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審査研究科	人間総合科学研究科
学位論文題目	Effect of prolonged culture and hypoxic conditions on in vitro neural differentiation potency of embryonic stem cell-derived neural stem cells (マウス胚性幹細胞由来神経幹細胞神経分化における低酸素および長期培養による影響の解析)
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Abstract of thesis

In this doctoral dissertation, LUKMANTO DONNY describes the development of a new method to induce neural cells from embryonic stem (ES) cells. The content is summarized as follows.

Background and purpose:

After birth the central nervous system (CNS) retains very limited regenerative capacity not only in physiological conditions but also even after injury or under degenerative diseases. Nowadays, it is expected that neural cell transplantation would be a novel therapy for CNS diseases. Neural stem cells (NSCs) are stem cells which have the capacity to self-renew and give rise to the three major cell types of the CNS (neurons, astrocytes and oligodendrocytes). However, endogenous NSCs are stem cells with very limited regenerative capacity owing to its quiescent nature.

Pluripotent stem cells (PSCs), such as embryonic stem (ES) cells, are expected to be the source for neural cells because of their capacity to generate NSC intermediary stage. In addition, ES cells have been utilized as an excellent model for the study of neural development. However, current methods of neural induction from PSCs do not generate neural cells exclusively. Moreover, the addition of developmental signals at certain specific times to mimic

development led to complexity and higher cost of the protocols. Therefore, it is required to develop a simple and cost-efficient neural induction method to generate ES cell-derived neural stem cells (ES-NSCs) that based on the principle of neural development.

Neural development is a dynamic process that is regulated through time, i.e., neural-to-glia switch is observed under the prolonged culture of brain slice-derived radial glia cells. On the other hand, microenvironment such as low oxygen tension (hypoxia) is reported to enhance glial differentiation of NSCs. Despite these accumulating knowledges, the dynamic changes of ES-NSCs under the effects of prolonged cell culture and hypoxic conditions are still obscured. Therefore, the author aimed to simplify the method to induce neural cells from PSCs and to clarify the effects of prolonged culture and hypoxic conditions on ES-NSCs.

Materials and methods:

Sox1 is a gene which encodes a transcription factor and functions primarily in neurogenesis. The author used Sox1-GFP mouse ES cells as PSCs, in which GFP is expressed under *Sox1* promoter. Neural induction was basically performed with the serum free embryoid body (SFEB) method without addition of any exogenous signals. ES-NSCs were isolated through cell sorting by Sox1 (GFP) expression. In order to determine whether the dynamic *in vitro* changes of ES-NSCs was similar to *in vivo* progression, the author defined the early-, middle- and late-passaged ES-NSCs to be equivalent with the timing of *in vivo* neural development of mouse, and examined the effects of environmental factors in the *in vitro* culture on early-, middle- and late-passaged ES-NSCs. Neural markers were analyzed with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunostaining. In addition, the expression of Sox1 was measured with fluorescence activated cell sorting (FACS). The expression of HIF-1α was measured by immunostaining and Western blot. Inhibition of HIF-1α expression was performed by treatment with small interfering RNA targeting HIF-1α (si-HIF-1α). Gene expression related to developmental signals was analyzed with qRT-PCR. Neural cells' apoptosis was detected with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. Finally, statistical analyses were performed through unpaired Student's t-test or Turkey-Kramer test after a one-way analysis of variance. The software used for the statistical analyses was GraphPad Prism 7.0 software program.

Results:

The author effectively isolated the neuroectoderm cell population derived from ES cell aggregates by using a simple method combined of SFEB culture and cell-sorting with Sox1 as a marker. Next, the author demonstrated that the isolated cells showed *in vitro* temporal neural specification which resulted in distinct cell fate after neural differentiation. In details, the early-passaged ES-NSCs gave rise to neurons, whereas late-passaged ES-NSCs gave rise to glial cells similarly to the *in vivo* dynamic changes during the neural development. Remarkably, hypoxic treatment induced the neural differentiation of ES-NSCs but did not affect the cell fate. Under hypoxic conditions, early-passaged ES-NSCs showed upregulation of neuronal markers, whereas late-passaged ES-NSCs showed the upregulation of a glial marker. Expectedly, HIF-1α expression was upregulated in hypoxic conditions. The knockdown of HIF-1α expression impaired the neuronal differentiation of early-passaged ES-NSCs under hypoxic

conditions. Thus, the author proved that HIF-1α is involved in the induced effects by hypoxic treatment on the neural differentiation of ES-NSCs.

Discussion:

The author demonstrated that cell sorting technology could be utilized to compensate the addition of exogenous signals during early neural induction from PSCs. More importantly, the author showed the distinct effects of prolonged culture and hypoxic conditions on the neural differentiation of ES-NSCs, i.e., prolonged culture was involved in the cell fate after neural differentiation, while hypoxic conditions efficiently promoted neural differentiation. In addition, the author suggested that loss of Sox1 expression might contribute to the loss of neurogenic capacity of NSCs due to prolonged culture whereas HIF-1α expression is responsible for upregulation of neural differentiation. Taken together, the author proposed that careful consideration about time and oxygen tension should be taken into account in the *in vitro* induction of neural cells from PSCs for clinical utilization of PSC-derived neural cells.

Although the results in this dissertation were derived from mouse ES cells, it will be highly likely applicable for human ES cells and iPS cells. In the fields of basic research, the neural cells derived from human iPS cells can be applied for drug discovery of many kinds of neural disease. As a matter of fact, many kinds of disease-specific iPS cell line derived from patients suffering from neural disease have been generated so far. On the other hand, in the fields of clinical application a marker-expressing PSC (e.g., Sox1-expressing ES cells) is not appropriate for clinical use of the cells derived from them, and thus the author discussed that another method such as staining of cell-surface molecule should be developed for application in the clinic.

Abstract of assessment result

General comments:

In vitro induction of specific differentiated cells from pluripotent stem cells such as ES cells and iPS cells has been tried and developed in many fields mainly for applications in regenerative medicines and sometimes for drug discovery. The author developed a new method to effectively induce neural cells from ES cells, in which prolonged culture and hypoxic conditions functioned as key factors. Of note, the method developed by the author is much more cost-effective compared to the conventional methods which require many expensive supplemental factors.

Assessment:

The final examination committee conducted a meeting as a final examination on June 11, 2020. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

Conclusion:

The final examination committee approved that the applicant is qualified to be awarded Doctor of Philosophy in Medical Sciences.