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Bidirectional Synaptic Connection between Primary and Stem Cell-Derived Neurons in Co-Culture Device*

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Abstract— **Regenerative medicine is expected to be a potent therapeutic option for the disorders or injuries of the central nervous system. However, little is known about how the newly formed neurons derived from grafted stem cells integrate into the host established tissue. The aims of this study are to make functional connection between primary neurons and stem cell-derived neurons via chemical synapses and maintain the connection for a long time in *in vitro*. We employed an *in vitro* co-culture device to cultivate two different neuronal populations and evaluate the interaction between them. Mouse cortical neurons and P19 cell-derived neurons were co-cultured in the co-culture device. The synchronous activities were maintained for at least 4 weeks. Evoked responses to electrical stimulation suggested that bi-directional connections were formed between cortical and P19-derived neurons. The responses were changed after pharmacological treatment. These results showed that cortical neurons and P19 cell-derived neurons formed bidirectional synaptic connections via glutamate receptors and the connection was maintained for at least 4 weeks.**

I. Introduction

Stem cell transplantation in central nervous system is a promising therapy for neurodegenerative diseases (e.g. Alzheimer disease and Parkinson disease). A goal of the therapy is to replace the damaged tissue with newly formed neurons derived from grafted stem cells. Many studies using animal models were performed to estimate the efficacy of stem cell therapy [1-3] in terms of animal behaviors or electrical activity of grafted neurons. However, with conventional approaches, it is hard to evaluate the differentiation process of grafted stem cells and the functional integration of young neurons into host neural networks. It is important to know how grafted young neurons integrate into host established network, taking account of the aim of stem cell transplantation.

Co-culture system is suitable for evaluating the interaction between primary cultured cells and stem cell-derived cells. Recently, co-culture devices fabricated with photolithography techniques were employed for co-culturing two different neuronal populations. In previous study, it was reported that mouse cortical neurons (CX) and neurons differentiated from P19 embryonal carcinoma cells, one of pluripotent cell lines, were successfully co-cultured on the co-culture device, and these neurons showed synchronous activity [4]. However, the co-culture device has not achieved long-term co-culture and evaluation of the connection between CX and P19-neurons.

In this study, we developed a new co-culture device for co-culturing primary neurons and stem cell-derived neurons. The aims of this study are as follows: 1) to maintain the connection between CX and P19-neurons until the co-cultured neuronal network develops to the mature stage, 2) to form the bidirectional connections via chemical synapses.

II. Materials and Methods

A. Co-culture Device Fabrication

The co-culture device is composed of a PDMS chamber and an MEA substrate. The chamber had ring-shaped structure (5 mm inner diameter, 7 mm outer diameter and 4 mm in height). Inside and outside of the chamber, named inner compartment and outer compartment, respectively, were interconnected with 36 micro-tunnels. A micro-tunnel was 750 μm in length, 30 μm in width and 5 μm in height, and allowed the axons of the neurons to pass though the tunnels and prevented the soma from passing through it. In addition, 32 electrodes were placed in the inner compartments, other electrodes in the outer compartment.

The PDMS chamber was fabricated by soft lithographic technique. The fabrication process is shown in Fig. 1. A soft-lithography master was developed on a silicon wafer through two-stage process. At the first stage, 36 strips (30 μm in width, 5 μm in height) were developed using SU-8 (3005, MicroChem), a negative photoresist. At the second stage, 2 concentric circles (100 μm in height) were developed using SU-8 (3050, MicroChem). Next, polydimethylsiloxane (PDMS, Silpot 184; Dow Corning Toray) prepolymer was casted on the master and cured using a hotplate. The PDMS was peeled off the master, and the ring-shaped structure was formed using biopsy punches.

The MEA substrate was coated with 0.1% w/v Polyethyleneimine solution (Wako) more than 8 hours and rinsed with sterile water 4 times before the PDMS chamber was placed on it. Next, the co-culture device was coated with 50 ng/ml laminin (Life Technologies) 30 minutes after alignment.

B. Cell Culture

Mouse cortices were taken from embryonic ICR mice (E16). The cortices dissociated with 0.25% Trypsin (Life Technologies), and CX were obtained. The CX were seeded into the outer compartment at a concentration of 5000

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cells/mm^2. Culture and neuronal differentiation of P19 cells were performed based on the protocols described previously [4]. Briefly, undifferentiated P19 cells were cultured in alpha minimum essential medium (aMEM; Life Technologies) supplemented with 10% fetal bovine serum and 1 % penicillin-streptomycin (Life Technologies). While P19 cells were differentiated into neural cell lineage, P19 cells were aggregated in a bacteriological grade dish (BD) in P19 cells-culture medium containing 1 μM all-trans retinoic acid (Sigma), and embryoid bodies (EBs) were formed in the dish. Four days after neural induction, EBs were dissociated by 0.25% trypsin-EDTA (Life Technologies) and seeded into the inner compartment at the same concentration of CX. P19 cells were seeded 4 days after CX seeding. Both types of cells were co-cultured in Neurobasal medium (Life Technologies) supplemented with a set of B27 supplement (Life Technologies), 2 mM GlutaMAX (Life Technologies) and 1% pe-st. The co-culture device was incubated in a humidified incubator maintained at 37 ºC and 5% CO_2. Half of the culture medium was replaced every 3 days with fresh one.

C. Immunohistochemistry

Double staining for anti-beta III tubulin (B3T) and anti-synapsin I (SYN) was performed to confirm axons entering the micro-tunnels. Co-cultures were immunostained 2 weeks after P19 seeding. Cells were fixed with 4% paraformaldehyde (Wako) in phosphate buffered saline (PBS; Life Technologies) for 30 minutes at room temperature. After fixation, cells were permeabilized for 30 minutes in 0.25% Triton X-100 (Calbiochem) in PBS. Subsequently cells were blocked in 4% Blocking agent (DS-pharma) for overnight at room temperature. After blocking, primary antibody solution was loaded into the co-culture device. Primary antibody solution contained mouse anti-beta 3 tubulin antibody (Abcam) and rabbit anti-synapsin I antibody (Millipore) for overnight at 4ºC. After washing with PBS, appropriate secondary antibodies were loaded into the co-culture device for 2 hours at 4ºC. Goat Alexa fluo-488 anti-mouse IgG (A-11001; Molecular probes) and Goat Alexa fluo-546 anti-rabbit IgG (A-11010; Molecular probes) were used as secondary antibody. Finally, the nuclei were counterstained with DAPI. Fluorescent images were acquired with inverted fluorescence microscopy (IX-71; Olympus) attached with a cooled CCD camera (Hamamatsu photonics).

D. Extracellular Recording and Stimulation

MEA recording was performed with MEA recording and stimulation system previously described [5]. The analogue signal detected from the electrodes was amplified X20 by pre-amplifier (NF) and filtered (100 - 2000 Hz, NF). Subsequently the signal was amplified X1000 by main-amplifier (NF) and sampled (25 kHz, 12 bits, National Instruments). When analyzing the evoked responses to electrical stimulation, SALPA algorithm [6] was employed to suppress stimulation artifact. Spikes of cultured neurons were detected with simple threshold method. Threshold was set at 5 times the noise level estimated by *AdaBndFlt* method previously described [7].

Electrical stimulation was applied to evaluate the direction of connection between CX and P19-neurons. Electrical stimulus was applied at 3 s interval from each electrode sequentially. The electrical pulse was single biphasic pulse (1 ms at +1 V, followed by 1 ms at -1 V). Moreover, NMDA receptor antagonist, 50 μM D-(-)-2-amino-5-phosphonovaleric acid (APV; Sigma), and AMPA receptor antagonist, 50 μM 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX; Sigma), were supplemented with culture medium sequentially.

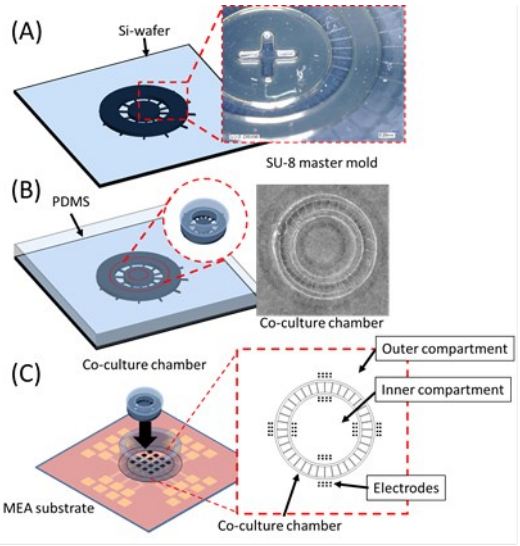

Fig. 1 Fabrication steps of co-culture device. The SU-8 master mold was developed on a silicon wafer (A). Next, the co-culture chamber was fabricated by casting of a PDMS prepolymer on the master mold (B). Finaly, the co-culture chamber was aligned on an MEA substrate(C). The inner and outer region of the ring-shaped chamber had 32 electrodes each.

III. Results

A. Connection between Cortical and P19 Neurons

CX were adhered to the outer compartment and extended their neurites. The neurites of CX passed through the micro-tunnels and reached the inner compartment. P19-neurons also extended their neurites. To confirm the neurites outgrowth along the micro-tunnels, CX and P19-neurons were stained with anti-B3T and anti-SYN antibodies. The nuclei were counterstained with DAPI. Fig. 2 shows the fluorescence images of co-cultured network around the micro-tunnels. B3T positive area was observed along micro-tunnels. SYN positive area was observed near the entrance of the micro-tunnels. DAPI staining showed that few cell bodies entered into the micro-tunnels.

B. Spontaneous Activity of Co-Cultured Neurons

Synchronous activity between CX and P19-neurons was observed 1 week after P19 cells had been seeded (weeks in vitro; WIV). The synchronous activities were maintained for at least 4 weeks more than 80% of all samples (n = 13). Fig. 3 shows the spontaneous activity of co-cultured neuronal network at 4 WIV. The waveforms of the firings of CX and P19-neurons were burst patterns (Fig. 3A). Network-wide synchronization was observed in Fig. 3B. Fig. 4 shows the change of electrical properties. The activities of CX were observed from more than 30% of electrodes in CX area. Meanwhile, the activities of P19-neurons were observed from

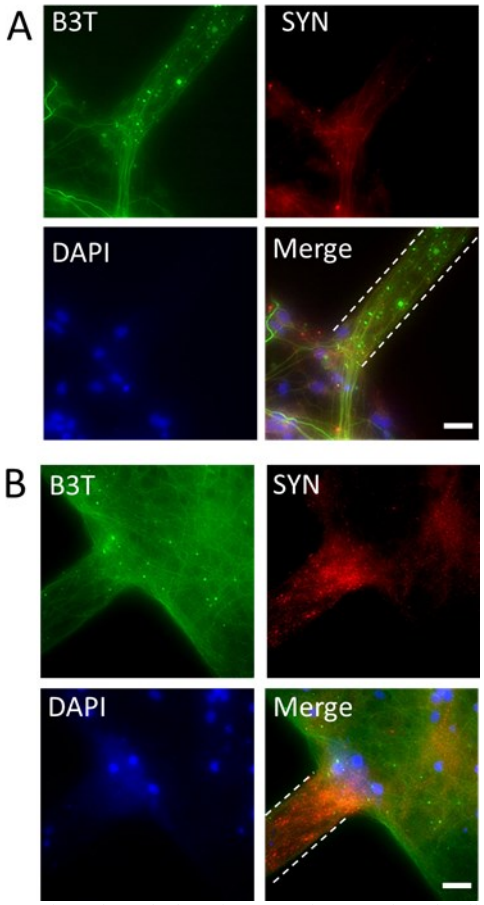

Fig. 2 Connections through the micro tunnels. Co-cultured cells were stained using antibodies against beta-3-tubulin (B3T; green) and synapsin I (SYN; red) 2 weeks after P19 cells seeding. The nuclei were counterstained with DAPI (blue). P19 neurons (A) and CX (B) extended axons into micro-tunnels. Synapse formation was observed near the edge of the micro-tunnels. Scale bar : 30 μm.

approximately 15%. As shown in Fig. 4B, firing rate of CX increased while firing rate of P19-neurons was unchanged.

C. *Evoked Responses of Co-cultured network*

To confirm the direction of the connections between CX and P19-neuosn, stimuli were applied from electrodes in each compartment. Figure 5 and 6 show the representative waveforms of evoked responses and peri-stimulus time histograms, respectively. Evoked responses of CX were elicited by application of stimulation to P19-neurons (Fig. 5A and Fig. 6 upper panel), and vice versa (Fig. 5B and Fig. 6 lower panel). In Fig. 6, the vertical axis and the horizontal axis indicate spike number per bin and time, respectively. The number of responses was reduced in the presence of NMDA receptor antagonist (APV). The number of responses increased for approximately 40 ms and maintained more than 300 ms (red line). NMDA receptor blockade (green line) shortened the duration of responses and decreased the number of responses. The responses of P19-neurons were markedly reduced with APV treatment compared to CX. Moreover, no response was observed with NMDA and AMPA receptor blockade (blue line).

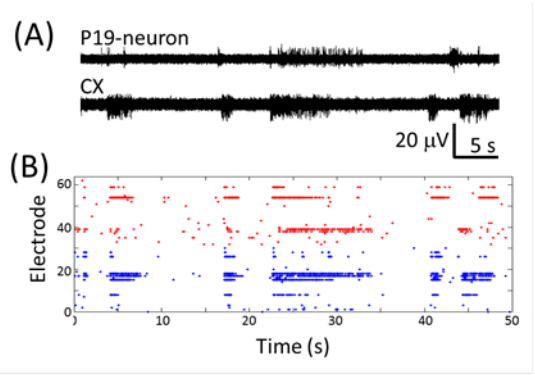

Fig. 3 Spontaneous activity of CX and P19 neurons. (A): Representative waveform of spontaneous activity recorded from CX and P19-neurons. (B): A raster plot of 50 s recording. Synchronous bursting of CX (blue) and P19-neurons (red) were observed.

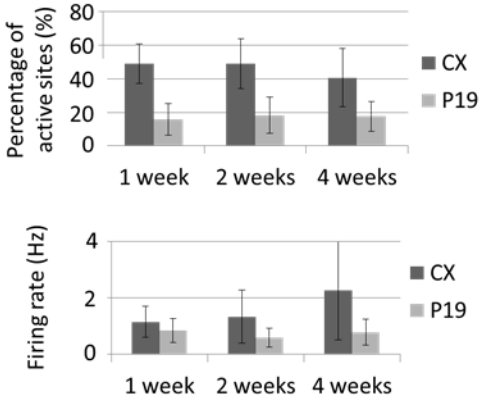

Fig. 4 Changes in spontaneous activity. (A): Percentage of active site. The active site in P19-neuron area was maintained at 15% for 4 weeks. (B): Mean firing rate of 1200 s recording. While the firing rate of CX was slightly increased, that of P19-neurons was maintained at 0.7 Hz. (mean ± SEM, 13 cultures)

IV. Discussion

A. *Functional Connection between CX and P19-neurons*

In this study, we performed immunofluorescence staining and extracellular recording to evaluate the connection between CX and P19-neurons. First, it is suggested that functional connection between CX and P19-neurons was formed. As shown in Fig. 2, CX and P19-neruons extended their neurites into micro-tunnels and formed synapses on the entrances of the micro-tunnels. Moreover, synchronous activity between CX and P19-neurons was observed from electrodes (Fig. 3). These results indicate that CX and P19-neurons are able to form functional connection via chemical synapses in the co-culture device.

Second, using our co-culture device, the functional connection is maintained for at least 4 weeks. As shown in Fig. 4, percentage of active sites and firing rate of P19-neruons were unchanged for 4 weeks. In previous study, cultured neuronal networks displayed the activity patterns of the mature network 3 or 4 weeks after seeding [8, 9]. Hence, we are able to evaluate the activity changes of co-cultured neuronal network from developing stage to mature stage using our device.

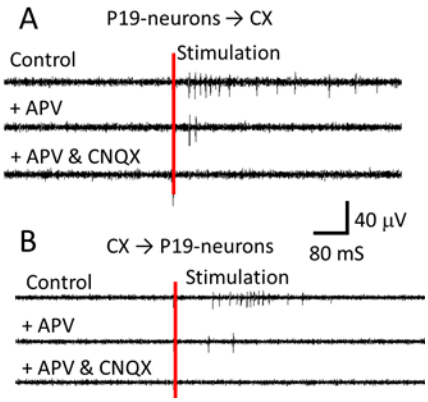

Fig. 5 Evoked response of CX and P19 neurons. Evoked response of CX elicited by stimulation to P19-neurons (A) ware observed, and vice versa (B). The responses were reduced by the NMDA receptor antagonist. Pharmacological blockade of NMDA and AMPA receptor completely eliminated the responses.

In contrast to the above findings, previous study reported that few activities were observed from co-cultured P19-neurons 3 weeks after seeding [4]. These differences might be due to the different amount of culture medium. Our co-culture device is designed to hold larger amount of the culture medium than previous one. Therefore, it is suggested that our device is able to prevent P19-neurons from damage by the changes of culture conditions (e.g. osmotic pressure and the concentration of glutamate). Further studies of the cell viability labeling and the chemical composition of culture medium are needed to confirm our hypothesis.

B. Direction and Property of the Connection

In order to assess the direction of functional connections, we recorded responses to electrical stimulation. Moreover, NMDA and AMPA receptor blockers were added to the culture medium to examine whether the transmission between CX and P19-neurons is via chemical synapses or not. The results showed that CX and P19-neurons formed bi-directional connection via NMDA and AMPA receptors. Evoked responses of one neuronal population were observed when the other neurons were stimulated. This result demonstrated that the connections between CX and P19-neurons were bi-directionally.

In addition, NMDA receptor antagonist APV reduced the response frequency and shortened the duration of responses, and blockade of AMPA receptor by CNQX abolished the responses. These results suggested that CX and P19-neurons interconnected via the ionotropic glutamate receptors. Previous study reported that functional NMDA and AMPA receptors were expressed in P19-neurons [10]. Our results are consistent with previous report.

V. Conclusion

We evaluated the functional connection between CX and P19-neurons using MEA recording and pharmacological blockade. The results showed that these neurons interconnected via chemical synapses and the connection maintained for at least 4 weeks. Therefore, this co-culture system is suitable to study the integration of newly formed neurons into established neuronal network *in vitro*.

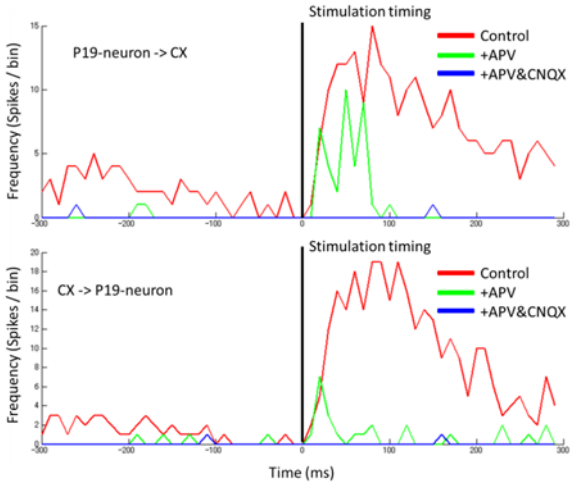

Fig. 6 Spike frequency before and after stimulation. Spike frequency of CX increased after P19-neurons were stimulated (upper panel), and vice versa (lower panel). In the control state, the response to stimulation continued more than 300 ms (red line). The NMDA blockade shortened the duration of responses and decreased the response frequency (green line). Moreover, when both NMDA and AMPA receptor were blocked, no response was observed.

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