PERFUSION-BASED MICROFLUIDIC DEVICE FOR THREE-DIMENSIONAL DYNAMIC PRIMARY HUMAN HEPATOCYTE CELL CULTURE IN THE ABSENCE OF BIOLOGICAL OR SYNTHETIC MATRICES OR COAGULANTS

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ABSTRACT

We present a perfusion-based microfluidic device for three-dimensional (3D) dynamic primary human hepatocyte cell culture in the absence of biological or synthetic matrices or coagulants. The microfluidic device was used to promote and maintain 3D tissue-like cellular morphology and cell-specific functionality by restoring membrane polarity and hepatocyte transport function *in vitro*.

KEYWORDS: Primary human hepatocyte, 3D dynamic cell culture, Perfusion-based microfluidic device

INTRODUCTION

In the past decade, progress has been made in the development of cell culture configurations and systems to restore hepatocyte membrane polarity and enhance hepatocyte function *in vitro* for longer time periods. Some of the cell configuration and systems, such as hepatocyte sandwich cultures of rats and humans between two layers of collagen or Matrigel™ overlay [1], three-dimensional (3D) primary rat hepatocyte culture and perfused hepatocyte culture systems [2, 3], indeed provide some degree of enhancement of hepatocyte cell performance by restoring cell polarity relative to conventional cultures in maintaining viable cell cultures with some phenotypic relevance.

Various methodologies have also been developed and applied to promote prolonged expression of drug metabolizing enzymes, liver-specific functions and cell viability *in vitro*. These include (i) the use of modified culture media [4, 6] (ii) co-cultures [3, 5, 6] and (iii) the use of various extracellular matrix (ECM) to promote 3D cellular organization [6, 7]. However, mimicking the complex *in vivo* liver architecture and microenvironmental interactions that are critical for successful long-term hepatocyte cultures remains a challenge. Also, while a few of the approaches highlighted above show limited improvement of hepatocyte cell function, they do not restore tissue-like liver architecture or the formation of an extended, anatomosing bile canalicular network that would be needed for the restoration of membrane polarity in 3D. In addition, previous literature does not describe the formation of 3D tissue-like cellular structures that promote restoration of membrane polarity in 3D as evidenced by the formation of extended bile canalicular structures and transport function in a microfluidic device without the addition of biological or synthetic matrices or coagulants.

In this paper, we describe a perfusion-based microfluidic device that supports the dynamic 3D culture of primary human hepatocytes *via* independent perfusion microchannels and virtual suspension of cells on bottom patterned microstructured supports in the absence of biological or synthetic matrices or coagulants.

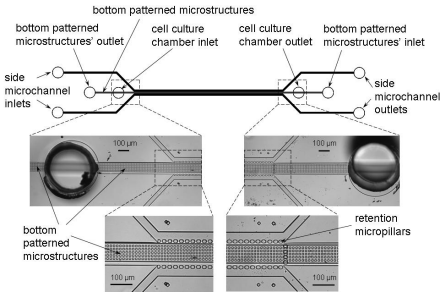

Figure 1: Schematic diagram of perfusion-based microfluidic device.

EXPERIMENTAL

The perfusion-based microfluidic device, which is similar to the one described by Toh *et al.* [8] and Ong *et al.* [9], consisted of a cell culture chamber centered between two side microchannels (Fig. 1). A series of retention micropillars was designed around the cell culture chamber for cell culture media to perfuse into the chamber *via* the two side microchannels. Unlike previous devices [8, 9], the bottom of cell culture chamber was patterned with microstructures for cell growth and support. Also, cell culture media could be perfused into the cell culture chamber *via* this bottom patterned microstructures. Soft lithography [10, 11] was used to fabricate the device which was made from two poly(dimethylsiloxane) (PDMS) replicas (Fig. 2).

Cyropreserved primary human hepatocytes were purchased from XenoTech, LLC (Lenexa, KS, USA). Lots of hepatocytes were prequalified for cell attachment and morphology by plating cells on BD BioCoat™ Collagen I Cellware. Hepatocytes were routinely cultured in serum-free MFE media commercially available from XenoTech, LLC.

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit for mammalian cells (Molecular Probes, *Inc.*, Eugene, OR, USA) was used to determine viability (live and dead) of the primary human hepatocytes inside the microfluidic device. Also, formaldehyde-fixed cultures were immunostained using antibodies against hepatocyte cell surface proteins, MRP2 and connexin 32. In addition, in order to verify the functionality of MRP2 transporter protein inside the perfusion-based cell cultured primary human hepatocytes, MPR2 substrate (5 µM 5-(6) carboxy-2'7' dichlorofluorescein diacetate solution in cell culture media) was perfused through the device.

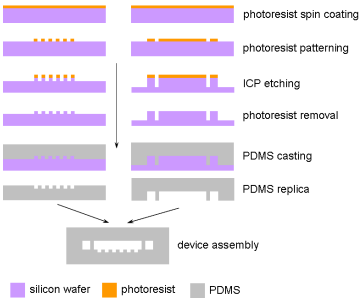

Figure 2: Schematic diagram depicting the fabrication steps for the perfusion-based microfluidic device.

RESULTS AND DISCUSSION

Flow dynamics of the device was experimentally characterized (Fig. 3). After two weeks of perfusion-based cell culture, primary human hepatocytes remained viable (> 90%) (Fig. 4a). Also, after dissembling the device, it was found that hepatocytes arranged in 3D morphology and fused tightly together to form 3D tissue-like cellular morphology without addition of matrices or coagulants (Fig. 4b) while after two weeks static cell culture, most of the hepatocytes were dead (Fig. 4c) and readily dispersed when the device was dissembled (Fig. 4d).

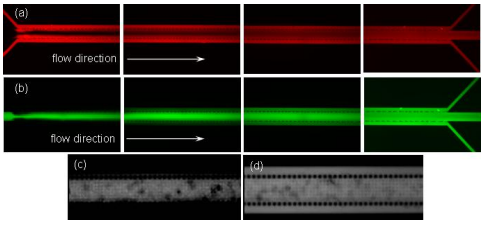

Figure 3: (a) Sulforhodamine B fluorescent dye perfusing from two side microchannels into empty cell culture chamber. (b) Carboxyfluorescein fluorescent dye perfusing from empty cell culture chamber into two side microchannels. (c) Dextran-rhodamine conjugate flowing through bottom patterned microstructures and it did not perfuse or leak into cell culture chamber and two side microchannels. (d) Dextran-rhodamine conjugate flowing through cell culture chamber and two side microchannels. Primary human hepatocytes were seeded and cultured for three days inside cell culture chamber before flow characterization experiments depicted in (c) and (d).

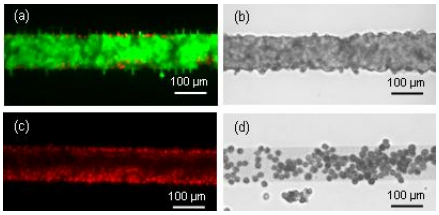

Figure 4: Two week primary human hepatocyte cell culture inside microfluidic devices. (a) and (b) Perfusion-based cell culture. (c) and (d) Static cell culture. (a) and (c) Fluorescent images of live (green)/dead (red) cell staining. (b) and (d) Microscopic images of hepatocytes after device disassembly.

In conventional two-dimensional (2D) cell culture, expression of MRP2 protein when present is observed as tiny unconnected dots between some hepatocytes (Fig. 5a) illustrating limited bile canalicular structures formation and limited transport function (Fig. 6a). On the contrary, perfusion-based microfluidic devices were shown to influence tightly fused, 3D tissue-like cellular morphology formation (Fig 4b) that promoted cell membrane polarity restoration (Fig. 5b and 5c), and transport function restoration (Fig. 6b). Cell membrane polarity was evidenced by extended 3D bile canalicular structures formation shown by expression of bile canalicular marker MRP2 (Fig. 5b and 5c) and gap junction protein connexin 32 (Fig. 5d). MRP2 protein is responsible for transport function from cells into bile canalicular structures. Transport function, often referred to as Phase III of drug metabolism, is critical for drug metabolites removal or active transport of drug compounds into liver cells. Primary human hepatocyte specific function in the device was demonstrated by MRP2 transport function assay where fluorescein diacetate was passively absorbed by hepatocytes and actively transported *via* MRP2 transport protein into extended bile canalicular structures (Fig. 6b). To our best of

knowledge, such extended 3D bile canalicular structures formation (membrane polarity restoration) and primary human hepatocytes transport function demonstration in a microfluidic device have not be demonstrated or reported before.

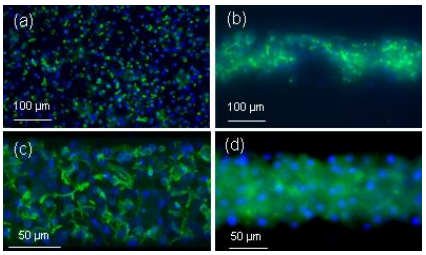

Figure 5: Immunofluorescent staining of cultured primary human hepatocytes inside microfluidic devices. (a) – (c) MRP2 protein. (d) Connexin 32 protein. (a) Seven day 2D static cell culture in BD collagen coated 96 well plate showing polarity was not being restored. (b) and (c) Seven day perfusion-based cell culture inside devices with microstructures at the bottom of cell culture chamber. FITC (green) fluorescent showing extended bile canalicular structures formation and polarity restoration. DAPI (blue) fluorescent showing cells' nuclei. (c) Maximum intensity projection image from sliced confocal images. (d) FITC (green) showing expression of gap junction protein connexin 32. Cell nuclei were stained with DAPI (blue).

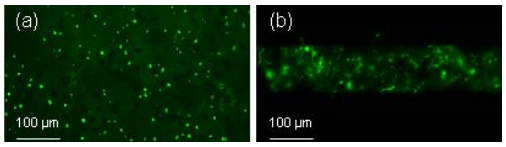

Figure 6: MRP2 hepatocyte transport function assay. (a) Seven day 2D static primary human hepatocyte cell culture in BD collagen coated 96 well plate showing limited transport function. (b) Seven day perfusion-based primary human hepatocyte cell culture inside microfluidic device with microstructures at the bottom of cell culture chamber showing active transport of fluorescent dye by MRP2 protein into extended bile canalicular structures.

CONCLUSION

The ability of primary human hepatocytes to support their *in vivo* functions while being cultured *in vitro* has high importance in tissue engineering applications and evaluating therapeutic candidates. For the first time, we demonstrated that a microfluidic device that enables the restoration of membrane polarity and transport function *in vitro* without the addition of biological or synthetic matrices or coagulants.

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