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Analysis of Local Tissue-Specific Gene Expression in Cellular Micropatterns

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While cellular micropatterning approaches are employed extensively in cell biology and tissue engineering, only a limited number of methods for analysis of local function in the context of a complex, microfabricated environment are currently available. The present study develops a novel strategy for analysis of local tissue-specific function in cellular micropatterns. Model hepatocytes (HepG2 cells) were seeded onto silane-modified glass slides containing robotically printed arrays of collagen type I. These model hepatocytes formed cell arrays with individual cell cluster dimensions (150 or 500 μm) corresponding in size to the printed collagen spots. Non-parenchymal cells (3T3 fibroblasts) were added to hepatocellular micropatterns to create heterotypic cocultures. Expression of hepatic phenotype in HepG2 cells was first verified by traditional techniques including intracellular staining and ELISA for albumin. In order to evaluate local liver function in the cellular microarray, individual array members composed of ~400 hepatocytes were retrieved using laser capture microdissection and analyzed with real-time reverse transcriptase (RT)-polymerase chain reaction (PCR). Hepatic function was assessed based on expression of four genes associated with differentiated liver phenotype: albumin, transferrin, α-fetoprotein, and α1-antitrypsin. "Titration" experiments, carried out to identify the smallest population of HepG2 cells yielding detectable mRNA levels and RT-PCR signals, showed that extraction area of 12 500 μm^2 (corresponding to ~70 cells) provided detectable gene expression signals. All four liver-specific genes were routinely evaluated after extraction of ~400 HepG2 from the micropatterned surfaces. Significantly, selective retrieval and subsequent analysis of tissue-specific function was demonstrated for hepatic cells micropatterned alone and in coculture with non-parenchymal cells. In the future, methods described in this study will offer the possibility to investigate dynamic and reciprocal interactions between two or more cell types positioned on a microfabricated cell culture surface. We also envision the proposed approaches to be ideally suited for cell analysis in the context of combinatorial microenvironment.

Multiple external stimuli including surrounding matrix proteins, neighboring cells, and soluble factors define cellular microenvironment in vivo and contribute to the state of cellular differentiation and morphogenesis.[1−3] Aspects of this microenvironment need to be recapitulated to maintain a tissue-specific phenotype of primary cells cultured in vitro. For example, freshly isolated hepatocytes de-differentiate rapidly, losing hallmark indicators of tissue (liver)-specific function including albumin secretion, urea synthesis, and cytochrome P450 enzymatic activity.[4,5] However, tissue-specific function of liver cells can be improved in vitro through introduction of necessary microenvironment components by culturing these cells on basement membrane-derived gels (e.g., Matrigel),[6] in a collagen sandwich configuration[7] or in a coculture with another cell type.[8,9]

While the importance of recreating cellular interactions is clear, traditional cell culture methods offer limited insight into the respective contributions of microenvironment components to the overall cellular phenotype. Microfabrication and micropatterning approaches are emerging as important tools for dissecting the effects of the microenvironment on cellular behavior by orchestrating cell−matrix, cell−cell, and cell−solution interactions in a well-controlled fashion.[10−15] For example, Singhvi et al. cultured hepatocytes on surfaces containing cell-adhesive (laminin) microdomains surrounded by nonfouling regions composed of poly(ethylene glycol).[13] These authors demonstrated that liver-specific differentiation of these cells could be controlled by varying the

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size of the protein attachment region. Bhatia and co-workers used photolithography to micropattern collagen (type I) and to subsequently form hepatocyte−fibroblast cocultures on patterned protein domains.[16] This micropatterning strategy allowed rational analysis of the effects of homotypic and heterotypic cell−cell interactions on the expression of hepatic phenotype.[17]

In addition to precisely controlling the cellular microenvironment through microfabrication methods, there is increasing interest in developing high-throughput, multiplexed cell culture methods for expedited discovery of inducers of cellular differentiation.[18−20] Such "discovery" cell culture tools are particularly important for the study of primary human cells, where the supply of cells is limited, or for investigation of stem cells where composition of the microenvironment conducive to lineage-specific differentiation is yet unknown. Robotic microarraying of ECM proteins,[20,21] small-molecule libraries,[19] or biomaterials[18] has recently been employed to create multiple microenvironment conditions on the same cell culture surface.

While the approaches for exposing cells in the same culture dish to different biological stimuli are emerging rapidly, methods for linking cell function to the local microenvironment primarily utilize immunocytochemical staining and reporter genes.[18,20] Limited use of other, more powerful and informative molecular biology tools (e.g., reverse transcriptase−polymerase chain reaction (RT-PCR) or DNA microarrays) for monitoring of cells in the microfabricated environment may be explained, in part, by the need to retrieve cells from the surface prior to performing these analyses. Recently, tools for selective retrieval of desired cells from heterogeneous tissue have become available. Laser microdissection tools are microscopy-based instruments enabling fluorescence or bright-field identification of cells followed by rapid retrieval based on either "catapult" or "capture" principles.[22−24] Laser-mediated cell retrieval employed in tandem with RT-PCR offers the possibility to retrieve cells of interest and to monitor gene expression levels in these isolated cell populations. Importantly, the appearance of tissue-specific function in cultured cells is closely connected with an increase in transcriptional activity of tissue-specific genes. For example, several reports described positive correlation between synthesis of liver-specific proteins (e.g., albumin) and an increase in the amount of mRNA encoding for these proteins.[25,26] Therefore, monitoring levels of liver-specific mRNA offers a method for establishing the liver phenotype in cultured hepatic cells.

Thus far, the combination of laser-mediated cell retrieval and RT-PCR has been primarily used in pathology experiments for isolating specific cells from tissue sections for downstream molecular analysis. In the present study, we demonstrate that these techniques can also be used to establish the tissue-specific phenotype of cells in the context of the local microfabricated environment. Protein microarraying, described by us earlier,[21] was used to pattern model hepatocytes (HepG2 human hepatoma cells) alone and in coculture with non-parenchymal cells (3T3 fibroblasts) on glass slides. Liver-specific phenotype in these micropatterned hepatic cultures was first verified by ELISA and intracellular staining for albumin. For analysis of tissue-specific gene expression, HepG2 cells were first extracted from specific locations on the surface using laser catapulting. The phenotype of extracted hepatocytes was then established based on real-time RT-PCR analysis of liver-specific gene expression. The approach we employed routinely yielded detectable mRNA levels for albumin, transferrin, α-fetoprotein, and α1-antitrypsin from ∼400 extracted HepG2 cells. "Titration" studies, performed to identify the smallest population of hepatocytes yielding detectable mRNA levels and RT-PCR signals, showed that gene expression could be observed based on as few as 70 cells. Significantly, hepatocyte extraction and liver-specific gene expression analysis were performed with equal success in micropatterned monocultures and cocultures containing 3T3 fibroblasts. We envision this methodology to be applicable for monitoring reciprocal interactions between multiple cell types residing in a microfabricated environment or for cell analysis in the context of combinatorial microenvironment.

EXPERIMENTAL SECTION

Chemicals and Materials. Glass slides (75 × 25 mm) were obtained from VWR International. (3-Acryloxypropyl)trichlorosilane was purchased from Gelest, Inc. Sulfuric acid, hydrogen peroxide, ethanol, collagen from rat tail (type I), monoclonal anti-human albumin antibody produced in mouse, and FAST OPD were obtained from Sigma-Aldrich. Goat anti-mouse IgG_{2a} Texas Red conjugated was purchased from Santa Cruz Biotechnologies, Inc. Phosphate-buffered saline (PBS) 10× was purchased from Cambrex. SuperScript III, random hexadeoxyribonucleotide (pd-(N)6) primers, RNaseOut (RNase inhibitor), dNTPs, Dulbecco's modified Eagles' medium (DMEM), minimal essential medium (MEM), sodium pyruvate, nonessential amino acids, and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies. Predeveloped TaqMan PCR assay (Assay-on-Demand), TaqMan Universal PCR Mastermix, lysis buffer, and 96-well optical reaction plates were obtained from Applied Biosystems. Goat anti-human albumin antibody, goat anti-human albumin antibody−HRP conjugate, and reference serum were obtained from Bethyl Laboratories, Inc.

Glass Surface Modification. Glass slides were cleaned by immersion in piranha solution consisting of a 1:1 ratio of an aqueous solution of 95% v/v sulfuric acid and 35% w/v hydrogen peroxide for 10 min. The glass slide was thoroughly rinsed with deionized water, dried under nitrogen, and kept in an oven at 200 °C before use. For silane modification, the glass slide was cleaned with an oxygen plasma chamber at 300 W for 5 min and then placed for 10 min in a solution containing (3-acrylopropyl)-trichlorosilane diluted in anhydrous toluene (20 μL/40 mL). The reaction was performed in a glove box filled with nitrogen to

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prevent moisturizing. The slides were rinsed with fresh toluene, dried under nitrogen, and cured at 100 °C for 2 h. Silane quality was assessed by ellipsometry and contact angle measurements. The silane-modified glass slides were stored in a desiccator before use.

Protein Microarraying. All proteins were dissolved in 1× PBS at 0.2 mg/mL concentration. Protein microarrays were contact-printed under ambient conditions on silane-modified glass substrata using a MicroCaster hand-held microarrayer system (Whatman Schleicher&Schuell) or GMS 417 robotic arrayer (Genetic Micro Systems, Inc.). In the case of MicroCaster, seven protein arrays of 6 by 12 spots were made in a 75 × 25 mm slide. The slide glass with the protein array was kept in a refrigerator before use, and the protein array was stable at least 1 month. The spot size was ~500 μm in diameter. Horizontal pitch was 1250 μm, and vertical pitch was 1500 μm. With the GMS 417 arrayer, 12 protein arrays of 20 by 20 spots were made in 75 × 25 mm slide. The quality of printed array was assessed by using a GMS 418 scanner (Genetic Micro Systems, Inc.) and Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss Inc.).

Cell Seeding and Culture. HepG2 cells were maintained in MEM supplemented with 10% FBS, 200 units/mL penicillin, 200 μg/mL streptomycin, 1 mM sodium pyruvate, and 1 mM nonessential amino acids at 37 °C in a humidified 5% CO_2 atmosphere. 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% CO_2 atmosphere. Cells were cultured until ~90% confluence and then passaged. Prior to cell seeding, 75 × 25 mm glass slides were cut into 25 × 25 mm glass pieces using a diamond scribing pen, sterilized with 70% ethanol, and washed with 1× PBS twice.

For cell seeding experiments, glass pieces, each imprinted with 144 collagen spots, were placed into wells of a conventional 6-well plate. Cell micropatterning and the coculturing process, shown in Figure 1A was described by us earlier.[21] In brief, collagen-patterned slides were first exposed to 3 mL of HepG2 cell suspension in culture medium at a concentration of 1×10^6 cells/mL. After 1 h of incubation at 37 °C, the medium containing unattached cells was removed and surfaces were washed twice with PBS. To introduce the second cell type, glass slides containing surface-bound arrays of HepG2 cells were exposed to 3 mL of fibroblast cell suspension at a concentration of 2.5×10^5 cells/mL. After a 2-h incubation at 37 °C, the fibroblast culture medium was removed and replaced with HepG2 culture medium. HepG2 and 3T3 fibroblast cocultures were maintained for 4 days before HepG2 cells became overconfluent.

Cell arrays formed on the glass slide were imaged by bright-field microscope (Carl Zeiss Inc.), and high-resolution images of individual cell clusters were obtained using Hitachi S3500N scanning electron microscopy operating at 5 kV of accelerating voltage. For scanning electron microscopy (SEM), imaging cells were fixed with 1% glutaraldehyde and sputter-coated with ~10 nm of Au−Pd.

Intracellular Albumin Immunostaining and Albumin ELISA. Cells were fixed with 2% paraformaldehyde 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 1× PBS) for 1 h at room temperature

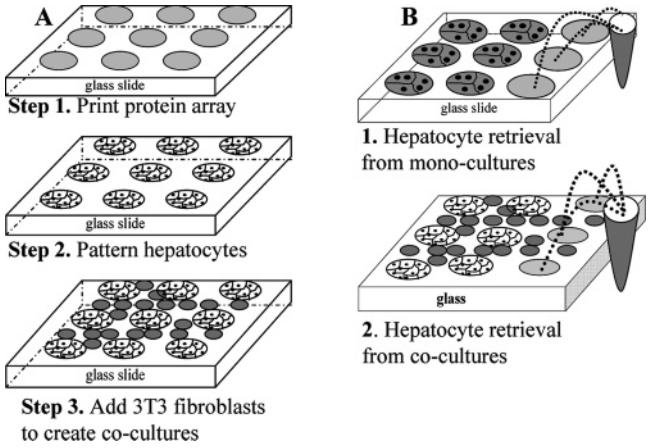

Figure 1. (A) Process flow diagram for creating hepatocellular micropatterns. Step 1: ECM proteins are robotically printed to define adhesive domains for hepatocytes. Selective adhesion of hepatocytes is ensured by silane modification of the glass surface. Step 2: Hepatocytes are seeded first, forming clusters on collagen spots. Step 3: Secondary cells are seeded, next creating a coculture. Location of both cell types is well defined. (B) Laser catapulting of hepatocytes from micropatterned surfaces prior to RT-PCR analysis. Analysis of two types of micropatterns was carried out. (1) Hepatocytes in monocultures were collected and compared to (2) hepatocyte coculture with 3T3 fibroblasts.

and exposed to 1:100 diluted monoclonal anti-human serum albumin antibody clone HSA-11 overnight at 4 °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 1× PBS three times for 5 min. All incubations were performed at room temperature if not specified. Stained cells were visualized and imaged using a confocal microscope (Zeiss LSM Pascal). Cell culture medium was collected everyday and analyzed for secreted albumin content. Albumin concentration was estimated using a standard kit from Bethyl Laboratories.

Extraction of Cells and Hepatic Gene Expression Analysis. Prior to laser catapulting, cells on glass slides were dehydrated, fixed with ice-cold 70% ethanol, and dried under nitrogen. Fixed cells were stored in an airtight container at −80 °C prior to catapulting and were catapulted within 3 weeks. Hepatocytes were retrieved from micropatterned surfaces by the PALM LMPC system (PALM Microlaser Technologies). Extracted cells were stabilized in 500 μL of 1× Applied Biosystems (AB) lysis buffer and stored at −20 °C. Total RNA was extracted from the cell lysates using a 6700 automated nucleic acid workstation (Applied Biosystems) according to the manufacturer's instructions. RNA from extracted cells was precipitated and resuspended directly in the reverse transcription reagents for complementary DNA (cDNA) synthesis. cDNA was synthesized using 100 units of SuperScript III, 600 ng of random hexadeoxyribonucleotide (pd(N)6) primers (random hexamer primer) 10 units of RNaseOut, and 1 mM dNTPs in a final volume of 40 μL with DNase-digested total RNA. The reverse transcription reaction proceeded for 2 h at 50 °C. After addition of 60 μL of water, the reaction was terminated by boiling for 5 min at 95 °C and cooling on ice. For each target gene, a predeveloped TaqMan PCR assay (Assay-on-Demand) was used. These assays contain 900 nM primers and 200 nM 6-carboxyflourescein-labeled TaqMan minor groove binder (MGB) probes. The MGB probes span an exon−exon boundary

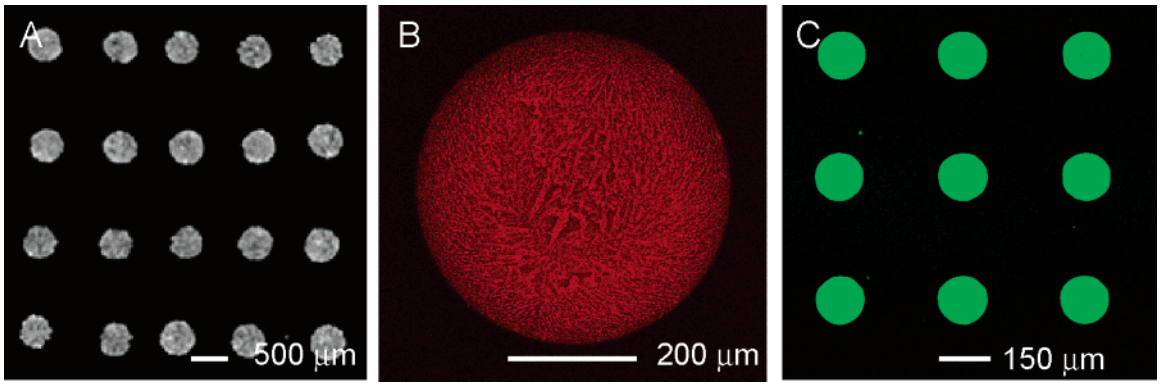

Figure 2. Protein microarraying. (A) Array composed of ~500-μm-diameter collagen spots imaged by a laser scanner. (B) Fluorescence microscopy image of an individual 500-μm-diameter collagen (type I) spot labeled with Cy 3. (C) FITC-labeled collagen microarray with individual spot dimensions of 150 μm. Horizontal and vertical pitch is 300 μm each.

to prevent detection of genomic DNA background. Each PCR reaction contained 20× Assay-on-Demand primer and probes for the respective TaqMan system and TaqMan Universal PCR Mastermix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 units of AmpliTaq Gold DNA polymerase, 0.25 units of AmpErase UNG, and 5 μL of the diluted cDNA sample in a final volume of 12 μL. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI Prism 7900 HTA FAST, Applied Biosystems). The manufacturer's standard amplification condition was used for real-time PCR: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. Fluorescent signals were collected during the annealing temperature and threshold cycle (C_t) values were extracted with a threshold of 0.04 and baseline values of 3−15. GAPDH was used as a housekeeping gene and an internal standard for relative quantification.

RESULTS AND DISCUSSION

The goal of this study was to establish a method for analysis of local liver-specific function of model hepatocytes cultivated on micropatterned substrates. Laser microdissection and real-time RT-PCR were used to retrieve hepatic cells from specific locations on the substrate and to monitor expression of liver-specific genes (see Figure 1B for the diagram of experiments). This methodology may be used in the future for analyzing cells stimulated in a combinatorial fashion (e.g., cells cultured on protein microarrays) or for monitoring reciprocal cellular interactions in microfabricated cocultures.

Surface Modification and Protein Micropatterning. Prior to protein micropatterning, glass slides were modified with acrylated silane ((3-acryloxypropyl)trichlorosilane). The thickness of the assembled silane was measured by ellipsometry to be 22.8 ± 1.3 Å, pointing to the formation of a monolayer or a double layer of silane molecules on the glass surface. Change in thickness was also associated with the water contact angle increasing from near zero (after O_2 plasma treatment) to 53 ± 2° after silane modification. The importance of the silane modification is discussed in the later sections of this article dealing with cellular patterning.

Robotic microarraying is a "direct write" technology that offers a simple means of patterning molecules on substrates. The high-throughput nature of this technology has recently been employed for creating ECM protein,[20] small molecule,[19] or biomaterials microarrays[18] in order to expose cells to multiple biological inducers in parallel. In the present study, redundant arrays of collagen (type I) were printed on glass slides (see Figure 2). This collagen type is abundant in the liver and has been used as a supporting matrix for culturing primary hepatocytes and stem cells.[25,27,28] Fluorescence microscopy was used to examine the quality of printed collagen spots. As seen in Figure 2, collagen deposited by physical adsorption onto moderately hydrophobic, silane-modified glass surfaces, formed circular microarray spots. Physical adsorption was robust and no smearing of protein spots was observed during sterilization or washing steps. Collagen spot dimensions of ~500 and 150 μm in diameter were printed by manual and robotic arrayers, respectively. The majority of cell retrieval and RT-PCR experiments were performed with HepG2 cells cultured on 500-μm collagen spots.

Hepatocellular Micropatterning. Cell−matrix and cell−cell interactions are central in maintaining differentiated liver phenotype in hepatocyte cultures.[7,17,29] Coculturing hepatocytes with non-parenchymal (supporting) cells in random and micropatterned configuration was shown to be of particular importance for maintaining liver function in vitro.[17,30] Creating a micropatterned coculture of hepatic cells with another cell type (described in Figure 1A) requires the following: (1) sequential seeding of the individual cell types and (2) preferential adhesion of hepatic cells onto protein microarrays. Thus, in addition to protein domains, this micropatterning scheme calls for another surface coating that has intermediate cell-adhesive properties, preventing adhesion of hepatocytes but allowing attachment of the non-parenchymal cells. In the prior micropatterning experiments, adsorption of BSA was used to prevent hepatocyte adhesion.[16,21] In the present study, BSA was replaced by a surface modification with an acrylated silane that was previously used by us as a coupling agent for hydrogel attachment.[15,21] As seen from images in Figure 3A,B,E,F, silane modification of the glass substrate combined with printing of collagen arrays allowed for highly specific attachment of HepG2 cells onto cell-adhesive protein domains. 3T3 cells added to these

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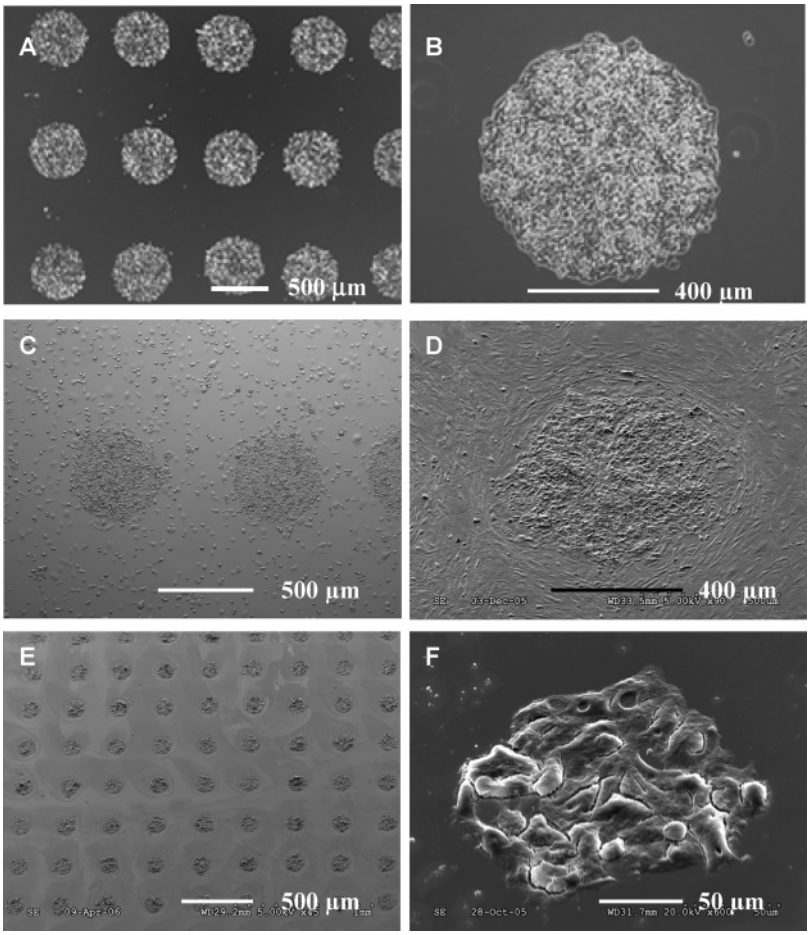

Figure 3. Formation of cellular micropatterns. (A) HepG2 cell array formed on 500-μm collagen spots ($\times$2.5 magnification). (B) Individual cluster formed on a 500-μm spot, composed of ~400 HepG2 cells ($\times$10). (C) HepG2 cells residing on 500-μm collagen spots and cocultured with 3T3 fibroblasts ($\times$5). (D) SEM image of a HepG2 cell cluster surrounded by 3T3 fibroblasts (3 days after seeding). (E) Array of HepG2 cells formed on 150-mm collagen spots. (F) Array of HepG2 cells formed on 150-μm collagen spot.

micropatterned surfaces attached onto silane-modified glass substrate and completed the coculture (see Figure 3C,D).

Albumin Synthesis in Micropatterned Hepatic Cultures. An important function of the liver is to synthesize and secrete plasma proteins such as albumin.[31] Therefore, albumin synthesis is one of the hallmark indicators of liver phenotype expression. Synthesis of albumin and other liver-specific proteins has also been positively correlated with the transcription of albumin mRNA, as well as other liver-specific mRNA.[25,26] The positive correlation between abundance of protein and protein-specific mRNA should, therefore, enable determination of tissue-specific phenotype based on the level of expression of tissue-specific genes.

Expression of hepatic function in micropatterned HepG2 cells was assessed by performing ELISA and intracellular staining for albumin. Immunocytochemical staining results shown in Figure 4A,B demonstrated the presence of intracellular albumin in HepG2 cell cultures. Importantly, Figure 4B shows that albumin synthesis was localized to hepatic cells and was not observed in the nonparenchymal 3T3 fibroblasts. Dynamics of albumin synthesis in mono- and cocultures of hepatocytes were monitored by collecting cell culture media and performing ELISA. As shown in Figure 4B, 3T3 fibroblasts do not synthesize albumin; therefore, production of this protein in a coculture can be safely attributed to hepatic cells. As shown in Figure 4C, secreted albumin concentration increased over time. However, this increase was due to proliferation of the transformed hepatic cells and not due to enhancement of liver phenotype. Another important factor obvious from Figure 4C is comparable levels of albumin secretion in HepG2 cells cultivated alone and in coculture with 3T3 fibroblasts. Given that previous reports by us[21] and others[16] demonstrated a near 10-fold increase in albumin synthesis in cocultures of primary hepatocytes, the equivalent levels of albumin in these two cellular systems, shown in Figure 4C, should be attributed to the use transformed hepatoma cells in this study. Unlike primary hepatocytes, HepG2 cells are highly proliferative and became overconfluent when cultured on 500- or 150-μm-diameter collagen islands for more than 4 days. In other experiments (data not shown), these cells were growth-arrested with mitomycin-C and cultured on collagen arrays for two weeks without noticeable loss in albumin synthesis. While HepG2 cells did not exhibit dedifferentiation and phenotype enhancement trends that were reported earlier for primary hepatocytes, these cells did express high levels of liver-specific function and proved highly useful as a cell model for validation of novel tools for tissue-specific phenotype determination.

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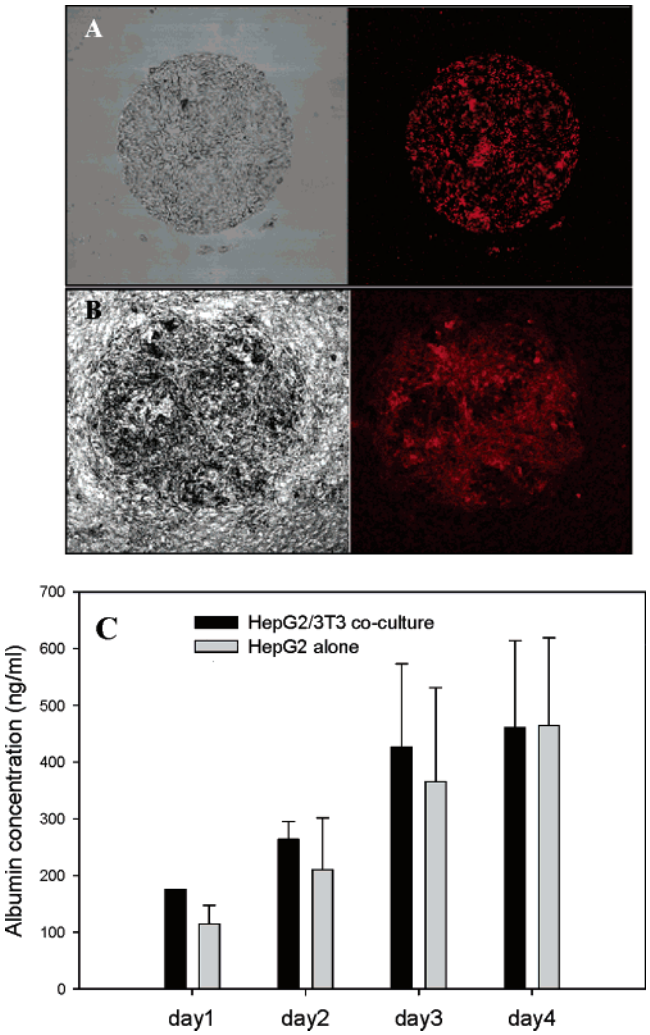

Figure 4. Traditional characterization of hepatic phenotype by immunocytochemical staining and ELISA. (A) A pair of bright-field and fluorescence images of a 500-μm cluster of HepG2 cells in monoculture stained for intracellular albumin ($\times$10). (B) Bright-field and fluorescence images of HepG2 cells cocultured with 3T3 fibroblasts stained for intracellular albumin ($\times$10). As seen from the images, albumin is only expressed in HepG2 cells. (C) ELISA for secreted albumin. Albumin secretion in micropatterned cocultures of HepG2 cells and 3T3 fibroblasts micropatterned was comparable to albumin synthesis in HepG2 cells cultured alone. ($n = 4$).

Cell Extraction and Hepatic Gene Analysis. A number of biological techniques have been developed to evaluate cells in a traditional culture environment (e.g., Petri dish or a tissue culture plate) and then adapted for analysis of cells in microfabricated or micropatterned cultures. Immunocytochemical staining and ELISA methods described in the previous section are some of the more common and robust cell analysis approaches. However, these traditional analytical approaches have several limitations in determining cell function in the context of local microenvironment. For example, the ELISA method cannot be employed in conjunction with a complex microenvironment where different levels of phenotype expression are expected from different groups of cells cultured on the same surface. Immunocytochemical staining is much more suited in this regard and can be used to determine local tissue-specific function; however, this method is limited to only a few intracellular proteins that can be monitored simultaneously. Moreover, it is not quantitative. New approaches are emerging for analysis of cell function in the context of a local microenvironment. For example, Thomas et al. described studies investigating how cell shape defined by a microfabricated environment affected induction of tissue-specific gene expression.[32] In that study, in situ RT-PCR was used to observe the presence of mRNA of several genes associated with the differentiated osteoblast phenotype.

Even more informative molecular approaches such as quantitative real-time RT-PCR and DNA microarrays are available for cell analysis; however, these techniques require that cells should be physically removed from culture surfaces. In the present study, we demonstrated that laser microdissection may be utilized to selectively remove hepatic cells from micropatterned substrates. Figure 1B presents two scenarios investigated here, whereby hepatic cells were extracted from micropatterned monocultures and from cocultures with 3T3 fibroblasts. Levels of liver-specific gene expression for both culture conditions were then compared. Significantly, as shown in Figure 1B, cells are collected directly into Eppendorf tubes and are immediately available for downstream molecular analysis. Figure 5A shows an array of four cell clusters of individual dimensions of 500 μm, where hepatic cells were selectively extracted from the upper row of array members while leaving the bottom row intact. Similarly, laser microdissection may be used to retrieve specific cell types from a micropatterned coculture where location of the individual cell types is precisely defined. As shown in Figure 5B, HepG2 cells could be selectively removed from the coculture without detaching the fibroblasts. In addition, it was important to ensure that collected cells did indeed express liver-specific proteins, as well as liver-specific mRNA. While the positive correlation between protein synthesis (translation) and mRNA production (transcription) has been reported,[26] posttranscriptional changes in mRNA abundance are also possible.[33] One way to connect protein synthesis to the presence of mRNA is to perform immunocytochemical staining in parallel with laser microdissection and RT-PCR. Figure 5C shows an example, where hepatic cell clusters were stained for intracellular albumin prior to catapulting. As seen from this image, catapulted cells did indeed contain albumin and, therefore, were expected to have detectable levels of albumin mRNA.

It should be noted that while cell extraction data presented here discusses catapulting from collagen type I, we have performed successful catapulting experiments with cells patterned on fibronectin, laminin, and poly(L-lysine). Therefore, a laser catapulting technique may be widely applicable for extraction of cells cultured on various proteins or ligands.

Gene expression analysis using real-time RT-PCR is one of the best methods for gene quantification. It is highly sensitive to the number of mRNA copies present in solution and is highly specific to the genes (sequences) to be analyzed.[34] In the present study, real-time RT-PCR (ABI TaqMan system) was used to analyze expression of four liver-specific genes (albumin, transferrin, α-fetoprotein, α1-antitrypsin) in hepatic cells extracted from micropatterned surfaces. GAPDH was used as a housekeeping

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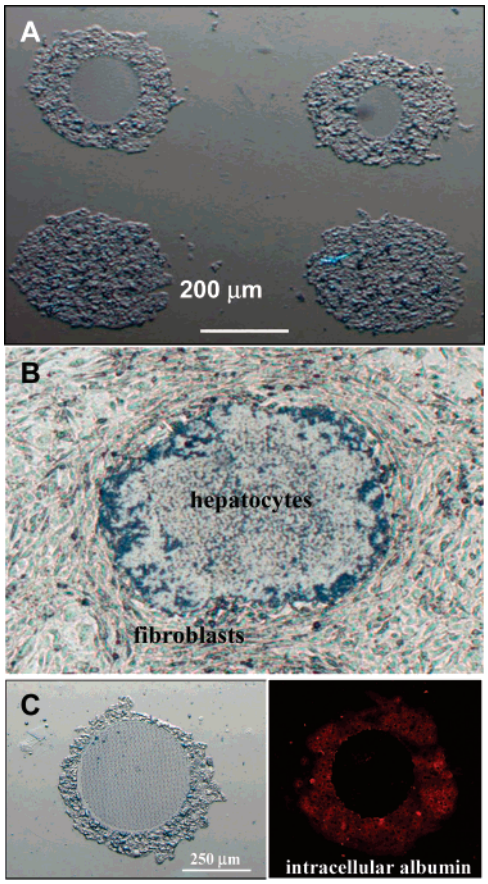

Figure 5. Laser-mediated retrieval of HepG2 cells from micropatterned environment. (A) Catapulting of HepG2 cells from the cell array. Cells were retrieved only from the upper row of array members, demonstrating ability to selectively remove desired cells from a micropatterned surface. (B) Selective retrieval of hepatic cells from a micropatterned coculture with 3T3 fibroblasts. HepG2 cell cluster is removed while the non-parenchymal cells remain on the surface. (C) Verifying expression of liver-specific function in hepatic cells prior to catapulting. SEM and fluorescence images demonstrating the presence of intracellular albumin in catapulted cells. Intracellular albumin was determined by immunostaining with anti-albumin antibody followed by staining with secondary antibody conjugated to Texas Red.

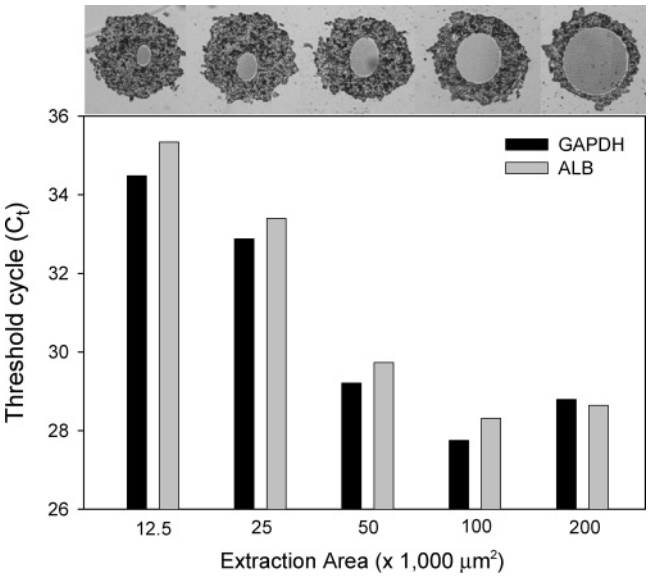

Figure 6. A "titration" experiment to determine smallest number of HepG2 cells that yields detectable gene expression signal. Cells extraction area was varied from 200 000 (far right, corresponding to ~400 HepG2 cells) to 12 500 μm^2 (far left, corresponding to ~70 HepG2 cells). Threshold cycle (C_t) values were monitored for expression of GAPDH housekeeping gene and the albumin gene.

gene for normalization. In these experiments, substrates containing micropatterned cell cultures were fixed by ethanol dehydration prior to cell retrieval. HepG2 cells, catapulted directly into microcentrifuge tubes as shown in Figure 1B, underwent RNA extraction and reverse transcription reactions to produce cDNA for gene expression profiling. For the RNA extraction, a 150 000−200 000 μm^2 area of cell cluster (see Figure 5A), corresponding to ~400 HepG2 cells, was catapulted from the glass substrate.

One of the questions investigated in this study was the number of hepatic cells required to observe detectable gene expression signals. "Titration" experiments varying the extraction area (i.e., number of extracted cells) were performed to answer this question. In a typical titration experiment shown in Figure 6, the extraction area was serially decreased by 2-fold from 200 000 to 12 500 μm^2. Real-time PCR was then performed on these different cell lysate samples to determine a threshold cycle (C_t)—an arbitrary value of the amplification cycle where target cDNA is first detected. As seen from the plot of C_t values versus the extraction area in Figure 6, larger amounts of extracted cellular material corresponded to less required amplification and to C_t values being lower. Therefore, the downward trend in C_t values, seen in the Figure 6, is to be expected. Assuming a hepatocyte of circular shape with diameter of ~15−20 μm, the smallest extraction area analyzed in our studies (~12 500 μm^2) corresponded to ~70−80 cells. This small number of cells provided a detectable real-time PCR signal with C_t values of 35 for both GAPDH and albumin genes. In the future, the cell number required for analysis may be decreased even further by using additional cDNA processing steps, for example, by preamplification of the target genes.[35]

It is important to note that the quality of cell catapulting and the amount of extracted mRNA may vary from one experiment to another. However, RT-PCR results are not normalized by the number of cells but by a housekeeping gene, human GAPDH in our experiments. The housekeeping gene serves as an internal reference that fluctuates up or down in accordance with the amount of catapulted cellular material and mRNA. This mode of normalization makes the gene expression analysis relatively insensitive to the precise number of the extracted cells. However, concerns over cell numbers will become prevalent as the population of extracted cells decreases. In the present paper, 300−400 hepatic cells were typically extracted for RT-PCR analysis. By performing the titration experiments shown in Figure 6, we determined that the number of hepatic cells (or the amount of mRNA) extracted in a typical experiment is severalfold higher than the minimum number of cells required to generate detectable signals of albumin and GAPDH gene expression. Therefore, the amount of mRNA extracted in our experiments is sufficiently large to result in gene expression signals that will remain stable over large variations in the number of extracted cells.

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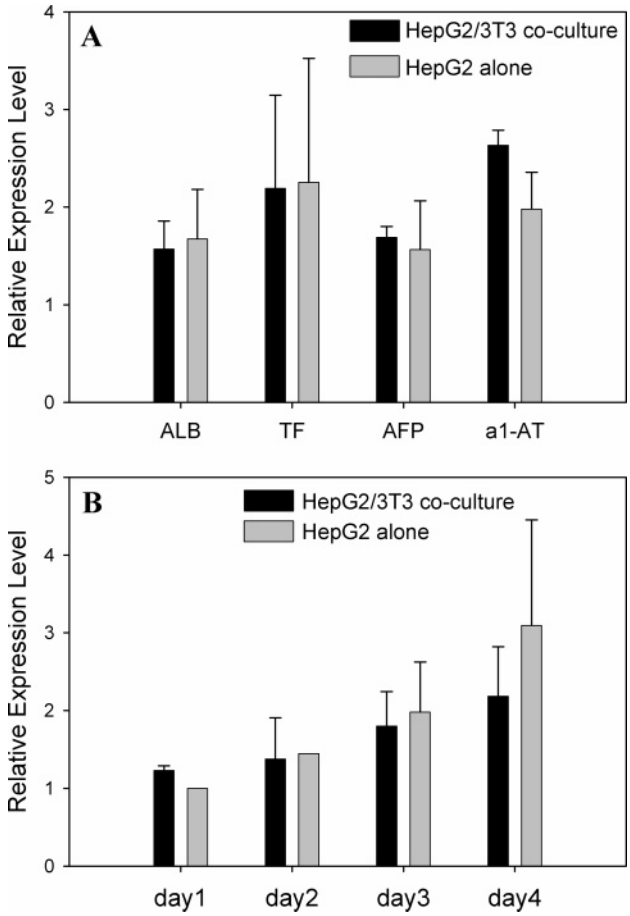

Figure 7. (A) Relative expression level of four genes associated with well-differentiated liver phenotype (albumin, transferrin, α-fetoprotein, α-1 antitrypsin). Gene expression was assessed for hepatic cells in micropatterned monocultures and in coculture with 3T3 fibroblasts. (B) Time-based analysis of expression of liver-specific genes in HepG2 cellular micropatterns.

After characterizing cell numbers required to perform analysis, levels of tissue-specific gene expression in micropatterned hepatic cells were monitored during four days in culture. To monitor gene expression over time, individual glass surfaces containing micropatterned cells were removed from culture media and fixed with ethanol. Hepatic cells were then extracted from mono- and cocultures at each time point and subjected to RT-PCR analysis. Figure 7A shows relative levels of expression for four liver-specific genes analyzed at day 3 in culture. These results are significant, as they demonstrate detection of robust expression of tissue-specific genes in micropatterned cells. Using the TaqMan system where fluorescent probes are conjugated to sequence-specific primers ensured that only target genes were amplified and detected. PCR amplification was gene as well as species specific so that no signals were observed when primer probes for human genes were used to analyze rat hepatocytes (results not shown). No significant difference in liver-specific gene expression of HepG2 cells cultivated alone and in cocultures with 3T3 fibroblasts was observed (see Figure 7A), corroborating the albumin ELISA results shown in Figure 4C. Similar lack of change in albumin gene expression was observed for these cells cultivated for four days on micropatterned surfaces (see Figure 7B). In our previous study of micropatterned cell cultures, primary hepatocytes cocultured with non-parenchymal cells synthesized 8 times more albumin after six days in culture then hepatocytes cultivated alone.[21] Once again, the lack of enhancement in liver-specific function should be attributed to the use of transformed hepatic cells in this study. Importantly, the RT-PCR technique is sensitive and has been used to detect differences in gene expression levels between samples of as small as 23%.[34] Given that fold differences are commonly observed during phenotype enhancement or dedifferentiation in hepatic cultures, quantitative real-time PCR has sufficient sensitivity to resolve these differences. In the time course study of hepatic gene expression shown in Figure 7B, micropatterned hepatic cells were removed from culture media and fixed for each time point.

CONCLUSIONS

The present paper describes a novel approach that allows phenotype analysis of micropatterned cells without losing the local microenvironment context. Laser microdissection and RT-PCR techniques were used in tandem to selectively retrieve and analyze hepatic cells cultured on protein microarrays. Expression of four liver-specific genes was routinely detected from ~400 hepatic cells retrieved from monocultures or cocultures with 3T3 fibroblasts. As few as 70 HepG2 cells generated detectable gene expression/PCR signals. Importantly, local hepatic phenotype could be determined with equal ease for HepG2 cells micropatterned alone or in coculture with 3T3 fibroblasts. While the present study only monitored levels of expression of four tissue-specific genes, a much larger number of genes may be analyzed using quantitative real-time RT-PCR. In addition to detecting mRNA encoding for liver-specific proteins, gene expression analysis may also be expanded to transcription factors associated with differentiated tissue phenotype.

Approaches described in this article will complement efforts currently underway in our group[36] and other laboratories that are aimed at designing novel microenvironment conditions for tissue-specific differentiation in mature and progenitor cells. The ability to monitor cell phenotype in the context of local environment will also facilitate the investigation of reciprocal interactions between multiple cell types cultured on the same surface.

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