Dynamic 3D micropatterned cell co-cultures within photocurable and chemically degradable hydrogels

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Abstract

In this paper we report on the development of dynamically controlled 3D micropatterned cellular co-cultures within photocurable and chemically degradable hydrogels. Specifically, we generated dynamic co-cultures of micropatterned murine embryonic stem (mES) cells with human hepatocellular carcinoma (HepG2) cells within 3D hydrogels. HepG2 cells were used due to their ability to direct the differentiation of mES cells through secreted paracrine factors. To generate dynamic co-cultures, mES cells were first encapsulated within micropatterned photocurable poly(ethylene glycol) (PEG) hydrogels. These micropatterned cell-laden PEG hydrogels were subsequently surrounded by calcium alginate (Ca-Alg) hydrogels containing HepG2 cells. After 4 days, the co-culture step was halted by exposing the system to sodium citrate solution, which removed the alginate gels and the encapsulated HepG2 cells. The encapsulated mES cells were then maintained in the resulting cultures for 16 days and cardiac differentiation was analyzed. We observed that the mES cells that were exposed to HepG2 cells in the co-cultures, generated cells with higher expression of cardiac genes and proteins as well as increased spontaneous beating. Due to its ability to control the 3D microenvironment of cells in a spatially and temporally regulated manner the method presented in this study is useful for a range of cell culture applications related to tissue engineering and regenerative medicine.

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Keywords

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1. Introduction

In the body, living cells communicate with each other in a microstructured 3D environment in response to soluble factors, extracellular matrix (ECM) molecules and intercellular contact dependent signals. Therefore, cell culture on 2D substrates, which has been employed in conventional biological research, does not adequately recapitulate the 3D nature of native cellular microenvironment (Pampaloni et al., 2007; Tibbitt and Anseth, 2009). In addition, spatially uniform and static materials lack the intricate spatial and temporal aspects of *in vivo* systems. Cells dynamically respond to the local microenvironment during diverse processes such as tissue morphogenesis, stem cell differentiation, cancer progression, and wound healing (Daley et al., 2008; Lopez et al., 2008). Therefore, recapitulating such dynamic microenvironments *in vitro* would have high potential impact in cell biology by providing an excellent model for systematic differentiation of stem cells and for understanding of tissue regeneration, ultimately leading to more rational tissue engineering strategies.

In the context of 3D microenvironment, one of the most important issues for stem cell differentiation is intercellular interaction including secreted soluble factors and contact dependent signals. Traditionally, co-culture systems have been employed to maintain cell function or to direct stem cell differentiation into desired cell types (Allon et al., 2012; Bigdeli et al., 2009; Cho et al., 2008; Fukumitsu et al., 2009; Hendriks et al., 2007; Lee et al., 2008; Ma et al., 2009; Seto et al., 2012). Microfabrication technologies have been used for generating patterned co-cultures for controlling intercellular interaction in the 2D icroenvironment (Kaji et al., 2011; Khetani and Bhatia, 2008; Trkov et al., 2010). In addition, a umber of methods have been developed to dynamically control intercellular interaction on 2D surfaces (Hui and Bhatia, 007; Jiang et al., 2003; Wright et al., 2007). However, none of these techniques can be applied to dynamic control 3D microenvironments.

Recently, several techniques have been reported to generate 3D microfabricated hydrogels (Billiet et al., 2012; Chung et al., 2012; Guillame-Gentil et al., 2010; Huang et al., 2011; Inamdar and Borenstein, 2011; Khetan and Burdick, 2011; Zorlutuna et al., 2012). For example, photolithography and stereolithography that utilize photocurable materials have been applied to construct hydrogels with 3D microarchitecture (Aubin et al., 2010; Chan et al., 2010; Hammoudi et al., 2010; Khetan and Burdick, 2010; Nichol et al., 2010; Qi et al., 2010; Zorlutuna et al., 2011). Alternatively, microfluidic devices have been used to fabricate microscale hydrogels such as particles (Dendukuri et al., 2006; Kim et al., 2011), microcapsules (Sugiura et al., 2007; Sugiura et al., 2005; Tan and Takeuchi, 2007), microfibers (Lee et al., 2010a; Shin et al., 2007; Yamada et al., 2012), and microtubes (Sugiura et al., 2008). Using these blocks, higher order structures were constructed by spontaneous assembly (Du et al., 2008; Gartner and Bertozzi, 2009; Nichol and

Khademhosseini, 2009), guided assembly (Chung et al., 2008; Lee et al., 2010b), hydrodynamic assembly (Bruzewicz et al., 2008), and molding (Matsunaga et al., 2011) of cells and hydrogels.

Stimuli-responsive hydrogels that utilize chemicals, light or heat stimulation are potentially applicable to dynamically control the 3D cellular microenvironment. For instance, Gillette *et al.* have reported a method to dynamically modify the structural properties of natural 3D ECM using calcium ion responsive alginate (Gillette et al., 2010). In addition, Anseth *et al.* have reported the use of photodegradable poly (ethylene glycol) (PEG) hydrogels for spatiotemporal control of 3D microenvironment (DeForest and Anseth, 2012; Kloxin et al., 2009; Kloxin et al., 2010). Despite these advantages the development of simple systems that avoid the need for advanced materials will be beneficial for the widespread use of this technology.

In this paper, we propose chemically degradable calcium alginate (Ca-Alg) hydrogel as biocompatible, simple and cheap material for dynamic control of 3D co-cultures. We applied our dynamic 3D micropatterning system to the co-culture of murine embryonic stem (mES) cells with human hepatocellular carcinoma (HepG2) cells (Fig. 1a), which is known to regulate the early stage differentiation of mES cells (Lake et al., 2000; Rathjen et al., 1999; Rathjen and Rathjen, 2003). The mES cells were encapsulated in micropatterned photocurable PEG hydrogels. Ca-Alg hydrogel containing HepG2 cells was then formed around the cell-laden PEG hydrogels. The micropatterned encapsulated mES cells and HepG2 cells were co-cultured for 4 days as a 1^{st} step in the dynamic culture system to induce mesoderm formation (Fig. 1b). Subsequently, the 3D co-culture microenvironment around the mES cells was dynamically changed such that the Ca-Alg hydrogel was degraded by exposure to sodium citrate solution and the HepG2 cells were removed. Then the resulting mES cells were cultured without HepG2 cells for 16 days as a 2^{nd} step to induce cardiac differentiation (Fig. 1c). Using this 3D co-culture platform, we investigated the effect of the dynamic co-culture on the differentiation of mES cells into cardiac lineage in comparison with continuous co-culture of the mES cells with HepG2 cells, and continuous monoculture of the mES cells without any HepG2 cell addition.

2. Materials and Methods

2.1. Materials

Sodium alginate, sodium citrate, and poly-D-lysine (Mw = 30–70 kDa) were purchased from SigmaAldrich (Wisconsin, USA). 4arm-PEG acrylate (Mw = 20 kDa) was obtained from Jenkem (Allen, USA). Photomasks were printed by CADart (Washington, USA). Light irradiation equipment was obtained from EXFO Photonic Solutions Inc. (Ontario, Canada) using a UV light source (Omnicure S2000).

2.2. Cell culture

Wild type mES cell line (R1) was cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM; Gibco) that was supplemented with 10% (v/v) ES cell qualified fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Sigma), and 1,000 U/ml of leukemia

inhibitory factor (Chemicon). The medium for mES cells was changed every day and the cultures were passaged every 2 to 3 days. HepG2 cells were maintained in DMEM supplemented with 10% FBS. The medium for HepG2 cells was changed every 3 to 4 days. The HepG2 cells were passaged upon reaching 90% confluency. All cells were handled under sterile tissue culture hoods and cultivated in a 5% CO_2 incubator at 37 °C.

2.3. Cell encapsulation and dynamic 3D micropatterned co-culture

mES cells were encapsulated in micropatterned PEG hydrogels on glass slides by photoinduced polymerization of acrylated PEG (Fig. 1b) (Du et al., 2008; Yanagawa et al., 2011). mES cells were trypsinized and suspended in 10% 4arm-PEG acrylate prepolymer solution containing 0.1% photoinitiator, 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure2959; CIBA Chemicals) in DMEM, at a concentration of 1×10^8 cells/ml. In order to enhance hydrogel attachment on the glass slides and to prohibit cell adhesion on to the glass slides, we coated glass slides with poly-D-lysine and sodium alginate. First, 500 μl of 5 μg/ml poly-D-lysine solution was placed on 25 × 25 mm cut glass slides, and then dried at 80 °C. After drying, 500 μl of 5 μg/ml sodium alginate solution in 0.1 mM HEPES buffer was placed on the glass slides coated with poly-D-lysine, and then dried at 80 °C. This double coated polymer layer presumably supports both of the PEG and Ca-Alg hydrogels due to the formation of interpenetrating polymer network during photopolymerization and electrostatic crosslinking with calcium ion, respectively. Also, negatively charged alginate layer prohibits the adhesion of the cells on to the glass slides. PEG hydrogel micropatterns (500 μm diameter × 300 μm height) containing mES cells were fabricated on the coated glass slides by exposing the prepolymer solution containing the cells to 13 mW/cm^2 UV light for 60 sec as previously described (Du et al., 2008). The hydrogels on the glass slides were washed with DMEM and then incubated in DMEM containing 10% FBS.

HepG2 cells were encapsulated in Ca-Alg hydrogels around PEG hydrogels containing mES cells on glass slides (Fig. 1b). HepG2 cells were trypsinized and resuspended in 1.5% sodium alginate solution in 1:1 mixture of DMEM and HEPES buffered saline (HBS) at a concentration of 2.5×10^7 cells/ml. Then, 50 μl of the cell suspension was added onto the PEG hydrogels and covered with a dialysis membrane (MWCO 12 to 14 kD, Spectrum Laboratories, Inc.). To this setup, we added 400 μl of 100 mM calcium chloride solution in HBS on the top of the dialysis membrane. After 1 min, the dialysis membrane was removed and the Ca-Alg hydrogels were soaked in 3 ml of calcium chloride solution for 10 min at 37 °C. The PEG and Ca-Alg hydrogels on the glass slides were washed with DMEM and incubated in DMEM containing 10% FBS. The encapsulated mES and HepG2 cells were cultured in a 6 well plate for 4 days as a 1^{st} step culture to induce early stage differentiation.

After the 1^{st} step culture, the Ca-Alg hydrogel was degraded by exposure to 50 mM sodium citrate solution in DMEM for 10 min at 37 °C. The PEG hydrogels on the glass slides were washed with alpha Minimal Essential Medium (α-MEM; Gibco) and incubated in α-MEM containing 15% (v/v) ES cell qualified FBS to induce cardiac differentiation. mES cells were subsequently cultured without HepG2 cells for an additional 16 days as a 2^{nd} step

culture (Fig. 1c). During this step, half volume of the medium in each well was changed on day 8 and on every day after day 10.

Dynamic 3D micropatterned co-culture experiment was carried out in comparison with continuous co-culture of the mES cells encapsulated in the PEG hydrogels with the HepG2 cells encapsulated in the Ca-Alg hydrogel, and continuous monoculture of the mES cells encapsulated in the PEG hydrogels without any HepG2 cell addition.

2.4. Evaluation of cell micropatterning and viability

To visualize the cultures during the dynamic 3D micropatterned co-culture, mES and HepG2 cells were stained with CellTracker Green and CellTracker Red (Invitrogen), respectively. For staining with CellTracker, the cells under adhesion culture condition were treated with CellTracker working solution of 40 and 20 μM for Green and Red, respectively, for 30 min. After washing with DMEM, the stained cells were incubated under growth conditions. A Live/Dead assay kit (Invitrogen) with calcein-AM/ethidium homodimer was used to quantify cell viability in the hydrogels. Images of the encapsulated cells were taken using a Nikon TE 2000U camera with a SPOT advanced software (SPOT Imaging Solutions).

2.5. Gene expression analysis by RT-PCR

Total RNA from mES cells was extracted using RNeasy Plus Mini Kit and QIAshredder (Qiagen). Following RNA extraction, primary cDNA synthesis and PCR amplification were performed by using SuperScript III One-Step RT-PCR System with Platinum Taq kit (Invitrogen). Primary cDNA was synthesized with 40 ng extracted RNA under 55 °C for 20 min. PCR amplification was carried out under the following conditions: 30 sec denaturing at 94 °C, 30 sec specific primer annealing temperature, and 45 sec extension at 68 °C in a PTC-100TM thermal cycler (MJ Research Inc). 1.2% (w/v) agarose gels with 0.4 μg/ml ethidium bromide were used to analyze the PCR products. Detailed information on the sequence of the primers and product sizes are described in Table S1.

2.6. Immunocytochemical staining and quantification

The hydrogel samples containing differentiated mES cells were fixed in 4% (v/v) paraformaldehyde (Sigma) for 45 min, washed three times with phosphate buffered saline (PBS), and then permeabilized with 0.1% Triton X-100 (Sigma). The nonspecific binding was blocked by incubation with 10% (v/v) goat serum (Sigma) in PBS. The primary antibodies, anti-sarcomeric-α-actinin (Abcam), and anti-cardiac-troponin I (Abcam) was diluted as 1:100 in 10% goat serum solution and incubated at 4 °C overnight. The secondary AF488- or AF594- conjugated antibodies (Abcam) were incubated at 4 °C overnight. Cell nuclei were counterstained by 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma). Fluorescence images of the immunostained samples were taken using the Nikon TE 2000U camera and a SPOT advanced software.

2.7. Evaluation of beating activity

In the study of cardiac differentiation, the PEG hydrogels containing beating colonies were counted on days 10, 12, 14, 16, 18 and 20. The hydrogels containing the beating colonies were counted throughout the entire area of the glass slides and percentage of the hydrogels

containing the beating colonies was calculated. Data were statistically analyzed using ANOVA and Bonferroni's post-hoc test by using a Prism 5.04 software (GraphPad).

3. Results

3.1. Dynamic 3D micropatterned co-culture

Dynamic 3D micropatterned co-cultures of mES cells with HepG2 cells were demonstrated using cells that were stained with cell tracker dyes (Fig. 2a). As it can be seen, mES cells (stained with CellTracker Green)were encapsulated in the micropatterned PEG hydrogels and were surrounded by HepG2 cells (stained with CellTracker Red)encapsulated in a layer of alginate hydrogel (Fig. 2a, Day 0). After 4 days, the Ca-Alg hydrogel was degraded by exposure to sodium citrate solution, and removed from the microarray of the PEG hydrogels containing the mES cells, demonstrating the dynamic control of the 3D co-culture microenvironment around the mES cells (Fig. 2a, Day 4). Dynamic 3D micropatterned co-culture of mES cells with HepG2 cells was successfully carried out without cross-contamination between the two cell types. As shown in Fig 2a, most HepG2 cells surrounding the PEG hydrogels were removed without disturbing the mES cells encapsulated within PEG hydrogels.

The encapsulated mES and HepG2 cells were highly viable after encapsulation in the PEG and alginate hydrogels, respectively (Fig. 2b, Day 0). The viability of mES cells was well maintained while proliferating, migrating, and forming cellular aggregates in the PEG hydrogels during 4 days of the co-culture period, and after degradation of the Ca-Alg hydrogel (Fig. 2b, Day 4).

3.2. Early stage differentiation of mES cells within 3D micropatterned co-culture

We investigated the effect of 3D micropatterned co-cultures on the early stage differentiation of mES cells in comparison with monoculture conditions (Fig. 3). Morphological differences were evident within 4 days between the co-culture and monoculture conditions. The mES cells in the co-culture conditions proliferated more than the cells cultured in the monoculture conditions, and formed embryonic body (EB)-like cell aggregates in the PEG hydrogels (Fig. 3a).

To study the effect of the co-cultures on early stage differentiation of mES cells, we analyzed gene expression of mES cells in the 3D micropatterned co-culture and monoculture on days 2 and 4 and compared the results with the undifferentiated mES cells as a control (Fig 3b). In both conditions, *Oct4* expression was downregulated over time with the co-culture group showing faster downregulation of *Oct4* gene compared to the monoculture group. Furthermore, primitive ectoderm marker, fibroblast growth factor 5 gene (*Fgf5*), which was barely detectable in mES cells, was expressed in the co-culture group from day 2, indicating that the early stage differentiation of mES cells occurred in the co-culture group earlier than in the monoculture group. It is known that *Fgf5* expression is upregulated upon the formation of primitive ectoderm from the inner cell mass (Haub and Goldfarb, 1991; Hebert et al., 1991), and the soluble factors secreted from HepG2 cells induce the conversion of mES cells to early primitive ectoderm-like cells (Rathjen et al., 1999; Rathjen

et al., 2003). Similarly, our results indicate that the co-culture of mES cells with HepG2 cells efficiently induces the early stage differentiation of mES cells.

3.3. Cardiac differentiation of mES cells through dynamic 3D micropatterned co-culture

We investigated the effect of dynamically exposing the mES cells to HepG2 cells on cardiac differentiation in comparison with continuous co-culture and monoculture conditions (Fig. 4a). Morphologically, we observed higher growth rates of mES cells in the dynamic and continuous co-cultures than that in the monoculture conditions (Fig. 4b) possibly due to the higher growth rate shown in the 1st step culture. In addition, mES cells in the dynamic co-culture and monoculture condition tended to grow out of the PEG hydrogels during the 2nd step culture (Fig. 4b). In contrast, mES cells in the continuous co-culture were retained within the PEG hydrogels that remained physically surrounded by the Ca-Alg hydrogel.

We examined the extent of cardiac differentiation of mES cells in various culture conditions by evaluating the gene expression of cardiac markers *Gata4* and *Nkx2.5* on days 4, 8, and 16 (Fig. 4c). In general, we observed an earlier expression of *Gata4* in the co-culture conditions compared to the monoculture conditions on day 4. Furthermore, enhanced expression of *Gata4* was found in all three culture conditions on days 8 and 16. We also observed enhanced expression of *Nkx2.5* in all three culture conditions over the time period.

To assess the tendency of the cells in the culture to exhibit cardiac cell function, we quantified the number of hydrogels that contained beating colonies. Beating colonies were visible in the dynamic and continuous co-culture conditions after day 12 but not in the continuous monoculture group (Movie S1 in Supporting Information). Some hydrogels contained multiple beating colonies in a single hydrogel unit (Movie S2 in Supporting Information). Among three different culture conditions, the highest number of beating colonies were observed in the dynamic co-culture condition on days 12 and 14 (Fig. 4d).

4. Discussions

J. Rathjen *et al*. (Rathjen et al., 1999) reported that conditioned medium derived from HepG2 cells stimulated the transition of mES cells to a cell population considered as the second pluripotent stage holding similar behavior to primitive ectoderm cells *in vivo* (Rathjen and Rathjen, 2001). After mES cells were treated with HepG2-conditioned medium, EB suspension culture was conducted using EB differentiation medium, resulting in the formation of multipotent mesodermal progenitors at the expense of ectoderm (Rathjen and Rathjen, 2001; Rodda et al., 2002). In this study, this methodology for selective early mesoderm formation was adopted to facilitate cardiogenic tissue generation by using our 3D dynamic co-culture system. In the 1st step culture, mES cells surrounded by the HepG2 cells were shown to spontaneously differentiate into primitive ectoderm-like cells expressing the *Fgf5* gene with downregulation of *Oct4* gene as shown in the previous study (Rathjen and Rathjen, 2001). Furthermore, the co-cultured group showed faster growth rate than that of monoculture group, which is also supportive of the formation of the primitive ectoderm, where rapid cell proliferation occurs before the gastrulation process (Pelton et al., 2002; Snow and Bennett, 1978). This result could lead to effectively triggering differentiation, in

that cell to cell contact is one of the crucial factors to derive pluripotent stem cells to the next advanced stage (Smith, 1992).

In the 2nd step of culture for triggering cardiogenesis of 3D-cultured cells, the gene expression profiles of *Gata4* and *Nkx2.5* displayed that cardiac lineage differentiation appeared to progress in all three culture conditions over the culture time period. However, significantly higher numbers of beating colonies were measured in the dynamic co-culture group. High expressions of *Gata4* and *Nkx2.5* in the monoculture condition are apparently contradictory to the low numbers of beating colony in the hydrogels, which were not even observed after day 14. This may be related to the low growth rate of the mES cells in the monoculture condition which could lead to a lack of abundant cell to cell contacts to function properly as a tissue. Likely, the continuous co-culture resulted in the lower possibility to contain beating colonies compared to the dynamic co-culture. Our observation led us to speculate the reason that the depletion of nutrients and oxygen, or accumulation of metabolic waste could occur in the continuous co-culture after day 10 in that mES cells were grown with being surrounded by the hydrogel fully packed with HepG2 cells that were also growing till the end of culture period. In addition, growth of mES cells into the outside of the PEG hydrogel in the dynamic co-culture during the 2nd step culture presumably induced more opportunity for the formation of beating tissue outside of the PEG hydrogel, while mES cells in the continuous co-culture continuously stayed in the PEG hydrogel because of the surrounding Ca-Alg hydrogel (Fig. 4b).

The cardiac differentiation of mES cells was confirmed by analyzing protein expression on day 16 (Fig. 5, enlarged images are also available as Fig. S1). We observed strong expression of sarcomeric α-actinin and cardiac troponin I in the dynamic and continuous co-culture conditions. Actin network formation was observed in the dynamic and continuous co-culture conditions. In contrast, we also observed expression of sarcomeric α-actinin and cardiac troponin I in the monoculture conditions; however, the density of the cells was very low and crosslinked actinin network was not observed in the monoculture conditions. This was probably due to the low number of cells after the 1st step culture. Even though the mES cells in the monoculture conditions differentiated into cardiac lineage as evidenced in the gene and protein expression, the low cell density after the 1st step culture probably resulted in significantly lower possibility to contain beating colony in the monoculture conditions in the late stage of 2nd step culture. As a comprehensive interpretation, the highest possibility to contain beating colonies in the dynamic co-culture can be explained by the two distinct factors: the soluble factor secreted from HepG2 cells in the 1st step culture and the removal of the Ca-Alg hydrogel containing HepG2 cells in the 2nd step culture.

We have demonstrated dynamic 3D micropatterned co-culture using photocurable PEG and chemically degradable Ca-Alg hydrogels. This platform is a useful experimental tool for investigating dynamic intracellular interaction in 3D culture conditions. One possible limitation of the proposed method is a lower probability for direct cell to cell contact in the hydrogel polymer network, which may restrict the migration of encapsulated cells. However, we minimized this limitation by encapsulating mES cells in high molecular weight PEG-4 arm-acrylate hydrogels, in which mES cells can form cellular aggregate (Schukur et al., 2013). The use of the other porous hydrogels with large molecular weight of

main polymer and low crosslinking density, in which cells can migrate, can be an alternative solution to address this issue.

Another potential limitation of the present system is the inertness of PEG gels, which limit the extent of cell-material interactions. It is known that processes such as cell adhesion, elongation, and differentiation are highly dependent on the properties of the encapsulating material. Thus the use of other photocurable hydrogels with enhanced biological properties (e.g. gelatin methacrylate (GelMA)) may be a solution to enhance such interactions (Anderson et al., 2004; Edalat et al., 2012; Nichol et al., 2010). In addition, for dynamic degradation of hydrogels, various degradation methods are available, including biological degradation (Lutolf and Hubbell, 2005), enzymatic degradation (Yamada et al., 2012), hydrolytic degradation (Li et al., 2011), and photodegradation (Kloxin et al., 2009). Use of these degradation methods in dynamic 3D micropatterned co-cultures creates new opportunities to recapitulate the dynamics in the body as more physiological model for dynamic control of 3D extracellular microenvironment.

5. Conclusions

We have developed dynamic 3D micropatterned co-cultures by using encapsulating cells within photocurable PEG and chemically degradable Ca-Alg hydrogels. This platform was applied to dynamically co-culture mES cells with HepG2 cells for cardiac differentiation. The photocurable PEG hydrogel enabled micropatterned encapsulation of mES cells, i.e. spatial control of 3D culture conditions, and chemically degradable hydrogel enabled changing the co-culture conditions at the specific time point, i.e. temporal control of 3D co-culture conditions. We observed that our dynamic co-culture system facilitated the early stage differentiation of mES cells during the 1st step culture, resulting in higher cardiac functionality shown after 2nd step culture. We believe that the proposed method is a potentially convenient approach to engineer the complexity of cell-cell interactions in 3D tissue construct in a spatially and temporally regulated manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SS, JMC, PZ, HB, and AK planned the research. SS and PZ designed the experimental protocol on the material chemistry, and SS, JMC, and HB designed experimental protocol on the biological part. SS and FY performed all experiments. SS, JMC, and FY processed and analyzed the data. SS and AK wrote the paper and AK supervised the research. All authors revised the manuscript and agreed on its final contents. This paper was supported by the Institute for Soldier Nanotechnology, National Institutes of Health (HL092836, DE019024, EB012597, AR057837, DE021468, HL099073), the National Science Foundation (DMR0847287), and the Office of Naval Research Young Investigator award. We thank Dr. Gulden Camci-Unal, Lina Schukur, Dr. Sang Bok Kim for their technical support.

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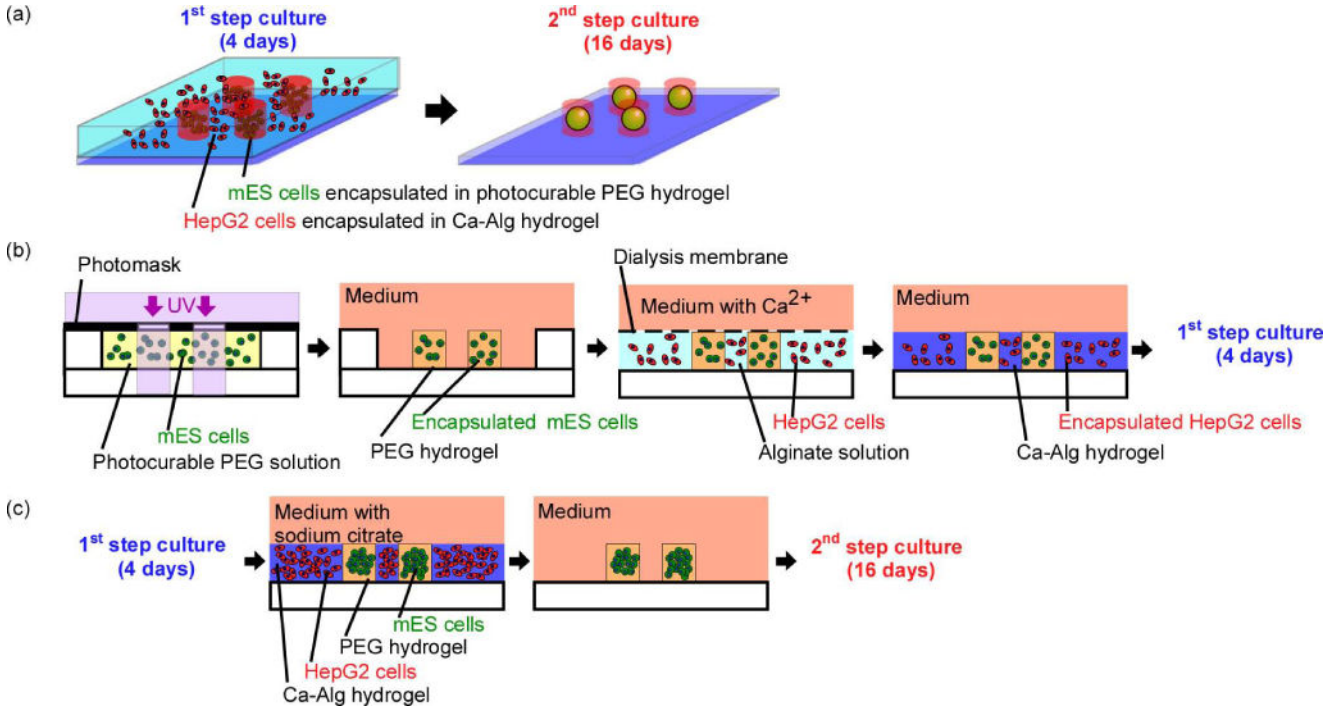

Fig. 1.

Schematic of the process for dynamic 3D micropatterned co-culture of cell-laden gels. (a) Schematic of the dynamic 3D micropatterned co-culture of mES cells in PEG hydrogel and HepG2 cells in alginate hydrogel. (b) Process for encapsulation of mES cells in PEG hydrogels in co-culture with HepG2 cells encapsulated in Ca-Alg hydrogel. (c) Process for degradation of Ca-Alg hydrogels, and removal of the HepG2 cells.

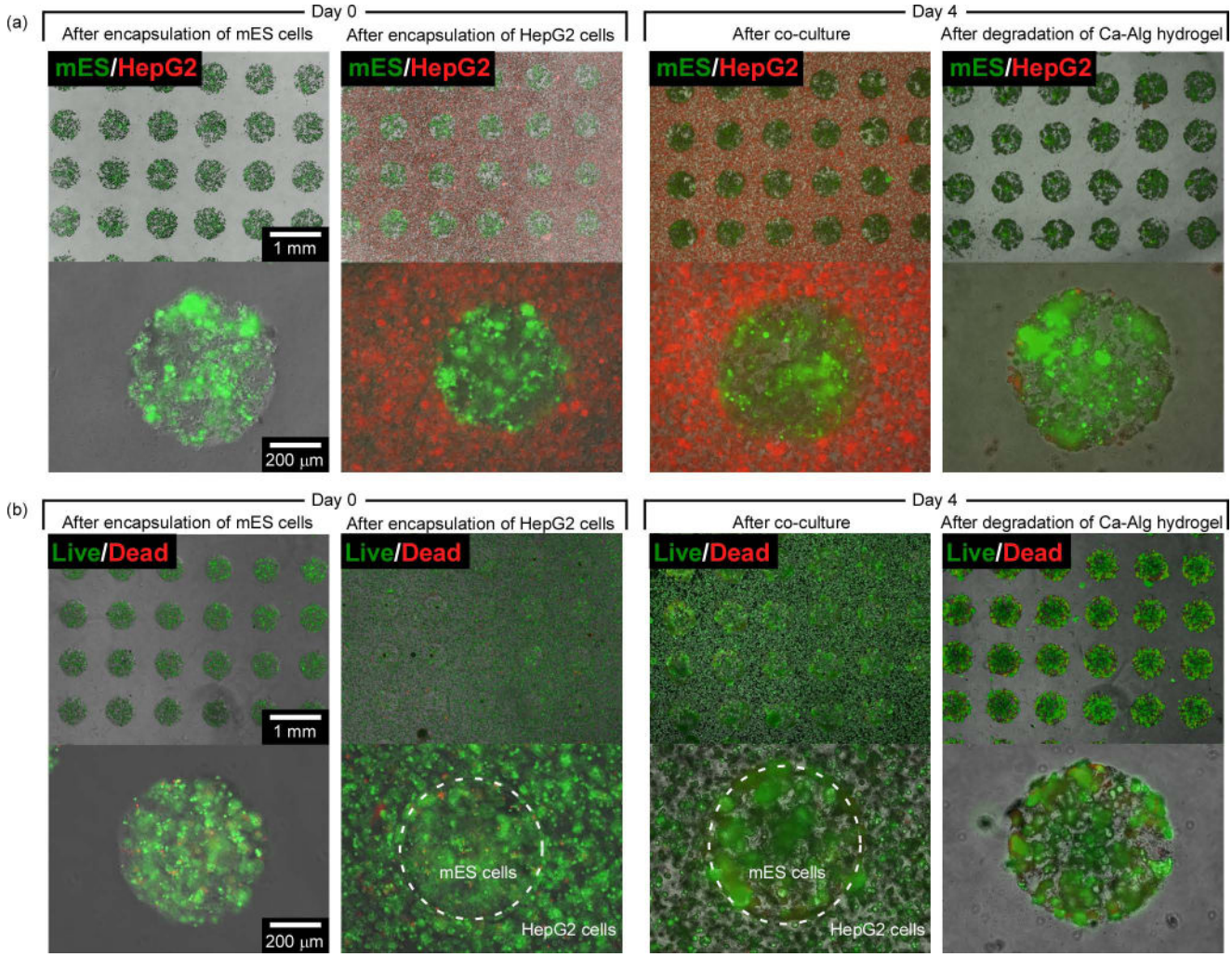

Fig. 2.

Dynamic 3D micropatterned co-culture in the photocurable PEG and chemically degradable alginate hydrogel. mES and HepG2 cells were encapsulated in the PEG and alginate hydrogels, respectively (Day 0). The mES and HepG2 cells were cultured for 4 days and the alginate hydrogel was subsequently degraded on day 4. (a) mES and HepG2 cells were labeled with CellTracker Green and CellTracker Red, respectively. (b) Live and dead cells were indicated by calcein-AM (green) and ethidium homodimer (red), respectively.

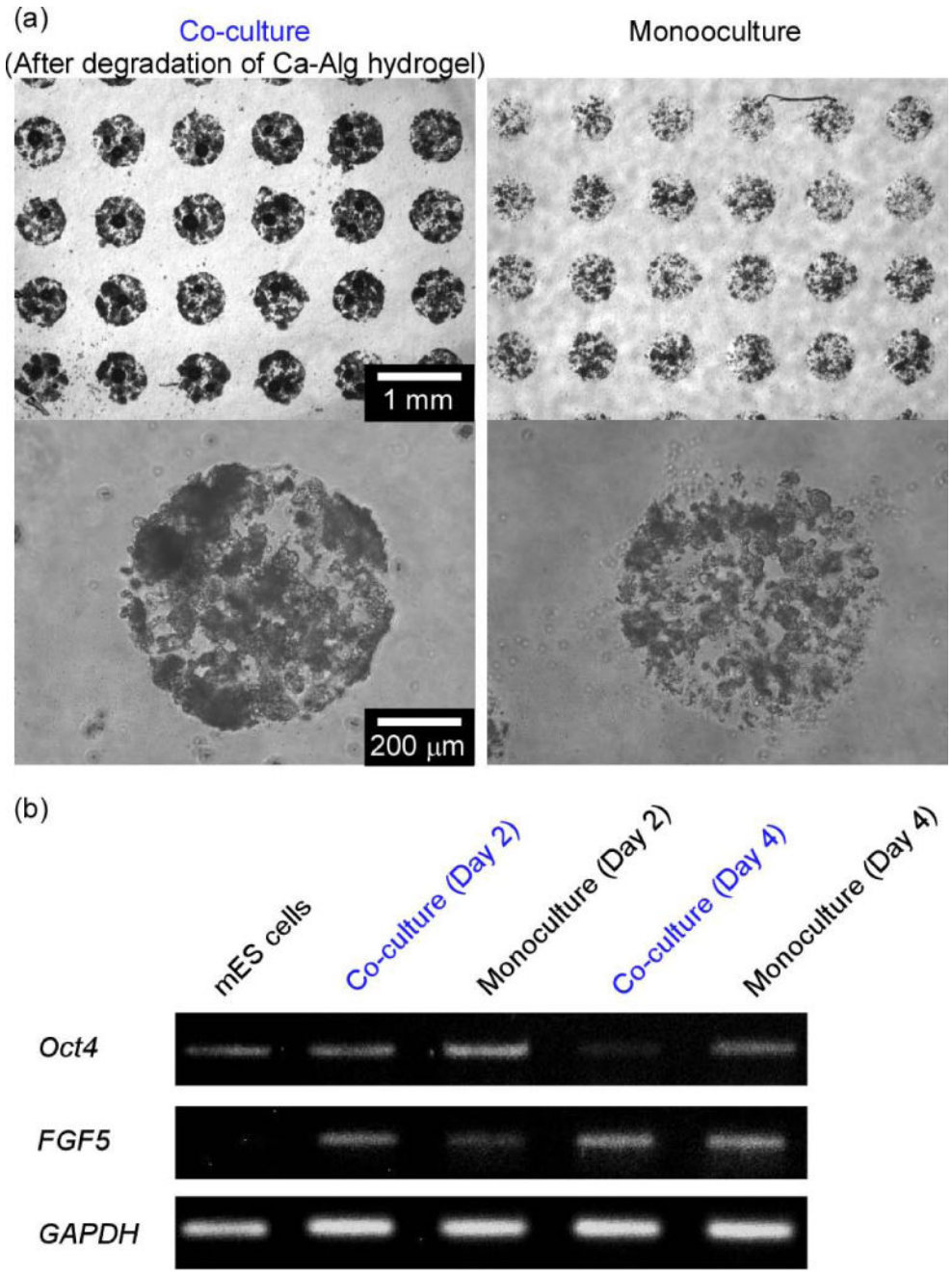

Fig. 3.

Morphology and gene expression of mES cells through 1st step of culture. (a) Microscope image of mES cells in PEG hydrogel after the 1st step of culture (Day 4). (b) Gene expression change through 1st step of culture.

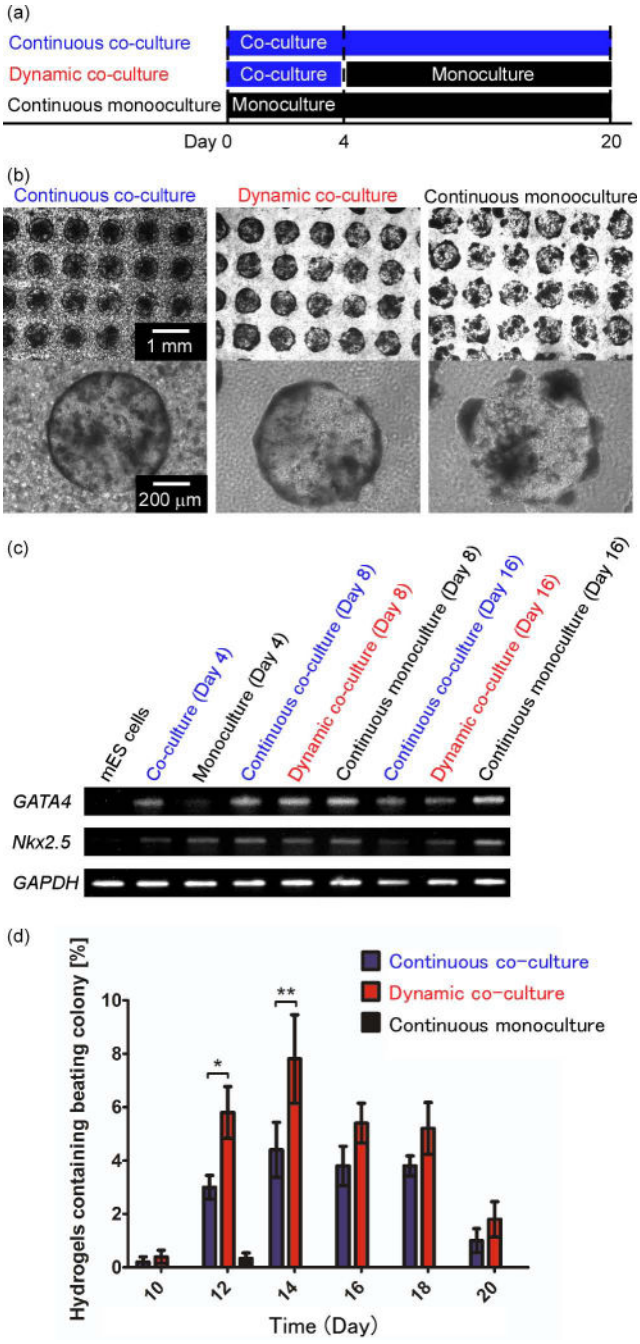

Fig. 4.

Morphology, gene expression, and beating characterization of mES cells through the 2nd step culture (i.e. continuous co-culture, dynamic co-culture, and monoculture). (a) Culture schedules for the continuous co-culture, dynamic co-culture, and monoculture (b) Microscope images of mES cells in the PEG hydrogel during the 2nd step culture (Day 12). (c) Cardiac gene expression in various culture conditions through the 2nd step culture. (d) Number of hydrogels containing beating colony in the three different culture conditions. Two-way ANOVA suggested significant effect of time and culture conditions on the number

of beating colonies ($p < 0.0001$). Statistically significant differences were determined employing the Bonferroni's multiple comparisons test and denoted as: *$p<0.05$ and **$p<0.01$. (N=5)

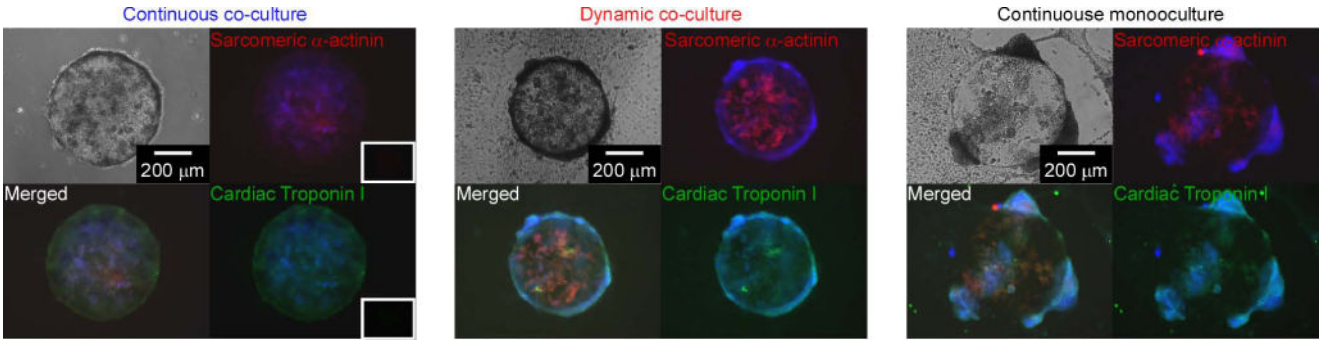

Fig. 5.

Immunocytochemical characterization of mES cells after 2nd step culture (day 16). Cardiomyogenic differentiation was identified by sarcomeric-α-actinin (red), troponin I (green) and DAPI (blue). Inset for continuous co-culture figure indicates negative control image stained only with secondary antibody.