Spheroid array of fetal mouse liver cells constructed on a PEG-gel micropatterned surface: upregulation of hepatic functions by co-culture with nonparenchymal liver cells†

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A spheroid array of fetal mouse liver cells, which comprise various immature cells, was constructed on a PEG-gel micropatterned surface and its hepatic activity and degree of differentiation induction were significantly upregulated by co-culture with nonparenchymal liver cells as feeder-cells.

The recent progress in the combination of cell culture and microfabrication technologies has stimulated the research on the development of new methods of cell culturing on chips for medical purposes.[1] Since primary hepatocytes play many important roles in various metabolic pathways *in vivo*, the possibility of using chips covered with hepatocyte arrays in cell-based assay systems as drug screening tools has been investigated.[2,3] However, hepatocytes are well known to lose much of their hepatic functions within the first 2 days of monolayer culturing.[4] Thus, the most crucial issues in cell or tissue culturing are long-term viability, and the upregulation and retention of cell functions on the supporting surface.[5] In order to solve these problems, we focused on the well-known fact that multicellular spheroids exhibit a characteristic *in vivo*-like morphology; this is attributed to the retention of the 3-D architecture and establishment of important cell–cell contacts. Actually, the spheroid patterned array culture of rat primary hepatic cells retains cellular activity for more than one month, if bovine aorta endothelial cells (BAECs) are used as feeder-cell in 100-μm patterned domains.[2,6]

Among the hepatic cells, fetal mouse liver cells (FMLCs) have been studied as a new material for growing artificial livers[7–9] and for liver-cell implantation,[10] because they are regarded as a suitable cell source for implantation and regeneration due to their genetic normality and potentially proliferative activity *in vitro*. Although some researchers have surveyed the hepatic activity of FMLCs in monolayer cultures[9] or 3-D cultures, such as gel encapsulation cultures[7] and cultures in a porous reticulated polyvinyl formal resin,[8] the spheroid formation of FMLCs and the culturing of such spheroids in a two-dimensional array have never been reported.

In this study, we tried to fabricate FMLC spheroid arrays on a PEG-gel micropatterned surface[6,11,12] and evaluate the activity of the FMLCs and the efficiency of the differentiation induction. FMLCs have a lower force of self-assembly than mature liver cells. If the micropatterned culture system applies to fetal liver cells as well as mature liver cells, it may well further the development of regenerative medicine. Interestingly, we found that the FMLCs formed spheroids on the constructed array under optimal experimental conditions, where the upregulation of the hepatic activity was accomplished by co-culture with nonparenchymal cells (NPCs) as feeder cells and the addition of a differentiation induction factor.

The PEG-gel micropatterned surface for fabricating the feeder-cell array was prepared on a glass support by the photolithography technique using a photomask, as shown in Fig. 1a, this is an improvement on our previous method[6,13] (Fig. S1, ESI†). The obtained PEG-gel micropatterned chip shown in Fig. 1b was soaked in PBS overnight prior to use, the exterior of the cavities in the chip was modified with cell-incompatible PEG gel, while the interior of the cavities was lined with cell-compatible glass. In order to construct the substrate-modified surface for fabricating the spheroid array, two

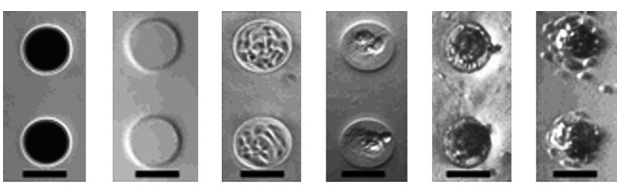

Fig. 1 Phase-contrast micrographs of the photomask, PEG-gel micropatterned surface, feeder-cell patterned arrays and spheroid arrays. (a) Photomask patterns with 100-μm metallic circles on a quartz glass plate. (b) PEG-gel micropatterned surface, where the interior of the cavities is glass surface, while the exterior of the cavities is a PEG-gel modified surface. (c) BAECs and (d) NPCs were arrayed as feeder-cell patterns on the surface (b). FMLCs spheroid arrays constructed on (e) BAECs- and (f) NPCs- patterned arrays at 16 days after seeding the FMLCs. All scale bars represent 100 μm.

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kinds of feeder-cell arrays were prepared on the constructed PEG-gel micropatterned surface using NPCs obtained from fetal mouse and bovine aorta endothelial cells (BAECs). NPCs and BAECs at a concentration of 600 cells/mm^2 were seeded onto the PEG-gel patterned surface, and the adhesion onto the glass area is shown in Figs. 1c and 1d, respectively. These cells were incubated at 37 °C with Dulbecco's modified Eagle's medium (DMEM) in a humidified atmosphere with 5% CO_2. DMEM containing 4,500 mg/L glucose was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Another type of substrate-modified surface was constructed by micropatterning collagen on a PEG-gel micropatterned surface using 0.1 mg/mL collagen solution in 0.02 N acetic acid.

In order to construct the FMLC spheroid arrays, FMLCs were isolated from C57BL/6 fetal mice (14 embryonic days) as described in the ESI†.[13] The FMLCs of mammalians such as a mice, which serve as a hematopoietic organ, comprise hematopoietic cells, progenitor hepatocytes and various types of nonparenchymal cells.[14] Purified FMLCs were suspended in Williams' medium E (WE) supplemented with 10% FBS, 0.1 μM dexamethasone, 0.1 μM insulin, 5,000 KIU/L aprotinin and 1% antibiotic-antimycotic. The FMLCs were seeded at various cell concentrations onto the constructed BAEC-, NPC-, and collagen-micropatterned PEG-gel surfaces. One hundred μL of culture medium supernatant were carefully substituted with the same volume of fresh medium every 2 days without removing the cells. At the 7th day of culture, a fully constructed spheroid array was obtained and the loose FMLCs were removed with all the medium, followed by washing in PBS. With any type of feeder cells, FMLC spheroids with a diameter of approximately 70 μm were formed in about 70% of the cavities (about 800 cavities) when FMLCs at a concentration of 1.1 × 10^5 cells/mm^2 were seeded onto the constructed surfaces (Figs. 1e and 1f). As the condition of the size of cell is 8 μm-diameter is added, the spheroid composed 700 cells. The FMLC cell concentration and culture period for forming spheroids on the constructed chips were approximately 10 times higher and longer, respectively, than those of other hepatic cells such as hepatic cancer cells[6,12] and mature hepatocytes.[2] Oncostatin M (OSM) was used as one of the differentiation induction factors for hepatocytes,[15] and 10 ng/mL OSM were added to the cultured FMLCs on the 1st day. In the case of FMLCs cultured with OSM, WE containing the same concentration of OSM was used for both the cell culture and the spheroid culture. The successful construction of FMLC spheroid arrays was demonstrated by several methods after the 7 days of culture on the PEG-gel surface.

The viability of FMLCs constituting the spheroids was assessed by fluorescent staining assay using SYTO® 10 and DEAD Red™ (Molecular Probes Inc., CA, USA), which are nucleic-acid staining regents for live and dead cells, respectively. Fig. 2 shows differential interference contrast (DIC) micrographs of stained FMLC spheroids at 21 days on BAEC, NPC, and collagen as the substrate in the presence (OSM(+)) and absence of OSM (OSM(−)).Although dead FMLCs were observed to some extent in spheroids with BAECs, almost all of the FMLCs in the constructed spheroids were confirmed to be still alive. Thus, it is concluded that the present microarray cultivation system maintained their viability at least 21 days of culture. Since it is hard to maintain the viability of monolayer-cultured FMLCs beyond a week,[16] these results indicate that the long-term culturing of FMLC was successful in spheroids on all substrate-modified surfaces.

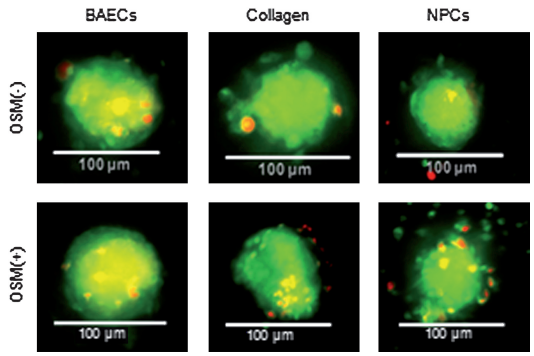

Fig. 2 DIC micrographs of the LIVE/DEAD double-stain assay of the constructed spheroids at 21 days of culture. Each spheroid was prepared on three different types of feeder layer, BAECs, NPCs and collagen with/without OSM.

The amount of albumin secretion, as one of the most important liver functions, from the constructed FMLC spheroid arrays was measured by sandwich enzyme linked immunosorbent assay (ELISA) using a Mouse Albumin ELISA Quantitation Kit according to the manufacture's protocol. The measurements were started from the 7th day of culture, and the cumulative albumin secretions were monitored at 9, 12, 16 and 21 days of culture. Fig. 3 shows the changes in cumulative albumin secretion from the constructed

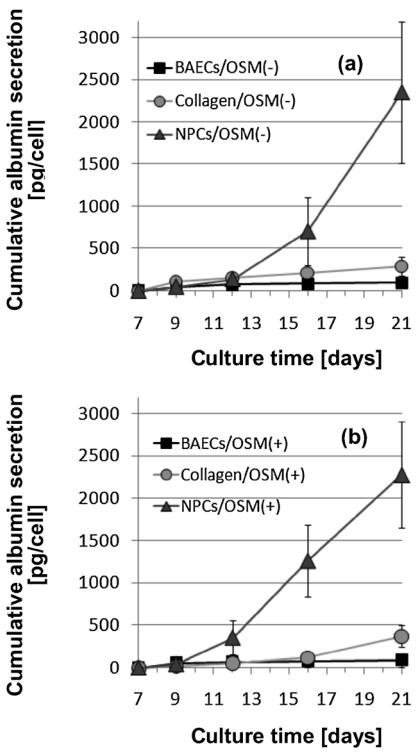

Fig. 3 Cumulative albumin secretion from the FMLC spheroid array on different substrates. The squares shows cultured spheroids on BAECs, the circles shows cultured spheroids on collagen, and the triangles shows cultured spheroids on NPCs. (a) Spheroids cultured without 10 ng/mL OSM (OSM(−), n = 3). (b) Spheroids cultured with OSM (OSM(+), n = 3).

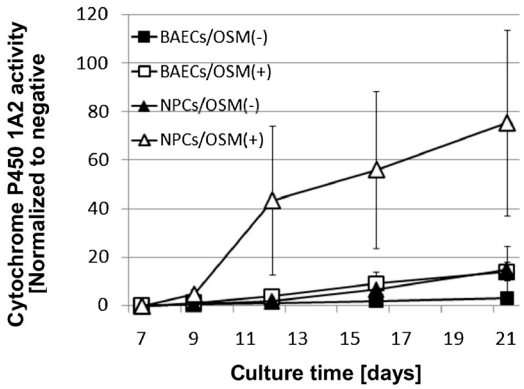

Fig. 4 CYP1A2 activity of FMLCs obtained from a spheroid array. The vertical axis represents the CYP1A2 activity normalized to negative control values ($n = 3$). The squares shows FMLCs derived from spheroids on BAECs, while the triangles shows FMLCs derived from spheroids on NPCs. The closed and open marks show OSM(−) and OSM(+), respectively.

FMLC spheroid arrays on different substrates. Fig. 3a shows the result in the absence of OSM, and the FMLC spheroid array on the NPCs showed a steadily large amount of albumin secretion, while that on the BAECs showed almost no secretion for the entire 21-day culture period. The FMLC spheroid array on collagen also showed a low amount of albumin secretion, at the same level as that on BAECs. The same tendency of albumin secretion level was observed in the presence of OSM, as shown in Fig. 3b. These results indicate that the amount of albumin secretion from FMLC spheroids on a PEG-gel micropatterned surface can be enhanced by using NPCs as the feeder-cell. Additionally, OSM did not regulate the amount of albumin secretion from the constructed spheroid arrays. The liquidity factor of the feeder cells might correlate to the differences in the albumin secretion activity. Almost no albumin secretion from the feeder layers and the FMLC monolayer cultured on a flat surface at the same cell concentration was confirmed (Fig. S2, ESI†).

In order to assess the differentiation induction of the constructed FMLC spheroid arrays, the cytochrome P450 (CYP) 1A2 activity, which appears only in the mature hepatocytes of mice or humans,[17] was evaluated using a P450-Glo™ Assays Kit and monitored at 9, 12, 16 and 21 days of culture, starting on the 7th day. Fig. 4 shows the CYP1A2 activity of the FMLC spheroids on two types of feeder cells in the presence and absence of OSM. In the case of the spheroid array with the BAECs as the substrate, almost no CYP1A2 activity was observed, although the activity of the spheroids with OSM (BSECs/OSM(+)) was slightly higher than that without OSM (BSECs/OSM(−)). Interestingly, in the case of the spheroids with NPCs as the substrate, the activity of the spheroids with OSM (NPCs/OSM(+)) was extremely high, while that of the spheroids without OSM (NPCs/OSM(−)) was low, at almost the same level as the spheroids with BAECs/OSM(+). These results indicate that NPCs and OSM can upregulate the CYP1A2 activity of the FMLC spheroids on the constructed arrays, indicating that constructed FMLC spheroid arrays have undergone a high degree of differentiation.

In conclusion, we succeeded in constructing a two-dimensional array of FMLC spheroids with uniform size and number on a PEG-gel micropatterned surface. The FMLC spheroids on the constructed array showed long-term viability and high hepatic activity. Especially, co-culturing with NPCs upregulated the hepatic activity of the FMLC spheroids. This novel cell-chip technology and the findings in this study could provide an interesting new approach to the construction of tissues and organs for regenerative medicine.

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Notes and references

1 (*a*) M. Rieke, E. Gottwald, K. F. Weibezahn and P. G. Layer, *Lab on a chip*, 2008, **8**, 2206–2213; (*b*) D. Kloss, M. Fischer, A. Rothermel, J. C. Simon and A. A. Robitzki, *Lab on a chip*, 2008, **8**, 879–884; (*c*) D. Falconnet, G. Csucs, H. Grandin and M. Textor, *Biomaterials*, 2006, **27**, 3044–3063.
2 H. Otsuka, A. Hirano, Y. Nagasaki, T. Okano, Y. Horiike and K. Kataoka, *ChemBioChems*, 2004, **5**, 850–855.
3 (*a*) S. D. Alvarez, A. M. Derfus, M. P. Schwartz, S. N. Bhatia and M. J. Sailor, *Biomaterials*, 2009, **30**, 26–34; (*b*) C. M. Nelson, J. L. Inman and M. J. Bissell, *Nature protocols*, 2008, **3**, 674–678.
4 A. P. Li, D. J. Beck and S. M. Colburn, *In Vitro Cell. Dev. Biol. A*, 1992, **28**, 673–677.
5 E. LeCluyse, P. Bullock and A. Parkinson, *Advanced Drug Delivery Reviews*, 1996, **22**, 133–186.
6 M. Ichino and Y. Nagasaki, *Photopolymer Science and Technology*, 2006, **19**, 451–454.
7 G. Underhill, A. Chen, D. Albrecht and S. Bhatia, *Biomaterials*, 2008, **28**, 256–270.
8 T. Ehashi, H. Miyoshi and N. Ohshima, *J. Cellular Physiology*, 2005, **202**, 698–706.
9 Y. Sakai, J. Jiang, N. Kojima, T. Kinoshita and A. Miyajima, *Cell Transplant*, 2002, **11**, 435–441.
10 I. J. Fox and J. Roy-Chowdhury, *J. Hepatology*, 2004, **40**, 878–886.
11 H. Moeller, M. K. Mian, S. Shrivastava, B. G. Chung and A. Khademhosseini, *Biomaterials*, 2008, **29**, 752–763.
12 K. Yoshimoto, M. Ichino and Y. Nagasaki, *Lab on a Chip*, 2009, **9**, 1286–1289.
13 H. Miyoshi, T. Ehashi, H. Ema, H. Hsu, H. Nakauchi and N. Ohshima, *ASAIO J.*, 2000, **46**, 397–402.
14 M. Nitou, Y. Sugiyama, K. Ishikawa and N. Shiojiri, *Exp. Cell Res.*, 2002, **279**, 330–343.
15 T. Hamazaki, Y. Iiboshi, M. Oka, P. Papst, A. Meacham, L. Zon and N. Terada, *FEBS Lett.*, 2001, **497**, 15–19.
16 H. Yu, B. Bauer, G. K. Lipke, R. L. Phillips and G. Van Zant, *Blood*, 1993, **81**, 373–384.
17 (*a*) D. Choudhary, I. Jansson, I. Stoilov, M. Sarfarazi and J. Schenkman, *Archives of Biochemistry and Biophysics*, 2005, **436**, 50–61; (*b*) T. Kinoshita and A. Miyajima, *Biochim. Biophys. Acta*, 2002, **1592**, 303–312.