

Neuronal Differentiation of NT2 Cells in Monolayer and Spheroid Cultures

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Abstract Metabolism and differentiation of cultured cells are influenced by changes in cellular morphology. In this study, we investigated the differences in cell proliferation and neuronal differentiation of NT2 cells in monolayer (2D) and spheroid (3D) cultures. In the monolayer culture, the cells adhered and extended on a tissue culture plate. For the spheroid culture, we fabricated a microwell chip comprising 195 circular microwells (600 μm in diameter) on a culture plate, and the surface was modified with polyethylene glycol to promote spheroid formation. The cells were aggregated in each microwell and formed spheroids within 24 hours of culture, and the spheroid morphology was maintained throughout the culture period. Although the cell proliferation ability in monolayer culture was higher than that in spheroid culture, the neuronal differentiation ability of NT2 spheroid culture was higher than that in monolayer culture. Furthermore, the neuronal differentiation of NT2 spheroids was dramatically enhanced by retinoic acid treatment. These results indicate that NT2 cell properties are influenced by differences in cell morphologies, and that spheroid culture is a promising technique to induce neuronal differentiation.

1 Introduction

The central nervous system manages neural communication and plays essential roles in maintaining normal physiology. Therefore, nerve cells have been used for various applications such as neural tissue engineering and pharmacological, toxicological, and fundamental cell biology studies. As one of the strategies for the success of such applications, it is important to control the neural differentiation of stem cells.

Among various stem cells including embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, mesenchymal stem cells (MSCs), and so on, human embryonal carcinoma cell line NTERA-2 clone D1 (NT2) cells are known as a typical cell model for neuronal differentiation *in vitro* (Serra *et al.*, 2007; Abolpour Mofrad *et al.*, 2016), and have been utilized for developmental neurotoxicity testing (Hill *et al.*, 2008) and neuronal transplantation studies (Kondziolka and Wechsler, 2008). NT2 cells can differentiate into neurons by treatment with retinoic acid (RA), an inducer of mammalian neural development (Coyle *et al.*, 2011). Furthermore, some researchers have reported that the neural differentiation of NT2 cells is promoted by the formation of spheroids (spherical multi-cellular aggregates), which are formed by the rearrangement and compaction of cell aggregates (Serra *et al.*, 2009). Herein, the control of spheroid size is an important issue, because it affects the differentiation fate of stem cells.

We have developed a microwell chip, in which microwells of several hundred micrometers are regularly fabricated on a culture substratum, as a culture platform for spheroid formation (Sakai *et al.*, 2010). Cells in the microwells form cell aggregates and then generate spheroids. Consequently, this technique facilitates control of spheroid size and produces a large number of homogenous spheroids.

In this study, we cultured homogenous NT2 spheroids using a microwell chip and then compared the differences in the cell proliferation and neuronal differentiation of NT2 cells using traditional monolayer and spheroid cultures. The goal of this study is to clarify the effects of cell morphology on NT2 cell properties.

2 Materials and Methods

2.1 Microwell chip

The chip contained 195 microwells in a triangular arrangement at a pitch of 660 μm on a polymethylmethacrylate plate (24 \times 24 mm) (Figure 1). Each circular microwell was 600 μm in diameter and 600 μm in depth, and its surface was modified with polyethylene glycol (PEG) to render it non-adhesive to cells.

This chip was prepared using microfabrication by a programmable micromilling system, along with chemical modification of the surface (Sakai and Takaichi, 2007).

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The fabricated chip was immersed in culture medium prior to use.

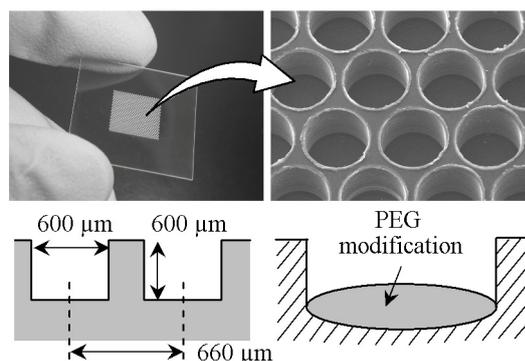


Figure 1. Schematic diagram of the microwell chip.

2.2 NT2 culture

NT2 cells (ECACC 01071221) were cultured as a uniform monolayer in a 100-mm tissue culture dish containing 10 mL Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

NT2 cells grown to confluence in a tissue culture dish were trypsinized (0.25% trypsin) and resuspended in culture medium. Following this, 1.95×10^5 cells were inoculated onto the chip placed in a polystyrene culture dish (diameter, 35 mm) containing 2 mL culture medium. Using this procedure, approximately 1000 cells were seeded in each microwell. For the monolayer culture, 1.95×10^5 cells were inoculated in the tissue culture dish (diameter, 35 mm) containing 2 mL culture medium. Furthermore, cells in both culture systems were cultured in two medium conditions, with and without 10 μ M retinoic acid (RA), to evaluate the effects of the neural development inducer.

The culture medium was changed every other day. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

2.3 Cell number and spheroid size

Cell numbers in both culture systems were determined at 1, 3, 5, and 7 d of culture using a modified DNA-40,6-diamidino-2-phenylindole (DAPI) fluorescence method (Nakazawa *et al.*, 2006). A standard curve was plotted for the DNA content using values obtained from a cell suspension, which was used to convert the DNA-DAPI fluorescence values into cell numbers.

To evaluate the changes in spheroid size over time, images of 50 spheroids formed in the microwell chip were obtained using a phase contrast microscope. Spheroid sizes were measured using a 2-dimensional image analysis program (Win ROOF). Each spheroid size was calculated by the equivalent circle diameter method.

2.4 Live/dead and hypoxia assays

Calcein-AM and propidium iodide (PI) were used to

identify the live and dead cells, respectively. The assay solution comprising calcein-AM and PI was prepared by adding 10 μ L of 1 mmol/mL calcein-AM stock solution and 15 μ L of 1.5 mmol/mL PI solution to 5 mL of phosphate buffered saline (PBS). NT2 spheroids were incubated with the assay solution for 15 min, and then washed with PBS. The double-stained cells were assessed using a fluorescence microscope.

Lox1 reagent was used to evaluate the hypoxic state in the spheroid. Culture medium containing 20 mg/mL Lox1 reagent was prepared as the assay solution. Spheroids were incubated in the assay solution for 24 h, and the stained spheroids were then examined under a fluorescence microscope.

2.5 Real-time PCR analysis

The neural differentiation patterns of NT2 cells were compared between the monolayer and spheroid cultures. Expression of typical gene markers of neural differentiation: nestin (NES) as neural progenitor cell marker; tubulin beta 3 (TUBB3) as a neuron marker; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, were analyzed using real-time polymerase chain reaction (PCR).

Cells in both culture systems were collected after 7 d of culture for gene expression analysis. The total RNA of each sample was extracted using a spin column according to the manufacturer's instructions. cDNA was synthesized from 0.2 mg total RNA via a high-capacity cDNA reverse transcription kit. The samples were then stored at -20°C until they were processed for real-time PCR analysis.

Real-time PCR was performed on an Applied Biosystems StepOne Real-Time PCR system using TaqMan Gene Expression Assay Kits. The comparative cycle time method was used to quantify gene expression according to the manufacturer's protocol. The expression levels of the target gene were normalized to those of GAPDH. Using the gene expression levels in the initially prepared NT2 cells as a reference, the expression levels of differentiation-related genes in the samples were calculated (the expression levels of the initially prepared NT2 cells were set as 1).

2.6 Statistical analysis

Data are presented as the mean \pm standard deviation (SD) and correspond to three time points. Statistical analysis was performed using a repeated-measures analysis of variance. $P < 0.05$ was considered statistically significant.

3 Results and Discussion

3.1 Changes in cell morphologies

Figure 2 shows the changes in cell morphologies in the tissue culture plate and microwell chip. In the tissue culture plate, the seeded NT2 cells immediately adhered

and extended on the plate. The cells then formed a monolayer morphology and covered the plate surface within 5 d of culture. Although the cells in RA(-) were layered with increased culture time, the monolayer morphology in RA(+) was maintained throughout the culture period.

In the microwell chip culture, the cells were aggregated in each microwell and formed spheroids within 24 hours of culture, and the spheroid morphology was maintained throughout the culture period. There was no difference in spheroid morphology with the presence or absence of RA.

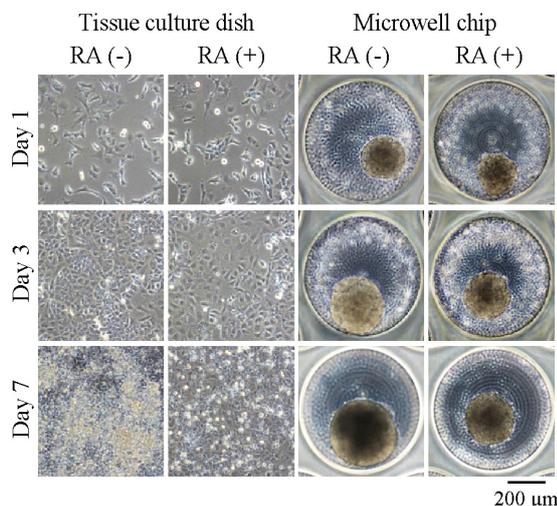


Figure 2. Changes in NT2 cell morphologies.

3.2 Cell proliferation

Figure 3 shows the changes in NT2 cell growth under the monolayer and spheroid cultures. In the RA(-) condition, the cell proliferation ability of the monolayer culture was approximately 5 times higher than that of the spheroid culture, indicating that the cell proliferation ability is deteriorated by the formation of 3D tissue structures. Generally, all cells in monolayer culture have potential for proliferation. However, cell cycles within a spheroid are different, and the cells in the periphery regions of spheroids have high proliferation ability, whereas the cells in the inner region hardly proliferate (Hirschhaeuser *et al.*, 2010). Consequently, the difference in the cell numbers that can proliferate may be reflected as the differences in cell numbers between the monolayer and spheroid cultures.

In both culture systems, RA treatment induced suppression of cell proliferation ability. RA is known to be involved in promoting cell differentiation as well as in cell cycle arrest (Schug *et al.*, 2007; Donato *et al.*, 2007). Our results indicate that these actions of RA are induced regardless of differences in cell morphology.

3.3 Analysis of spheroid properties

To understand the properties of NT2 spheroids, we evaluated changes in spheroid size and cell density within a spheroid.

In this study, the cells were seeded at the density of approximately 1000 cells/microwell. Consequently, the size of NT2 spheroids was approximately 200 μm at the initial stage of culture. Although the spheroid size increased via cell proliferation, spheroid growth in the RA(-) condition was higher than that in the RA(+) condition (Figure 4). This result corresponded well with observed cell proliferation profile (Figure 3).

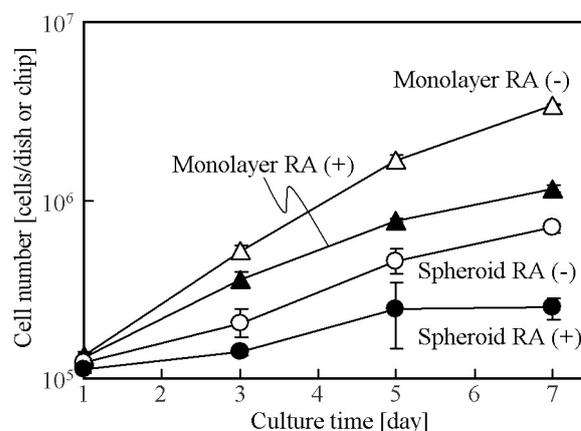


Figure 3. Growth curves of NT2 cells.

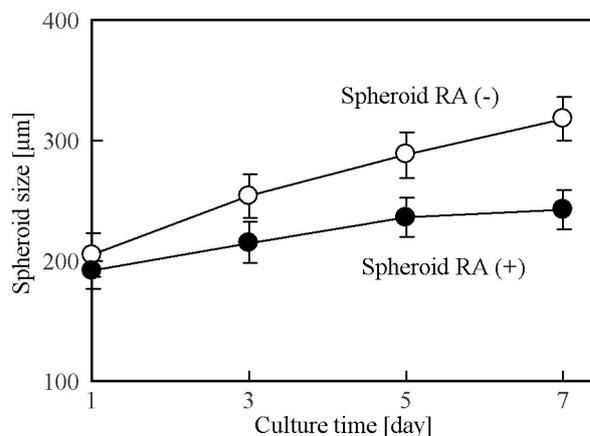


Figure 4. Changes in spheroid size.

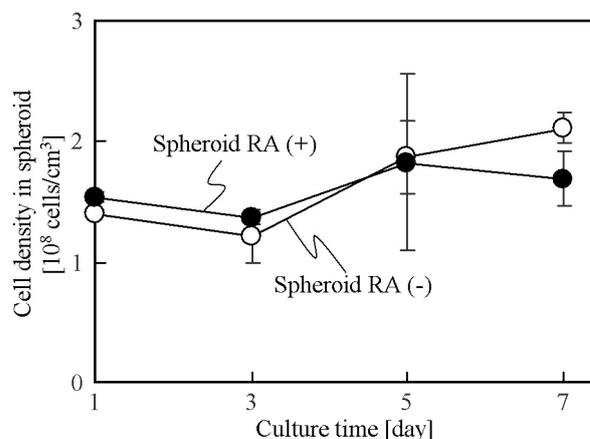


Figure 5. Changes in spheroid cell density.

The cell density in spheroids was calculated from the spheroid size and cell number. The changes in cell densities in spheroid were almost the same regardless of RA treatment, and these cell densities showed a

tendency to increase throughout the culture period (Figure 5). These results indicate that the packed state of cells in the spheroid is gradually enhanced with increasing culture period.

Because the spheroids are cell clumps, the oxygen concentration within a spheroid gradually decreases from the periphery to the center. Consequently, in large spheroids, a hypoxic region appears in the center of the spheroid, and it induces occurrence of cell necrosis (Hirschhaeuser *et al.*, 2010). To understand such spheroid properties, we evaluated the cell survival and oxygenation state within a spheroid at 3 and 7 d of culture (Figure 6).

Regardless of RA treatment, few dead cells (red) existed in the peripheral regions of spheroids, though most cells forming the spheroid consisted of viable cells (green).

The hypoxia probe, Lox1 reagent, produces fluorescence when the intracellular oxygen concentration is in the range of 2.5–10% O₂ (Zhang *et al.*, 2010). There were no appearances of the hypoxic domain at 3 d of culture. However, a hypoxic domain was slightly apparent in the center of the spheroid in the RA(+) condition at 7 d of culture, and a remarkable hypoxia state was observed in the large spheroids under RA(-) conditions. These results suggest that, in the case of NT2 cells, spheroids less than 350 μm can elude the occurrence of a cell death layer in the center of spheroid, although the hypoxia state is observed in large spheroids.

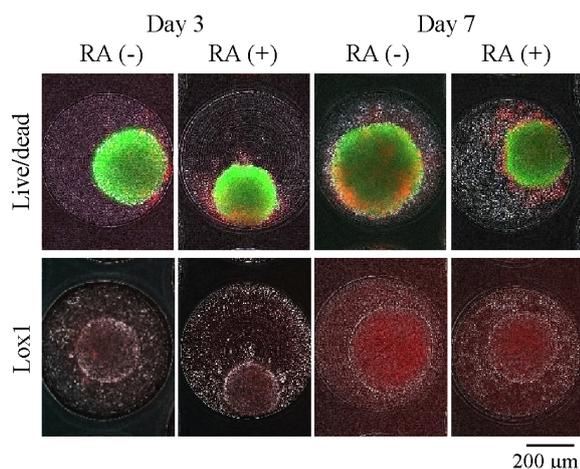


Figure 6. Staining of live (green) and dead (red) cells, and the hypoxic area (red) within the spheroid

3.4 Neural differentiation

Neurons are differentiated from stem cells via neural progenitor cells. In this study, the expression of typical neural differentiation marker genes (NES as a neural progenitor cell marker and TUBB3 as a neuron marker) was compared between monolayer and spheroid cultures after 7 d of culture (Figure 7).

In normal culture condition (without RA treatment), the expression levels of NES and TUBB3 in the monolayer were almost the same as those in the initially prepared cells, indicating that the monolayer culture maintains an undifferentiated state of NT2 cells. The

neural differentiation of NT2 cells was promoted by spheroid formation, and the expression levels of neural differentiation marker genes in the spheroid culture was approximately 2 times higher than those in the monolayer culture. Furthermore, the RA treatment enhanced neural differentiation in the monolayer and spheroid cultures. These results indicate that neural differentiation of NT2 cells can be accelerated by the synergistic effect of 3D tissue structure formation and stimulation with the inducer.

It is known that spheroid culture of ES/iPS cells has been widely used as a trigger for the initial process of stem cell differentiation (Kurosawa, 2007; Hsiao and Palecek, 2012). In this study, we demonstrated that the spheroid culture was effective to promote neural differentiation of stem cells. The mechanism that the spheroid culture can promote neural differentiation than monolayer culture is not clear. However, multi-cellular interactions through cell-cell contact and mechanical stress, and concentration gradients of various substances (oxygen, nutrients, and wastes) occur inside the spheroid. Therefore, the changes in such intercellular signals and internal environments resulting in the formation of 3D tissue structure may affect neural differentiation.

Furthermore, the microwell chip culture is a promising culture platform for the investigation of spheroid size effect, because it can control the spheroid size by the difference of seeding cell density. The evaluation of relationship between spheroid size and neural differentiation is our next target.

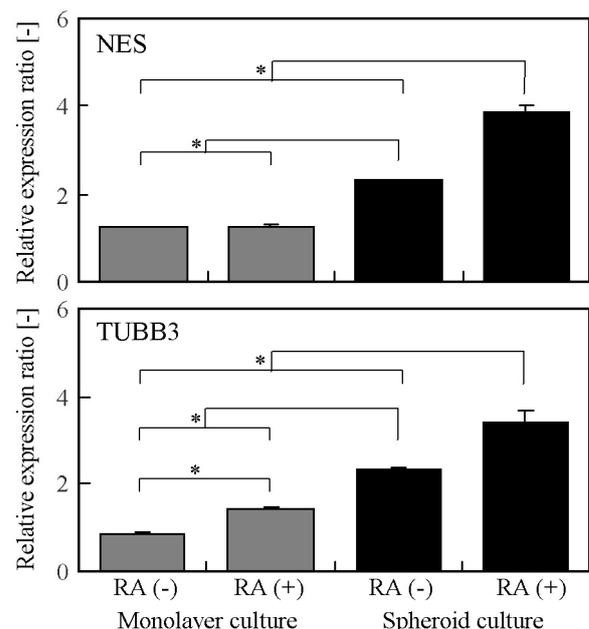


Figure 7. Expression levels of neural marker genes, NES and TUBB3, in monolayer and spheroid cultures. The expression levels of the initial cell suspension were set as 1.0. Error bars represent the SD. *P < 0.05

4 Conclusions

In this study, we established an array culture of homogeneous NT2 spheroids using a microwell chip.

Cell proliferation ability was decreased upon spheroid formation compared with the monolayer culture, whereas the neuronal differentiation of the spheroid culture was higher than that of the monolayer culture. Furthermore, the neuronal differentiation of NT2 cells was dramatically enhanced by the combination of spheroid formation and inducer (retinoic acid) treatment. From these results, we demonstrated that the NT2 cell properties were influenced by the difference in cell morphology and that spheroid culture is a promising technique to induce neuronal differentiation.

Acknowledgements

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