defining local surface composition and investigating cell−surface interactions. However, microarraying technology alone provides limited control over intercellular communications. For example, as seen in Figure 2E,F, collagen microarraying leads to organization of clusters of 15−20 hepatocytes on each cell-adhesive domain. To introduce control over intercellular contacts at a single-cell level while retaining advantages of protein microarraying, the latter technology was combined with PEG photolithography.

The surface modification procedure employing both protein printing and PEG photolithography is presented schematically in Figure 1B. In this process, PEG microwells are superimposed over printed protein arrays so that groups of ca. 9 wells become functionalized with cell-adhesive ligands. Both protein array and PEG microwell patterns are periodic; thus, no registration step is required. Figure 5A,B shows printed collagen-FITC regions covered with 30 × 30 μm PEG microwells. Individual wells were composed of cell-resistant PEG walls, 20 μm in width, and glass attachment pads. Cell-adhesive properties of the glass pads were modulated from more adhesive collagen-modified regions to less adhesive silane-presenting regions. When seeded, primary hepatocytes covered micropatterned surfaces conformally, interacting everywhere. However, given the equal opportunity to attach, cells selectively adhered to collagen domains and did not interact with PEG- or silane-modified regions of the glass surface. When unattached cells were aspirated after 1 h of incubation, hepatocyte patterns were revealed. As seen in Figure 5C,D, hepatocytes residing on printed collagen islands were now compartmentalized in PEG hydrogel wells. BSA modification was not necessary since little nonspecific attachment of hepatocytes onto acrylated silane-modified glass surfaces was observed. Effects achieved by the composite micropatterning procedure are best illustrated by Figure 5E where on the right side of the image, hepatocytes reside on a collagen island with cell−cell contacts unimpeded, whereas on the left, intercellular communications are limited by PEG regions. The main advantage of the combined protein and PEG micropatterning method is the ability to effectively decouple cell−cell and cell−surface interactions. This will allow us to precisely define the local microenvironment and to study the effects of the engineered stimuli on hepatocyte behavior.

Conclusions

The present paper describes a simple and effective cell patterning approach based on robotic microarraying of proteins. We have shown that hepatocytes and fibroblasts can be micropatterned on glass slides containing collagen arrays to form heterotypic co-cultures. These co-cultures maintained albumin and urea synthesis over the course of 2 weeks at levels comparable or superior to those reported in our previous studies of microfabricated co-cultures. Furthermore, robotic protein printing was combined with PEG photolithography to control individual cell−cell contacts of hepatocytes residing on collagen domains. While the present work only demonstrated

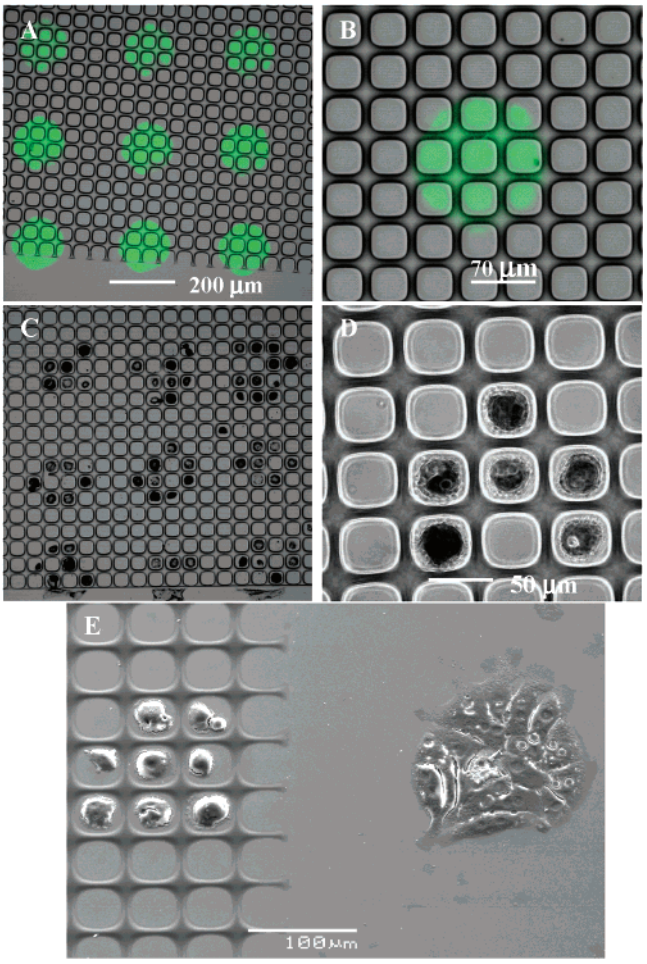

Figure 5. Combining robotic protein printing and PEG photolithography. The individual dimensions of PEG wells are 30 μm × 30 μm. (A) An array of PEG microwells is superimposed over robotically printed collagen islands. Discrete groups of ∼9 wells become functionalized with collagen (×100). (B) Higher magnification view of PEG regions micropatterned over a collagen island (×200). (C) Primary hepatocytes preferentially adhere to underlying collagen regions while becoming confined within PEG microwells (×100). (D) Hepatocytes residing on an individual collagen island (×400). (E) SEM micrograph of hepatocytes exhibiting drastically different adhesion behavior: on the right, cells residing on a collagen spot are permitted to form intercellular contacts, while on the left, cell−cell contacts are inhibited by the PEG micropattern.

confinement of hepatocytes inside PEG wells, in the future, PEG regions will be microfabricated to permit controlled contacts between adjacent cells. Combining PEG micropatterning with the ability to print arrays of multiple ECM proteins will allow simultaneous control of cell−cell and cell−surface interactions on a micrometer scale and evaluation of local effects of the engineered microenvironment on hepatocellular behavior. Therefore, we envision the micropatterning strategies presented here becoming valuable assets to the fields of hepatic tissue engineering and liver biology. Ultimately, robotic printing of ligands represents a method for designing combinatorial surfaces to evaluate cellular interactions in a systematic, rational, and parallel manner.

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gestions. Confocal microscopy was performed at CNY-6 Confocal Microscopy Core at Massachusetts General Hospital with assistance from Mr. Igor Bagayev. Scanning electron microscopy was performed at the W. M. Keck Microscopy Facility at the Whitehead Institute for Biomedical Engineering. Financial support for this work was provided by the National Institutes of Health (DK 43371, P41 EB002503 and T32GM07035).

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