Review

Neural stem cells and strategies for the regeneration of the central nervous system

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Abstract: The adult mammalian central nervous system (CNS), especially that of adult humans, is a representative example of organs that do not regenerate. However, increasing interest has focused on the development of innovative therapeutic methods that aim to regenerate damaged CNS tissue by taking advantage of recent advances in stem cell and neuroscience research. In fact, the recapitulation of normal neural development has become a vital strategy for CNS regeneration. Normal CNS development is initiated by the induction of stem cells in the CNS, i.e., neural stem cells (NSCs). Thus, the introduction or mobilization of NSCs could be expected to lead to CNS regeneration by recapitulating normal CNS development, in terms of the activation of the endogenous regenerative capacity and cell transplantation therapy. Here, the recent progress in basic stem cell biology, including the author’s own studies, on the prospective identification of NSCs, the elucidation of the mechanisms of ontogenic changes in the differentiation potential of NSCs, the induction of neural fate and NSCs from pluripotent stem cells, and their therapeutic applications are summarized. These lines of research will, hopefully, contribute to a basic understanding of the nature of NSCs, which should in turn lead to feasible strategies for the development of ideal “stem cell therapies” for the treatment of damaged brain and spinal cord tissue.

Keywords: neural stem cells, neurosphere, COUP-TF, spinal cord injury, embryonic stem cells, induced pluripotent stem cells

1. Introduction

It has long been thought that the adult mammalian central nervous system (CNS) does not regenerate after injury. As Ramón y Cajal wrote over 80 years ago, “once the development was ended, the fonts of growth and regeneration . . . dried up irreversibly”.1) This description is called, “Cajal’s dogma”. However, researchers are seeking to challenge this dogma by taking advantage of recent progress in bio-science and medicine, especially stem-cell technology.

Today, regeneration of the CNS involves various concepts, including i) the Regrowth of disrupted neuronal axons, ii) the Replacement of lost neural cells, and iii) the Recovery of lost neural function, all of which are essential components of CNS regeneration.2) On the other hand, how the regeneration of the CNS can be achieved is still an important and unanswered question. To meet this goal, I believe it is important to recapitulate at least some aspects of normal development. Notably, the development of the CNS is initiated early in development by the induction of neural stem cells (NSCs) or NSC-like precursor cells; this developmental stage is called neural induction. The fact that the entire CNS is generated from the NSCs that arise from this initial induction provides the rationale for utilizing NSCs to recapitulate CNS development in regenerating the damaged CNS.

NSCs are defined as undifferentiated cells that can both self-renew and generate the three major cell types that constitute the CNS, i.e. neurons, astro-
cytes, and oligodendrocytes, a characteristic known as multipotency. During ontogenic development, the names, morphology and differentiation properties of NSCs likely change.

In the early embryonic stage, NSCs exist as neuroepithelial stem cells (or matrix cells) in the embryonic neural tube, which undergoes symmetric division. Subsequently, at the onset of neurogenesis, NSCs exist as radial glia (or as matrix cells) in the ventricular zone (VZ) of the neural tube [Please see chapter 3 on the ontogeny of NSCs for further details]. Recent evidence has shown that NSCs continue to undergo neurogenesis in adult mammalian brain, including human brain. These findings, especially the availability of NSCs in the adult CNS, suggest potential therapeutic strategies for the regeneration of damaged CNS tissue using NSCs.

The transplantation of fetal neural tissue into patients with Parkinson's disease by the groups of Prof. Olle Lindvall and Anders Björklund at Lund University in Sweden represents a widely known successful example of first-generation cell therapy for a CNS disorder. Although the proof of the concepts showing the therapeutic effects of cell transplantation for Parkinson's disease was obtained in their studies, there remain the potential problems, including limited number of fetal tissues, difficulties in the validation of the grafted cells, ethical issues, and potential side effects including graft-induced dyskinesia. To overcome these problems, it is obvious that stem cell-based transplantation should be developed. In this review, I will summarize the recent research of stem-cell biology of the CNS and its application to CNS repair, including our own studies.

2. Basic NSC technologies and their application

I believe that the rapid progress in stem-cell biology of the CNS within the past 15 years has been achieved by the development of i) selective immuno- cytochemical markers, ii) selective culture methods, and iii) technologies for the prospective identification and isolation of NSCs and early precursor cells (neural stem/precursor cells, NS/PCs) (Fig. 1).

The selective marker molecules for NS/PCs include Musashil, Nestin, and some Sox family members, which are all strongly, though not exclusively, expressed in NSCs within the CNS. Musashil, a protein that is strongly expressed in the embryonic and postnatal mammalian CNS, was identified by my colleagues and I as a member of an evolutionarily conserved family of RNA-binding proteins. By taking advantage of this molecule's expression...
pattern, we were able to demonstrate the presence of NS/PCs in the periventricular area of the adult human brain. Nestin is an intermediate filament that is selectively expressed in NS/PCs in the CNS. Some members of the Sox-protein family are also known to be effective markers for these cells.

As a selective culture method for NS/PCs, neurosphere culture has been invaluable. By this method, NS/PCs can be expanded as a cell mass in a serum-free defined medium containing fibroblast growth factor 2 (FGF-2) and/or epidermal growth factor (EGF), using single dissociated cells derived from neural tissues. In this culture system, NS/PCs can proliferate in an undifferentiated state in vitro, which permits them to be expanded mitotically and harvested in bulk. However, we cannot identify the cells that will form neurospheres, when the cells are first plated. Only retrospectively, after the formation of the neurospheres, do we know that the cells that initiated the spheres were NSCs. Thus, further technological innovation was required for the prospective identification of NSCs.

Some researchers have addressed this issue by using flow-cytometry to identify cells recognized by combinations of antibodies against cell-surface antigens. For example, Uchida et al. isolated live human fetal NS/PCs using the surface antigen-based immunoselection of cells that had the phenotype CD133(+), CD34(−), and CD45(−). When these isolated cells were used to initiate neurosphere cultures, the progeny of the clonogenic cells could differentiate into neurons and glia both in vitro and in vivo. On the other hand, unfortunately, antibodies to NS/PC-selective marker molecules, such as Musashi1, Nestin, and Sox1, cannot be used to sort living NS/PCs, because these molecules are not cell-surface antigens. An alternative method for the prospective identification of NSCs was developed by constructing reporter genes using fluorescent proteins, including green fluorescent protein (GFP) and its derivatives, which are expressed by the promoters or enhancers of NSC-selective genes, including Nestin, Musashi-1, Sox genes, and Nucleostemin. One advantage of these NSC-selective fluorescent protein reporter genes is that they allow the activities and localization of NS/PCs to be observed in transgenic animals. For example, by using transgenic mice that express EGFP under the control of the second intronic enhancer of the nestin gene, it was revealed that NSCs exist as “radial glia” in the embryonic cerebral cortex, particularly during the neurogenic period.

On the other hand, EGFP is stable for too long to be used to evaluate changes that occur within a short period, such as a single cell cycle. To address this issue, we generated transgenic mice that expressed destabilized Venus (dVenus) under the control of the same driver as used for EGFP; i.e., the nestin second intronic enhancer (E/nestin:dVenus). Venus is a GFP derivative that shows a greatly accelerated maturation of the fluorescent protein at 37 °C and has a 10- to 100-fold stronger fluorescence than yellow fluorescent protein (YFP) in vitro. In the dVenus construct, a PEST amino acid sequence from mouse ornithine decarboxylase is fused to the C-terminus of Venus, thus targeting the protein for rapid, cell-cycle-independent intracellular degradation. Using these E/nestin:dVenus transgenic mice, we found that nestin expression is regulated in a cell-cycle-dependent manner during the neurogenic stage, when the brain wall thickens markedly. Thus, the transcription of nestin is likely to be coordinated with the stage-dependent or cell-cycle-dependent morphological alteration of NS/PCs.

In terms of applying these basic technologies, the harvestability of NS/PCs, achieved through in vitro expansion or the isolation techniques described above, is likely to make cell therapy for the damaged CNS more feasible than has ever been expected. In fact, pre-clinical and clinical studies have begun in which purified, non-genetically modified human NS/PCs, grown as neurospheres (hCNS-SCns), are being used to treat infantile neuronal ceroid lipofuscinosis (INCL), a fatal neurodegenerative disease caused by a deficiency in the lysosomal enzyme palmitoyl protein thioesterase-1 (PPT1).

3. Ontogenic changes in the differentiation potential of NSCs

How the diverse types of neurons and glia are generated by NSCs and progenitor cells during CNS development is an important question in the field of developmental neurobiology. The differentiation potentials of NSCs are known to be controlled by spatial and temporal regulatory mechanisms. During the ontogenic development of CNS, the following changes in the behaviors and differentiation potentials of NSCs occur:

i) Expansion phase: NSCs expand their popula-
tion by symmetric cell division, before they begin producing neurons. At this early phase, NSCs are called "neuroepithelial cells" or "matrix cell." They are columnar and touch the ventricle and pial surfaces during the cell cycle. During this neurogenic period, NSCs (radial glia/matrix cells) undergo asymmetric cell division, generating one self-renewing NSC and one daughter cell that becomes a neuron directly or produces neurons indirectly via intermediate progenitor cells.

ii) Neurogenic phase: Thereafter, NSCs begin to generate neuronal lineages through asymmetric cell divisions in the germinal ventricular zone (VZ). In the developing cerebral cortex, NSCs change their morphology in accordance with the thickening of the neural tube from a columnar to a radial shape, and come to be called "radial glia," which continue to contact both the ventricle and pia. The radial glia have long been known to produce cortical astrocytes, but recent data indicate that they may also divide asymmetrically to produce cortical neurons at the mid-gestation stage. The production of neurons precedes that of astrocytes, and the newly generated neurons migrate along radial glia toward the pial surface to settle in the cortical plate. One important feature of the NSCs at this neurogenic phase is that they do not produce glial cells even though they are exposed to gliogenic environmental factors, indicating that they have not acquired the competency to respond to these factors at this point.

iii) Gliogenic phase: After the major neurogenic period, NSCs acquire gliogenic competency and produce glial progenitor cells, which proliferate mostly in a second germinal zone, the subventricular zone (SVZ). By the postnatal stage, the radial glia have transformed into astrocytes and the VZ has disappeared, but some portions of the SVZ remain into adulthood, to become sites of adult neurogenesis.

Although such temporal changes in the differentiation potential of NSCs have been known for a long time, the underlying mechanisms have been largely obscure. To address this issue, we recently developed an in vitro culture system using mouse embryonic stem (ES) cells that recapitulate the temporal changes in the differentiation potential of NSCs (Fig. 2). Mouse ES cells can be induced to form neurally biased embryoid bodies (EBs) that contain a substantial number of NSCs, by incubating them in the presence of so-called "neural inducers," such as Noggin or a low concentration of retinoic acid (RA).
These NSCs expand to form primary neurospheres in the presence of FGF-2. When the primary neurospheres are grown under differentiation conditions, they predominantly give rise to early projection neurons, and essentially no glial cells. After a certain period, these primary neurospheres can be dissociated and passaged to form secondary neurospheres. These secondary neurospheres give rise to neurons (mostly interneurons), astrocytes, and oligodendrocytes. The neurons generated from the secondary neurospheres are mostly GABAergic interneurons, which are different from the neurons derived from primary neurospheres. If the secondary neurospheres are then passaged to form tertiary neurospheres, even more cells differentiate into glia than arose from the secondary neurospheres. Thus, this in vitro culture system appears to recapitulate the temporal changes in the differentiation potential of NSCs occurring in vivo.

Taking advantage of this in vitro culture system, we sought to identify key genes involved in the transition of the temporal identity of NSCs. For this purpose, we combined DNA microarray analysis and functional screening with the in vitro culture system. By DNA microarray analysis, we screened 20,280 genes, and found that 618 genes were expressed in primary neurospheres with expression levels that were more than twice as high as in secondary neurospheres. To narrow down the candidate genes involved in the temporal identity transition of NSCs, we examined their in vivo relevance. We found that 202 of the 618 genes were expressed in the mouse striatum on embryonic day 11 (E11) at a level that was more than twice that at postnatal day 1 (P1). We then performed lentivirus-mediated overexpression and/or knockdown of about 30 of the 202 genes, focusing on genes involved in epigenetic regulation, transcription factors, and other possible regulators (Fig. 3). We examined the effects of the overexpression and/or knockdown of these candidate genes on the differentiation of tertiary neurospheres, which produce differentiated cells that include about 20% neuronal cells and 80% astrocytes, under control conditions.

Among the candidate genes we examined, only the knockdown of Coup-tfI/II caused a significant change in phenotype, resulting in tertiary neurospheres with a high neurogenic potential. Further investigation in vitro and in vivo suggested that the Coup-tfI/II knockdown inhibits the neurogenic-to-gliogenic transition and causes sustained neurogenesis and the prolonged generation of early-born neurons.

Fig. 3. Screening of factors involved in the temporal identity transition of NSCs. Candidate factors involved in the temporal identity transition of NSCs were determined by combining DNA microarray analysis and functional screening with an in vitro culture system. See the text for details.
neurons. Interestingly, we also found that the Coup-
tfII/II knockdown prolongs the epigenetic silencing of
the astroglial gene, GFAP, which is normally down-
regulated during embryonic development, even in
secondary and tertiary ES cell-derived neurospheres.
COUP-TFII are evolutionarily conserved members
of an orphan nuclear receptor family. Interestingly,
Seven up (SVP), a Drosophila homolog of COUP-
tfII, is known to play essential roles in the tem-
poral identity switch of NSCs in the Drosophila
CNS. Thus, the COUP-TF/SVP family is likely
to regulate the temporal specification of NSCs in an
evolutionarily conserved fashion. Further studies to
elucidate the molecular mechanisms by which net-
works of transcription factors, including COUP-
tfII, modify the epigenetic status of key genes
to elicit the temporal specification of NSCs are
currently underway in my laboratory (Fig. 4).

4. Potential therapeutic applications of NS/
PC transplantation for the damaged CNS

In addition to the above-mentioned basic char-
acterization of NSCs, we have been investigating the
therapeutic application of NS/PCs. In particular, we
are focusing on the transplantation of NS/PCs
derived from various sources into spinal cord in-
jury (SCI) models. The pathophysiology of SCI
changes rapidly with time, after the primary me-
chanical trauma (Fig. 5), as follows. In the acute
phase i) hemorrhage, ischemia, and hypoxia occur
within seconds, ii) pro-inflammatory cytokines are
produced and glutamate cytotoxicity occurs within
minutes, and iii) free radicals and nitric oxide are
produced, and various proteases are activated within
hours. Subsequently, during the subacute phase, neu-
ral apoptosis, reactive astrogliosis, glial scar forma-
tion, and axonal de-myelination take place within
days. During the chronic phase, severe axonal de-
germination, cyst formation, and the permanent loss
of spinal function occur within weeks. Based on the
timeline of these events, we performed the transplan-
tation of NS/PCs 9 days after injury, i.e., after the
acute inflammatory phase and before the astroglial
scar becomes prominent. The chronic phase of
spinal cord injury is not likely to be appropriate for
therapeutic transplantation, due to the formation
of large cysts and the development of glial scarring,
which might inhibit axonal regeneration.

Fig. 4. Function of COUP-TF in the temporal identity transition of NSCs. During ontogenic development, NSCs undergo a
temporal identity transition from the neurogenic to the gliogenic stage. While primary neurospheres represent the state of
NSCs at the neurogenic phase, secondary neurospheres represent their state at the gliogenic phase. COUP-TF was found to
play a crucial role in this transition (Modified from Okano, H. and Temple, S. Ref. 43).
Recently, we also investigated the therapeutic effects of transplanting pluripotent stem cell-derived NS/PCs. Taking advantage of our recently established neurosphere-based culture system for mouse ES cell-derived NS/PCs, in which the primary neurospheres and passaged secondary neurospheres exhibit neurogenic and gliogenic potentials, respectively, we examined the effects of transplanting neurogenic or gliogenic NS/PCs on the functional recovery of a mouse model of SCI (Fig. 6). ES cell-derived primary and secondary neurospheres transplanted 9 days after contusive injury at the 10th thoracic spinal vertebral (T10) level exhibited neurogenic and gliogenic differentiation tendencies, respectively, which were similar to those seen in vitro.

Interestingly, we found that transplantation of...
the gliogenic secondary neurospheres, but not of the neurogenic primary neurospheres, promoted axonal growth – including that of the 5-HT-positive raphe-spinal tract – remyelination, and angiogenesis, and resulted in significant locomotor functional recovery after SCI, suggesting that gliogenic NS/PCs are effective for promoting recovery from SCI (Fig. 7).

Although the transplantation of primary neurospheres had no effect on the functional recovery from contusive SCI, it may be beneficial for neurological diseases in which particular types of early projection neurons are selectively lost.

These findings using mouse ES cells may contribute to the development of therapeutic methods using NS/PCs derived from other types of pluripotent stem cells, such as induced pluripotent stem (iPS) cells. In fact, we recently found that the neural differentiation protocol for mouse ES cells can be applied for mouse iPS cells. In this experiment, we generated secondary neurospheres from various mouse iPS cell clones and examined their neural differentiation capacity and teratoma-forming potential after transplantation into the brains of immunodeficient NOD/SCID mice. In collaboration with Prof. Shinya Yamanaka’s laboratory, we used 36 iPS cell clones, which differed in (1) their origin, (2) the presence or absence of c-Myc retroviral transduction, and (3) whether or not genetic selection for Nanog expression was used. While the NS/PCs generated from the various mouse iPS cells showed a similar neural differentiation capacity, they also exhibited enormously different teratoma-forming potentials. In fact, we found that the teratoma-forming propensities of secondary neurospheres vary significantly depending on the iPS cells’ tissue of origin. Secondary neurospheres from iPS cells derived from adult tail-tip fibroblasts (TTF-iPS cells) showed the highest propensity, whereas those from iPS cells derived from embryonic fibroblasts (MEF-iPS cells) and iPS-derived from gastric epithelial cells (Stm-iPS cells), showed the lowest; these findings were comparable to those from ES cells. Secondary neurospheres from iPS cells-derived from hepatocytes (Hep-iPS cells) showed an intermediate propensity.

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Based on these results, we concluded that the somatic tissue of origin of the iPS cells is a critical determinant of the tumorigenic potential of iPS-derived NS/PCs, and that their tumorigenic potential is related to the persistence of undifferentiated cells among the iPS-cell-derived cells. Before starting cell transplantation using iPS cell-derived cells, safety issues concerning the risk of tumorigenicity must obviously be addressed. On this ground, these findings should greatly contribute to the development of safe cell therapies for SCI patients using human iPS-derived NS/PCs in the near future (Fig. 8).

In fact, some clinical applications of fetal-derived NSCs have already been performed.\textsuperscript{30,61} However, one case report has described a donor-derived brain tumor following NSC transplantation in a patient with ataxia telangiectasia;\textsuperscript{30} this case likely resulted from the insufficient characterization of the safety issues associated with NSC transplantation. Meanwhile, a Phase 1 trial of the transplantation of human ES-derived oligodendrocyte precursor cells into SCI patients has been approved by the United States Food and Drug Administration following an intensive characterization of potential tumorigenesis in preclinical tests.\textsuperscript{62–64} However, the mechanisms of functional recovery (e.g. trophic effects, myelination, axonal sprouting, synaptogenesis, etc.) and the potential risks differ for each case. Intensive preclinical tests examining the safety and effectiveness of stem cell transplantation using appropriate designs and disease models are needed. The advantage, disadvantage and current situation of cell therapy using primary tissue-derived NS/PCs, ES-derived NS/PCs and iPS-derived NS/PCs are summarized in Table 1. The recent progresses of cell therapy for damaged CNS including neurodegenerative disorders (such as Parkinson’s disease, Huntington’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis) and acute lesions (such as ischemic or haemorrhagic stroke and SCI) are well summarized in other review articles.\textsuperscript{65–69}

### 5. Conclusion and perspectives

As described in this and other review articles,\textsuperscript{70,71} there is accumulating evidence that the recapitulation of normal developmental processes – including neurogenesis, gliogenesis, migration, axonal sprouting, synaptogenesis, and neuron-glia interactions, including myelination – is important for repairing the damaged CNS and functional recovery. This is true for transplantation strategies using NS/PCs\textsuperscript{4} or those involving the activation of endogenous NS/PCs for SCI.
Therefore, it is important to elucidate the regulatory mechanisms of normal CNS development, including its evolutionary aspects, in depth. In particular, even though neurogenesis persists in adults at specific sites, early projection neurons, such as midbrain dopaminergic neurons, do not regenerate in the adult mammalian CNS, including the brains of Parkinson’s disease patients. However, notably, in a disease model using a lower vertebrate, the salamander, in which the dopaminergic neurons are eliminated by 6-hydroxydopamine, midbrain dopaminergic neurogenesis does occur. Such a remarkable difference in regenerative capacity could be attributed to species differences of intrinsic ability of NSCs. Alternatively, it may be attributed to the microenvironment of the adult mammalian brain, which cannot maintain or support the differentiation of an early type of NSCs, which may correspond to the NSCs in primary neurospheres, compared with the microenvironment of the adult salamander brain. Thus, elucidation of the mechanisms for the maintenance and transition of early NSCs could open up new strategies for treating various neurological disorders in which early projection neurons are lost. Such investigations would also facilitate the development of pluripotent stem cell-mediated therapies for these disorders, by improving the methods for preparing the precursor cells to produce early projection neurons.

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| Advantage | Disadvantage | Clinical application |
|-----------|-------------|---------------------|
| Primary tissue-derived NS/PCs | Moderate ethical concerns about action process (depending on the nation) [ref. 67] | limited expandability [ref. 67] | Spinal cord injury (pre-clinical with non-human primates) [ref. 54] |
| | Less tumorigenic (except one report) [ref. 67] | limited availability [ref. 67] | infantile neuronal ceroid lipofuscinosis (Phase 1 trial finished) [ref. 29] |
| | various trophic effects [ref. 29] | limited patterning potential | Pelizaeus-Merzbacher disease (FDA-approved) |
| ES-derived NS/PCs | Unlimited expandability [ref. 67] | Ethical concerns about derivation process [ref. 67] | Spinal cord injury (human ES-derived oligodendrocyte progenitor cells; Phase 1 trial approved by FDA) [refs. 62–64] |
| | Broad patterning potential [ref. 67] | Genetic and epigenetic instability (+) | |
| | | Modestly tumorigenic | |
| iPS-derived NS/PCs | Derivation without ethical concerns [ref. 67] | Highly tumorigenic (depending on cellular origin of the iPS cells) [ref. 60] | Characterized only at non-clinical level [ref. 73] |
| | Unlimited expandability [ref. 67] | Genetic and epigenetic instability (+++) [refs. 60, 67] | |
| | Broad patterning potential [ref. 67] | | |
| | Intrinsic expression of disease-associated genes [ref. 67] | | |
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Profile

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