NOVEL CELL CULTURING SYSTEM WITH MICROMACHINED TEMPLATE FOR THE REALIZATION OF A COMPLEX ORGAN

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Abstract: Observing the native organ structures, the following three factors have to be satisfied to develop three-dimensional organic living structures in the *in-vitro* regenerative process: (a) appropriate arrangement of any specific cells according to the organ function, (b) appropriate generation of the extracellular matrix, and (c) appropriate induction of capillary blood vessels in the regenerated organ structure. Our group is developing a new culturing system, which could realize all these three factors. The cell culturing chip was fabricated on the silicon wafer with microfabrication techniques. Our immediate target is set to the regeneration of the liver lobule. The mixture of endothelial cells and liver cells is to be cultured on the system where cell culture medium is perfused. The perfused flow through the micro holes punched on the culturing chip would induce the generation of the capillary blood vessels among the cultured liver cells. The eventual goal is to demonstrate that our newly developed culturing system is useful in the regenerative medicine research and that micro fluid flow would induce the generation of capillary blood vessels in the stack of the cultured liver cells.

Introduction

For the creation of three-dimensional complex living organs in the *in-vitro* regeneration process, the following three factors have to be satisfied: (a) appropriate arrangement of any specific cells according to the organ function, (b) appropriate generation of the extracellular matrix, and (c) appropriate induction of capillary blood vessels in the regenerated organ structure. These factors are the environmental modulations due to cell-cell interactions, cell-extracellular matrix interactions, and nutrients delivery and waste removal processes, respectively.

To investigate the cell-cell interactions, the co-culture system with two or more cell types has to be developed and all these different types of cells should be properly disposed in the order of 10–100 μm. For this purpose of the cell patterning, the standard microelectromechanical systems (MEMS) techniques could be applied [1,2]. The co-culture of parenchymal cells with mesenchymal cells could elucidate the cell-extracellular matrix interactions [3,4]. The maximal thickness of the regenerated tissues is limited to several hundred micrometers because of oxygen diffusion limitations without capillary networks [5]. Especially for the regenerated tissues of vital organs of high oxygen consumption, such as heart and liver, the construction of the capillary blood vessels are essential. Furthermore, the fluid-dynamic forces due to the extracellular flow could be the effective stimulation for the cultured cells [6–11]. Therefore, the perfusion culturing system is practical to exert some fluid-dynamic stimulation to the cultured cells.

Our group is developing a new culturing system to fulfill all three factors stated above. The cell culturing chip was fabricated on the silicon (Si) wafer with photolithographic techniques. Our immediate target is set to the regeneration of the liver lobule. The mixture of endothelial cells and hepatocytes is to be cultured on the system where cell culture medium is perfused. The perfused flow through the micro holes punched on the culturing chip would stimulate the cultured cells and induce the generation of the capillary blood vessels among the cultured hepatocytes. The eventual goal is to demonstrate that our newly developed culturing system is useful in the regenerative medicine.

Materials & Methods

Culturing system. The cell culturing system was developed applying the perfusion culturing technique (Figure 1). Cell culture medium was circulated with a tubing pump, and it flowed out from the micro openings on the Si culturing chip of 11 mm in diameter. The Si

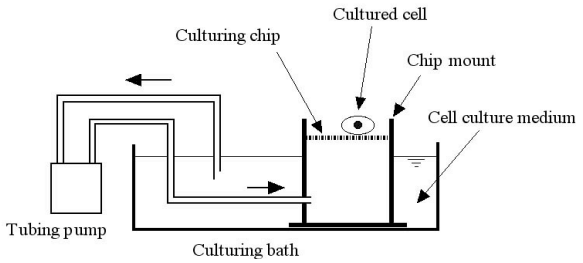

Figure 1: Schematic figure of the perfusion cell culturing system

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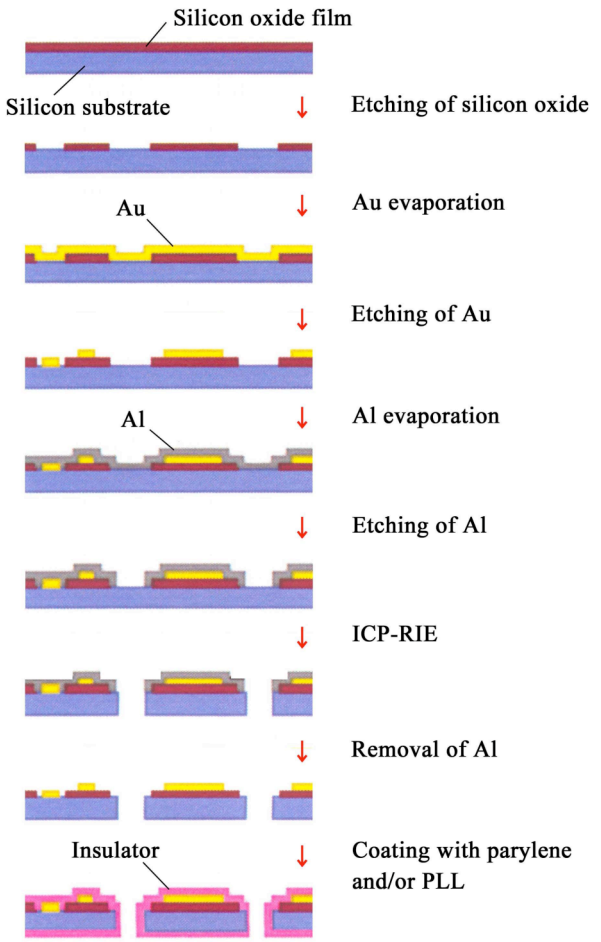

Figure 2: Micromachining process of the culturing chip

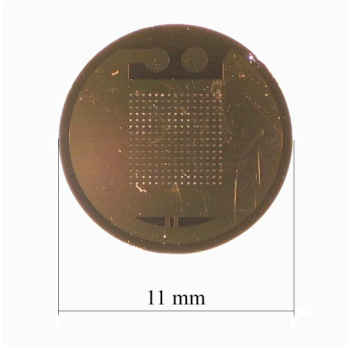

Figure 3: Culturing chip after micromachining

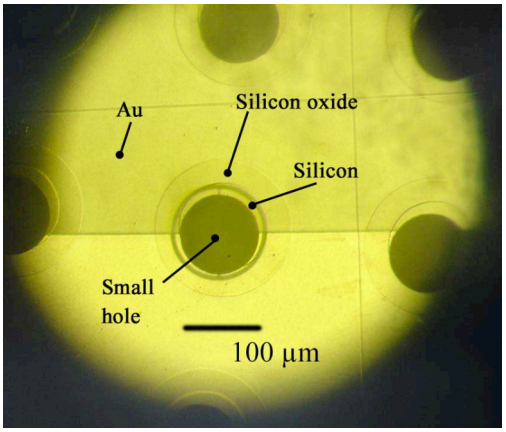

Figure 4: Holes on the culturing chip

chip was fabricated through standard photolithographic techniques (Figure 2). Gold (Au) layer was deposited and patterned on a 2.5-inch thermal-oxidized Silicon wafer. Au could be used as an electrode with the Si substrate. Electric potential would be exerted between Au and Si layers for the manipulation of endothelial cells onto the edge of the micro openings on the Si culturing chip. The holes were penetrated through the wafer using the ICP-RIE (inductively coupled plasma reactive ion etching) technique. A Si culturing chip has 15 rows and 15 columns holes of 100 µm in diameter (Figures 3 and 4). Finally parylene (poly-para-xylylene) and type I collagen coatings were implemented to achieve electric isolation and good cell compatibility, respectively.

Preparation and culturing of cells. First, the toxicity of the Si chip was confirmed using murine melanoma-derived B16-BL6 cells. These cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University, Japan. B16-BL6 cells were cultured and maintained in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) and 1% antibiotic-antimycotic solution (Gibco). For the evaluation of the toxicity of the Si chip, only the parylene coating was conducted without that of type I collagen. B16-BL6 cells were suspended in culturing medium at a concentration of $1x10^4$ cells/ml, and 1.5 ml of this solution was added to the Si chip and 2 ml culture medium was added. B16-BL6 cells were cultured for 3 days with the culturing chip under the static culturing condition. Cells were cultured in the incubator at 37°C containing 5% CO_2. Second, human umbilical vein endothelial cells (HUVECs) were tried for the preliminary research aiming at the co-culture of HUVECs and hepatocytes. HUVECs were provided by Cascade Biologics, Inc (Portland, OR, USA). Cell cultures were maintained with HuMedia-EG2 (KURABOU, Osaka, Japan) containing 2% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor (hEGF), 5 ng/ml human fibroblast growth factor-B (hFGF-B), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 µg/ml amphotericin B and 10 µg/ml heparin. For the culturing of HUVECs, the collagen coating was conducted to the Si culturing chip in addition to the parylene coating. The culturing chip was immersed in 50 µg/ml type I collagen (Nippon Meat Packers, Inc, Osaka, Japan) for 1 hour and rinsed in phosphate buffered saline (PBS). HUVECs were suspended in culturing medium at a concentration of $1x10^5$ cells/ml, 3 ml of this solution was added to the Si chip. After 60 min culturing, 20 ml culture medium was added. HUVECs were cultured with the Si culturing chip for 4 days and photographed using a phase contrast microscope (DIAPHOT, Nikon, Japan) and a CCD camera (PDMC II, Polaroid, USA).

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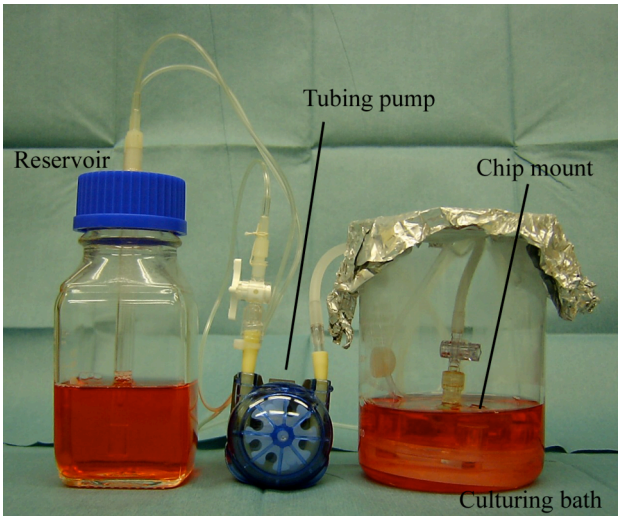

Figure 5: Perfusion culturing system during HUVEC culturing

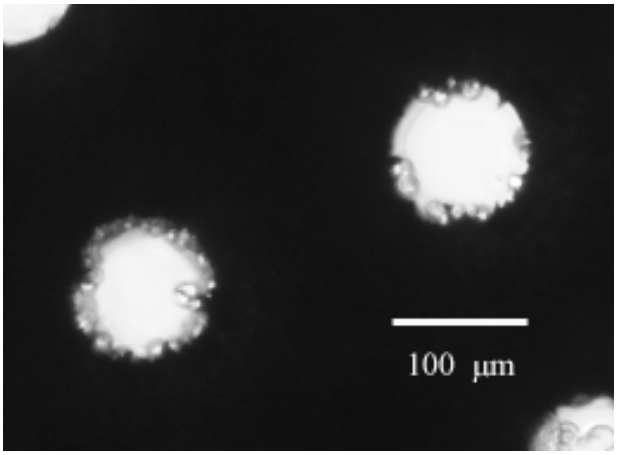

Figure 6: B16-BL6 cells cultured on the developed culturing chip

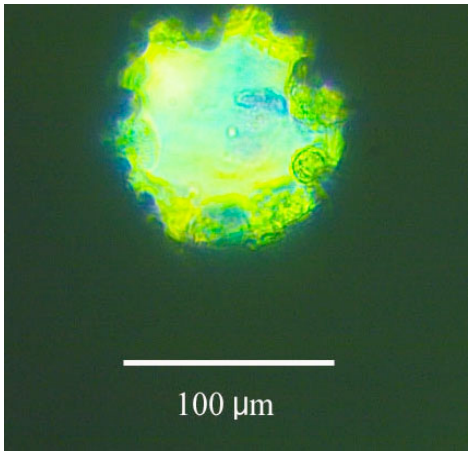

Figure 7: HUVECs cultured on the developed culturing chip

Preliminary results

The developed system is shown in Figure 5. The micromachined Si chip was placed on the chip mount in the perfusion culturing system, and B16-BL6 cells and HUVECs were cultured to confirm the system performance. As shown in Figure 6, B16-BL6 cells were attached on the Si chip, and it shows that the microfabricated Si chip and parylene coatings are nontoxic. Then HUVECs were tried and the result is shown in Figure 7. In this figure, HUVECs were stained with 0.4% trypan blue (Sigma, St.Louis, USA) for 5 min. In this preliminary study, any electric potential was not provided to dispose the cultured cells. HUVECs were, therefore, supposed to spread on the whole surface of the culturing chip as well as on the inner surface of the holes of the chip. At present, the co-culture of HUVECs and hepatocytes has been tried on our newly developed culturing system.

Conclusion and Future work

The new cell culturing system fabricated using MEMS techniques has been developed. This system would realize the co-culture of several different types of cells and also the fluid-dynamic stimulation using the perfusion technique. In this study, the preliminary results of culturing of B16-BL6 cells and HUVECs on our system were confirmed.

As future work, the disposition of HUVECs onto the edge of the micro openings on the Si culturing chip would be tried by providing the electric potential between the Si substrate and the Au layer. Also we are now developing another design of the culturing chip, and the self-assembled monolayers of alkanethiols on the Au layer with fibronectin [12–14] would be applied to this new designed culturing chip for more secure attachment of HUVECs onto the edge of the micro openings.

After confirming the manipulation of HUVECs onto the edge of the micro openings, the mixture of HUVECs and hepatocytes would be cultured. Then the induction of the capillary blood vessels in the cultured hepatic liver cells would be tried and the function of the regenerated hepatic lobule would be evaluated in terms of the viability of cells and its enzyme secretion.

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