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Monitoring the differentiation process of human iPS cells to cerebral cortical neurons by LC-MS/MS media analysis

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1. Overview

Human induced pluripotent stem cells (hiPSCs) can differentiate into any cell type in the body. They consequently hold great promise as a source of cells for applications in regenerative medicine and drug discovery. To contribute to development of hiPSCs technologies, we have aimed to monitor the hiPSCs differentiation through multi components analysis using triple quadrupole LC-MS/MS.

Figure 1 Monitoring of culture supernatant components

2. Introduction

Alzheimer's disease (AD) is a neurological disorder in which the death of brain cells causes memory loss and cognitive decline. Due to increasing of the number of patients with AD, drug development for AD has been requiring. To facilitate drug development for AD, human induced pluripotent stem cells (hiPSCs) has drawn attentions because disease-specific cells from patients can be used for *in vitro* model of AD by differentiation into cerebral cortical neurons. However, it needs long term culture process to differentiate hiPSCs into cerebral cortical neurons, there is no verification method during differentiation without cell disruption. Therefore, we have aimed to monitor the differentiation through multi components analysis using triple quadrupole LC-MS/MS.

3. Methods

3-1. Differentiation of hiPSCs to cerebral cortical neurons

hiPSCs were maintained in StemFit® AK02N medium on iMatrix-511 coated surface, and 6 days before of differentiation culture, three chemicals were added to medium for pre-culture. After pre-culture, cells were dissociated and seeded into 96-well U bottom plates to form neurosphere and cultured in DMEM/F-12 based differentiation medium for 12 days. Neurospheres were replaced on Poly-L-Ornithine and Laminin coated well plates and cultured for 14 days to differentiate to cerebral cortical neurons.

3-2. Biological analysis

Cultured cells were collected at day -6, 0, 12 and 26 for qRT-PCR analysis. Reactions were carried out in triplicate, and data were analyzed by using the comparative (ΔΔCt) method. At the end of culture, cells were fixed for immunocytochemistry staining. Markers analyzed for these analysis were described at Figure 5 and 6.

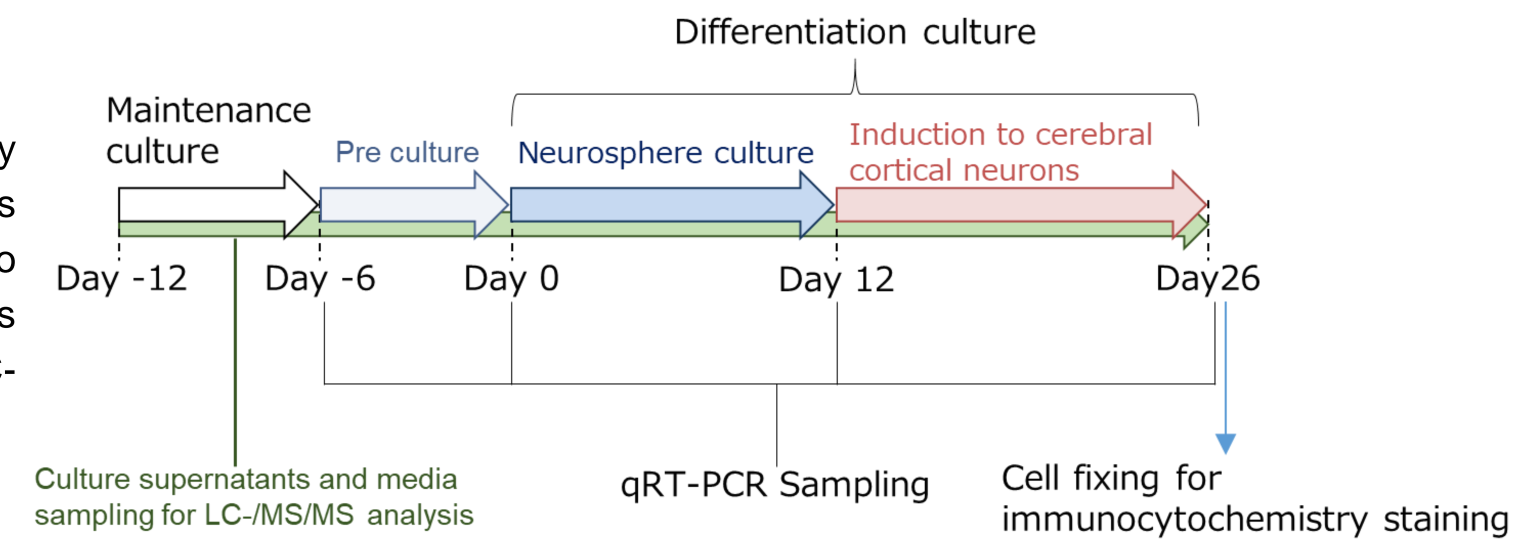
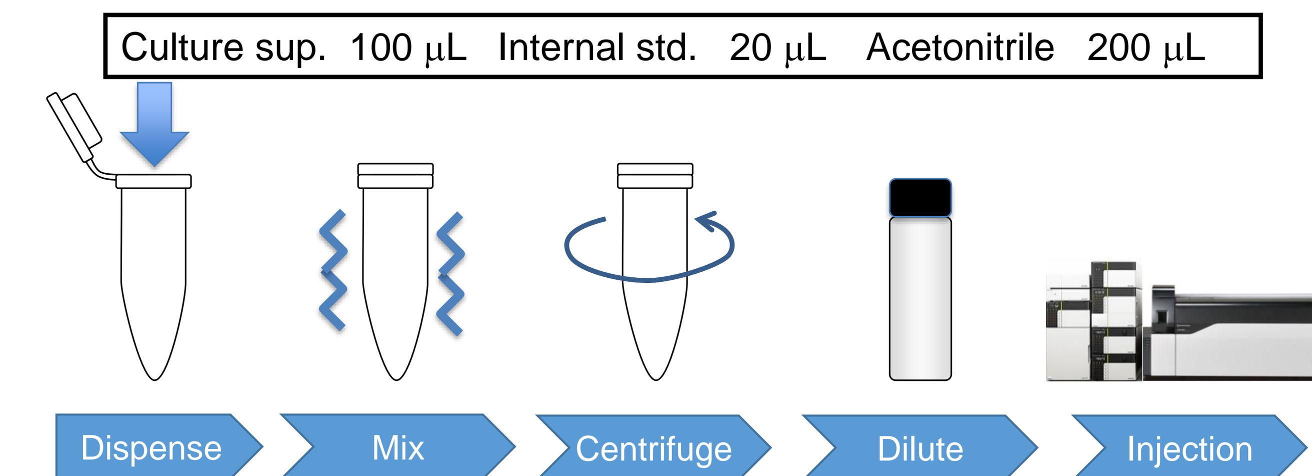


Figure 2 Experimental flow

3-3. Culture supernatant analysis by LC-MS/MS

Culture-supernatants were collected at every medium change and stored at -80℃ until use. 2-Isppropylmalic acid solution (20 µL) , which is an internal standard, was added to the supernatant (100 µL) , and acetonitrile (200 µL) was also added to the mixture to precipitate proteins. Precipitated proteins were removed by centrifugation (15,000 rpm for 15 min) and the supernatant was diluted with ultrapure water (10-fold dilution) and used as the sample for LC-MS/MS. The culture-supernatants were analyzed in triplicates. Before every medium change fresh media were collected and stored as blank samples.



LCMS-8050

LC/MS/MS Method Package For Cell Culture Profiling ver. 2 (including all the parameters necessary for LC-MS/MS analysis for 126 compounds including internal Std.) (Figure 4).

Figure 3
Flow of LC-MS/MS analysis

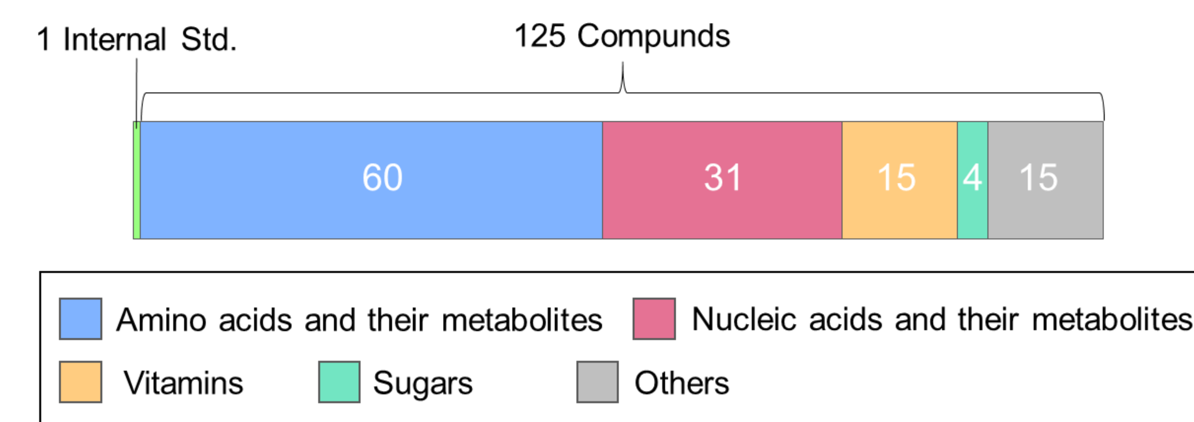


Figure 4 Breakdown of compounds registered in the method

4. Results

4-1 Biological analysis for evaluation of hiPSCs differentiation

Time course profiles of each gene markers were examined to evaluate differentiation culture. During pre culture and differentiation culture, expression levels of *Oct-3/4* were become lower compared to its level of maintenance culture. The expression levels of *FOXG1* become highest on day 12 and showed the same level of expression at day 0 and 26. The expression levels of *MAP2* showed a tendency to become higher with culture process. *CTIP2* was expressed higher at day 26 (Figure 5).

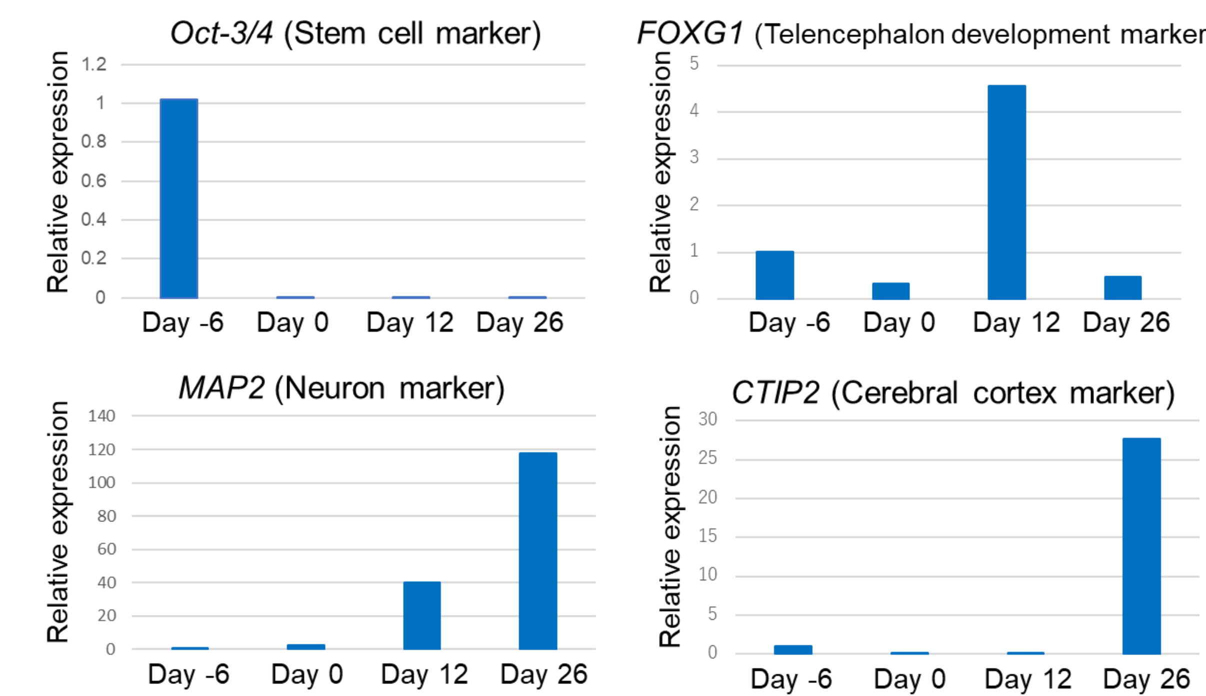
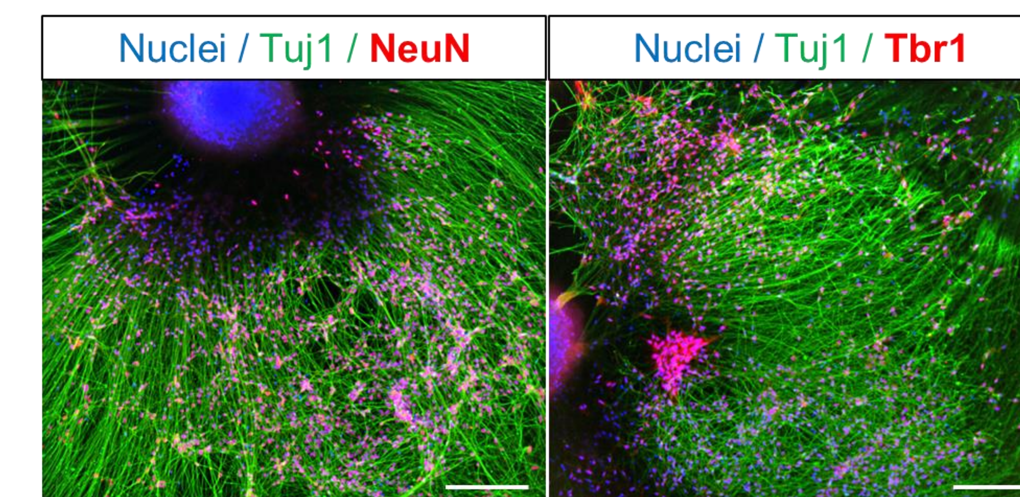


Figure 5 Time course profiles of gene markers

We next examined expression of protein markers by immunocytochemistry staining (Figure 6). Expression of all protein markers was detected from cells at the end of culture.



※Tuj1：Brain neuron marker, NeuN：Mature neuron marker, Tbr1：Cerbral cortex marker

Figure 6 Immunocytochemistry analysis of cultured cells
(Scale bars : 200 µm)

These results suggested that hiPSCs was lost its pluripotency during pre culture, and cells were differentiated into neural progenitor cells of the telencephalon after neurosphere culture. At the end of differentiation culture, cells were differentiated into mature cerebral cortical neurons. Thus differentiation of hiPSCs into cerebral cortical neurons was evaluated by qRT-PCR and immunocytochemistry staining analysis.

4-2 Time course change of culture supernatant components

After evaluation of differentiation, we examined time course change of culture supernatant components. In this differentiation process, there were 4 steps of a culture process, such as maintenance culture, pre-culture, neurosphere culture, and differentiation into cerebral cortical neurons (Figure 2). Some components changed their trends of increasing or decreasing when the culture step was changed to the next one (Figure 7).

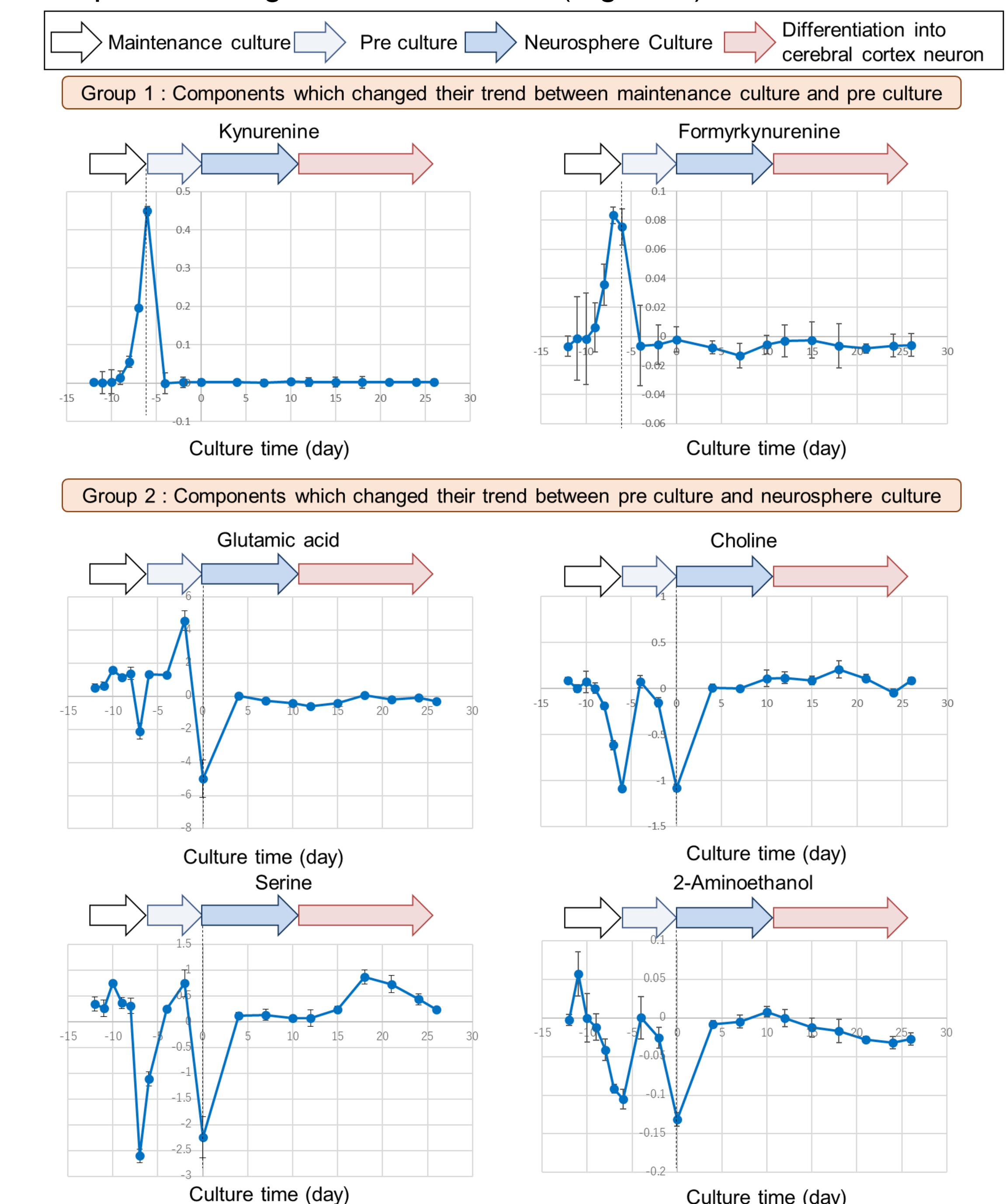


Figure 7 Time course profiles of components (※The vertical axis represented the difference between area ratio of supernatants and blanks by internal Std.)

Kynurenine has an important role for maintenance of undifferentiated state of hiPSCs and Formylkynurenine is a precursor of Kynurenine. Components at Group 2 have been reported their relationship with neurotransmission. These results suggested that monitoring consumed or secreted components in differentiation culture is useful for evaluation of whether neural differentiation is going well or not.

5. Conclusion

- Culture supernatant components analysis by LC-MS/MS reveals trend change of during culture process for hiPSCs differentiation to neurons.
- Culture supernatant monitoring has a potential to be a candidate of non-invasive monitoring method for hiPSCs differentiation to neurons.

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