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A neurospheroid network-stamping method for neural transplantation to the brain

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ABSTRACT

Neural transplantation therapy using neural stem cells has received as potential treatments for neurodegenerative diseases. Indeed, this therapy is thought to be effective for replacement of degenerating neurons in restricted anatomical region. However, because injected neural stem cells integrate randomly into the host neural network, another approach is needed to establish a neural pathway between selective areas of the brain or treat widespread degeneration across multiple brain regions. One of the promising approaches might be a therapy using pre-made neural network *in vitro* by the tissue engineering technique. In this study, we engineered a three-dimensional (3D) tissue with a neuronal network that can be easily manipulated and transplanted onto the host brain tissue *in vivo*. A polydimethylsiloxane microchamber array facilitated the formation of multiple neurospheroids, which in turn interconnected via neuronal processes to form a centimeter-sized neurospheroid network (NSN). The NSN was transferable onto the cortical surface of the brain without damage of the neuronal network. After transfer onto the cortical tissue, the NSN showed neural activity for more than 8 days. Moreover, neurons of the transplanted NSN extended their axons into the host cortical tissue and established synaptic connections with host neurons. Our findings suggest that this method could lay the foundation for treating severe degenerative brain disease.

1. Introduction

The transplantation of neuronal suspension or neural stem cells has been developed to recover the functionality of damaged neural tissue in neurodegenerative diseases [1–9]. These transplantation therapies were mainly developed to replace degenerating dopaminergic neurons in Parkinson's disease [10–13]; however, they are not suited for recovering the intrinsic connections in brain, because the graft neurons randomly create a network with host neurons. To connect selective areas of the brain or replace a neural pathway of degenerated neural tissue, the transplantation of fetal neural tissue grafts has been employed [14–16]. This method is the classical technique of transplantation, but it cannot be applied to replace widespread degenerated tissue due to the limited size of the graft. Moreover, the dissection of optimal neural tissue grafts from fetal donor brain is technically difficult, and ethical concerns limit this approach. Consequently, it is crucial to develop a simple method for transplanting a pre-made neural network *in vitro* to damaged regions of the brain. Although the fabrication of neuronal networks by two-dimensional (2D) patterning neurons on a microarray has been reported previously [17–22], they cannot be transplanted onto the brain because of their fragile, thin, 2D-structure, limiting the ability to peel them from a culture plate without physical or chemical treatment.

In this paper, we describe a method to fabricate centimeter-sized, robust neurospheroid network (NSN) to connect wide-ranging areas of the brain (Fig. 1a and b). The NSN was formed by culturing multiple neurospheroids in a polydimethylsiloxane (PDMS) microchamber array; these spheroids were eventually interconnected to each other via a large number of neuronal processes and thus having a centimeter-sized robust network. We also developed a simple technique for transplanting NSN onto specific brain areas at a high transfer rate, termed the NSN-stamping method (Fig. 1c). We here investigate that the neurons of NSN can extend their axons into the host brain and establish synaptic connections with host cortical neurons.

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2. Materials and methods

2.1. Fabrication of the PDMS microchamber array

The master for preparing the PDMS replica was fabricated with SU-8 photoresist (MicroChem Co.) on a silicon wafer by using photolithography [23]. We fabricated the PDMS replica with customized hole-size and center-to-center spacing (Supplemental Figure S1). The replica was molded by casting the liquid prepolymer composed of a mixture of 10:1 silicon elastomer and a curing agent (Sylgard 184; Toray). The mixture was cured at 70 °C for 2 h, and then, the PDMS mold was peeled from the silicon wafer and cleaned with ethanol.

2.2. NSN formation

The cerebral cortices of Wistar rats (embryonic days 17–19) were dissected and cultured as previously described [24–26]. In brief, the cortices was dissociated with papain (Worthington Biochemical Corp.) and triturated through a pipette. Dissociated cells were suspended in a mixture of Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 (1:1) (DMEM/F-12; Invitrogen) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum (Japan Bioserum), and B27 serum-free additive (Invitrogen). The resultant cell suspension was plated at a density of 4×10^6 cells/ml on a PDMS microchamber array, and then, the superfluous cells were washed away after 30 min by changing the medium. The formed neurospheroids were maintained for 1–2 weeks in 5% CO_2 at 37 °C. Cell viability of the NSN was determined by using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen). The NSN was then observed under a fluorescence microscope (Axio Observer Z1; Zeiss). All rats were maintained in accordance with the policies of the University of Tokyo Institutional Animal Care and Use Committee.

2.3. Immunocytochemistry

Cultured neurospheroids were fixed for 1 h with 4% paraformaldehyde (Muto Pure Chemicals Co., Ltd.) in phosphate-buffered saline (PBS; Sigma–Aldrich), permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 5% bovine serum albumin in PBS for 1 h for nonspecific binding sites. They were then reacted with a 1:500 dilution of mouse anti-MAP2, Alexa Fluor® 488 conjugated monoclonal antibody (Millipore), 1:1000 dilution of rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP; Convance) or 1:500 dilution of rabbit polyclonal antibodies to neurofilament (Sigma) at 4 °C overnight. Following a brief rinse in PBS, the cultured neurospheroids or NSN were incubated with Alexa Fluor 568 goat anti-rabbit IgG (1:500; Invitrogen) at room temperature for 2 h and finally washed five times with PBS. After staining with Hoechst 33342 (1:1000; Invitrogen), the cultured neurospheroids or NSN were observed under a fluorescence microscope (Axio Observer Z1; Zeiss) and confocal laser microscope (LSM710; Zeiss).

2.4. $[Ca^{2+}]_i$ imaging

The NSN was observed for $[Ca^{2+}]_i$ fluxes. Fluo-4/AM, a Ca^{2+}-sensitive fluorescent dye (Invitrogen) was dissolved in DMSO (Kanto Chemical Co., Inc.) to a concentration of 1 mM and used as a stock solution. This solution was diluted to a final concentration of 2.5 μM with basal salt solution (BSS) consisting of 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 20 mM HEPES–NaOH (pH 7.4). The NSN was incubated with the Fluo-4/AM solution at 37 °C for 60 min. After washing out excess dye with BSS, the cultured neurospheroids were observed by fluorescence microscopy (Axio Observer Z1; Zeiss). The changes in the intensity were measured by using an image analysis system (Axiovision; Zeiss).

2.5. NSN transfer onto a PEI-coated glass plate with the NSN-stamping method

After 14 days of culture, PDMS microchambers with an NSN was flipped and placed onto a polyethyleneimine (PEI; Sigma)-coated glass plate, and the culture medium was added to the glass plate. After 24-h incubation in 5% CO_2 at 37 °C, only the PDMS microchamber array was peeled off. The transfer rate was determined by counting the number of transferred neurospheroids on the PEI-coated glass plate per initial number of neurospheroids in the NSN. The transferred NSN was observed under a fluorescence microscope (Axio Observer Z1; Zeiss).

2.6. NSN buildup

For NSN tissue buildup, we prepared two NSNs. A PDMS microchamber array (first) with an NSN was carefully placed with tweezers on another PDMS microchamber array (second) under microscopy. After 24 h of culture, the first PDMS microchamber array was peeled from the second one. The NSNs were labeled with CellTracker Green or Orange (Invitrogen) before transfer and observed under a fluorescence microscope (Axio Observer Z1; Zeiss).

2.7. NSN transplantation onto cortical tissue

Whole brain and cerebral cortical tissues were dissected from postnatal (day 20) or neonatal (day 3) Wistar rats. A PDMS microchamber array with an NSN was handled with tweezers and carefully placed facedown on the cortical surface; then, static culture was established after addition of the medium. After 24-h incubation in 5% CO_2 at 37 °C, only the PDMS microchamber array was peeled off. The NSN was labeled with CellTracker Orange (Invitrogen) before transfer and observed under a fluorescence stereoscopic microscope (Lumar.V12; Zeiss). The NSN buildup was

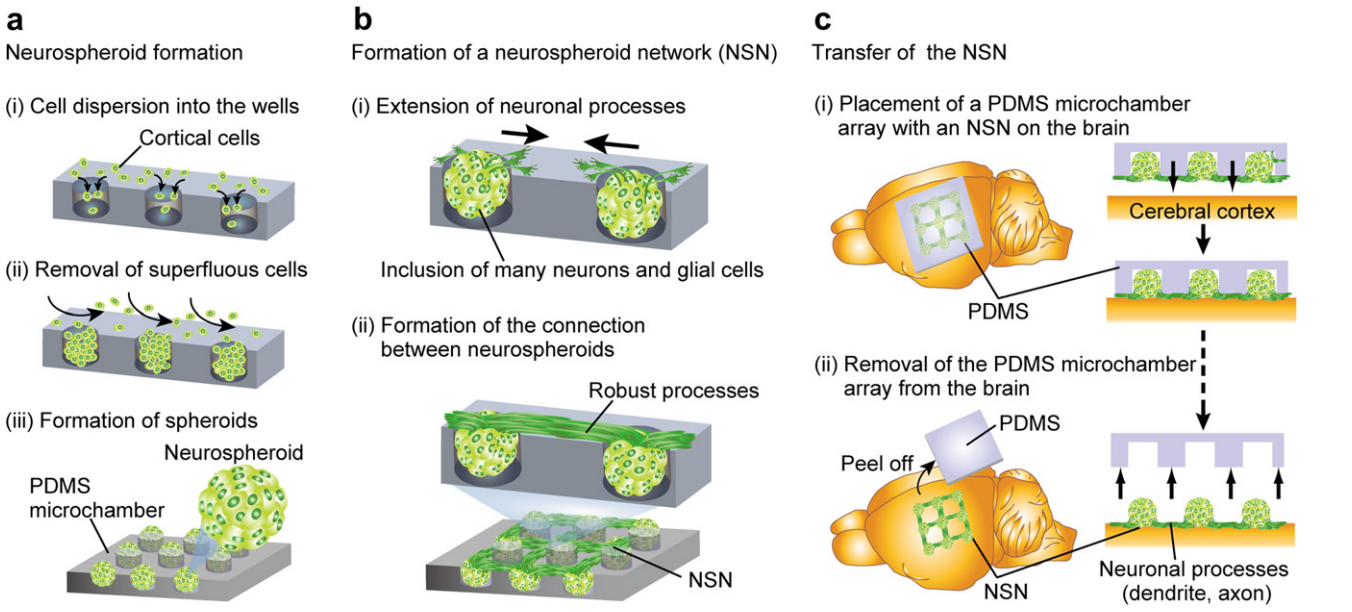

Fig. 1. Schematic illustration of the protocol to fabricate an NSN and transplant it onto brain tissue. (a) Fabrication of neurospheroids of primary cortical cells on a PDMS microchamber array. (b) Formation of an NSN, showing tight connections of neural processes. (c) Transfer of the NSN formed on the PDMS microchamber array onto rat brain by using the NSN-stamping method.