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Micropatterned coculture of hepatocytes on electrospun fibers as a potential *in vitro* model for predictive drug metabolism

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ABSTRACT

The liver is the major organ of importance to determine drug dispositions in the body, thus the development of hepatocyte culture systems is of great scientific and practical interests to provide reliable and predictable models for *in vitro* drug screening. In the current study, to address the challenges of a rapid function loss of primary hepatocytes, the coculture of hepatocytes with fibroblasts and endothelial cells (Hep-Fib-EC) was established on micropatterned fibrous scaffolds. Liver-specific functions, such as the albumin secretion and urea synthesis, were well maintained in the coculture system, accompanied by a rapid formation of multicellular hepatocyte spheroids. The activities of phase I (CYP3A11 and CYP2C9) and phase II enzymes indicated a gradual increase for cocultured hepatocytes, and a maximum level was achieved after 5 days and maintained throughout 15 days of culture. The metabolism testing on model drugs indicated that the scaled clearance rates for hepatocytes in the Hep-Fib-EC coculture system were significantly higher than those of other culture methods, and a linear regression analysis indicated good correlations between the observed data of rats and *in vitro* predicted values during 15 days of culture. In addition, the enzyme activities and drug clearance rates of hepatocytes in the Hep-Fib-EC coculture model experienced sensitive responsiveness to the inducers and inhibitors of metabolizing enzymes. These results demonstrated the feasibility of micropatterned coculture of hepatocytes as a potential *in vitro* testing model for the prediction of *in vivo* drug metabolism.

1. Introduction

The modern drug discovery and development has long been interested in identifying high-throughput screening systems to predict the *in vivo* disposition of new chemical entities. A stable and effective *in vitro* model would allow investigators to reduce the use of animals in drug testing, to eliminate false lead candidates, and to reduce the incidence of costly late-stage failures during clinical trials. The liver performs many complex functions within the body including the biotransformation of drugs and xenobiotics through high levels of metabolizing enzymes. Thus, the development of an *in vitro* hepatocyte culture system is of great scientific and practical interests to evaluate the preclinical metabolism and toxicity and to identify potential complications for drug candidates [1]. Hepatoma cell lines, such as HepG2 cells indicate an entire lack or a very low level of many important drug metabolizing enzymes and transporters [2], and thus freshly isolated primary hepatocytes constitute a common model for *in vitro* drug metabolism testing [3].

Primary hepatocytes are one kind of polarized cells and undergo a rapid loss of their cuboidal morphology within 2–3 days in a monolayer culture, accompanied by a reduction in the expression of metabolizing enzymes [4]. Attempts have been made to explore a reliable culture of primary hepatocytes for studying drug metabolism and responsiveness to inducers or inhibitors of metabolizing enzymes. One of the strategies is the establishment of three-dimensional (3D) hepatocyte culture by loading cells between two layers of Matrigel or entrapping cells in hydrogels, to reflect more closely the *in vivo* structure of cells surrounding by extracellular matrices (ECMs) [5]. But the barriers of Matrigel or hydrogel would affect the availability of test compounds to hepatocytes and the mass exchange in the drug screening process [6]. Alternatively, hepatocyte spheroids were established in hanging drop and microfluidic culture system to maintain the hepatocyte morphology and functions [7]. Schutte et al. cultured primary rat hepatocytes on a microspace plate for use in drug metabolism tests over 72 h. Hepatocytes retained their natural cuboidal morphology and showed stable expression of metabolizing enzymes and high sensitivity to an model drug acetaminophen [4]. It should be noted that the neighboring cells and surrounding ECMs determine the morphology and differentiation functions of hepatocytes [8], but an optimal physical, chemical and biological microenvironment has not been identified to maintain high-level cellular functions.

Hepatic lobule is the basic unit of liver and consists primarily of hepatocytes, endothelial cells (ECs), fibroblasts, hepatic stellate cells,

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and Kupffer cells. Thus, significant enhancement in hepatocyte phenotype and function has been achieved after coculture of hepatocytes with nonparenchymal cells [9]. Yamada et al. cocultured primary rat hepatocytes with fibroblasts on a hydrogel fiber-based scaffold, and a significant increase in the albumin secretion and urea synthesis was observed because of heterotypic and homotypic cell-cell interactions, compared with conventional monolayer culture and single cultivation in hydrogel fibers [10]. In addition, fibroblasts are not in physical contact with hepatocytes in native liver, and the space of disse resides between fibroblasts and hepatocytes [11]. It was indicated that the random coculture of hepatocytes and fibroblasts restricted hepatocytes move and hampered the formation of spheroids [12]. Thus, micropatterned scaffolds were developed for hepatocyte coculture to confine the cell growth and ECM deposition in patterned regions [13]. Khetani et al. cocultured hepatocytes with non-parenchymal cells on micropatterned areas of a tissue culture plate (TCP), where hepatocytes grew on defined areas as islands, surrounded by fibroblasts which served as feeder cells. It revealed a noticeably improved performance in detecting hepatotoxic drugs when compared with standard sandwich hepatocyte culture [14,15]. However, the two-dimensional (2D) patterned coculture is not sufficient to achieve efficient cell−cell interactions and the formation of 3D hepatic lobule-like microtissues *in vitro*.

Electrospinning is currently the only technique that allows the fabrication of continuous fibers with diameters ranging from several micrometers down to a few nanometers, which have found wide applications in biomedical field [16]. In our previous study, patterned fibrous mats were constructed with distinct ridges and grooves after collection on a glass substrate patterned with an electrically conductive circuit [17]. To reassemble into an *in vivo*-like structure, micropatterned coculture of hepatocytes was established by precise assembly of cell-loaded patterned fibrous mats, in which hepatocytes were located in patterned regions separately from other types of cells [18,19]. In the current study, the micropatterned coculture of hepatocytes with fibroblasts and ECs was investigated to resemble heterotypic micro-organoids of hepatic lobules. The activities of phase I enzymes CYP3A11 and CYP2C9 and phase II enzymes were determined on the cocultured hepatocytes, along with the mRNA levels of these enzymes. Five drugs with hepatic metabolism covering above enzymes were chosen to predict the clearance rates by the *in vitro* cocultured hepatocytes, and the correlations with the observed data *in vivo* were evaluated. Additionally, the inhibition and induction of enzyme activities are the key mechanisms in drug-drug interactions. Thus, the metabolic activities of cocultured hepatocytes were examined by using specific inducers and inhibitors to above enzymes.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol)-poly(DL-lactide) (PELA, Mw = 42.3 kDa, Mw/Mn = 1.23) was prepared by bulk ring-opening polymerization of lactide/poly(ethylene glycol using stannous chloride as the initiator [20]. Lactosylated poly(DL-lactide) (lac-PLA, Mw = 7.6 kDa, Mw/Mn = 1.32) was prepared by bulk ring-opening polymerization of lactide using pentaerythritol as the core and stannous octoate as the initiator, followed by conjugation with lactobionic acid [18]. Dimethyl sulfoxide (DMSO), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), tetramethylrhodamine isothiocyanate (TRITC)-phalloidin, and glutathione were procured from Sigma-Aldrich (St. Louis, MO), and fluorescein diacetate was from Molecular Probes (Carlsbad, CA). Goat antirat albumin antibody was received from Abcam (Cambridge, UK), and goat antirabbit IgG-aminomethylcoumarin acetate (AMCA) was from Innova Biosciences Ltd. (Innova, UK). Rabbit antimouse collagen I antibody, mouse antihuman collagen IV antibody, mouse antigoat IgG-FITC, and goat antimouse IgG-TRITC were obtained from Biosynthesis Biotechnology Co., Ltd. (Wuhan, China). 7-Benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxycoumarin (7-HC), and 7-methoxy-4-trifluoromethylcoumarin (MFC) were acquired from Tianjin Heowns Medicine Co. Ltd. (Tianjin, China). Tolbutamide, S-warfarin, midazolam, testosterone, acetaminophen, rifampicin, ketoconazole, and probenecid were purchased from Dalian Meilun Medicine Group Co. Ltd. (Dalian, China). All other chemicals and solvents were of reagent grade or better, and received from Chengdu Kelong Reagent Co. (Chengdu, China), unless otherwise indicated.

2.2. Construction of patterned fibrous scaffolds

The patterned scaffolds for loading hepatocytes, fibroblasts and ECs were constructed as described previously [19]. Briefly, a photomask containing parallel strips of 100 and 200 μm wide and gaps between the strips of 300 and 400 μm wide was fabricated by E-beam mask lithography system (Mark 40, CHA Industries, Fremont, CA). Micropatterned collectors were constructed on a glass template patterned with silver circuit by a photolithography process (Suss Mircotec MA6, Germany). PELA and Lac-PLA blends at the weight ratio of 1/1 were dissolved in chloroform and fed into an electrospinning system at 0.6 mL/h by a syringe pump (Zhejiang University Medical Instrument Company, Hangzhou, China). The electrospinning was performed at a voltage of 20 kV using a high voltage statitron (Tianjing High Voltage Power Supply Company, Tianjing, China), and fibers deposited on the patterned collector with a thickness of 400 μm and a strip/gap width of 200/300 μm were used for hepatocyte loading. Additionally, PELA fibers were obtained on patterned collectors with the strip/gap widths of 200/300 μm and 100/400 μm for loading fibroblasts and ECs, respectively. Patterned mats were punched into disks of 15 mm in diameter to fit the well size of a 24-well TCP.

2.3. Establishment of patterned hepatocyte coculture

Primary hepatocytes were isolated from livers of adult rats using collagenase perfusion procedure as described previously [21]. Male Sprague-Dawley rats weighing 120–150 g were from Sichuan Dashuo Biotech Inc. (Chengdu, China), and all animal protocols were approved by the University Animal Care and Use Committee. Swiss mouse embryo fibroblasts NIH3T3 and human umbilical vein ECs were from American Type Culture Collection (Rockville, MD). The patterned coculture of hepatocyte with fibroblasts and ECs (Hep-Fib-EC) was established in 24-well TCPs as described previously [19]. Briefly, hepatocytes, fibroblasts, and ECs were seeded on patterned mats at a cell density of 2×10^5, 3.0×10^5 and 1.5×10^5 cells/cm^2, respectively. The patterned fibrous mats with cells loaded were rightly stacked by fitting the bulges of one patterned mat nearly into the dents of another mat, ensuring a close contact with each other. The patterned coculture system was incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, MD), supplemented with 10% of fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Gibco BRL, Grand Island, NY), and the culture medium was refreshed every 2–3 days. Patterned coculture of hepatocyte with fibroblasts (Hep-Fib) was established as described above, and hepatocyte culture on the patterned mats was set as the control.

2.4. Characterization of patterned scaffolds and coculture

The patterning features of electrospun fibrous mats were observed by an optical microscope (Nikon Eclipse TS100, Japan). Fiber morphologies in the patterned areas were investigated by a scanning electron microscope (SEM, FEI Quanta 200, The Netherlands) equipped with a field-emission gun (20 kV) and a Robinson detector after 2 min of gold coating to minimize the charging effect. The fiber diameter was measured from SEM images as described previously [17]. The distribution