Developmental Plasticity of CNS Stem Cells:

Implications for Neurodevelopmental/Psychiatric Diseases

Abstract

Interest in neurodevelopmental and psychiatric diseases, including autism spectrum disorders, and metal disorders including schizophrenia, continues to increase in the medical science as well as with respect to social issues. However, very few effective biomedical therapies are available for the treatment of such conditions, which may be attributable to the lack of knowledge of the basic principles governing the development, shaping and operation of the human brain. These deficits in scientific understanding drew my interest to the physiological regulation of the development of the central nervous system (CNS), leading to the acquisition of higher brain functions. I hypothesize that impairment of these processes leads to the development of various neurodevelopmental and psychiatric diseases. As a first step, I have sought to experimentally investigate the temporal regulation of developmental plasticity of stem cells in the CNS, and was able to recapitulate the “first neuron and glia generation” principle in vitro by inducing the neural differentiation of mouse embryonic stem cells. In introducing this work, I will also suggest how these findings might help to deepen our understanding of the human brain and diseases of the CNS.

Introduction:

The Rationale to investigate CNS stem Cells

The human brain, which is composed of a trillion cells including neurons and glia (the name “glia” refer to astrocytes, oligodendrocytes and so on), is one of the most complex biological organizations on this planet. Cellular communities such as neuronal networks and neuron-glia interactions have enabled us to acquire higher brain functions. I believe that various neurodevelopmental and psychiatric diseases develop because of impairment of these brain functions, leading to serious social problems and economic burdens. At present, the media has provided us with a huge amount of information about neurodevelopmental/psychiatric diseases, including autism spectrum disorders such as Asperger syndrome, Savant syndrome and attention deficit hyperactivity disorder (ADHD), as well as related mental disorders, such as schizophrenia, from the aspects of both medical science and social issues. People who suffer from these kinds of neurodevelopmental/psychiatric disorders tend to have difficulties in their learning abilities or social skills (Osumi, 2006, chap.1). However, it is also interesting to notice that a certain population of people/children

with neurodevelopmental disorders are gifted with higher abilities in other fields such as art, photographic memory, imagination, etc. These people with autism who are endowed cognitively with “higher functioning” are diagnosed as having high-functioning autism (HFA) (Rao et al., 2008, p.353). Historically, people who have had great impacts on human civilization, such as Isaac Newton, Albert Einstein, Wolfgang Amadeus Mozart, Ludwig van Beethoven, and Lewis Carroll, are said to have suffered from HFA or Asperger syndrome (Osumi, 2016, chap.1). It is also unknown why several kinds of neurodevelopmental/psychiatric diseases occur simultaneously in the same individuals. I suspect that this situation results from the ambiguity of diagnosis for these diseases, due to the lack of basic understanding of these diseases. To identify concrete mechanisms leading to these diseases and to develop their innovative therapeutic methods, the normal developmental processes in the human brain must first be studied and then contrasted with those of patient-specific developmental processes. To understand these processes, I came to be interested in the stem cells present in developing and adult central nervous system (CNS), so-called “CNS stem cells,” which produce a wide variety of cells within the CNS (Temple, 2001, p.113). Particularly, as a first step to finding a way to

understand and cure neurodevelopmental/psychiatric diseases, I have tried to confirm the temporal regulation of the differentiation ability (which I term “developmental plasticity”) of CNS stem cells using actual data obtained from experiments that I performed during the summer of 2016.

The precise mechanisms of how a wide variety of neuronal and glial cells are produced during CNS development largely remain unknown. Previous studies have shown that both neurons and glia are generated from CNS stem cells in spatially and temporally regulated fashions. It is also known that a wide variety of neuronal subtypes are generated according to their spatial and temporal identities. The developing neural tube (a prototype of the CNS) is known to subdivided into distinct domains along two perpendicular axes, i.e., anteroposterior (A-P) and dorsoventral (D-V) axes, and each region produces a specific subtype of CNS stem cells (Imaizumi et al., 2015, p.1010). Currently, regional identities of CNS stem cells can be obtained *in vitro* during neural differentiation of pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), by modulating signals involved in development of A-P and D-V axes (Imaizumi et al., 2015, p.1011).

Regarding the temporal specification of CNS stem cells, the order of “first neuron and glia generation” is widely conserved across both CNS regions (Okano and Temple, 2009, p.112). However, precise mechanisms for how CNS stem cells acquire their temporal identities remain largely elusive. In the present study, I could confirm the “first neuron and glia generation” principle in a dish utilizing neural differentiation of mouse ESCs through improvements of the previous protocol (Okada et al., 2008, p. 3087). This method will be applied, in combination with genome editing and patients-specific iPSCs, to investigate functions of genes that are potentially involved in the specification of CNS stem cells and to model neurodevelopmental/psychiatric diseases (Okano and Yamanaka, 2014, p.6) to finally understand and conquer these diseases.

Experimental Procedures

ESCs Culture and Neural Induction

Mouse ESCs (EB3) were cultured as reported previously (Niwa et al., 2002, p.1527). Their neural differentiation was performed using a protocol similar to that of a previously report (Okada, 2008, p.3087). Formation of primary, secondary and

tertiary neurospheres and subsequent their differentiation was performed according to the previous method (Okada et al., 2008, p.3087). However, I made the following modification: undifferentiated mouse ESCs were dissociated and used for embryoid bodies (EBs) formation in the presence of 150 nM LDN193189 (StemRD), instead of Noggin protein.

Immunocytochemistry and Cell Counting

Immunocytochemical analysis of cultured cells was performed according to a previous report (Imaizumi et al., 2015, p.1018) using anti-β-TUBULIN III (Sigma-Aldrich) and anti-GFAP (DAKO Cytomation) as the primary antibodies and observed using a LSM-710 confocal laser microscope (Carl Zeiss). The number of β-TUBULIN III-positive neurons, GFAP-positive glia and total cells (Hochest33342-positive cells) was determined by counting the number of each type of cell present in 10 visual fields for each slide. For the statistical analysis, the Student *t*-test was used (*$P < 0.05$, ***P< 0.001).

Results and Discussions

Maintenance of mouse ESCs and neural induction

To examine the effects of temporal changes in the differentiation potential of CNS stem cells, I took advantage of a neural induction system consisting of mouse ESCs (Fig.1), using the EB3 line, which carries a Blasticidin (an antibiotic)-resistant gene under the promoter of Oct4 gene (a pluripotent stem cell marker gene) for maintenance of undifferentiated and pluripotent state. To induce the differentiation of EB3 cells, I replaced the medium with Blasticidin-free one and allowed embryoid bodies (EBs) to form. To obtain efficient neural induction during EBs-formation, I tried to block the BMP signaling pathway, which inhibits neural development (Smith and Harland, 1992, p.829). Noggin is an endogenous factor involved in neural induction by blocking the BMP signal (Zimmerman et al., 1996, p.599); thus, a recombinant Noggin protein was added to cell cultures to induce EBs to preferentially adopt a neural fate in a previous study (Okada et al., 2008, p.3087). Noggin-treated EBs allow the induction of CNS stem cells, which can then be expanded using the neurosphere method. However, the recombinant Noggin protein is an expensive reagent; thus, I added a cheaper inhibitor of the BMP signaling pathway, LDN193189, which is a small chemical compound that can inhibit BMP type I receptors ALK2/3 (Yu et al., 2008, p.1363). The replacement of Noggin with

LDN193189 worked well, and I was able to efficiently induce primary neurosphere formation from LDN193189-treated EBs.

Changes in Differentiation Potential of CNS stem cells during passage

Next, I examined whether the passage of primary neurospheres into secondary and tertiary neurospheres could result in a change in the differentiation potential of CNS stem cells (Figs. 2 and 3). Consequently, I was able to obtain the following data based on the three independent experiments: i) primary neurospheres almost exclusively gave rise to neurons, ii) the glial cell differentiation potential of CNS stem cells increased significantly during passages of neurospheres (Primary < Secondary, Tertiary) (*P < 0.05, ***P< 0.001), iii) the neuronal differentiation potential of CNS stem cells decreased significantly during cell passages (Primary > Secondary, Tertiary) (*P < 0.05, ***P< 0.001). Interestingly, these results obtained in vitro using mouse ESCs-derived CNS stem cells were consistent with the *in vivo* situation, i.e., “first neuron and glia generation” (Temple, 2001, p.112).

Evidence showing early CNS stem cells lack a response to gliogenic signals

Since the primary neurospheres were almost exclusively composed of neurons, but not glia, in the experiments described in Figure 1, it is likely that “early”

CNS stem cells lack the competency to respond to gliogenic signals (glia-inducing factors). Alternatively, it is possible that the amount of these factors) was insufficient to promote glial differentiation. To investigate these possibilities, I examined the effects of BMP-2, a well-known glia-inducing factor (Nakashima et al., 1999, p.479), on the glial differentiation of primary neurospheres in the same experiments shown in Figs 2 and 3. Interestingly, primary neurospheres still preferentially gave rise to neurons even in the presence of exogenously added BMP-2. Thus, early CNS stem cells have little competency to respond to gliogenic signals, consistent with the “first neuron and glia generation” principle.

Notably, a recent report charactering patients-specific iPSCs derived from Rett syndrome (a neurodevelopmental disease resulting in mental retardation and autistic behavior (Hargberg et al., 1983, p.471-479)) indicates that glial differentiation potential is prematurely induced in patients-derived early CNS stem cells (Andoh-Noda, 2015, p.1). This would provide an evidence showing the investigation of temporal identity of CNS stem cells is essential for understanding neurodevelopmental/psychiatric diseases. How their impaired temporal identity could lead to these diseases would be important issues to be clarified in the future.

Perspectives: Advanced research I can do by applying this method

In the present study, I treated and characterized wild type ESCs-derived neurospheres. The method used in the present study is a fundamental strategy for every kind of experiment using ESC/iPSCs-derived neurospheres, including those derived from human patient-derived iPSCs. Using this method, I will be able to analyze the relationship between the differentiation potential and the functions of specific genes using transgenic or genetically modified ESCs and iPSCs. Thus, the present technology is likely to contribute to the fundamental understanding of neurodevelopmental/psychiatric diseases, which I hope to continue studying in the future.

Conclusions

1. The temporal changes in the differentiation potential of CNS stem cells-derived mouse ESCs were recapitulated in a manner that reflected those occurring in vivo during CNS development.
2. I could develop an improved method for the neural induction of mouse ESCs

using a small chemical BMP-inhibitor.

3. The current method will be useful for the investigation of neurodevelopmental/psychiatric diseases by analyzing the developmental plasticity of CNS stem cells using patient-derived iPSCs.

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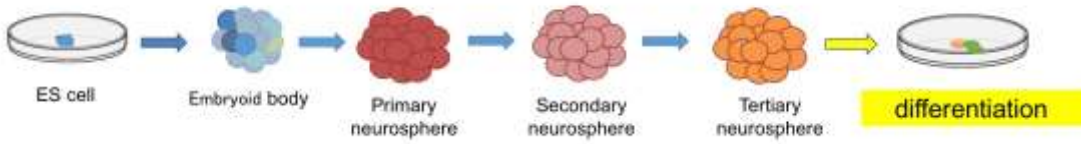

Figure 1. Induction of CNS stem cells from mouse ESCs by forming EBs and neurospheres.

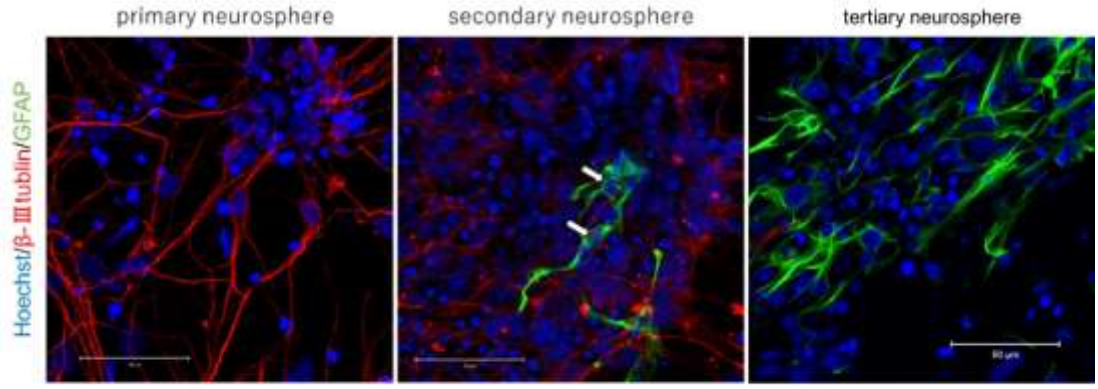

Figure 2. Differentiation of primary (left), secondary (middle), and tertiary (right) neurospheres into βIII-TUBULIN-positive neurons (red) and GFAP-positive glia (green). Quantitative data are shown in Fig.3.

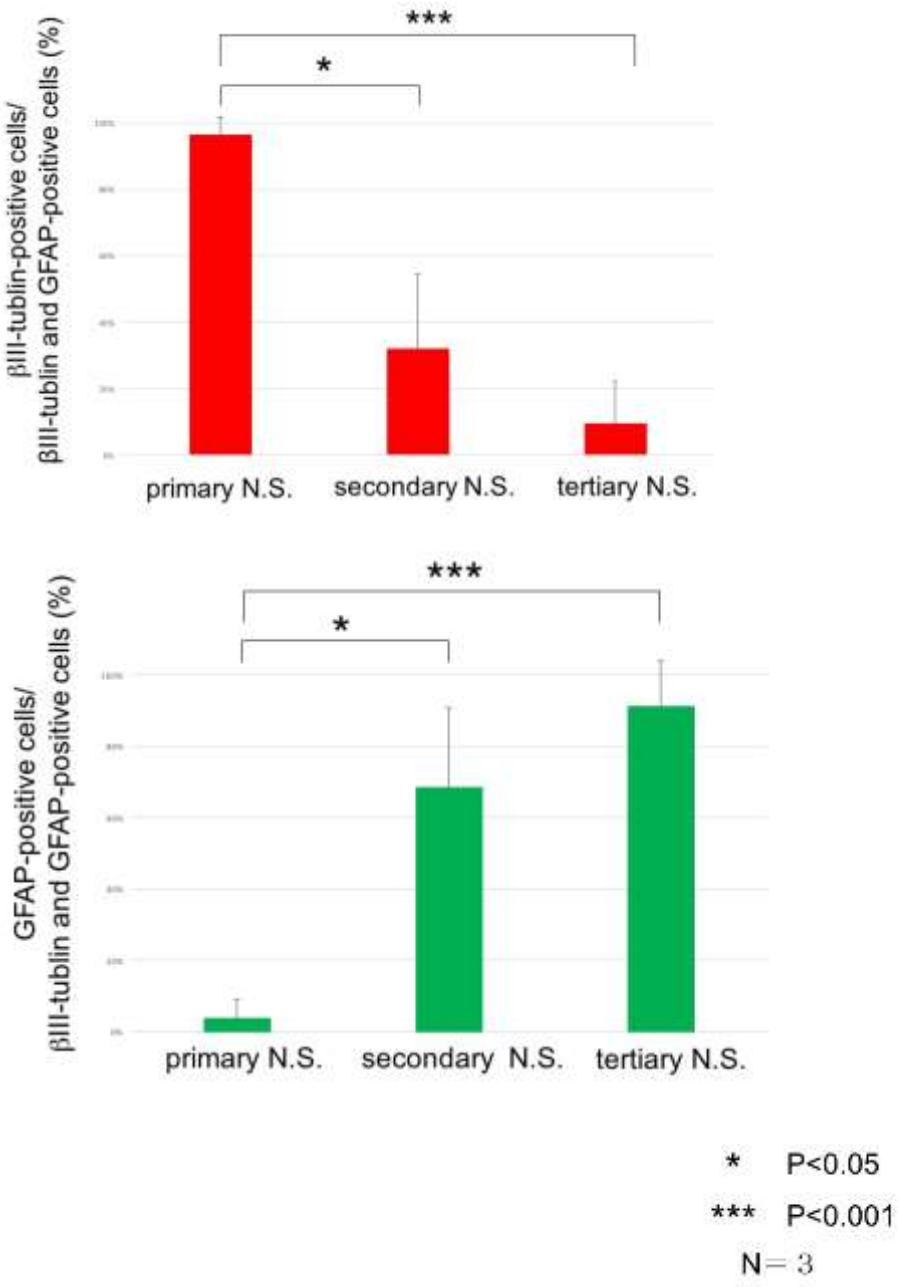

Figure 3. Ratio of neuronal (upper panel) or glial (lower panel) differentiation of mouse ESCs-derived primary, secondary and tertiary neurospheres based on the immunocytochemical data.

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