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Three-dimensional hydrogel culture conditions promote the differentiation of human induced pluripotent stem cells into hepatocytes

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Abstract

Background aims. Human induced pluripotent stem cells (hiPSCs) are becoming increasingly popular in research endeavors due to their potential for clinical application; however, such application is challenging due to limitations such as inferior function and low induction efficiency. In this study, we aimed to establish a three-dimensional (3D) culture condition to mimic the environment in which hepatogenesis occurs *in vivo* to enhance the differentiation of hiPSCs for large-scale culture and high throughput BAL application. *Methods.* We used hydrogel to create hepatocyte-like cell (HLC) spheroids in a 3D culture condition and analyzed the cell-behavior and differentiation properties of hiPSCs in a synthetic nanofiber scaffold. *Results.* We found that treating cells with Y-27632 promoted the formation of spheroids, and the cells aggregated more rapidly in a 3D culture condition. The ALB secretion, urea production and glycogen synthesis by HLCs in 3D were significantly higher than those grown in a 2-dimensional culture condition. In addition, the metabolic activities of the CYP450 enzymes were also higher in cells differentiated in the 3D culture condition. *Conclusions.* 3D hydrogel culture condition can promote differentiation of hiPSCs into hepatocytes. The 3D culture approach could be applied to the differentiation of hiPSCs into hepatocytes for bioartificial liver.

Key Words: *cell behavior, hepatocyte, human induced pluripotent stem cells, hydrogel*

Introduction

Liver failure is associated with high morbidity and mortality and is the seventh leading cause of death worldwide [1]. The bioartificial liver (BAL) system is a cell-based external artificial biological device that has synthetic functions and biotransformation activities that are similar to those of the liver [2]. However, the shortage of primary human hepatocytes, the xenotransplantation-related disadvantages of porcine cells and the limited metabolic function of immortalized hepatic cell lines preclude the widespread acceptance of the BAL system.

Human induced pluripotent stem cells (hiPSCs) that are reprogrammed from a diverse range of cell types, such as hair follicle mesenchymal stem cells, peripheral blood mononuclear cells and skin fibroblasts, possess unlimited self-renewal capability and the potential to differentiate into all three germ layers [3–5]. The successful generation of functional hepatocytes from hiPSCs could be a potential cell source for BAL devices. Using a step-wise method and cocktails of growth factors/cytokines to promote hiPSCs to hepatocytes is a current protocol. However, lower induction efficiency and significantly lacking liver function are the greatest obstacle to application. To enhance differentiation efficiency, human serum was used to replace fetal bovine serum (FBS) to mimic the environment of hepatogenesis in our previous study. We found human serum, particularly that acquired relatively soon after hepatectomy, can enhance the differentiation efficiency and functionality [6]. These

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results suggested that conditions that mimic early organogenesis could enhance hepatocyte differentiation and the functionality of hiPSCs.

Recently, several studies have demonstrated that three-dimensional (3D) culture conditions create a pragmatic microenvironment and mimic *in vivo* development, enhancing hepatocyte differentiation and the functionality of human embryonic stem cells (hESCs) and hiPSCs compared with 2-dimensional (2D) culture conditions [7–9]. Various 3D culture conditions have been used, including the formation of self-aggregated spheroids on low-attachment surfaces [8,10], the encapsulation of hepatocytes in alginate and the embedding of cells in synthetic biomaterials [11]. Compared with the other two conditions, synthetic biomaterials provide 3D structures, extracellular matrix (ECM)-mimicking stiffness and an environment that facilitates the diffusion of nutrients and cellular growth factors [12–14]. Nanofiber hydrogel comprises a animal-free synthetic biomaterial that can mimic native ECM functions and thus support the adhesion and differentiation of hiPSCs. This gel is made by interweaving a self-assembling polypeptide, and the pore size is 50–200 nm. These peptides are completely synthetic, thus avoiding the potential pathogenicity of animal-derived materials. This 3D nanofiber hydrogel is critical for meeting future demands because of the advantages of biocompatibility and retrieval and the lower risk of immunogenic reaction. Thus, embedding cells in a 3D biomaterial construct including a nanofiber scaffold is expected to provide a microenvironment to improve the induction efficiency of hepatocyte differentiation for BAL applications [12,15,16].

Recent advances in 3D culture techniques have enabled the development of promising scaffolds for the differentiation of hiPSCs. However, to the best of our knowledge, few studies have applied this nanofiber hydrogel to hiPSC differentiation, and few have tried to determine how this completely synthetic hydrogel influences cell activities and differentiation. In this study, we focused on the cell-behavior and differentiation properties of hiPSCs in a synthetic nanofiber scaffold and designed the fabrication method in 3D culture condition.

Methods

Ethics approval and consent to participate

This study was approved by the institutional review board of Tianjin Third Central Hospital, Tianjin, China (file no. 13010). All patients provided written informed consent. The methods were carried out in accordance with the approved guidelines. All experimental protocols were approved by the institutional review board.

Maintenance of hiPSCs cultures

Three human iPSC lines were used for hepatic differentiation (hiPSCs-HF1, hiPSCs-HF2 and hiPSCs-EC1). hiPSCs-HF1 and hiPSCs-HF2 were induced from IMR-90 human fibroblasts using *Oct4, Sox2, Klf4 and c-myc*. hiPSCs-EC1 were induced from urine of renal epithelial cells using *Oct4, Sox2, Klf4 and c-myc*. In our laboratory, theses hiPSC cell lines were routinely passaged on a feeder layer of mitomycin-C (Roche)–inactivated mouse embryonic fibroblasts. Briefly, cells were maintained on an inactivated feeder layer and were cultured in hiPSC medium (Dulbecco's Modified Eagle Medium [DMEM]/F12 supplemented with 20% knockout serum replacement, 8 ng/mL basic fibroblast growth factor [bFGF], 1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine and 0.1 mmol/L 2-mercaptoethanol; all from Thermo Fisher Scientific) at 37°C in 5% CO_2. Before differentiation, cells were harvested and separated indigestion solution containing Dispase (Stemcell Technologies), and they were then transferred to plates coated with Matrigel (BD Biosciences) grown in mTesR1 (Stemcell Technologies).

Hepatic differentiation of hiPSCs

Before differentiation, hiPSCs were used to form embryoid bodies (EBs). Feeder-free hiPSCs were digested to a single-cell suspension by Accutase (Stemcell Technologies), and they were cultured in AggreWell 400 plates (Stemcell Technologies; ~ 1.2×10^6 cells/well) for 24 h, and EBs (consisting of ~1000 cells) were later transferred to Ultra Low Cluster Plates (Corning) and cultured for another 24 h in DMEM/F12 supplemented with 20% knockout serum replacement, 1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine and 0.1 mmol/L 2-mercaptoethanol. In the first stage, EBs were cultured on Matrigel-coated well plates (~100 EBs/well) and maintained in DMEM/F12 supplemented with 100 ng/mL recombinant Activin-A and 100 ng/mL bFGF (all from R&D Systems). The concentration of fetal bovine serum (FBS) was 0% for the first 24 h, 0.2% for the second 24 h and 2.0% for the last 24 h. In the second stage, the differentiated cells were further induced to hepatoblasts in DMEM/F12 media containing 10% FBS, 1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine, 1% dimethyl sulfoxide (Sigma-Aldrich) and 100 ng/mL hepatocyte growth factor (HGF; R&D Systems) for the following 8 days. In the third stage, hepatoblasts were switched to maturation media containing 10% FBS, 1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine and 0.1 μmol/L dexamethasone (Sigma-Aldrich) for 3 days. For 3D differentiation, we initiated the differentiation of the cells in the same condition as the 2D culture until the first day of the