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Liver-Specific Functional Studies in a Microfluidic Array of Primary Mammalian Hepatocytes

Bartholomew J. Kane,† Michael J. Zinner,† Martin L. Yarmush,‡ and Mehmet Toner*,‡

Department of Surgery, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115, and Surgical Services and Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School and Shriners Hospital for Children, 51 Blossom Street, Boston, Massachusetts 02114

Nearly half a billion dollars in resources are lost each time a drug candidate is withdrawn from the market by the Food and Drug Administration (FDA) for reasons of liver toxicity. The number of late-phase drug developmental failures due to liver toxicity could potentially be reduced through the use of hepatocyte-based systems capable of modeling the response of in vivo liver tissue to toxic insults. With this article, we report progress toward the goal of realizing an array of primary hepatocytes for use in high-throughput liver toxicity studies. Described herein is the development of a 64 (8 × 8) element array of microfluidic wells capable of supporting micropatterned primary rat hepatocytes in coculture with 3T3-J2 fibroblasts. Each of the wells within the array was continuously perfused with medium and oxygen in a nonaddressable format. The key features of the system design and fabrication are described, including the use of two microfluidic perfusion networks to provide the coculture with an independent and continuous supply of cell culture medium and oxygen. Also described are the fabrication techniques used to selectively pattern hepatocytes and 3T3-J2 fibroblasts within the wells of the array. The functional studies used to demonstrate the synthetic and metabolic capacity of the array are outlined in this article. These studies demonstrate that the hepatocytes contained within the array are capable of continuous, steady-state albumin synthesis (78.4 μg/day, $\sigma = 3.98$ μg/day, $N = 8$) and urea production (109.8 μg/day, $\sigma = 11.9$ μg/day, $N = 8$). In the final section of the article, these results are discussed as they relate to the final goal of this research effort, the development of an array of primary hepatocytes for use in physiologically relevant toxicology studies.

Liver toxicity is the most common reason for withdrawal of drug candidates by the Food and Drug Administration (FDA). Recent examples of FDA actions taken against drug candidates for issues of liver toxicity include Duract (Wyeth-Ayerst Laboratories, withdrawal, 1998), Rezulin (Park-Davis, withdrawal, 2000), and Serzone (Bristol-Meyers Squibb, selective withdrawal, 2003).[1] Each of these medications was withdrawn after they had been approved by the FDA for use in the general public. With the average cost to obtain FDA approval for a drug candidate estimated at $403 million (2000 dollars), withdrawal of a medication due to liver toxicity represents a tremendous loss of resources.[2] The number of late-phase drug developmental failures due to liver toxicity could potentially be reduced through the use of hepatocyte-based systems capable of modeling the response of in vivo liver tissue to toxic insults. The goal of this research is the realization of an array of primary human hepatocytes for use in high-throughput liver toxicity screening. With this article, we report progress toward realizing that goal. We have successfully developed a nonaddressable, continuously perfused microfluidic array of primary rat hepatocytes capable of supporting long-term liver-specific synthetic function.

The hepatocytes within each well were surrounded by 3T3-J2 fibroblasts. The effects of coculturing primary hepatocytes with nonparenchymal cell (NPC) such as fibroblasts have been extensively studied and have been shown to confer distinct advantages over culturing hepatocytes alone. First reported by Langerbach et al.[3] and Michalopoulos et al.[4] in 1979, coculture techniques have been shown to aid in maintaining hepatocyte viability and support the performance of many liver-specific functions.[5,6] These coculture techniques have been shown to support albumin synthesis,[6−8] urea production, and detoxification via the cytochrome P450 enzyme complex.[9−15] Murine 3T3-J2 fibroblasts have been shown to reliably increase albumin synthesis

* Corresponding author. Phone: (617) 371-4883. Fax: (617) 523-1684. E-mail: mtoner@sbi.org.

† Brigham and Women's Hospital.

‡ Harvard Medical School and Shriners Hospital for Children.

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rates (100−360 μg/10^6 cells/day)[16−18] and thus were chosen as the first cell type to be incorporated into the coculture construct supported within the wells of the array.

Further, the relative placement of the hepatocytes and fibroblasts within each well was controlled through the use of micropatterning. The use of micropatterning coculture techniques has been shown to confer distinct advantages over random coculture by enabling control over the heterotypic cell−cell interactions between the primary hepatocytes and the adjacent fibroblasts. Control over these heterotypic cellular interactions through micropatterning has enabled researchers to maximize protein synthetic and metabolic functions within coculture constructs.[16−19]

To the authors' knowledge only three other research efforts have targeted the development of microscale arrays containing hepatocytes. Two of these efforts were limited by the use of human cancer or fetal cell lines.[20,21] The third effort demonstrated primary rat hepatocytes culture in a microfluidic array format, but did so in an array architecture that was not clearly amenable to the development of independent fluidic addressing.[22] The system described herein enables long-term primary hepatocyte culture and will allow integration with existing microfluidic technology.

MATERIALS AND METHODS

Array Design. The system, shown in Figures 1 and 2, was an 8 × 8 array of 3.25 mm diameter wells rendered in poly(dimethylsiloxane) (PDMS). Each well within the array was comprised of two chambers. The primary chamber was 150 μm in height and contained micropatterned primary rat hepatocytes in coculture with 3T3-J2 fibroblasts. The bottom surface of each primary chamber was comprised of glass that has been micropatterned with collagen to enable selective hepatocyte adhesion. The collagen patterns were comprised of 40 μm diameter circles that were in a two-dimensional array format with 70 μm center-to-center spacing in both directions. Each of these 40 μm collagen islands enabled selective adhesion of the primary hepatocytes. Surrounding each of the collagen circles were the 3T3-J2 fibroblasts. This primary chamber was in continuity with a microfluidic network that allowed the cells to be continuously perfused with cell culture medium, thereby supplying them with metabolic substrate and removing the resultant metabolic byproducts.

The secondary chamber of each well was 100 μm tall and was separated from the primary chamber by a 100 μm thick membrane of PDMS. This secondary chamber was connected to its own microfluidic network that carried heated (37 °C), humidified room air with 10% carbon dioxide. This second gas perfusion network enabled diffusion of oxygen through the PDMS membrane and the cell culture medium to the cells without the formation of bubbles in the culture medium. With this second perfusion network it was possible to reduce the dependency of cellular oxygen delivery on the flow rate of the cell culture medium.

To allow ease of system seeding, the array was designed to be operated in both capped and uncapped modes. In the uncapped mode, the medium perfusion channels and wells were not covered by the PDMS-based gas perfusion network. With the gas perfusion network removed, the wells of the array could be brought into direct contact with cell-containing medium. It was this direct contact with medium in the uncapped mode that enabled the wells of the array to be seeded with the cells of interest. Once the

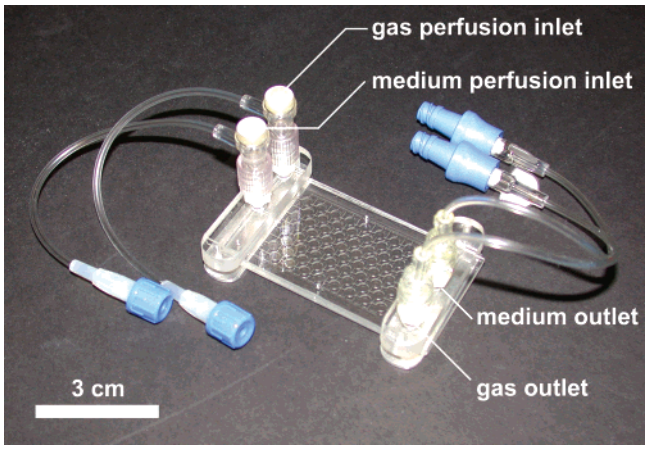

Figure 1. Photograph of array prototype including the interface used to clamp the glass slides and connect to the microfluidic channels contained between the slides. The perfusion inlets are shown on the left side of the array, with the gas inlet on top. The perfusion outlets are shown on the right side of the array with the gas outlet on the bottom.

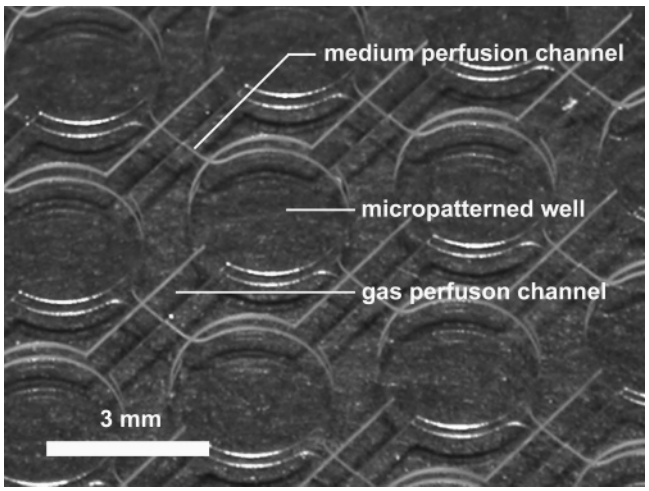

Figure 2. Perspective view photograph of the gas and medium perfusion channels within the array following surface modification and prior to cell seeding. Cell culture medium channels (200 μm × 100 μm) allow sequential perfusion of individual wells from the upper left to the lower right positions in this image. The gas perfusion channels (500 μm × 100 μm) are aligned orthogonally to the medium perfusion channels.

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system was seeded with both primary hepatocytes and fibroblasts, it was possible to clamp the gas and medium perfusion channels together between two 3 in. × 1.5 in. glass slides. This clamped configuration enabled the cells to be perfused continuously via closed gas and medium channels.

Access to the two perfusion networks was enabled by holes drilled through the glass slides with a diamond drill bit (1.6 mm diameter, no. 852-016, DiamondBurs.Net, LLC, Tucker, GA). Two custom interface manifolds were then used to provide a leak-proof connection from the medium perfusion networks to the syringe pump and the gas perfusion network to the oxygen source. The manifolds also provided mechanical compression of the PDMS layers between two glass slides to prevent leakage of cell culture medium and gas while the system was in the closed perfusion configuration.

Array Fabrication. The array was constructed by combining soft lithographic techniques with traditional microlithographic techniques. For the sake of clarity, the fabrication process can be divided into three phases. In the first phase, the medium and gas perfusion networks were constructed using a soft lithographic molding technique. In the second phase, a glass slide was selectively patterned with positive photoresist to allow subsequent collagen patterning. The medium perfusion network was then physically bonded to that glass slide. During the third and final phase of the fabrication process, a collagen surface modification process was performed to enable selective hepatocyte attachment to the glass substrate. These three fabrication phases are fully described below.

In the first phase of the fabrication process, the PDMS layers containing both the well and channel geometries were fabricated using a modification to a previously documented molding process.[23] The multilevel molds were comprised of two patterned layers of an epoxy-based photoresist (NANO SU-8 50, MicroChem, Newton, MA) supported on 4 in. silicon wafers (UniversityWafers.com, South Boston, MA). The first step in construction of the SU-8 molds was to clean the surface of the silicon wafers. An initial chemical cleaning was accomplished by immersing the wafers in piranha solution (1:3, sulfuric acid/30% hydrogen peroxide, Mallinekrodt Baker Incorporated, Phillipsburg, NJ) for 10 min. Following chemical cleaning, the silicon wafers were placed in a dehydration oven at 200 °C for 30 min. This was followed by oxygen plasma etching process to further remove organic materials from the surface (PX-250, March Instruments Incorporated, Concord, CA, power = 0.3 W/cm^2, oxygen flow = 50%, time = 180 s). A uniform layer of SU-8 with a goal thickness of 100 μm was then formed on the clean wafers by pouring approximately 10 mL of liquid SU-8 on the wafer and spinning it at 500 rpm for 10 s followed by 1000 rpm for 30 s. The SU-8 was then soft baked at 65 °C for 10 min, followed by 100 °C for 30 min. The SU-8 was selectively exposed to UV light using a contact mask aligner (2001 CT mask aligner with Ultra-Sense power supply, Quintel Corporation, San Jose, CA, power = 16 mW/cm^2, time = 40 s) and a 10 μm resolution emulsion photomask on a Mylar film (Advanced Reproductions Corporation, North Andover, MA). A postexposure bake was then performed on a hot plate at 65 °C for 3 min and 95 °C for 10 min. The patterns were then developed with immersion in developer (Thinner P Microposit thinner, Shipley Company, Marlborough, MA) at room temperature for 20 min. The wafer containing the developed SU-8 geometries was then rinsed in a second reservoir of developer before being subjected to a final bake on a hot plate at 65 °C for 3 min and 95 °C for 10 min. That step completed construction of the first layer of the mold, representing both the medium perfusion channels that interconnected the wells and alignment marks for relative positioning of the second SU8 layer. To complete construction of the mold, the same SU-8 deposition, baking, exposure, and development sequence was repeated to form a second 100 μm layer. This second layer represented the wells of the array.

Once the mold was completed, degassed liquid PDMS was poured over the surface of the wafer and an unpatterned 0.5 mm thick Mylar film was placed on top of the PDMS. A second, unpatterned wafer was then placed on top of that Mylar film. This sandwich was then placed between two 6 in. × 6 in. × 0.25 in. thick aluminum plates. These plates were placed on top of a hot plate before an unquantified force was applied across the two plates with a standard, 12 in. woodworking C-clamp. This force served to evacuate the excess liquid PDMS from the mold cavity and to create thru-holes corresponding to the wells of the array. The PDMS was allowed to cure in this configuration for 6 h at 60 °C before it was removed from the press apparatus thereby completing fabrication of the medium perfusion channel network.

The same SU-8 lithography process was used to create the single layer mold for the gas perfusion channel. Once the mold for the gas channels was completed, a relatively thick layer (~1.5 cm) of PDMS was poured over the channel geometries of the mold. The PDMS was then cured by placing it in a convection oven at 65 °C for 6 h. No clamping force was applied to the top surface of the mold in that case. Once cured, the block of PDMS was removed from the mold. A preformed, thin (100 μm) PDMS membrane was then bonded to the surface of the block, thereby forming an enclosed network of gas perfusion channels. This bonding process occurred after a brief oxygen plasma etch was performed on the PDMS surfaces to be bonded (PX-250, March Instruments Incorporated, Concord, CA, power = 0.3 W/cm^2, oxygen flow rate = 2%, time = 30 s). The PDMS channel network was then placed on a hot plate at 85 °C for 10 min to potentiate bonding of the membrane to the perfusion network. Once the membrane was bonded in place, four via channels were formed in the block using a custom-sharpened 21 gauge blunt tip needle with a luer hub (NE-211PL-10, Small Parts Incorporated, Miami Lakes, FL).

To initiate the second phase of the fabrication process, glass slides (3 in. × 1.5 in., 2947 Micro Slides, Corning Glass Works, Corning, NY) were cleaned using the chemical and plasma methods described above. Each slide was then coated with approximately 1.2 μm of positive photoresist (AZ 1512, Clariant Corporation, Somerville, NJ) by pouring approximately 5 mL of liquid resist on the surface of the slide and then spinning the slide first at 600 rpm for 10 s, then at 3000 rpm for 30 s. This spin coating was followed by a preexposure bake in a convection oven at 95 °C for 30 min. The photoresist was then exposed using the same contact mask aligner (power = 16 mW/cm^2, time = 6 s) and a 10 μm resolution emulsion photomask on a Mylar film. The

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photoresist patterns were then developed by immersion in positive photoresist developer (AZ 400K developer dilute 1:3, Clariant Corporation, Somerville, NJ) for 80 s. Following this step, a postdevelopment bake was performed in a convection oven at 120 °C for 30 min. To reduce the photoresist thickness to approximately 0.6 μm, the slides underwent a second oxygen plasma etching step using the same plasma etching plant (power = 0.3 W/cm^2, oxygen flow = 50%, time = 300 s). The thickness of the resulting photoresist film was then measured with a surface profile meter (Dektak^{3}ST surface profiler, Veeco Instruments Incorporated, Santa Barbara, CA) to ensure that the end thickness was less than 1 μm.

Next, the cured medium perfusion network rendered in PDMS was aligned and bonded to the patterned glass slide. To bond the PDMS network to the glass slide, both underwent a brief oxygen plasma etch (power = 0.3 W/cm^2, oxygen flow rate = 2%, time = 30 s). Once the plasma step was completed, the PDMS layer was aligned to the corresponding photoresist patterns on the glass slide using a custom-built alignment tool. The alignment tool was comprised of a square (3.5 in. × 3.5 in.) aluminum frame overlying a Plexiglas base (3.5 in. × 7.0 in.). The aluminum frame was connected to the base through four micropositioning stages. One rotational (40 mm metric rotary stage, Edmund Optics Incorporated, Barrington, NJ) and three linear micropositioning stages (center drive 45 mm square linear positioning stage, Edmund Optics Incorporated) were used to provide four axes of relative position between the perfusion network and the patterned glass slide. Once alignment was obtained using this device, the PDMS layer and the glass slide were brought into contact. They were then heated to 65 °C for 10 min to potentiate bonding of the PDMS to the glass surface.

After the patterned PDMS sheet was adherent to the glass surface, the final phase of construction was initiated. During that phase, collagen was selectively attached to the exposed glass surface of the wells to allow physical localized adhesion of the hepatocytes during the subsequent seeding process. This selective attachment of collagen through silane immobilization represents an adaptation of a previously published technique.[19,24,25] The process is schematically illustrated in panels a and b of Figure 3. To allow the selective attachment of collagen to the glass surface, the slides with patterned photoresist were first rinsed twice in deionized water and then blown dry with compressed nitrogen gas. Silane was then immobilized on the exposed glass surfaces by immersing the slides in a 2% v/v solution of *N*-[3-(trimethoxysilyl)propyl]-ethylenediamine (104884, Sigma-Aldrich Inc., St. Louis, MO) in water for 30 s before they were rinsed twice in deionized water. The slides were then blown dry with nitrogen gas before being dried in a vacuum oven at 100 °C and −25 mm of Hg for 10 min. The dry slides were then immersed in an aqueous solution of 1:1:8 phosphate-buffered saline (PBS, ×10)/gluteraldehyde (25%)/deionized water at room temperature for 1 h at room temperature. The next step in the surface modification process was to rinse the slides in PBS. The slides were then immersed in an aqueous solution of 0.625 wt % type I collagen at 37 °C for 30 min. Prior to immersion of the slides, the pH of the collagen was changed to 5.0 by adding a small amount of dilute (1 N) sodium hydroxide as needed. To remove the photoresist pattern and leave the collagen attached to the unprotected glass surface, each slide was placed in an ultrasonic bath (FS20H, Fisher Scientific, Pittsburgh, PA) containing acetone at room temperature for 3 min. Once the photoresist was removed, the slides were rinsed vigorously twice in deionized water before being placed in sterile Petri dishes without a drying step. These surface-modified slides were then stored at a temperature of 8 °C for a minimum of 12 h. This 12 h dwell step allows time for the acetone absorbed by the PDMS to diffuse away and thereby avoid its known cytotoxic effect on the hepatocytes and fibroblasts. Surface-modified slides such as these have been stored in Petri dishes for up to 6 months before being successfully seeded, closed, and perfused.

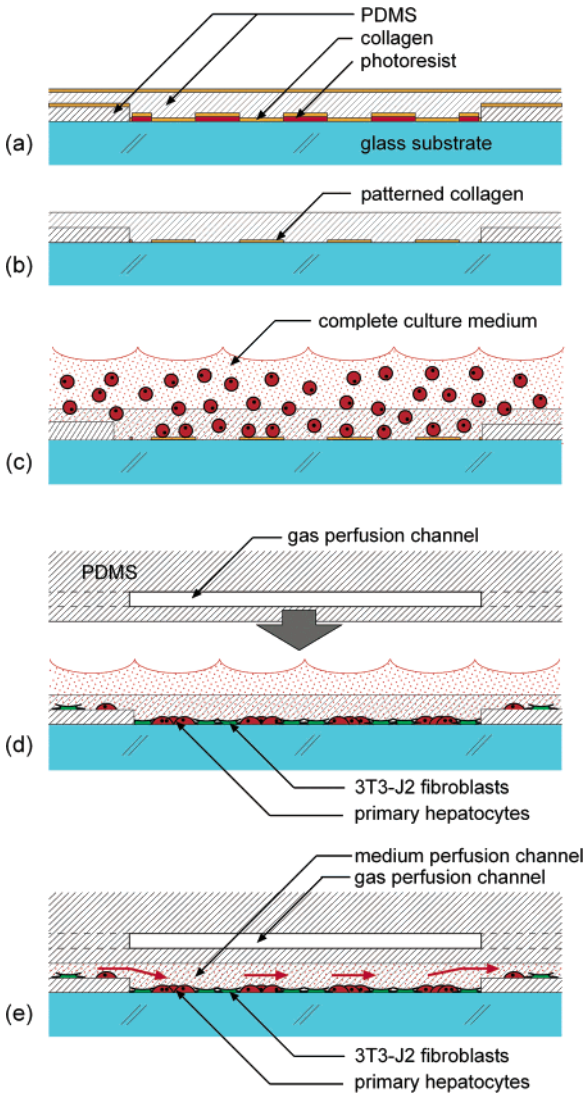

Figure 3. Schematic diagram of selected steps in the array fabrication and seeding processes. Panels a and b demonstrate the method used to selectively pattern the bottom surfaces of the wells with collagen. Panel c demonstrates how the system was seeded with primary hepatocytes and 3T3-J2 fibroblasts. Panels d and e show how the system is capped and perfused continuously with cell culture medium and humidified room air.

Cell Seeding and Perfusion Circuit Management. Once the fabrication process was completed, the arrays were seeded with

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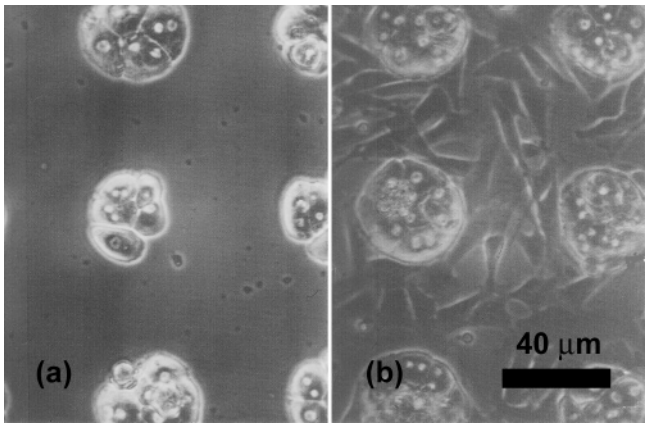

Figure 4. Phase contrast micrograph (20× objective) of the coculture within the wells of the array. Hepatocytes adherent to the collagen micropatterns on post-seeding day 1 are shown alone in panel a and on post-seeding day 3 surrounded by 3T3-J2 fibroblasts in panel b.

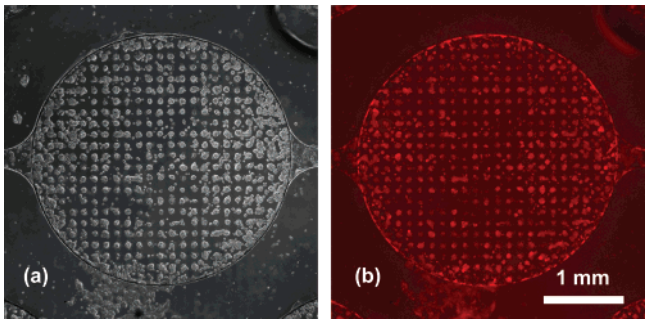

Figure 5. Micrographs (1× objective) of the coculture within a single well of an array on post-seeding day 1, prior to being seeded with fibroblasts and capped with the gas perfusion channels. A phase contrast image is shown on the left in panel a. A fluorescent image of the same hepatocytes stained with CellTracker orange CMRA dye (C34551, Molecular Probes, Eugene, OR) is shown on the right in panel b.

primary rat hepatocytes and fibroblasts. This seeding process is outlined in panels c and d of Figure 3. To seed the system, the glass slide containing the medium perfusion network and the collagen-patterned wells was placed at the bottom of a custom-made 3 in. × 1.5 in. PDMS well contained within a standard Petri dish. Once in the 3 in. × 1.5 in. well, the slide was immersed in phosphate-buffered saline (PBS) containing 0.05% bovine serum albumin at 37 °C for 1 h. This solution served to increase the selectivity of hepatocyte adhesion to the collagen patterns. This BSA-containing PBS solution was then aspirated off before the slide was rinsed twice with PBS. The slides were then covered with 4.5 mL of Dulbecco's modified Eagle medium (DMEM, 11995-040, Invitrogen Corporation, Carlsbad, CA) containing primary rat hepatocytes at a concentration of 0.75×10^6 cells/mL. These hepatocytes were obtained from 2−3 month old female Lewis rats weighing 180−200 g (Charles River Laboratories, Wilmington, MA) using a modified version of the collagenase-based liver perfusion technique developed by Seglen.[26,27] Since the hepatocytes were denser than DMEM, the cells settled onto the glass slide and contacted the areas of open glass that had been modified with collagen. The slides were then placed in an incubator containing humidified room air with 10% CO_2 at 37 °C for 40 min. After 40 min, the DMEM was aspirated off and the slides were again perfused with 4.5 mL of DMEM containing hepatocytes at the same concentration. After this second perfusion step, the slides were again incubated for 40 min before the solution was aspirated off and the slides were perfused with 4.5 mL of serum containing DMEM. These slides were then incubated overnight. The hepatocytes bound to the 40 μm collagen circles are shown in panel a of Figure 4 on post-seeding day 1. A low-magnification micrograph of an entire well is shown in Figure 5. Also on post-seeding day 1, the cell culture medium was sampled, then aspirated off completely, and replaced with 4.5 mL of serum containing DMEM with 3T3-J2 fibroblasts at a concentration of 0.75×10^6 cells/mL. These slides were then allowed to incubate overnight. The serum containing DMEM was again sampled and then changed on post-seeding day 2. The following day (post-seeding day 3), the top surfaces of the PDMS layers on the glass slides were scraped twice with a sterile glass slide to remove the cells at the interface between the fluid networks and the gas perfusion networks. At that time eight slides were capped with the gas perfusion networks. The capping procedure enabled the cells within the wells to be perfused with serum containing medium and humidified room air. To provide a constant flow rate of medium to the systems, each of them was attached to a 10 mL syringe (Hamilton GASTIGHT 1000, Hamilton Company, Reno, NV) containing complete medium. These syringes were loaded into a programmable pump capable of supporting 10 individual syringes (PHD2000, Harvard Apparatus, Holliston, MA). The pump and the individual 10 mL syringes were kept in the incubator along with the arrays in order to eliminate temperature gradients. The pump was set to a constant flow rate of 1 μL/min so that 1.44 mL of medium flowed through the wells of each array per day. The medium flowed out of each array through a single channel that was connected to a 2 mL cryogenic storage vial (430659, Corning Costar Corporation, Cambridge, MA) through a 21 gauge threaded female luer plug (NE-211PL-25, Small Parts Incorporated, Miami Lakes, FL). The fluid was removed from the vial each day and stored at −80 °C. To supply the cells with oxygen, one end of the gas perfusion network was attached to the house vacuum line and the other end was left open to the environment of the incubator. With this method, the flow rate through the gas perfusion network is not quantified or controlled. The incubator was set to maintain the internal temperature at 37 °C and keep the carbon dioxide concentration of the humidified air at 10%.

Four arrays were maintained in an open configuration to serve as controls for the closed and perfused arrays. After the top surfaces of the slides were scraped to remove the cells from the interface between the fluid networks and the gas perfusion networks, four of them were not capped with the gas perfusion networks. These slides were maintained in the 3 in. × 1.5 in. PDMS wells contained within standard Petri dishes. The control devices were immersed in 4.5 mL of complete medium that was changed daily. A 1.5 mL volume of the medium was sampled from each of the four arrays each day and stored at −80 °C until the intentional termination of the study at day 32.

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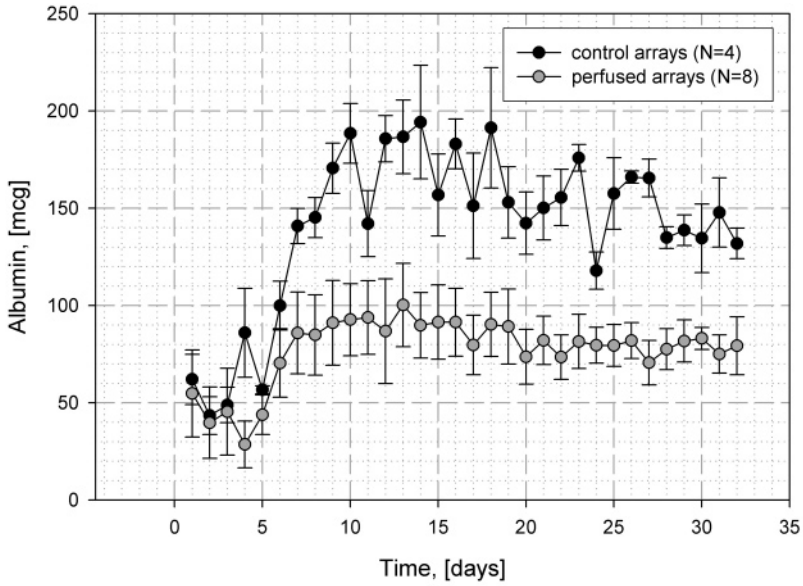

Figure 6. Albumin synthesis within a series of microfluidic arrays. The average daily albumin concentration within eight closed and continuously perfused devices is represented by the gray circles. The average daily albumin concentration within four open, control devices is represented by the black circles. The error bars indicate standard deviation.

Quantitative Assays. Medium samples were collected daily and stored at −80 °C for subsequent analysis of urea and albumin content. Albumin synthesis was determined using a previously described enzyme-linked immunosorbent assay (ELISA).[28] Purified rat albumin (55952, MP Biomedicals, Aurora, OH) and horseradish peroxidase conjugated IgG antibodies fractionated to rat albumin (55776, MP Biomedicals) were used for this assay.

The urea measurements were performed using a colorimetric chemical assay (no. 0580, Urea Nitrogen Direct, Stanbio Laboratory, Boerne, TX). The optical density values for both the urea and the albumin assays were determined using a 96-well plate reader (VERSAmax tunable microplate reader, Molecular Devices, Sunnyvale, CA) controlled by a commercially available software package (SOFTmax PRO, Molecular Devices, Sunnyvale, CA). Fibroblast production of urea and albumin was shown to be below the detection level of both assay methods.[29]

Statistics and Data Analysis. To determine an individual albumin values, three measurements were made from the same sample of effluent collected from an individual array on a single day. The average of these three values was then used to determine the albumin concentration for the effluent from that individual array on that day. The same method was used to determine the daily urea concentrations from each of the arrays in the study. Once the daily albumin and urea concentrations from each device were determined, the daily average values and standard deviations for the two groups were determined using the average function in a spreadsheet software program (Microsoft Excel, Microsoft Corp, Redmond, WA). The data were then plotted using SigmaPlot 2001 graphing software (Systat Software, Inc., Point Richmond, CA). Each data point in Figures 6 and 7 represents the mean value with the error bars representing the standard deviation.

RESULTS

Microscopy. To verify that the hepatocyte contained within the wells of the array were viable, the wells of the array were microscopically monitored throughout the course of the study. Because each of the arrays were perfused by a single syringe and were attached to a single effluent receptacle, as a unit they could be removed from the syringe pump setup and microscopically inspected. During the first 2 days of closed perfusion, the cells demonstrated geometric features known to be consistent with viable in vivo primary hepatocytes; polygonal morphology, distinct nuclei and nucleoli, and well-demarcated borders.[30] The adherent hepatocytes both before and after (post-seeding days 1 and 3, respectively) fibroblast seeding can be seen in Figure 4. Some hepatocytes and fibroblasts were observed in the channels between the wells of the array. After 4 days under closed perfusion conditions, cells continued to maintain these features. However, at that time it became apparent that the primary hepatocytes were spreading beyond the borders of the 40 μm collagen islands. After 6 days under closed perfusion conditions, the fibroblast continued to proliferate and began to overlap the patterned hepatocytes. Because of this overlap, it was not possible to clearly distinguish the borders of the hepatocyte islands from the surrounding hepatocytes using microscopic evaluation. Further, it was not possible to clearly determine if the hepatocytes continued to demonstrate polygonal morphology, distinct nuclei, and well-demarcated borders. Throughout the remainder of the perfusion trial, the cells in the coculture continued to adhere to the bottom surface of the wells.

Synthetic Function. One of the critical functions of in vivo hepatocytes is the synthesis of albumin, a protein of 585 amino acids known to play critical rolls in binding and transport of drugs, maintenance of colloid osmotic pressure, and scavenging of free radical. Albumin production has been used as a functional marker

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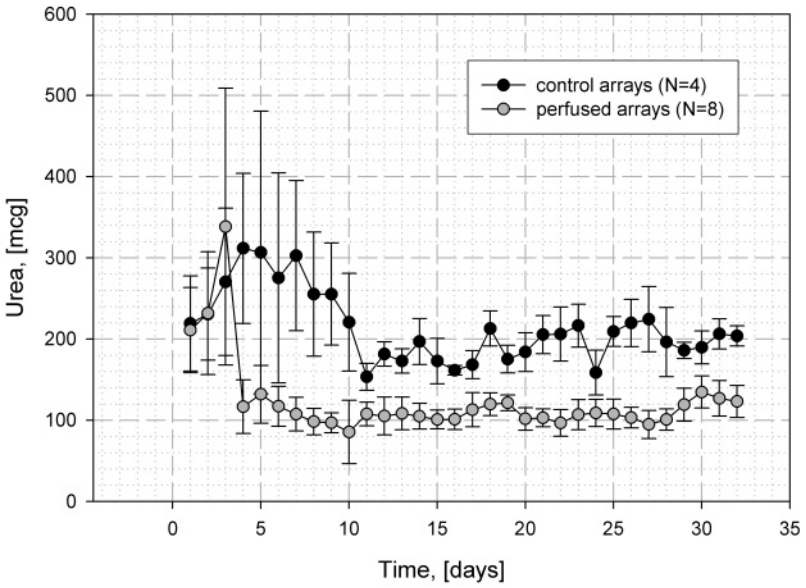

Figure 7. Urea production within a series of microfluidic arrays. The average daily urea concentration within eight closed and continuously perfused devices is represented by the gray circles. The average daily urea concentration within four open, control devices is represented by the black circles. The error bars indicated standard deviation.

for health of hepatocytes in culture. To determine that the hepatocytes contained within the arrays were performing liver-specific protein synthesis, the stored effluent was evaluated for albumin concentrations using a colorimetric ELISA assay described above.

In Figure 6, the average daily albumin concentrations measured in the effluent of the eight perfused arrays are plotted over the course of the study. Also shown are the average daily albumin concentrations of the four open control devices. Here, albumin production rates can be seen to increase for approximately 7 days before reaching a relatively constant synthesis rate. The average daily albumin production in the perfused arrays from day 20 until the study was intentionally terminated at day 32 was determined to be 78.4 μg/day ($\sigma = 3.98$ μg/day, $N = 8$). This is 53.1% of the average albumin production measured within the control devices over the same time interval, 147.6 μg/day ($\sigma = 16.3$ μg/day, $N = 4$).

Metabolic Function. Like albumin synthesis, urea production serves a critical role in the human body. Highly toxic ammonia is formed when amino acids are broken down in the body. To prevent fatal accumulation of ammonia, carrier molecules and enzymes contained within hepatocytes converts ammonia into urea, which is much less toxic and can be effectively cleared through the renal system. Like albumin, urea production rates have been used as a marker for the functional capacities of hepatocytes in culture. In Figure 7, the average daily urea concentrations measured in the effluent of the eight perfused arrays are plotted over the course of the study. Also shown are the average daily urea concentrations of the four open control devices. Here, urea production rates demonstrate variability throughout the first 10 days of the study. After post-seeding day 10, the cell mass within both the control devices and the perfused devices demonstrate a relatively constant urea production rate. The average daily urea production in the perfused arrays from day 20 until the study was intentionally terminated at day 32 was determined to be 109.8 μg/day ($\sigma = 11.9$ μg/day, $N = 8$). This is 54.8% of the average urea production measured within the control devices over the same time interval, 200.4 μg/day ($\sigma = 17.0$ μg/day, $N = 4$).

DISCUSSION

The long-term goal of this project is to realize an array of primary hepatocytes for use in high-throughput liver toxicity studies. With the results described above, we show that it is possible to support metabolic and synthetic function in micropatterned primary rat hepatocytes within a perfused, microfluidic array. Figure 6 demonstrates that the array is capable of supporting steady-state protein synthesis in the form of relatively constant albumin synthesis from day 20 to the intentional termination of the study on day 32 (78.4 μg/day, $\sigma = 3.98$ μg/day, $N = 8$). Similarly, Figure 7 demonstrates that the array is capable of supporting steady-state metabolic function in the form of relatively constant urea production from day 20 to day 32 (109.8 μg/day, $\sigma = 11.9$ μg/day, $N = 8$). These results underscore the idea that a microfluidic platform can be used to perform long-term coculture of primary hepatocytes. These results also show that the hepatocytes in such a system are capable of long-term protein synthesis and detoxification functions that are relatively repeatable from array to array. Further, the results demonstrate the possibility of micropatterning hepatocytes within a microfluidic device to provide a degree of control over the heterotypic interactions between the cells in coculture.

Another point of interest that may be gleaned from Figures 6 and 7 is the difference in albumin and urea production rates in the perfused arrays when compared to those of the control arrays. The steady-state albumin synthesis rate within the perfused arrays was found to be 53.1% of the open, control devices. A similar result was determined in the case of urea production. The steady-state urea production within the perfused arrays was measured at 54.8% of the open, control arrays. Four potential sources for this discrepancy are apparent. First, it is possible that hepatocytes and fibroblasts within the perfused device experience reduced meta-

bolic substrate delivery when compared to that of the control devices. The perfused devices are exposed to a volume of 1.44 mL of continuously flowing medium over a 24 h period. In contrast, the open arrays must be immersed in 4.5 mL of static medium in order to completely cover them. The data presented in Figures 6 and 7 has been normalized for the volume of medium used in the two configurations. However, the issue remains that the cells in the open devices have been exposed to 3 times the amount of metabolic substrate when compared to that of the cells within the perfused arrays. Similarly, the volume of distribution for diffusion of metabolic byproducts is 3 times larger in the open arrays when compared to that of the perfused arrays. A second potential reason for the discrepancies in albumin and urea production could be the result of reduced delivery of oxygen to the cells within the perfused device. Since a PDMS membrane is present between the gas and medium perfusion networks and the flow rate of the oxygen carrying gas was not controlled, the amount of oxygen diffusing to the cells in the perfused arrays may be reduced when compared to that of the cells in the open arrays.

The third possible reason for the differences in albumin and urea production could be the exposure of the cells within the perfused arrays to shear stress. This is unlikely though as the hepatocytes and fibroblasts contained within each of the wells of the array were exposed to a wall shear stress much less than that shown to be detrimental in prior studies. In 2001, Tilles et. al. demonstrated that cocultured primary hepatocytes and fibroblasts in a flat plate bioreactor were not adversely effected by wall shear stress values less than 0.33 dynes/cm^2.[30] The wall shear stresses in each well of the array were determined to range between 1.5×10^{-3} dynes/cm^2 at the inlet of the well to 0.05×10^{-3} dynes/cm^2 at the midpoint of the well. A fourth potential reason for the discrepancies in albumin and urea production could be the result of differences in concentration of soluble mediators in the medium of the perfused and the uncapped devices.

Further studies are needed to clearly determine the reasons for the discrepancies in albumin and urea production between the perfused arrays and the control devices. With the current array design, it will be possible to execute the required studies. In the current array, the delivery of nutrients and the removal of metabolic byproducts is a function of the cell culture medium flow rate. Similarly, the shear stress to which each cell is subjected is a function of the medium flow rate and the location of that cell within the well. However, with the dual perfusion networks, the oxygen delivery is not a primary function of the medium flow rate. Oxygen delivery is primarily dependent upon the oxygen concentration and the flow rate in the gas perfusion channels. With this dual perfusion system, it is possible to control oxygen delivery independently from nutrient delivery, metabolic byproduct removal, and shear stress. This feature will allow determination of the optimal perfusion flow rate for nutrient delivery and metabolic byproduct removal independently from the rate at which oxygen is supplied to the cell mass. Similarly, it will be possible to perform a set of studies where the shear stress that the cells are exposed to is varied independently from the rate of oxygen delivery. With those capabilities it would be possible to experimentally determine the medium and gas perfusion rates that optimize many hepatocyte-specific functions of interest.

With this article, we have shown that it is possible to support micropatterned primary rat hepatocytes in coculture with 3T3-J2 fibroblasts inside a 64 (8×8) element array of microfluidic wells. What is more, these hepatocytes demonstrate stable, liver-specific function in the form of relatively constant albumin and urea production rates. Building upon these encouraging results, we are currently working to realize an array of primary human hepatocytes for use in high-throughput studies of human liver physiology. We have undertaken studies to determine the feasibility of supporting primary human hepatocyte within the current array design.

One of the fundamental limitations of the microfluidic array described here is that it does not allow the individual wells of the array to be accessed in an addressable mode. Specialized microfluidic valves need to be incorporated into the system to allow the formation of distinct physical and chemical environments within the individual wells of the array. The development of an array with integrated microfluidic valves for controlling microfluid flow is currently underway.

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