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Preparation of stripe-patterned heterogeneous hydrogel sheets using microfluidic devices for high-density coculture of hepatocytes and fibroblasts

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Here we demonstrate the production of stripe-patterned heterogeneous hydrogel sheets for the high-density 3D coculture of multiple cell types, by using microchannel-combined micronozzle devices. The prepared hydrogel sheet, composed of multiple regions with varying physical stiffness, regulates the direction of proliferation of encapsulated cells and enables the formation of arrays of rod-like heterotypic organoids inside the hydrogel matrix. We successfully prepared stripe-patterned hydrogel sheets with a uniform thickness of ~100 μm and a width of several millimeters. Hepatoma cells (HepG2) and fibroblasts (Swiss 3T3) were embedded inside the hydrogel matrix and cocultured, to form heterotypic micro-organoids mimicking *in vivo* hepatic cord structures. The upregulation of hepatic functions by the 3D coculture was confirmed by analyzing liver-specific functions. The presented heterogeneous hydrogel sheet could be useful, as it provides relatively large, but precisely-controlled, 3-dimensional microenvironments for the high-density coculture of multiple types of cells.

[**Key words:** Coculture; Microfluidic device; Hydrogel; Hepatocyte; Alginate; Tissue engineering]

Cultivation of mammalian cells in a controlled 3-dimensional (3D) environment is an essential technology for medical, biological, and bioengineering applications, including cell-based drug screening assays, preparation of complex tissue models, and physiological studies of cells (1–3). Unlike conventional 2-dimensional (2D) plate culture, cells reorganized into 3D aggregates can recover inherent physiological characteristics because of their similarity to *in vivo* tissues. Homotypic cell–cell contact is one of the most critical features for maintaining cellular function as observed in spheroid-based cell culture (4). For cells that are prone to lose their desired differentiated/undifferentiated characteristics *in vitro*, 3D culture techniques are highly effective (5). In addition, recent studies have revealed that heterotypic cell–cell interactions are critical, as demonstrated in the cocultivation of stem cells with feeder cells (6,7). Various state-of-the-art coculture techniques have been proposed, including micropatterning on planar substrates (8,9), formation of heterogeneous spheroids (10,11), stacking of cell sheets (12,13), and microchannel-based cultivation (14,15). These techniques not only mimic the morphology of *in vivo* tissues but also induce the upregulation of differentiated cell function and improved polarity.

Hydrogels can provide ideal microenvironments for 3D cell cultivation, because of the efficient supply of oxygen and nutrition to the encapsulated cells through permeable matrices. Small-size hydrogel materials, several hundreds of micrometers in diameter or width, are often employed to ensure high cell viability and function. Hydrogel microbeads (16), fibers (17,18), and planar patterns (19,20) are frequently employed, and have been prepared using microfluidic devices, photolithography, and micromolding techniques. Assembly of these unit structures with different types of cells is a reasonable strategy for realizing a 3D coculture platform (21) and for constructing relatively large tissue models. In addition to the homogeneous hydrogels, attempts have been made to form patterned, heterogeneous hydrogel materials. Hydrogel beads (22), fibers (23,24), and planar sheets (25) composed of several regions with different physicochemical properties have been prepared using microfluidic technologies. Compared to beads and fibers, hydrogel sheets are advantageous in the formation of complex cell assemblies resembling *in vivo* tissues. Such thin planar structures can ensure the efficient diffusion-based supply of oxygen and nutrition to the cells located inside the hydrogel matrix. In addition, planar morphologies are potentially useful as building blocks for constructing large-scale 3D tissue models via stacking. However, the encapsulation of multiple types of cells into the micropatterned matrices of anisotropic hydrogel sheets and its applications, has not been fully investigated to date.

In this study, we propose a microfluidic device for producing stripe-patterned anisotropic hydrogel sheets, which can encapsulate multiple types of cells at high densities with precisely-controlled positions. Fig. 1 shows a schematic of the microfluidic system used to prepare the stripe-patterned hydrogel sheets and

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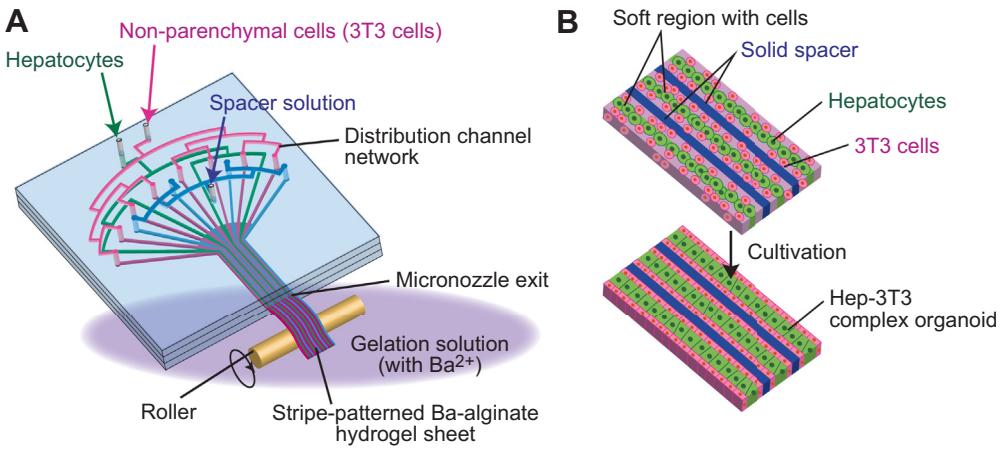

FIG. 1. Schematic showing the preparation procedure of the stripe-patterned heterogeneous hydrogel sheet (A) and formation of heterotypic micro-organoids in the soft/solid hydrogel sheet (B).

the behaviors of encapsulated cells. Hydrogel precursor solutions of sodium alginate (NaA) with or without cells are continuously introduced into a flat micronozzle-combined microfluidic device having 3 inlet-channel networks. The flow of these solutions is branched into multiple streams and recombined alternately, and then extruded through the micronozzle exit to the outer aqueous solution with a gelation agent, resulting in the formation of stripe-patterned hydrogel sheets. We employed propylene glycol alginate (PGA), a non-gelling ester derivative of alginate, together with NaA, to form the cell-containing soft regions; PGA can prevent the shrinkage of alginate during the gelation process, whereas keeping the low degree of gelation. The cells are encapsulated in the soft regions of the sheet, whereas the solid regions prevent the cells from growing in the horizontal direction, resulting in the formation of rod-like heterotypic micro-organoids. In this study, we encapsulated hepatocytes and fibroblasts to prepare heterotypic tissue models mimicking the hepatic microstructures found *in vivo*, and examined whether coculture with fibroblasts positively affects hepatocyte-specific functions.

MATERIALS AND METHODS

Cell culture HepG2 cells (human hepatoma cells, RIKEN BRC, Tsukuba, Japan) were cultured in minimum essential medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 100 unit/mL penicillin, and 0.1 mg/mL streptomycin (Sigma) in a CO_2 incubator at 37°C with 5% CO_2. Swiss 3T3 (embryonic mouse fibroblast) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) with the same supplements under the same conditions. Confluent cells were harvested by trypsin/EDTA treatment. For distinguishing cell positions in the hydrogel sheets, Swiss 3T3 cells were stained red by using the PKH26 Fluorescent Cell Linker Kit (Sigma).

Microchannel fabrication and design Polydimethylsiloxane (PDMS) microfluidic devices were fabricated using standard soft lithography and replica molding techniques, as described elsewhere (26). Fig. 2 shows the design of a microfluidic device for preparing the stripe-patterned hydrogel sheets. Three PDMS plates were bonded to form the microdevice; a top plate with the distribution channel networks connected to inlet 2 or 3, a middle plate with the distribution channel networks connected to inlet 1 and the micronozzle structure (width of 2.5 mm and depth of 50 μm), and a flat bottom plate. There were 3 inlets: inlet 1 for the hydrogel precursor solution with hepatocytes, inlet 2 for that with fibroblasts, and inlet 3 for that without cells but with a higher precursor concentration (spacer solution).

Preparation of cell-encapsulating alginate hydrogel sheets HepG2 and Swiss 3T3 cells were independently suspended in an aqueous solution of 0.3% sodium alginate (NaA; Wako, Osaka, Japan) and 0.9% propylene glycol alginate (PGA; Wako), supplemented with 0.9% NaCl, 0.05% atelocollagen (Koken, Tokyo, Japan), 1% BSA (Rockland Immunochemicals, Gilbertsville, PA, USA), and 10 mM HEPES (Life Technologies, Carlsbad, CA, USA), at concentrations of 3×10^7 and 8×10^7 cells/mL for HepG2 and Swiss 3T3 cells, respectively. As the spacer solution, 2.0% NaA solution supplemented with 0.9% NaCl, 0.05% atelocollagen, 1% BSA, and 10 mM HEPES was used. These cell suspensions and the spacer solution were continuously introduced into the microchannel by using syringe pumps (KDS200, KD Scientific, Holliston, MA, USA). The flow rates of the HepG2 cell suspension, 3T3 cell suspension, and spacer solution were 15, 20, and 25 μL/min, respectively. The flow of the HepG2 cell suspension, introduced from inlet 1, was divided into 16 streams, each of which was sandwiched by flows with the 3T3 cells. The flow of the spacer solution separated the flows of cells. The divided and recombined flows with array patterns were extruded through a thin micronozzle (50 μm × 2.5 mm) into a bath with a gelation solution (20 mM $BaCl_2$ supplemented with 110 mM NaCl and 10 mM HEPES). To obtain straight hydrogel sheets with a uniform thickness, a roller with a

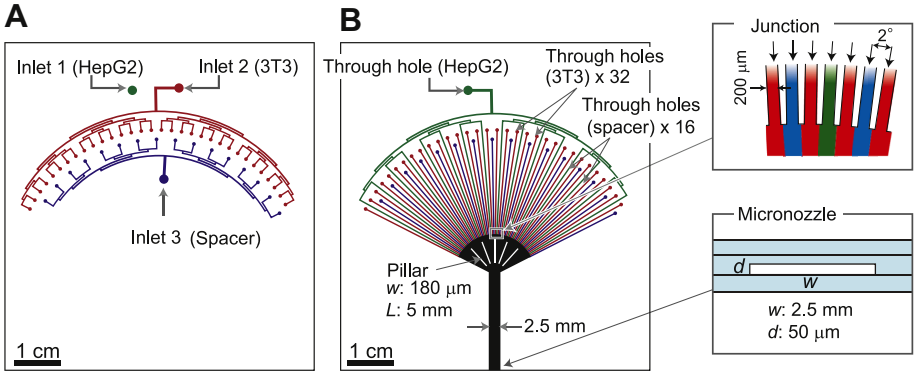

FIG. 2. Microchannel design for preparing stripe-patterned hydrogel sheets. This device was fabricated by layering 3 PDMS plates; a top (A), a middle layer (B), and a flat bottom layer.