学位論文内容の要旨
（Summary of Dissertation）

博士の専攻分野の名称　博士（医　学）　　氏 名　　范彬
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学 位 論 文 題 名
Establishment of a Novel Method for Differentiating into Dopaminergic Neurons Using Charged Hydrogels
（基質荷電を用いたドパミン作動性神経細胞への新規分化法及び治療法の確立）

Background and Objectives

Parkinson's disease (PD) is a long-term neurodegenerative disease of mainly the central nervous system that affects both the motor system and non-motor systems. The classic symptoms of PD result from selective degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc).

For now, cell replacement therapy is considered as a suitable treatment to replace the lost neurons. And differentiation of human induced pluripotent stem cells (iPS cells) to DA neurons has been suggested to provide such a model system.

The aim of this study was to develop the protocol based on substrate to cultivate the cells. I developed the new substrate using an electrically charged hydrogel so that allowed to generate very consistent numbers of matured DA neurons from human iPS cells lines which could generate dopamine.

Materials and Methods

The charged hydrogel was made by cationic monomer, 3-(acryloylaminopropy)-trimethylammonium chloride (APTMA), anionic monomer, 2-acrylamido-2-methylpropane sulfonic acid (NaAMPS), and dimethylacrylamide (DMA) was employed as a neutral monomer.

The iPS cells were plated on PS dish (polystyrene) and hydrogels in stem cell induction medium AK02N. From day 0 to day 12, DA neural induction was performed using Glasgow's MEM (GMEM) supplemented with 8% KnockOut Serum Replacement (KSR), 0.1 mM MEM nonessential amino acids, 100 mmol/L sodium pyruvate, and 0.1 mM 2-mercaptoethanol. Then 100um LDN193189, 500um A-83-01, 1mM Purmorphamin，100ug/ml fibroblast growth factor 8 (FGF8) and 3mM CHIR99021 were added in different time pried to induce DA progenitor cells (DAPs). After day 12, change the medium to the neural differentiation medium containing Neurobasal medium supplemented with B27 supplement, 2 mM L-glutamine, 10 ng/ml glial cell line-derived neurotrophic factor (GDNF), 200 mM ascorbic acid, 20 ng/ml brain-derived neurotrophic factor (BDNF), and 400 mM bucladesine (dbcAMP) were added in this time. Then cultured with the neural differentiation medium until day 28.

I have checked the expression levels of mRNA and protein which are involved in DA differentiation by using RT-PCR and western blot. The expression level of L-dopa and dopamine was checked by ELISA. The pathways expression was checked by RNA sequence, A Gene Ontology (GO) enrichment test and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Statistical analysis: Graphical data are presented as means and standard deviation. Student's t-test was used for comparisons, with $P < 0.05$ considered significant.

Results

1. The physical properties of the charged hydrogel

A series of synthetic hydrogels that focus on having the concentration of cationic and anionic charge was developed in my thesis. They were constructed in different ratios. The zeta potential charge analysis shows that the whole gels possessed negative charge, and was from -41 mV to -6mV. And the modulus was from 25 kPa to 80 kPa.

2. The charged hydrogels efficiently induced DA neurons

First, I tested cell culture on multiple hydrogels with varying ratios of cationic, anionic, or neutral monomers.

On most hydrogels, cells either failed to adhere and proliferate, forming spheres on the gel surface or underwent cell death. However, on C1A9 and C2A8 hydrogels, cell attachment and proliferation were observed.

Next, I found that on hydrogels, tyrosine hydroxylase *(TH)* mRNA expression was significantly higher compared to the PS control. A concomitant decrease in the mRNA levels of stemness-related marker genes octamer-binding transcription factor 4*(Oct 3/4)* and homeobox protein *(Nanog*) levels decreased by 50% and 55%, respectively. Additionally, neural differentiation markers polysialylated neuronal cell adhesion molecule *(PSA-NCAM)* and microtubule-associated protein 2 *(MAP2)* increased in the gel group. Markers for DA neuron's differentiation, such as, aromatic l-amino acid decarboxylase (*AADC)* and dopamine transporter *(DAT)*, as well as midbrain differentiation markers like atrial natriuretic peptide-converting enzyme (*CORIN)*, nuclear receptor related 1 *(NURR1)* and forkhead box A2 *(FOXA2)* increased in the gel samples. In western blot analysis, TH was detected at day 12 in PS dish group, while it could be observed at day 7 in gel groups. Besides, in day 28, the expression level of TH and AADC were increased in both gels. These results indicate that DA differentiation of iPS cells was promoted on charged gels.

3. DA cells differentiated on charged gels show an early enhancement in dopamine production capacity.

Next, I examined the functionality of differentiated DA cells by assessing their L-dopa and dopamine production capabilities through both *in vitro* and *in vivo* experiments. The concentrations of L-dopa and dopamine in the culture medium were measured using ELISA. Specifically, the expression level of L-dopa and dopamine in gel group was higher than the PS group. *In vivo* assay, the result was similar.

In RNA sequence analysis, I calculated the fold change by dividing the gene expression of each differentiated sample by that of the undifferentiated control. These results indicate that the stem cell characteristics of hiPS_day0 were lost during differentiation, and neuronal differentiation was strongly promoted in all three groups.

Pathways significantly activated in C1A9 and C2A8 included many related to neuronal differentiation, such as catecholamine secretion and dopamine transport. Additionally, the database for annotation, visualization and integrated discovery (DAVID) analysis using the gene list with more than a two-fold increase which were visualized as an interactive graph using REduce + VIsualize Gene Ontology (REVIGO). These results suggest that iPS cells induced to differentiate on hydrogels show more advanced neuronal differentiation and enhanced differentiation towards DA neurons.

Discussion

The most significant differences between the hydrogel and PS dish are surface charge and stiffness. The hydrogel is considerably softer than the PS dish. In this thesis, I observed an early promotion of neural differentiation on the hydrogel compared to the PS dish, suggesting that substrate stiffness may have influenced this outcome. Additionally, surface charge may provide distinct stimuli to adherent cells by affecting protein adsorption from the culture medium or proteins secreted by the cells, or by directly influencing membrane potential. The stiffness and charge promoted the differentiation of iPS cells into DA neurons.

Conclusion

In summary, I found that the C1A9 and C2A8 gels could induce the differentiation of DA neurons more efficiency, and this phenomenon may be referred to as hydrogel-activated promotion. One limitation of this study is that the essential chemical and/or physical factor(s) of the C1A9 and C2A8 gels were not directly described and 100% precisely controlled, so further study is required.