Microstructured multi-well plate for three-dimensional packed cell seeding and hepatocyte cell culture

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In this article, we present a microstructured multi-well plate for enabling three-dimensional (3D) high density seeding and culture of cells through the use of a standard laboratory centrifuge to promote and maintain 3D tissue-like cellular morphology and cell-specific functionality *in vitro* without the addition of animal derived or synthetic matrices or coagulants. Each well has microfeatures on the bottom that are comprised of a series of ditches/open microchannels. The dimensions of the microchannels promote and maintain 3D tissue-like cellular morphology and cell-specific functionality *in vitro*. After cell seeding with a standard pipette, the microstructured multi-well plates were centrifuged to tightly pack cells inside the ditches in order to enhance cell-cell interactions and induce formation of 3D cellular structures during cell culture. Cell-cell interactions were optimized based on cell packing by considering dimensions of the ditches/open microchannels, orientation of the microstructured multi-well plate during centrifugation, cell seeding density, and the centrifugal force and time. With the optimized cell packing conditions, we demonstrated that after 7 days of cell culture, primary human hepatocytes adhered tightly together to form cord-like structures that resembled 3D tissue-like cellular architecture. Importantly, cell membrane polarity was restored without the addition of animal derived or synthetic matrices or coagulants. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4892978>]

I. INTRODUCTION

Unforeseen hepatotoxicity is a major reason for the after-market removal of drugs. The failure to detect toxicity earlier in drug development is in part due to the routine use of two-dimensional (2D) culture systems that poorly model *in vivo* physiology. Two-dimensional cell culture on flat substrates has been the de facto method for *in vitro* cell culture for decades as it offers simplicity and ease of use. Unfortunately, 2D cell culture formats typically lead to rapid loss of native (*in vivo*) cell functionality and membrane polarity, a phenotype that favors cell proliferation. There is an increasing awareness that drug toxicity may result from the inhibition of hepatic drug transporters. Also, it is believed that culture models that promote the reorganization of cells into three-dimensional (3D) *in vivo*-like structures often better model the tissue microenvironment and may provide physiologically relevant data. A common goal for mimicking the *in vivo* cell microenvironment is to bridge the gap between the use of animals and cellular monolayers. A simple starting point to accomplish this goal is the introduction of 3D or perfusion cell culture.

In recent years, several culture models have been developed to promote *in vivo* 3D cell behaviour *in vitro*. These models include the use of cellular spheroids, microcarriers, tissue-engineered models with biomimetic or structural scaffolds and organotypic explant cultures.^{1–6} Among them, cellular spheroids are one of the simplest 3D cell culture models because in the

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absence of an adherent culture substrate most cell types tend to aggregate when cultured in close proximity. Cellular spheroids can be created from various single culture or co-culture techniques such as hanging drop, electric, magnetic or acoustic force cell aggregation enhancement, micromolding, spinner flask, or rotating system.^{1,7-9} Also, wells with a concave bottom and/or with a ultra-low attachment surface are also being used to create cellular spheroids.^{1,7,10,11} On the other hand, in perfusion cell culture models, microfluidic-based devices are the predominant system for enabling dynamic cell culture with fluid perfusion to mimic the *in vivo* cell culture microenvironment.¹²⁻²² Microfluidic-based devices typically consist of a middle cell culture microchannel sandwiched between two side perfusion microchannels to enable access to culture media.¹⁶⁻²² Cells are introduced into the middle cell culture microchannel in a controllable way so that they are closely packed. Cells are retained in the middle channel by a series of retention micropillars that permit exposure to culture media. There are also reports that describe inoculating the microfluidic device with pre-formed spheroids²³⁻²⁵ or the use of coagulant chemistry as a mechanism to aggregate cells in a 3D format.^{20,21} Thus, in both 3D and perfusion cell culture models, cell-cell and cell-extracellular matrix (ECM) interactions are critical for maintaining long-term viability and function *in vitro*. However, even with increasing activities in the 3D and perfusion cell culture models, there are still needs for developing tools and methods that better reconstitute the *in vivo* cell culture microenvironment to improve cell organization and enhance cell functions.

In this article, we present a microstructured multi-well plate for enabling 3D high density cell seeding and culture through the use of a standard laboratory centrifuge. The packing of cells during seeding promoted and maintained 3D tissue-like cellular morphology and cell-specific functionality *in vitro*. Each plate well has a microstructured bottom comprised of a series of ditches/open microchannels (Fig. 1). Cells were first seeded in the microstructured wells of the multi-well plate by standard pipetting. The multi-well plate was then centrifuged to tightly pack the cells in the ditches to enhance 3D cell-cell interactions during the culture period. Also, pump-free membrane-based perfusion technology could be integrated into the multi-well plate to enable pump-free perfusion cell culture to better mimic the *in vivo* cell culture microenvironment.²⁶

II. EXPERIMENTAL DETAILS

A. Device fabrication, assembly, and sterilization

The ditches/open microchannels on the bottom of poly(dimethylsiloxane) (PDMS) wells were fabricated by soft lithography (Figs. 1(a) and 1(b)). Briefly, features on a plastic photo mask were first transferred onto a silicon wafer using standard photolithographic process. The photoresist-defined silicon wafer was then used as a mold for casting a PDMS prepolymer (10: 1 w/w) (Sylgard[®] 184, Dow Corning Corporation, Midland, MI, USA). After curing at room temperature for at least 24 h to minimize shrinkage after curing, the PDMS replica was carefully

FIG. 1. (a) Schematic diagram of the top view of a PDMS replica with a 3×3 array of ditches/open microchannels. (b) Schematic diagram and microscopic image of the cross-sectional view of typical PDMS replicated ditches/open microchannels. (c) Schematic diagram of the exploded cross-sectional view of the assembly of a multi-well plate with the ditches/open microchannels on the bottom (only one well was depicted).

peeled away from the silicon wafer. Next, the PDMS replica was attached to the bottom of a standard flat bottom 96-well holey (bottomless) microplate (Corning Incorporated, Corning, NY, USA) using a double-sided pressure sensitive adhesive (PSA) sheet (ARcare 90106[®], Adhesive Research, Inc., Glen Rock, PA, USA) (Fig. 1(c)). Finally, before each cell culture experiment, assembled microstructured multi-well plates were sterilized with 300 μ l of 70% ethanol for 30 min, washed twice with 300 μ l of deionized water and air dried the assembled microplates in a sterile cell culture hood for 15 min.

B. Cell packing optimization using C3A cells

We first thawed and cultured cryopreserved C3A cells, a derivative of HepG2/C3A human hepatoblastoma cell line (CRL-10741TM, American Type Culture Collection (ATCC), Manassas, VA, USA), in a sterile cell culture flask (Product # 430641, Corning Incorporated) in Eagle's Minimum Essential Medium (EMEM) (ATCC[®] No. 30-2003, ATCC) supplemented with 10% fetal bovine serum (FBS) (Catalog No. 16000-077, Invitrogen Corporation, Carlsbad, CA, USA) and 1% Penicillin-Streptomycin (Catalog No. 15140-163, Invitrogen Corporation) in a CO₂ HEPA incubator (Model 3130, Forma Scientific, Inc., Marietta, OH, USA) at 37 °C, 95% humidity, and 5% CO₂. Next, we seeded C3A cells with various cell densities from the cell culture flask into individual microstructured wells of the assembled microplate in 120 μ l of Minimum Essential Medium (MEM) (Catalog No. 41090-036, Invitrogen Corporation). We then centrifuged the loaded microplate at 100 g for 5 min to study the cell packing performance.

C. Primary human hepatocytes culture

Cryopreserved primary human hepatocytes were thawed according to manufacturer's recommendations (Lot No. Hu4175, Invitrogen Corporation) and seeded into individual microstructured wells of the assembled microplate with ditches/open microchannels in 120 μ l of Corning[®] Hepatocyte Maintenance Medium. Next, loaded microplates were centrifuged at 100 g for 5 min. Primary human hepatocytes were cultured at 37 °C, 95% humidity, and 5% CO₂ for 7 days. In a control experiment, primary human hepatocytes were seeded and cultured for 7 days on a collagen coated flat PDMS surface using the standard cell culture protocol.

D. LIVE/DEAD[®] viability/cytotoxicity assay kit

We used the LIVE/DEAD Viability/Cytotoxicity Assay Kit for mammalian cells (Molecular Probes, Inc., Eugene, OR, USA) to determine viability of the cultured cells following the standard protocol. Briefly, cells cultured inside the microstructured wells were incubated with the fluorescent dye mixture for 15 min at 37 °C inside a cell culture incubator followed by the phosphate-buffered saline (PBS) buffer wash. Fluorescent live and dead staining images were collected using a Zeiss Axiovert 200 inverted fluorescence microscope equipped with an epifluorescence condenser and camera system (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

E. Immunofluorescence staining of cultured hepatocytes

After 7 days of cell culture, formaldehyde-fixed cultures were immunostained using antibodies against hepatocyte cell surface multidrug resistant protein 2 (MRP2) protein. The choice of this marker allowed us to define the impact of 3D cell culture on restoration of cell polarity. Briefly, after 7 days of cell culture, cultured primary human hepatocytes were first washed 3 times with 200 μ l of PBS buffer per wash. Then, they were incubated with 200 μ l of 3% formaldehyde for 15 min at room temperature. After another 3 times wash of 200 μ l of PBS buffer per wash, cells were permeabilized with 200 μ l of 1% Triton X-100 solution for 15 min at room temperature. Next, cells were washed twice with 200 μ l of washing buffer (0.1% Tween-20 in PBS) per wash. In order to block nonspecific binding, cells were incubated for 30 min in 200 μ l of blocking buffer (0.1% Tween-20 and 0.5% goat serum in PBS). After nonspecific binding blocking, cells were incubated overnight with 9 μ g/ml primary Rabbit anti-MRP2 polyclonal antibody (Abcam, Inc., Cambridge, MA, USA) in 1: 100 dilution of blocking buffer at

4°C. After 4°C incubation, the sample was washed three times with 200 μl of washing buffer per wash and incubated with secondary antibodies conjugated to fluorescein isothiocyanate (FITC) (494/518 nm) fluorescent label in 1: 100 dilution of blocking buffer for 1 h at room temperature. Unbound antibodies were washed away by washing the cells 3 times with 200 μl of washing buffer per wash. Finally, fluorescent images were acquired with Zeiss Axiovert 200 inverted microscope equipped with an epifluorescence condenser.

F. MRP2 transporter functional assay

In order to verify the functionality of MRP2 transporter protein inside the cultured primary human hepatocytes, after 7 weeks of cell culture, MRP2 substrate (5 μM 5-(6) carboxy-2'7' dichlorofluorescein diacetate solution in cell culture media) was added and incubated with the primary human hepatocytes for 20 min at 37°C. Carboxy-2'7' dichlorofluorescein diacetate was absorbed by the cells and metabolized. The metabolites were actively excreted by MRP2 transporter protein complexes into bile canalicular structures. Efflux of fluorescent metabolites was monitored by a fluorescent microscopy revealing the bile canalicular structures inside the cell aggregates.

III. RESULTS AND DISCUSSION

A. Cell packing optimization using C3A cells

The optimization of cell packing was based on dimensions of the ditches/open microchannels, orientation of the microplate during centrifugation, cell seeding density, and the centrifugal force and time. Since cell-cell and cell-ECM interactions are critical for maintaining long-term viability and function *in vitro*, the dimensions of the ditches were designed to mimic sinusoidal microstructure of the liver and to promote *in-vivo*-like cell-cell interactions during cell culture. The dimensions of microchannels were designed to be 100 μm wide $\times$ 70 μm tall and 120 μm center-to-center spacing (5 cells across and 3 cells deep).²⁷ Also, in order to maintain cell viability and reasonable cell seeding time, the centrifugal force and time were chosen at 100 g for 5 min, which was based on the recommended values from commercial cell culture protocol (Cell Seeding Protocol for Cryopreserved Primary Human Hepatocytes, Invitrogen Corporation).

When the ditches of the wells were aligned parallel to the direction of rotation, C3A cells were forced to both ends of the ditches leaving large empty spaces in the middle of the ditches (Fig. 2(a)). On the other hand, when the ditches were aligned perpendicular to the direction of rotation during centrifugation, C3A cells were tightly packed inside the ditches with very little open spaces between cells (Fig. 2(b)). Also, the confocal image confirmed that cells were packed tightly in 3D layers providing a cell culture microenvironment that enhanced cell-cell interaction (Fig. 2(c)). Next, we investigated the impact of cell seeding density on cell packing performance. We found that 1.8×10^5 cells/well resulted in an excellent and consistent cell

FIG. 2. C3A cell packed inside the 100 μm wide $\times$ 70 μm tall and 120 μm center-to-center spacing ditches/open microchannels on the bottom of a PDMS well of a 96-well microplate after cell seeding and centrifugation at 100 g for 5 min. Ditches were aligned (a) parallel or (b) perpendicular to the direction of rotation. Cell seeding density was 1.2×10^5 cells/well. (c) Confocal image of the packed cells inside two ditches after centrifugation with the ditches aligned perpendicular to the direction of rotation. Cell seeding density was 1.8×10^5 cells/well.

FIG. 3. C3A cell packing inside the $100\text{ }\mu\text{m}$ wide $\times$ $70\text{ }\mu\text{m}$ tall and $120\text{ }\mu\text{m}$ center-to-center spacing ditches/open microchannels on the bottom of a PDMS well of a 96-well microplate after cell seeding and centrifugation at 100 g for 5 min . Ditches were aligned perpendicular to the direction of rotation. Cell seeding densities were (a) 0.6×10^5 cells/well, (b) 1.2×10^5 cells/well, and (c) 1.8×10^5 cells/well.

packing performance with approximately 5 cells across and 3 cells deep packed inside the ditches although 0.6×10^5 cells/well and 1.2×10^5 cells/well cell seeding density were also acceptable with very little open spaces in the ditches (Fig. 3). Thus, in all the cell culture experiments, 1.8×10^5 cells/well seeding density was used and the ditches were aligned perpendicular to the direction of rotation for optimal cell-cell packing and interaction.

B. Primary human hepatocytes culture

After 7 days of cell culture, primary human hepatocytes adhered tightly together to form cord-like structures that resembled 3D tissue-like cellular architecture without addition of matrices or coagulants (Fig. 4(a)). Hepatocyte membrane polarity was evaluated by monitoring the expression of MRP2, a hepatobiliary transporter critical in the efflux of drug metabolites. In conventional 2D cell culture, the expression of MRP2 transporter protein was occasionally observed as tiny unconnected dots between two adjacent hepatocytes illustrating the limited formation of bile canalicular structures (Fig. 4(c)). In contrast, culturing hepatocytes using the described cell seeding and packing method promoted the formation of tightly fused, 3D tissue-like cellular morphology (Fig. 4(a)) and restored cell membrane polarity (Fig. 4(b)) without the addition of animal derived or synthetic matrices or coagulants. Formation of 3D extended bile canalicular structures of the cultured primary human hepatocytes was confirmed with confocal microscopy (Fig. 4(d)). Cultured primary human hepatocytes were immunofluorescent stained for MRP2 transporter protein. Extension of bile canalicular structures illustrated in the xz and yz maximum intensity projection images demonstrated 3D character of cellular cords.

We further evaluated the long term cell culture capability using the microstructured multi-well plate. After 7 weeks of cell cultured in the microstructured multi-well plate, primary human hepatocytes tightly fused together into multicellular cord-like structures inside each ditch/open microchannel (Fig. 5(a)) and remained viable (Fig. 5(b)). These multicellular cord-

FIG. 4. (a) Bright field and (b) and (c) immunofluorescent staining images of MRP2 transporter protein of primary human hepatocytes after 7 days of cell culture. Cell seeding density was 1.8×10^5 cells/well. (a) and (b) Primary human hepatocytes were packed inside the $100\text{ }\mu\text{m}$ wide $\times$ $70\text{ }\mu\text{m}$ tall and $120\text{ }\mu\text{m}$ center-to-center spacing ditches/open microchannels on the bottom of a PDMS well of a 96-well microplate after cell seeding and centrifugation at 100 g for 5 min . Ditches were aligned perpendicular to the direction of rotation. (c) Primary human hepatocytes were seeded and cultured on a collagen coated flat PDMS surface of a PDMS well of a 96-well microplate. Cell seeding density was 0.6×10^5 cells/well. (d) Maximum intensity projection images for the xy , xz , and yz planes from sliced confocal images after 2 weeks of primary human hepatocyte cell culture. Fluorescein isothiocyanate (FITC) (green) fluorescent showing extended bile canalicular structure formation and polarity restoration. 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI) (blue) fluorescent showing cell nuclei.

FIG. 5. (a) Bright field image, (b) live/dead cell staining, and (c) MRP2 hepatocyte transporter functional assay illustrating active transport of fluorescent dye by MRP2 transporter protein into extended bile canaliculi structures after 7 weeks of primary human hepatocytes cell culture in the microstructured multi-well plate. Cell seeding density was 1.8×10^5 cells/well.

like structures were separated by empty gaps. Formation of such empty gaps can be explained by fusion/densification of the packed cells during long period of cell culture. We further demonstrated the primary human hepatocyte transporter function using carboxyfluorescein diacetate, a substrate for the MRP2 transporter. The bile canaliculi MRP2 transporter is responsible for the efflux of drug metabolites into bile and transport function is critical for removal of drug metabolites or the active transport of drug compounds into cells. Carboxyfluorescein diacetate is passively absorbed by the cultured primary human hepatocytes, metabolized and fluorescein diacetate is actively effluxed via MRP2 transporter protein into extended bile canaliculi structures (Fig. 5(c)). This function is largely driven by restoration of cell polarity resulting from the formation of tightly fused, 3D tissue-like cellular structure (Fig. 5(a)).

IV. CONCLUSIONS

We present a multi-well plate with microstructured bottom for enabling 3D cell seeding and hepatocyte cell culture to promote and maintain 3D tissue-like cellular morphology and cell-specific functionality *in vitro*. This multi-well plate can be incorporated with the pump-free membrane-based perfusion technology to enable pump-free perfusion cell culture in order to better mimic the *in vivo* cell culture microenvironment.

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