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# Induced pluripotent stem cell-derived hepatocytes and endothelial cells in multi-component hydrogel fibers for liver tissue engineering

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ABSTRACT

Liver tissue engineering requires a suitable cell source, methodologies to assemble the cells within their niche microenvironments in a spatially defined manner, and vascularization of the construct *in vivo* for maintenance of hepatocyte viability and function. Recently, we have developed methods of encapsulating cells within separate domains in multi-component hydrogel fibers and methods of assembling fibers to form 3D-patterned tissue constructs. In the present work, we have combined these approaches to encapsulate hepatocytes and endothelial cells within their specific niches, and to assemble them into endothelialized liver tissue constructs. The hepatocytes and endothelial cells were obtained in parallel by differentiating human recombinant protein-induced human pluripotent stem cells, resulting in a construct which contained genetically identical endothelial and parenchymal elements. We were able to demonstrate that the presence of endothelial cells in the scaffold significantly improved hepatocyte function *in vitro* and facilitated vascularization of the scaffold when implanted in a mouse partial hepatectomy model. The *in vivo* studies further asserted that integration of the scaffold with host vasculature had occurred, as demonstrated by the presence of human albumin in the mouse serum.



## 1. Introduction

There is a high demand for liver transplantation treatment due to its success; however, the scarcity of donor organs means that it cannot be available for every patient. Hepatocyte transplantation is an alternative method that holds great promise for the treatment of liver disease, where the transplanted cells reduce the load on the existing host cells and help in the recovery or regeneration of the host liver.

Many approaches have been studied for the rescue of liver failure such as extracorporeal devices (reviewed in Refs. [1,2]) and cell-based therapies [3,4]. Cell-based therapies have a great potential to treat acute and chronic liver failure and the cell source for the therapies could be from adult stem cells or pluripotent stem cells. Mesenchymal stem cells from bone marrow and umbilical cord blood [5], as well as human embryonic stem cells [6–8] have been successfully differentiated into functional hepatocytes. Induced pluripotent stem cells (iPSCs) generated by reprogramming fibroblasts [9] have been postulated to be an excellent cell source for liver tissue engineering [10] and have been differentiated to hepatocytes by several groups [11–13].

In addition to the issue of cell source, delivery of hepatocytes to the liver requires the appropriate vehicle and form. One of the systems that has gained attention is the implantable tissue engineered liver construct. Hepatocytes have a self-assembling property that helps them to form spheroids. The formation of spheroids on low-attachment surfaces and on Matrigel enhanced the functional characteristics of the hepatocytes when compared to 2D collagen surface [14–18]. Similarly, hepatocyte spheroids cultured in 3D biomaterial constructs showed better functionality than cells grown on 2D substrate [19–24]. Encapsulation of hepatocytes in alginate [20,21], methylated collagen [22,23] or synthetic materials [24] formed spheroids with improved cell–cell interactions and hepatocyte functions. Thus, hepatocytes were encapsulated in the permissive matrix of polyelectrolyte complex hydrogel fibers in the present work, to allow them to self assemble into 3D structures for their optimal function.

The liver contains several cell types, the majority being hepatocytes and endothelial cells. Co-cultures of hepatocytes with non-parenchymal cells have been reported to improve hepatocyte function by means of reciprocal signaling interactions between the

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two cell types [25–28]. The effectiveness of these interactions relied on spatial patterning of the cells with respect to each other [26]. Based on our earlier work with interfacial polyelectrolyte complex (IPC) fiber scaffolds, where fibers are drawn from the interface between two oppositely charged polyelectrolytes [29,30], we recently established proof-of-concept for co-culture of cells within multi-interfacial polyelectrolyte complex (MIPC) fibers [31,32]. MIPC fibers are drawn from two or more interfaces, resulting in segregated domains within single fibers. In the current work, these multi-component fibers allowed us to spatially define hepatocytes and endothelial in separate compartments, with the objective of reproducing the ideal 3D microenvironment for their reciprocal interactions.

We first differentiated human iPSCs to hepatocytes and endothelial cells, by adapting the existing differentiation strategies [5,8,12,13,33–37]. These differentiated cells were encapsulated in fibers drawn by interfacial polyelectrolyte complexation (IPC) that were subsequently assembled to form tissue-like constructs. We then characterized the performance of these constructs, in terms of hepatocyte function and integration with host tissue, in a mouse partial hepatectomy model.

## 2. Materials and methods

### 2.1. iPSCs culture

iPSCs used in this report were generated by reprogramming human foreskin fibroblasts with recombinant proteins as described earlier [38]. hESC-qualified Matrigel (BD Biosciences, NJ, USA) was used as the substrate for the maintenance of the iPSCs culture.

### 2.2. Differentiation of human iPSCs to hepatic cells

Hepatocyte differentiation was carried out according to the three-stage protocol reported by Takata et al. [39], with slight modifications. iPSCs were seeded at 70–80% confluence and were cultured in serum-free RPMI 1640 medium (Invitrogen, CA, USA) supplemented with B-27 (Invitrogen, CA, USA) and rh-activin-A (100 ng/mL) (R&D systems, MN, USA) for 3 days. Subsequently, differentiated mesendodermal cells were treated with HGF (20 ng/mL) (R&D systems, MN, USA) for 5 days to generate hepatic cells. To achieve final hepatic cell maturation, cells were cultured for 5 days in hepatocyte culture medium (HCM; Lonza, Walkersville, MD) supplemented with oncostatin M (10 ng/mL) (OSM; R&D systems, MN, USA). Cells were maintained in HCM with no oncostatin M after hepatic cell maturation.

### 2.3. Differentiation of human iPSCs to endothelial cells

We designed a two-step protocol to differentiate the stem cells into endothelial cells. The first stage involved differentiation of the stem cells to the mesodermal lineage. In the second stage, the mesodermal cells were differentiated into endothelial cells. During the first stage, iPSCs were cultured for 7 days with RPMI 1640 media supplemented with insulin–transferrin–selenium A (1% v/v) (Invitrogen, CA, USA), non-essential amino acids (0.1 mM) (Invitrogen, CA, USA), L-glutamine (2 mM) (Invitrogen, CA, USA), 2-mercaptoethanol (0.1 mM) (Invitrogen, CA, USA), penicillin and streptomycin (each 100 U/mL) (Invitrogen, CA, USA), rh-activin-A (100 ng/ml), rh-BMP-4 (25 ng/ml) (R&D systems, MN, USA), rh-bFGF (4 ng/ml) (R&D systems, MN, USA), rm-Wnt-3a (50 ng/ml) (R&D systems, MN, USA) and 10 μM SB431542 (Tocris Bioscience, MO, USA). The mesodermal cells were further differentiated to endothelial cells for 7 days with EndoGro media (Millipore, MA, USA). The EndoGro media contains rh-VEGF (5 ng/ml) and rh-FGF (5 ng/ml). We supplemented the second stage of differentiation with VEGF (50 ng/ml) (Millipore, MA, USA) and rh-bFGF (10 ng/ml) (R&D systems, MN, USA). The differentiated cells were maintained with EndoGro media without excess VEGF and FGF. Human umbilical vascular endothelial cells (HUVEC) were obtained from ATCC (CRL-1730).

### 2.4. Encapsulation of cells in multi-component hydrogel fibers

Water soluble chitin (WSC), prepared from crab chitin (Sigma–Aldrich), and sodium alginate (Sigma–Aldrich) were used at a concentration of 10 mg/ml as polycation and polyanion, respectively. WSC containing galactose (WSC-Gal) and collagen (WSC-Col) were used to provide niche microenvironments for the hepatocytes and endothelial cells respectively (see Supplementary Information for preparation of WSC-Gal and WSC-Col).

The hydrogel was formed by assembly of polyelectrolyte fibers as described previously [40]. Each polyelectrolyte fiber, formed by multi-interfacial polyelectrolyte complexation (MIPC) [31], consisted of four separate domains. Differentiated hepatocytes and endothelial cells were suspended in WSC-Gal and WSC-Col solutions at a density of $1 \times 10^4$ cells/μl, respectively. The solutions were dispensed in the layout of WSC-Col solution flanked by two 20 μl droplets of sodium alginate solution, which were in turn flanked by two 20 μl droplets of WSC-Gal solution on the outside. Each polyelectrolyte droplet was brought to meet its adjacent droplets, creating four interfaces which were fused to create a nascent polyelectrolyte fiber with four domains. Consequently, this led to the formation of a fiber with endothelial cells in the two center domains, flanked by hepatocytes in the outer domains. Multiple polyelectrolyte fibers were drawn simultaneously, fused and spooled to make a hydrogel. The resulting hydrogel was fused further by immersing sequentially in 0.5% WSC in PBS, 0.5% alginate in PBS, and the process was repeated. Finally, the fused hydrogel was removed from the spooling device and transferred to the culture medium. For single culture control, WSC-Col solution was used without the endothelial cells.

### 2.5. Immunostaining

Cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min. The cells were permeabilized with PBS containing 0.1% triton X-100 (Sigma–Aldrich, MO, USA) for 10 min and used as described earlier [41]. Suitable secondary antibodies conjugated with a fluorophore were used and the samples were visualized under a fluorescence microscope.

Mice liver slices containing the implanted cell/scaffold constructs were fixed with 4% paraformaldehyde solution overnight at 4 °C, dehydrated sequentially in 15% (w/v) sucrose solution in PBS overnight, followed by 30% sucrose in PBS overnight, embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (Sakura, The Netherlands) and cryosectioned. Liver sections were immunostained with specific antibodies as described earlier [41]. The list of antibodies used in the study is given in Table 1A.

### 2.6. RNA extraction, reverse transcription, polymerase chain reaction (PCR) and real-time PCR

Total RNA from the cells were extracted using RNeasy Mini Kit (Qiagen, CA, USA) or with TRIZOL reagent (Invitrogen, CA, USA). The RNA concentration was determined using NanoDrop 2000 (Thermo Scientific, MA, USA). For real-time PCR, Taqman assays (Life Technologies, NY, USA) were used, as listed in Table 1B.

### 2.7. Flow cytometry

Flow cytometry was carried out as described earlier [41]. Briefly, a suspension of single cells was fixed with formaldehyde and permeabilized with PBS containing 0.1% Triton X-100. The specific antibodies used for flow cytometry are listed in the table. The stained cells were analyzed in a BD LSRII flow cytometry analyzer at the Biopolis Shared Facility, Singapore.

### 2.8. Human albumin ELISA

The concentration of albumin secreted into the media was assayed using the human albumin enzyme linked immunosorbent assay (ELISA) kit. The assay was carried out as described in the manufacturer's instructions (Cygnus Technologies, NC, USA). Assays were performed on samples obtained from three independent cultures and were performed in duplicate. A standard curve was prepared for the determination of the albumin concentration in the samples.

**Table 1**
List of antibodies and PCR assays used in the study.

| (A) List of antibodies | |
|---|---|
| Antibody | Source |
| Sox17 | R&D Systems, MN, USA |
| GATA4 | Abcam, Cambridge, UK |
| FOXA2 | Abcam, Cambridge, UK |
| E-CAD | Abcam, Cambridge, UK |
| AFP | Abcam, Cambridge, UK |
| ALB | Sigma-Aldrich, MO, USA |
| vWF | Abcam, Cambridge, UK |
| CD-31 | Abcam, Cambridge, UK |
| (B) List of Taqman assays | |
| Gene | Assay# |
| GAPDH | Hs99999905-M1 |
| GATA4 | Hs00171403-m1 |
| SOX17 | Hs00751752-s1 |
| HNF4a | Hs00604435-m1 |
| ALB | Hs00910225-m1 |
| V-ECAD | Hs00901463-m1 |
| PECAM-1 | Hs00169777-m1 |