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Microfabricated device for co-culture of sympathetic neuron and iPS-derived cardiomyocytes

Akimasa Takeuchi, *Member, IEEE,* Kenta Shimba, Yuzo Takayama, *Member, IEEE,* Kiyoshi Kotani, *Member, IEEE,* Jong-Kook Lee, Makoto Noshiro, *Member, IEEE,* and Yasuhiko Jimbo, *Member, IEEE*

***Abstract*— Induced pluripotent stem (iPS) cell-derived cardiomyocytes (iPS-CMs) has been expected as a cell source for therapy of serious heart failure. However, it is unclear whether the function of iPS-CMs is modulated by the host sympathetic nervous system. Here we developed a device for co-culture of sympathetic neurons and iPS-CMs using microfabrication technique. The device consisted of a culture chamber and a microelectrode-array (MEA) substrate. The superior cervical ganglion (SCG) neurons were co-cultured with iPS-CMs in a microfabricated device, which had multiple compartments. Several days after seeding, synapses were formed between SCG neurons and iPS-CMs, as confirmed by immunostaining. Spontaneous electrical activities of the SCG neurons and the iPS-CMs were observed from the electrode of the MEA substrate. The beat rate of iPS-CMs increased after electrical stimulation of the co-cultured SCG neurons. Such changes in the beat rate were prevented in the presence of propranolol, a β-adrenoreceptor antagonist. These results suggest that the microfabricated device will be utilized for studying the functional modulation of iPS-CMs by connected sympathetic neurons.**

I. Introduction

Stem-cell derived cardiomyocytes have great therapeutic value for heart failure such as ischemic heart disease or myocardial infarction [1]. Especially, induced pluripotent stem (iPS) cells [2, 3] were expected as a cell source for autogenous transplantation [4]. Recently, interest for iPS cells has focused on efficiency during cardiac differentiation, functional evaluation of the differentiated cardiomyocytes, or tumorigenic transformation. However, little is known whether the sympathetic nervous system (SNS), a component of the autonomic nervous system (ANS), innervates the iPS cell-derived cardiomyocytes in the same manner as the SNS controls the non-damaged cardiac tissue. Hence, to establish the cell based therapy for heart failure, it essential for studying the innervation of the intravital sympathetic neurons to iPS cell-derived catiomyocytes.

In vitro co-culture system is one of approaches for evaluating the innervation of intravital neurons to cardiac-differentiated cells. We have previously carried out compartmentalized co-culture of sympathetic neurons and cardiac-differentiated P19.CL6 cells using microfabrication techniques to examine whether sympathetic neurons modulate differentiated cardiomyocytes [5]. Our previous work demonstrated that compartmentalized co-culture using microfabrication technique was helpful for studying the signaling pathway from sympathetic neurons to differentiated cardiomyocytes. However, the technique has not been employed for developing co-culture of sympathetic neurons and iPS cell-derived cardiomyocytes.

In this study, we developed a device for co-culture of the superior cervical ganglion (SCG) neurons, which are sympathetic neurons, and iPS cell-derived cardiomyocytes (iPS-CMs) using microfabrication techniques. Then, the functional relationship of the two components was evaluated in terms of changes in the beat rate of the iPS-CMs after applying electrical stimulation to co-cultured SCG neurons.

II. Materials and Methods

A. Culture Device Fabrication

The co-culture device consisted of a microelectrode- array (MEA) substrate [6] and a culture chamber, which were fabricated using photolithography. The latter contained 2 compartments, 16 microcompartments and 16 microchannels (Fig. 1). One of the compartments, compartment A, was 2.0 mm in width, 3.0 mm in length and 2-3 mm in depth. The compartment A contained 16 microcompartments, which were about 50-70 μm in diameter and 70 μm in depth. The other compartment, compartment B, was 2.5 mm in width and 3.0 mm in length, and had a reservoir which was 2.5-3.0 mm in diameter. The microcompartments were connected to compartment B via 16 microchannels (approximately 50 μm in width and 5 μm in height).

Figure 2 shows fabrication process of the co-culture chamber. The co-culture chamber was formed using two types of soft-lithography masters, named masters A and B (Fig. 2A and 2B). First, each master was produced through a two-stage process using negative photoresists (SU-8

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A. Takeuchi, K. Shimba, K. Kotani, and Y. Jimbo are with the Graduate School of Frontier Sciences, The University of Tokyo, Chiba, 277-8563 Japan (phone: +81-047-136-4635; fax: +047-136-4648; e-mail: takeuchi@ bmpe.k.u-tokyo.ac.jp)

Y. Takayama is with the Graduate School of Information Science and Technology, The University of Tokyo, Tokyo, 113-8656 Japan.

J. K. Lee is with the Department of Cardiovascular Regenerative Medicine, Osaka University Graduate School of Medicine, 1-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

M. Noshiro is with Kitasato University, Kitasato 1-15-1, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan.

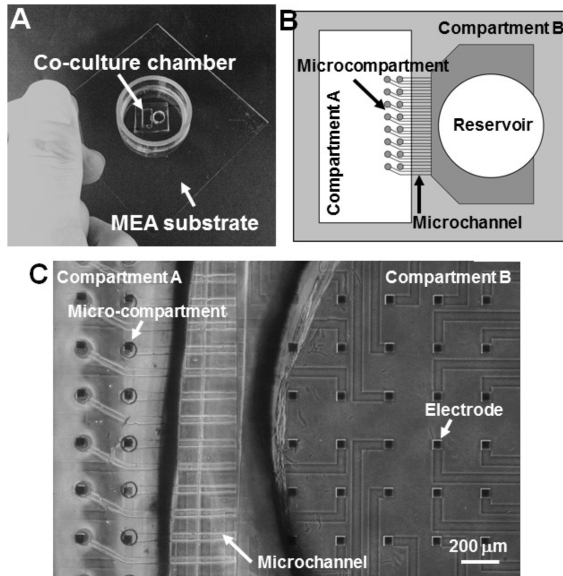

Fig. 1 Co-culture chamber on the MEA substrate. (A) Sixty-four electrodes were separated to two groups, which were 16 and 48 electrodes groups. Each electrode measured 50×50 µm. (B) Schema of the co-culture chamber. The chamber consisted of compartments A and B, which were connected with 16 microchannels. Compartments B had a reservoir. (C) Close-up of the middle part of compartment B: 48 electrodes were located so as to allow extracellular recordings from iPS-CMs. The reservoir in compartment B confined an aggregate of beating colony of iPS-CMs. Compartments B was 70 µm in height.

3005 and SU-8 3050, Microchem Corp.). Next, 2- to 3-mm-thick polydimethylsiloxane (PDMS, Dow Corning Toray Co., Ltd.) was casted on the master A. After a baked PDMS was released from the master, a reservoir and compartment A were formed on the PDMS brock using a punch and a scalpel, respectively (Fig. 2C). Then, 70-µm-thick PDMS was spin-coated onto the master B (Fig. 2D). The PDMS block was placed on the coated PDMS before the coated PDMS were baked at 80 ºC for 1 h. The released co-culture chamber was released from the master B, and was placed on the MEA substrate so that the center of a microcompartment was placed over that of an electrode.

B. Culture Preparation

The SCG neurons taken from 1- to 3-day-old Wistar rats were dissociated by the protocol previously described [7]. iPS-CMs (ReproCardio2, ReproCELL Inc.) were seeded into a 96 well multiwell plate (round-bottom, ReproCELL Inc.) and cultured for 3-4 days before they were seeded into the device. Then, aggregates including beating iPS-CMs were formed in the multiwell plate.

The culture device was coated with ReproCoat (ReproCELL Inc.) and filled with culture medium before seeding of the SCG neurons and iPS-CMs.

Dissociated SCG neurons were seeded into 16 microcompartments in the compartment A using a glass pipette under microscopic observation [7]. On the other hand, an aggregate containing beating iPS-CMs was seeded into the reservoir of compartment B. In addition, separation of the iPS-CMs from the SCG neurons was confirmed.

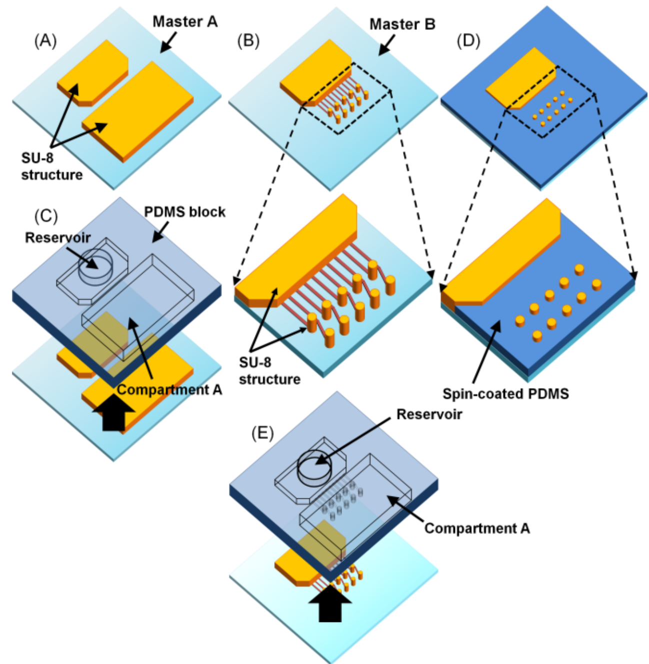

Fig. 2 Process of co-culture chamber fabrication. (A and B) Soft-lithography masters were fabricated by photolithography. (A) A PDMS block was formed on the master A. (B) Compartment A and a reservoir of compartment B were formed after the PDMS block was released from the master. (D) Unhardened 70-µm-thick PDMS was spin-coated on the master B. (E) The PDMS block was put on the spin-coated PDMS, and they was baked at 85 ºC for 60 minutes. Co-culture chamber was released from the master B. Then, the chamber was arranged on an MEA substrate, so that the positions of the microcompartments were located over the positions of the electrodes on the MEA substrate.

The culture medium in this study contained 80 %($^V/_V$) ReproCardio Culture Medium (ReproCELL Inc.) and 20 %($^V/_V$) fetal bovine serum, supplemented with a set of 25 ng/ml 2.5S Nerve Growth Factor (Invitrogen Corp.), 2 mM Glutamax (Invitrogen Corp.), and 1% Penicillin-Streptomycin (Invitrogen Corp.). The medium was replaced every 48 hours.

C. Extracellular Recording

Spontaneous electrical activities of the SCG neurons and iPS-CMs were recorded using a system including the MEA substrate [6]. The settings of the system were based on our previous study [5, 7]. The MEA substrate in a temperature- and CO_2-level- controlled box (37 °C, the CO_2 concentration at 5%) was used to measure spontaneous electrical activities of the cell cultures.

D. Electrical Stimulation and Evaluation of the Functional Connection between Sympathetic neurons and iPS-CMs

Functional connection was estimated from the changes in the beat rate of the iPS-CMs after application of electrical stimulation to co-cultured SCG neurons. First, spontaneous electrical activities of the iPS-CMs were recorded for 3 minutes. Then pulse-train stimulation (10 Hz, 1200 pulses) was applied to the co-cultured SCG neurons in all of the microcompartments via the electrodes of the MEA substrate. The pulse constituted the stimulation was set to biphasic square pulse (1 ms at +1 V, followed by 1 ms at -1 V). The applied current flowed from each electrode under a microcompartment to the reference electrode. After stimulation had been discontinued, spontaneous activities of the iPS-CMs were recorded for 60 s.

The beat rate of the iPS-CMs was obtained by counting the number of spontaneous electrical activities recorded form the electrode on the MEA substrate. The frequency change ratio was calculated by dividing the beat rate after the stimulation by that before the stimulation.

In addition, functional synaptic connection between the SCG neurons and iPS-CMs was examined by pharmacological blockade using 1 μM Propranolol, a β-adrenoceptor antagonist.

III. Results

A. Co-culture developed on the microfabricated device

After seeding of SCG neuron, they adhered to each microcompartment. In the microcompartment, cell bodies of the SCG neurons were confined near the electrodes of the MEA substrate. Neurites of the SCG neurons passed through the microchannels and reached compartment B containing the iPS-CMs (Fig. 3a). On the other hand, an aggregate including beating iPS-CMs adhered to the bottom of the compartment B.

Next, we examined whether the SCG neurons formed presynaptic zones upon contact with axons on iPS-CMs using immunostaining with two antibodies. One antibody recognized synapsin I, a synaptic vesicle-associated protein, (8) and the other recognized cardiac troponin-I, a cardiac regulatory protein that controls the calcium-mediated interaction between actin and myosin. (9) The formation of synapses on neurites crossing iPS-CMs was confirmed at 9 days after initiation of co-culture (Fig. 3b).

B. Spontaneous Electrical Activities and Evoked responses

The SCG neurons generated spontaneous electrical activities, which were observed as spikes containing positive and negative peaks (Fig. 4a). On the other hand, spontaneous electrical activities of iPS-CMs were observed in synchronization with the contraction of the iPS-CMs, and contained two components. One was spike corresponding to depolarization of the iPS-CMs, and the other was small-amplitude change corresponding to repolarization of them (Fig. 4b).

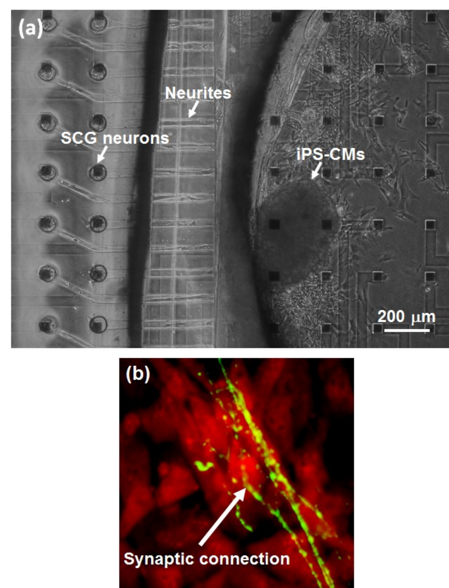

Fig. 3 Co-culture of SCG neurons and iPS-CMs on the microfabricated device. (a) iPS-CMs co-cultured with SCG neurons adhered to the bottom of the reservoir in the compartment B, and displayed spontaneous contraction. SCG neurons were confined to each microcompartment in the compartment A. (b) Fluorescence immunostaining for synapsin I and cardiac troponin-I. iPS-CMs were stained with the anti-cardiac troponin-I antibody (red), and the SCG neurons with the anti-synapsin I antibody (green). Synapses of the SCG neurons were formed on iPS-CMs.

Evoked responses were elicited after application of electrical stimulation to the SCG neurons. Evoked responses of the neurons were observed as spikes from several electrodes immediately after electrical stimulation was applied to the SCG neurons via electrodes in the microcompartments (Fig. 4c). No contraction of iPS-CMs was triggered when the SCG neurons were stimulated, as confirmed by microscopic observation.

C. Changes in the beat rate of iPS-CMs after electrical stimulation of Co-cultured SCG neurons

Synchronized spontaneous beating of the iPS-CMs was maintained after the electrical stimulations had been applied and discontinued. Then, the beat rate of the iPS-CMs increased after electrical stimulation was applied to the co-cultured SCG neurons (Fig. 5). In addition, no significant increase in the beat rate after electrical stimulation was observed in the presence of propranolol, a β-adrenergic receptor antagonist (Fig. 5).

Discussion

A. Functional connection between the SCG neurons and iPS-CMs

The important findings of this study were that intravital sympathetic neurons formed synapses on the iPS-CMs and that application of electrical stimulation to sympathetic neurons resulted in elevation in beat rate of co-cultured iPS-CMs. Our previous study had shown that sympathetic neurons modulate beat rate of cardiac-differentiated P19.CL6

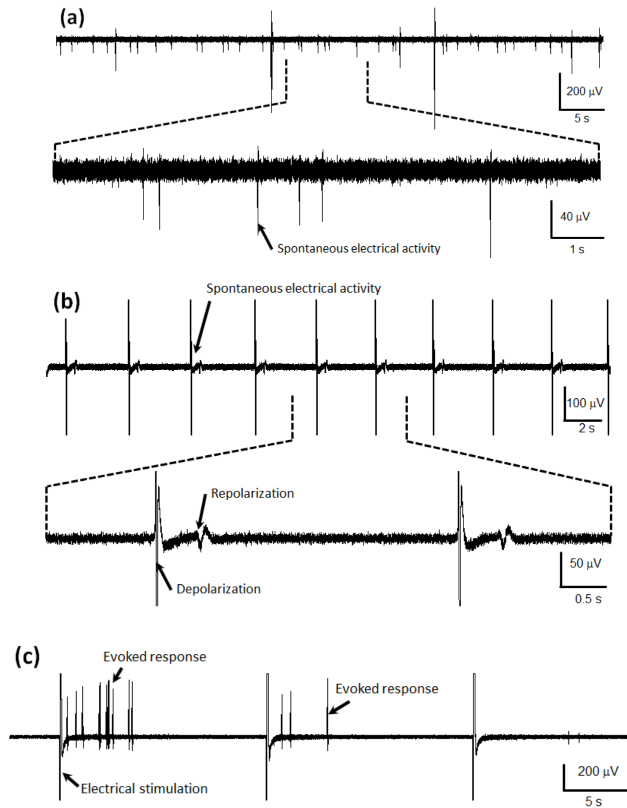

Fig. 4 Spontaneous electrical activity obtained from co-culture of the SCG neurons and iPS-CMs. (a) Nine days after aggregate seeding, spike-shaped spontaneous electrical activities were detected from the electrodes under each microcompartment which SCG neurons were cultured. (b) Spontaneous electrical activities were recorded almost synchronously in the surrounding electrodes with the beating of the iPS-CMs colony. (c) Evoked responses of the SCG neurons. In addition, no electrical activities of the iPS-CMs were triggered when electrical stimulation was applied to the SCG neurons (data not shown).

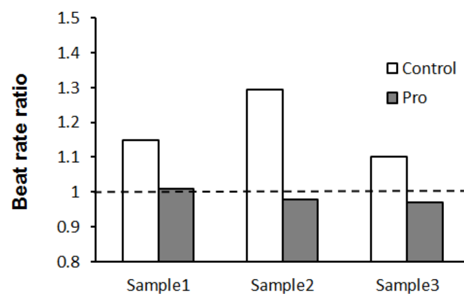

Fig. 5 Changes in beat rate after application of electrical stimulation to co-cultured SCG neurons. The SCG neurons were stimulated with 1200 pulses at the frequencies of 10 Hz (Control). Furthermore, increases in the beat rate after stimulation were not observed after treatment with propranolol (Pro).

cells, which were model cell of the cardiac differentiation. However, it remained unclear whether intravital sympathetic neurons controlled the beat rate of human iPS cell-derived cardiomyocytes, either in vivo or in vitro. Results in the present study suggest that intravital sympathetic neurons connected to iPS-CMs were adrenergic and that released noradrenaline from the sympathetic neurons activated β-adrenergic receptor in iPS-CMs. Moreover, results of pharmacological blockade suggest that propranolol blocked the activation of the receptor by noradrenaline. Earlier study reported that iPS-CMs expressed β-adrenergic receptor [ref], and their work supports our suggestions.

Moreover, our previous study reported that the changes in the beat rate of P19CMs after stimulation were affected only by the pulse frequency of the stimulation [5]. Hence, the beat rate of iPS-CMs might be also affected by the parameter of the electrical stimulation to the SCG neurons. Further study will be necessary to examine this possibility.

Conclusion

A compartmentalized co-culture method employing a MEA substrate was developed to examine the functional connections between sympathetic neurons and iPS cell-derived cardiomyocytes. First, the formation of synapses between sympathetic neurons and iPS-CMs was confirmed by immunostaining with antibodies against synapsin I and cardiac troponin-I. Second, changes in the beat rate of the iPS-CMs were triggered by application of electrical stimulation to co-cultured sympathetic neurons.

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