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A Survey of the Molecular Basis for the Generation of Functional Dopaminergic Neurons from Pluripotent Stem Cells: Insights from Regenerative Biology and Regenerative Medicine

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Additional information is available at the end of the chapter

1. Introduction

Animals that possess regenerative abilities are widespread in the animal kingdom [1]. Hydra, planarian, zebrafish, newt and axolotl are known prominent species, and the cellular aspects of the stem cell system for regeneration are well elucidated [2]. However, few animals can be used to investigate the molecular basis of neuronal regeneration, in spite of the presence of prominent regenerative animals, as mentioned above. Planarians, for instance, can regenerate a functional brain after amputation in a few days, even from non-brain tissue [3,4]. Newts can regenerate several tissues and organs (*i.e.,* lens, limbs, jaws, hearts and tails) with recovery of function and physiology after injury or tissue removal [5-8]. These animals achieve regeneration of missing nervous system utilizing stem cells. However, it is difficult to regenerate nervous system in mammalians, including human beings, although these animals possess neural stem cells. Therefore, regenerative animals provide unique opportunities to investigate the generation and utilization of stem cells to repair lost or injured tissue in non-regenerative animals. On the other hand, the successful derivation of neural cells from human embryonic stem cells (ESCs) [9] and induced pluripotent stem cells (iPSCs) [10,11] under *in vitro* conditions provides a new experimental strategy for clinical translation. In other words, although human beings lack regenerative abilities, the new clinical strategy of "regenerative medicine," including cell-transplantation therapy, has been developed to recover lost neural functions by using stem cells. This research field has become a greatly advancing scientific field worldwide.

In this chapter, we focus on the molecular systems of generation of functional dopaminergic (DA) neurons *in vivo* and/or *in vitro* in regenerative and non-regenerative animals. The first topic investigates how regenerative animals recruit new DA neurons from stem cells after injury. The second topic explores how to generate DA neurons from mammalian ESCs and iPSCs under *in vitro* conditions. The third topic evaluates clinical applications for human neural disease, especially Parkinson's disease.

2. DA neuronal regeneration in regenerative animals

Freshwater planarians, *Plathelminthes*, have a primitive central nervous system (CNS) that is composed of a well-organized brain and a pair of ventral nerve cords [12]. Large-scale expression analyses have revealed that many neural genes involved in vertebrate brain development and function are also expressed in distinct domains of the planarian CNS [13,14]. These results indicate that the planarian CNS is functionally regionalized according to a discrete expression of neural-specific genes.

We recently discovered that the planarian CNS contains dopamine, serotonin, γ-aminobutyric acid (GABA) and acetylcholine, which are known to be present in mammalians, and that the planarian nervous system constitutes particular neural networks and functions [15-19]. Since planarians possess pluripotent stem cells throughout their entire bodies, their CNS can be completely regenerated along with recovery of morphology and function after amputation [20].

Recently, two different processes of DA neuroregeneration in the planarian *Dugesia japonica* have been described. One involves DA neuroregeneration accompanied by brain regeneration after artificial amputation [15,21]. The other involves DA neuronal regeneration after selective degeneration of DA neurons by the DA neurotoxin 6-hydroxydopamine (6-OHDA) [22]. Although both of these processes are achieved by manipulating pluripotent stem cells, the systems of the processes are different between these regenerative processes [23]. In this section, we focus on these two processes of DA neuroregeneration in planarians, one of prominent regenerative animals.

2.1. Brain regeneration and DA neuroregeneration in planarians

Planarians can regenerate a functional brain within 7-10 days after amputation. This regenerative process can be divided into at least five steps: (1) anterior blastema formation, (2) brain rudiment formation, (3) pattern formation, (4) neural network formation and (5) functional recovery. Each step is defined by sequential gene expression alterations that are similar to those observed in mammalian brain development [20].

2.1.1. The early stage of brain regeneration after amputation

The first step of head regeneration after amputation is wound closure, which involves adhesion of the dorsal and ventral tissues, thereby inducing activation of *noggin-like gene A* (*DjnlgA*) at the edge of the amputated site [24]. Subsequently, a blastema (a mass of cells derived from

pluripotent stem cells) is formed by mitogen-activated protein (MAP) kinase activation 24 hours after amputation [25,26]. It has been revealed that blastema cells are supplied from the post-blastema region via mitosis from G2-phase pluripotent stem cells. Activation of c-Jun-N-terminal kinase (JNK) after amputation induces G2/M transition and supplies blastema cells. Subsequently, activation of extracellular signal-related kinase (ERK) signalling is required for blastema cells to exit the undifferentiated state and enter the differentiation state in order to form the brain rudiment [27]. Similarly, activation of ERK signalling facilitates exit from self-renewal and regulates differentiation signals, as in mouse ESCs [28]. These findings indicate that planarian stem cells and mouse ESCs may possess a similar molecular basis for cell fate determination.

After the formation of the brain rudiment, the wnt and bone morphogenic protein (BMP) signaling pathways regulate brain polarity along the anterior-posterior axis [29,30] and the dorso-ventral axis [31,32], respectively. Therefore, pluripotent stem cells are regulated by various signals in spacio and temporal manners to form the brain. Similarly, wnt and BMP signaling pathways also regulate polarity of neural tube formation in mammalian early development [33,34].

2.1.2. The stem cell system of DA neuroregeneration in the head regeneration process

During head regeneration, DA neurons begin to appear in future brain regions starting three days after amputation. On day 5, the number of new DA neurons increases and axons start to extend. On day 7, brain regeneration is complete along with complete reconstruction of DA neurons (Figure 1A) [15]. Other neurotransmitter-synthesizing neurons, such as GABAergic, octopaminergic (OA) and cholinergic neurons are also regenerated in a similar manner from pluripotent stem cells during brain regeneration [17-19]. Recently, we revealed that the numbers of each type of different brain neurons are maintained in a constant ratio that is dependent on body size in intact planarians [21]. For instance, the ratio of DA neurons to OA neurons is 2:1 in intact planarian brains. Interestingly, in the early stage of brain regeneration, the ratio is larger than 2:1; however, it is gradually restored to 2:1 during brain regeneration. The ratio among different neuronal cell types fluctuates in the early stages of regeneration and is gradually restored to the original ratio. These data suggested that non-cell-autonomous mechanisms utilized to adjust the ratio among different types of brain neurons [21].

2.2. DA neuronal regeneration after selective DA lesions

We recently established an experimental model to investigate cell-type specific regeneration following selective degeneration by 6-hydroxydopamine (6-OHDA), a DA neurotoxin [22]. According to our observations, DA neurons are completely degenerated within 24 hours after 6-OHDA-administration. Newly generated DA neurons begin to appear in the brain four days after 6-OHDA-induced lesions. Thereafter, the number and the axons of DA neurons gradually recover over a period of several days. Finally, DA neurons are completely recovered within 14 days after 6-OHDA-induced lesions (Figure 1B). Bromodeoxyuridine (BrdU)-pulse and chase experiments indicate that newly generated DA neurons are derived from proliferative stem cells that enter the S-phase in the trunk region and migrate to the head region from the trunk region

without entering the M-phase (*i.e.*, they remain in the G2-phase) and then give rise to DA neurons in the head region (Figure 1C). In addition, histological analyses support the hypothesis that pluripotent stem cells may directly give rise to differentiated DA neurons in planarians (Figure 1D, E). This observation suggests that G2-phase proliferating stem cells can respond to degeneration of DA neurons and are committed to DA neurons in planarians [22].

Although most vertebrates show low regenerative capabilities, newts have powerful regenerative abilities among adult vertebrates. Surprisingly, adult newts regenerate brain tissue after partial brainectomy in spite of having a complex brain structure [35,36]. Recent reports indicate that newts maintain the neurogenic potential to repair lost midbrain DA neurons, even in the adult state [37]. Although proliferative abilities are essentially quiescent in the midbrain of adult newts, quiescent ependymoglia cells are activated by the degeneration of midbrain DA neurons. Additionally, activated ependymoglia cells start to proliferate and differentiate to DA neurons to repair lost DA neurons. This neurogenic potential is activated under conditions of injury-responsive cell-replacement and not under homeostatic conditions [38,39]. These reports indicated that these regenerative animals possess unique stem cells system to regenerative missing DA neurons.

3. Strategies for generation of DA neurons in non-regenerative animals

Regenerative animals easily regenerate lost brain and neural tissues by maturating stem cells. Regenerative animals provide us unique ideas to generate neural tissue from mammalian pluripotent stem cells such as ESCs and iPSCs.

Although the capacity for brain formation is present during the developmental stage among animals, including human beings, it is difficult to regenerate missing neurons and brain tissue in the adult state. It has been reported that neurogenesis homeostatically occurs in the restricted regions of the adult mammalian brain such as hippocampus and subventricular zone [40,41]. However, it remains controversial whether dopaminergic neurogenesis/neuroregeneration occurs in the adult mammalian midbrain [42,43] and whether neurogenic potential is "lost" or "quiescent" in the adult mammalian brain. Regardless, this potential is not adequate to recover missing neurons and brain tissue in the adult mammalian brain. If human beings had an adequate regenerative potential, some types of neural disorders and brain injuries might be self-curable. However, it is difficult to self-repair neurodegenerative disorders. Therefore, it is strongly expected that neuronal differentiation techniques will contribute for therapeutic applications, such as cell-transplantation therapy using ESCs/iPSCs.

Parkinson's disease (PD) is a candidate disease for the expected application of cell-transplantation therapy. PD is an intractable neurodegenerative disorder that arises from the progressive death of DA neurons in the substantia nigra pars compacta. Although human beings lack adequate abilities to regenerate DA neurons, techniques to generate midbrain DA neurons from ESCs/iPSCs *in vitro* have been developed and are being improved for use in cell-transplantation therapy (Figure 2).
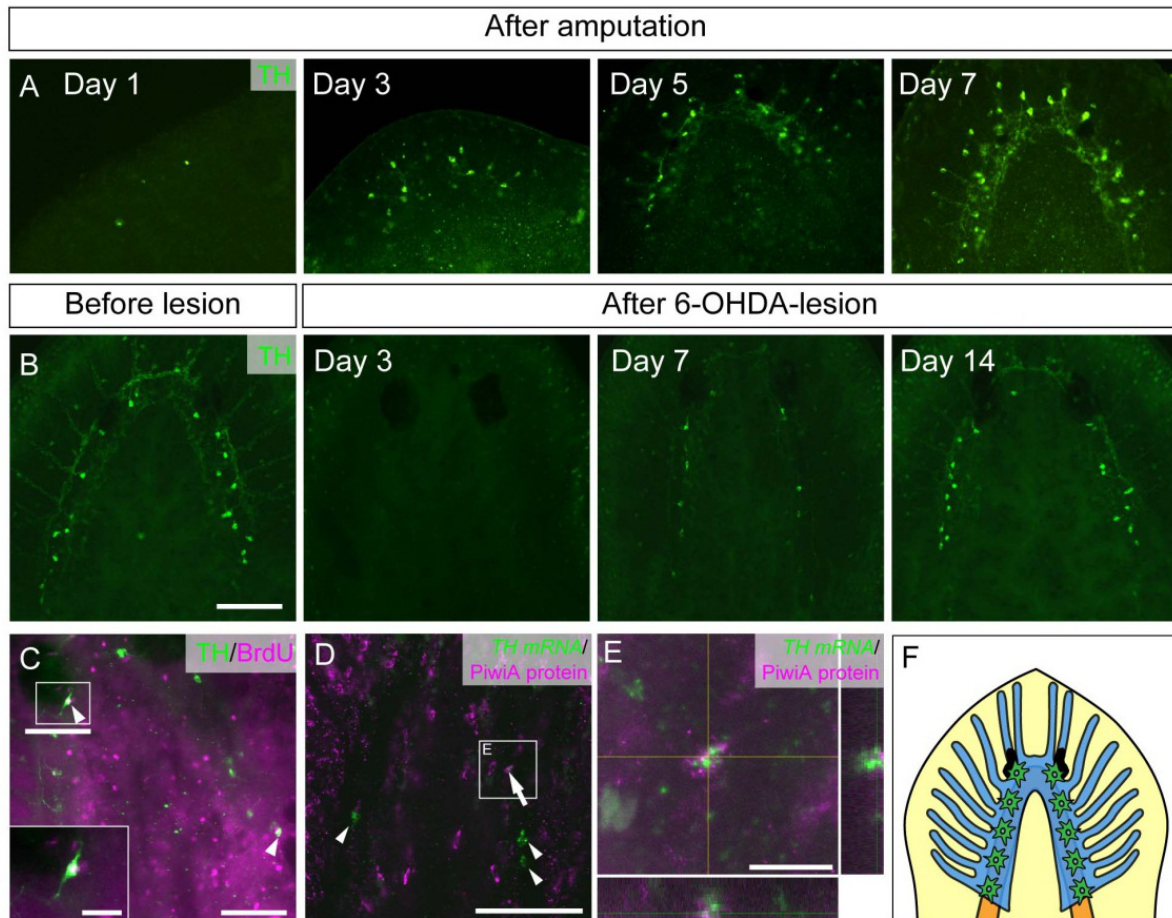
Figure 1. A) The regeneration process of DA neurons after decapitation. Immunofluorescence of brain DA neurons one day, three days, five days and seven days after decapitation. (**B**) The process of DA neuronal regeneration in the brain after 6-OHDA-induced-lesions. Immunofluorescence of brain DA neurons in an intact brain three days, seven days and 14 days after 6-OHDA-administration. (**C**) BrdU-incorporation is detected in newly generated DA neurons five days after 6-OHDA-administration. Double-immunofluorescence of tyrosine hydroxylase (TH; green) and BrdU (magenta). The arrowheads indicate BrdU/TH-double positive neurons. (**D, E**) Double-fluorescence of *TH mRNA* (green) and PiwiA proteins (a marker for pluripotent stem cells; magenta) seven days after 6-OHDA-administration. Scale bars: 200 µm (**A-D**), 50 µm (high magnification image in C) and 50 µm (**E**). (**F**) A schematic drawing of the planarian brain nervous system and an image of the distribution of DA neurons. The blue color represents the brain. The orange color represents ventral nerve cords (VNCs). The green cells are DA neurons.

3.1. Induction of midbrain DA neurons *in vitro* from ESCs/iPSCs

In 2000, it was reported that stromal cell-derived inducing activity (SDIA) strongly promotes neural induction in mouse ESCs co-cultured with mouse PA6 stromal cells under serum-free conditions without growth factors [44]. SDIA-induced neurons contain high amounts of DA neurons and are integrated into the 6-OHDA-lesioned mouse striatum after transplantation. Additionally, it has been proven that functional DA neurons can be differentiated from primate and human ESCs using SDIA [45,46]. Therefore, the establishment of the SDIA method opened new fields for both basic neuroscience research and therapeutic applications.
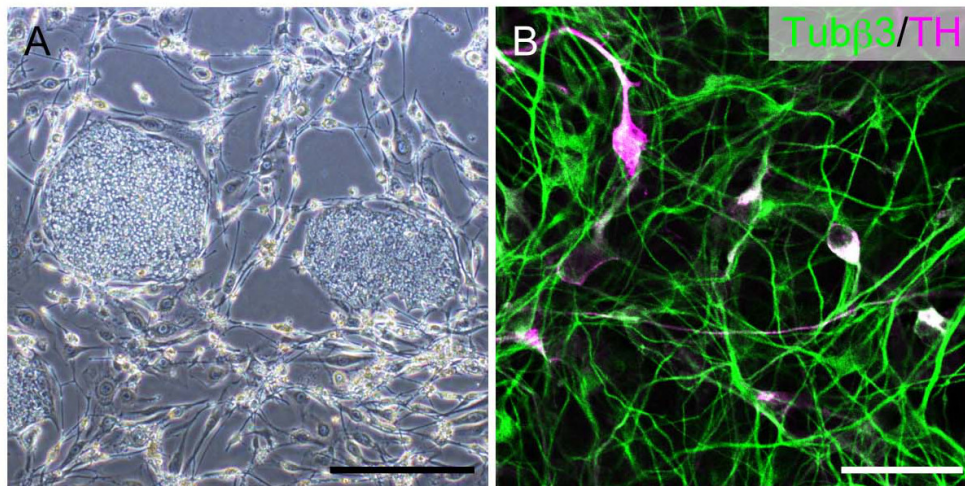
Figure 2. A) A phase contrast image of colonies of undifferentiated human iPSCs on SNL feeder cells. (**B**) DA neurons derived from human iPSCs differentiated using the SFEB method *in vitro*. Double-immunofluorescence of ßIII-tubulin (TubßIII), a neuronal marker (green), and TH, a marker of DA neurons (magenta). Scale bars: 500 μm (**A**) and 50 μm (**B**).

Recently, the molecular mechanisms of mammalian brain development have become better understood. The expression and secretion of patterning factors facilitate neural induction and define anterior-posterior and dorso-ventral patterning of the mammalian brain. For instance, mammalian midbrain development is governed by fibroblast growth factor 8 (FGF8) and Sonic hedgehog (Shh), which are locally expressed at the midbrain-hindbrain boundary and the ventral neural tube, respectively [47]. The floor plate is located along the ventral midline of the neural tube and is known to function as a signaling center during brain development and a source of midbrain DA neurons [48,49]. Midbrain DA neuronal specification is regulated by several transcription factors, including Lmx1a, FoxA2, Nurr1 and Pitx3 [50-53]. Therefore, cellular aspects of brain development provide ideas for improving the differentiation methods of authentic neural identity and subtype specification, such as differentiating DA neurons from ESCs/iPSCs *in vitro*.

Recently, neural lineage commitment has been improved with dual inhibition of SMAD signaling by bone morphogenic protein (BMP) inhibitor (noggin and dorsomorphine) and transforming growth factor-ß (TGF-ß)/activin/nodal inhibitor (SB431542). These combinations promote efficient neural induction of both human ESCs and iPSCs in serum-free, floating cultures of embryoid body-like aggregates (SFEB) and stromal (PA6) feeder co-cultures [54,55]. In addition, GSK3ß inhibitor (CHIR99021) strongly activates wnt signaling and induces the Lmx1a expression in FoxA2-positive floor plate precursors and the neurogenic conversion of human ESC-derived midbrain floor plates towards DA neurons [56]. This differentiation method mimics mammalian brain development.

Recently, human ESCs/iPSCs maintenance and differentiation have been advanced in order to achieve clinical translation, which results in chemically defined conditions and the elimination of animal-derived components and the need for feeder cells [57,58].

4. Towards the clinical application of ESCs/iPSC-derived DA neurons in Parkinson's disease

Clinically, PD is estimated to affect approximately 1% of the population over 65 years of age, and PD patients often exhibit muscle rigidity, tremors, bradykinesia and akinesia. Currently, the primary clinical treatment for PD is dopamine replacement therapy using L-dihydroxy-phenylalanine (L-DOPA) and/or dopamine receptor agonists. Although pharmacotherapy temporarily improves parkinsonian symptoms, the efficacy of pharmacotherapy is gradually lost over long-term treatment, and the wearing-off phenomenon, the on-off phenomenon and drug-induced dyskinesia develop. In addition, the progression of the degeneration of midbrain DA neurons cannot be delayed. Therefore, new strategies such as cell transplantation therapy are expected to recover lost DA neurons.

The first clinical trial of cell transplantation using human fetal ventral midbrain in PD patients was performed in the latter half of the 1980's [59]. Currently, over 400 PD patients have been evaluated in this clinical trial. Some PD patients who underwent grafting have exhibited drastic improvements in movement symptoms. However, strict ethical problems remain regarding the use of human fetal cells for the treatment of human disease. Therefore, efficient methods need to be developed for differentiating DA neurons from ESCs/iPSCs, instead of using human fetal cells, as described above. In addition, the efficiency and safety of ESCs/iPSCs-derived DA neurons should be evaluated *in vivo* experiments using animal models before conducting human trials. In general, DA neurotoxins such as rotenone, 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are used to create parkinsonian animal models with rodents and non-human primates. In particular, since the pathological symptoms and brain anatomy of monkeys are similar to those of humans, the outcomes of monkey trials strongly contribute to realizing human trials.

4.1. Preclinical trials of human ESCs/iPSC-derived DA neurons for PD

In 2005, it was reported that intra-striatal primate ESC-derived DA neurons survive and function in the putamen in MPTP-lesioned primate parkinsonian models [60]. In that study, positron emission tomography imaging revealed that [^{18}F]-F-DOPA uptake increased in grafted monkeys 14 weeks after transplantation. In addition, the neurological scores of the grafted monkeys improved in comparison with that observed in sham-operated monkeys starting from 10 weeks after transplantation. This study is the first report to indicate the functional efficiency of grafted DA neurons derived from primate ESCs and has opened up the possibility for transplantation therapy using ESC-derived DA neurons.

Meanwhile, human ESC/iPSC-derive DA neurons have the potential to improve motor function in PD model rats after intra-striatal grafting [56,61,62]. However, some problems, such as tumorigenicity, remain in clinical trials. We recently reported that long-term neural maturation (> 28 days) of human ESCs reduces tumorigenicity after grafting in primate parkinsonian models. In addition, motor symptoms are also improved by grafting human ESC-derived DA neurons that have matured over a long term (42 days). These results suggest that human ESC-derived DA neurons that are differentiated for appropriate terms strongly

contribute to both reducing the risk of tumor genesis and improving parkinsonian motor dysfunction [46]. Recently, we first reported that human iPSC-derived DA neurons that are differentiated under feeder-free and serum-free conditions survived in an MPTP-lesioned primate parkinsonian model for six months [63]. This report may support the therapeutic potential of human iPSCs for future clinical trials.

5. Stem cell utilization *in vivo* and *in vitro* for non-regenerative animals: Lessons from regenerative animals

Although the number of regenerative animals used to investigate DA neuronal regeneration is limited, the cellular mechanisms of DA neuronal regeneration are starting to be understood [22,39]. The cellular and molecular mechanisms for DA neuronal regeneration in regenerative animals provide new ideas for generation of DA neurons in non-regenerative animals. Our histological analysis of regeneration indicates that pluripotent stem cells may directly give rise to differentiated DA neurons in the planarian head region (Figure 1D, E) [22]. In addition, we have not yet obtained direct evidence for the presence of neural stem cells in planarians. First, we have not observed proliferating cells in the brain, either during regeneration or in intact brains. Second, the expression of the planarian *musashi* family of genes supports the above hypothesis. *Musashi*, an RNA binding protein, is expressed in neural stem cells in various animals[64]. Although three *musashi*-like genes (*DjmlgA-C*) have been isolated from planarians, the expressions of these genes are detected in the planarian CNS and are not eliminated by X-ray irradiation [65]. These results indicate that planarian *musashi*-like genes are expressed in differentiated cells, not in proliferative stem cells. Based on these observations, we speculate that the neural stem cell system most likely evolved at a later stage of evolution [66].

In the case of brain regeneration, the brain rudiment is formed inside of the blastema. The cells that participate in blastema formation already exist from the proliferative state, and a portion of these cells start to form the brain rudiment [25,26]. Therefore, the commitment of DA neurons may immediately occur after brain rudiment and brain pattern formation.

In the case of DA neuronal regeneration after 6-OHDA-induced lesions, G2-phase stem cells are recruited into DA neurons in the brain. Since the presence of neural stem cells were not clarified in planarians, the key roles for differentiation into DA neurons from pluripotent stem cells would function in G2-phase in the cell cycle. The neurons remaining in the brain after 6-OHDA-induced lesions may play an important role in sensing the loss of DA neurons and recruiting G2-phase stem cells into DA neurons [22]. Recent findings using eukaryote cells indicate that cell fate determination of either self-renewal or differentiation occurs during the G2/M phase in the cell cycle [67]. Therefore, the results of our study concerning the cellular system of DA neuronal regeneration are supported by these observations. Therefore, G2-phase stem cell strongly contributed to DA neuronal regeneration by response to extrinsic environment in planarians. However, we have not yet understand a clear answer regarding which

signal molecules contribute to the recruitment of G2-phase stem cells to DA neurons in planarians.

In the case of newts, another regenerative animals, quiescent ependymoglia cells can sense degeneration of DA neurons, and re-enter into the cell cycle to restore lost DA neurons [39]. In this case, DA receptor-expressing surrounding cells (*i.e.*, ependymoglia cells) respond to the degeneration of DA neurons via DA receptor signaling, and contribute to regenerate DA neurons. Therefore, the responsibility of stem cells to extrinsic environment is important for DA neuronal regeneration in both planarians and newts.

The findings from regenerative animals may provide any idea to generate DA neurons from ESCs/iPSCs of non-regenerative animals. Recent strategies for DA neuronal induction from ESCs/iPSCs have based on mimicking midbrain development, that is, the activation of morphogenic factors and transcriptional regulation. In addition, the establishment of 3-dimensional (3D) culture systems strongly contributes to mimicking the complicated organogenesis and lead to the acquisition of sub-regional identities because the cells respond to extrinsic signals [57, 69].

Recently, Kirkeby *et al.* developed an induction method for human ESC-derived DA neurons using dual-SMAD inhibition with embryoid body formation. The gene expression profile and transplantation aspects of human ESC-derived DA neurons can be recaptured in the human fetal midbrain [70], suggesting that 3D cultures are suitable for mimicking brain organogenetic processes in authentic midbrain DA neurons.

In addition, our findings in planarians may contribute to improving strategies of cell-transplantation therapy. That is, how to integrate ESCs/iPSC-derived grafting neurons into the host brain. We found that commitment to DA neurons in stem cells occurs during the G2-phase of the cell cycle. Which state of committed cells can be easily incorporated into lesioned regions and whether the location of commitment activates important factor(s) in the incorporation of committed cells into appropriate positions should be considered. Additionally, both planarians and newts showed drastic behavioral recovery according to DA neuronal regeneration [22,37]. This suggested that regenerated DA neurons contributed to functional recover by integrating to the existing neural circuit. We speculate that this phenomenon may provide to the any ideas how to integrate ESCs/iPSC-derived neurons into existing neural circuit after grafting and contribute to recover the motor function. We will attempt to answer several important questions in our future works. A deeper understanding of the answers to the above questions may provide unique clues regarding not only how to commit DA neurons in vitro, but also how to optimize cell-transplantation therapy in the future.

6. Conclusion

Stem cell research is an interesting field in basic science due to its potential therapeutic applications. It is now extensively studied to investigate that the cellular and molecular

mechanisms of DA neuronal regeneration in many regenerative animals *in vivo* have been described over the last several years, although numerous unclear issues remain. Important aspects learned from regenerative animals suggest that the cell sources and stem cell systems of DA neuronal regeneration may reflect several concepts in the achievement of regeneration, even among regenerative animals. Therefore, it is difficult to find a common rule for DA neuronal regeneration among animals. However, this trend in regenerative biology will be more emphasized by discoveries made in future studies of regenerative animals, and will provide hints about more efficient utilization of stem cells towards clinical application. Importantly, studying the diversity of regenerative contexts in different animals may contribute to highlighting diverse concepts and attractive clues for investigating the generation of DA neurons *in vivo* and *in vitro* in regenerative medicine.

Acknowledgements

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References

[1] Agata K, Inoue T. Survey of the differences between regenerative and non-regenerative animals. Development Growth and Differentiation 2012;54(2) 143-152.

[2] Tanaka EM, Reddien PW. The cellular basis for animal regeneration. Developmental Cell 2011;21(1) 172-185.

[3] Agata K, Watanabe K. Molecular and cellular aspects of planarian regeneration. Seminars in Cell and Developmental Biology 1999;10(4) 377-383.

[4] Reddien PW, Sánchez Alvarado A. Fundamentals of planarian regeneration. Annual Review of Cell and Developmental Biology 2004;20 725-757.

[5] Eguchi G. Cellular and molecular background of wolffian lens regeneration. Cell Differentiation and Development 1988;Suppl. 147-158.

[6] Brockes JP, Kumar A. Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nature Review Molecular Cell Biology 2002;3(8) 566-574.

[7] Tanaka EM. Regeneration: if they can do it, why can't we? Cell 2003;113(5) 559-562.

[8] Kurosaka H, Takano-Yamamoto T, Yamashiro T, Agata K. Comparison of molecular and cellular events during lower jaw regeneration of newt (*Cynops pyrrhogaster*) and West African clawed frog (*Xenopus tropicalis*). Developmental Dynamics 2008;237(2) 354-365.

[9] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391) 1145-1147.

[10] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5) 861-872.

[11] Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858) 1917-1920.

[12] Agata K, Soejima Y, Kato K, Kobayashi C, Umesono Y, Watanabe K. Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers. Zoological Science 1998;15(3) 433-440.

[13] Cebrià F, Nakazawa M, Mineta K, Ikeo K, Gojobori T, Agata K. Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. Development Growth and Differentiation 2002;44(2) 135-146.

[14] Mineta K, Nakazawa M, Cebria F, Ikeo K, Agata K, Gojobori T. Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. Proceedings of the National Academy of Sciences of the United States of America 2003;100(13) 7666-7671.

[15] Nishimura K, Kitamura Y, Inoue T, Umesono Y, Sano S, Yoshimoto K, Inden M, Takata K, Taniguchi T, Shimohama S, Agata K. Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. Developmental Neurobiology 2007;67(8) 1059-1078.

[16] Nishimura K, Kitamura Y, Inoue T, Umesono Y, Yoshimoto K, Takeuchi K, Taniguchi T, Agata K. Identification and distribution of tryptophan hydroxylase (TPH)-pos-

itive neurons in the planarian *Dugesia japonica.* Neuroscience Research 2007;59(1) 101-106.

[17] Nishimura K, Kitamura Y, Umesono Y, Takeuchi K, Takata K, Taniguchi T, Agata K. Identification of glutamic acid decarboxylase gene and distribution of GABAergic nervous system in the planarian, *Dugesia japonica*. Neuroscience 2008;153(4) 1103-1114.

[18] Nishimura K, Kitamura Y, Inoue T, Umesono Y, Taniguchi T, Agata K. Characterization of tyramine ß-hydroxylase in planarian *Dugesia japonica*: Cloning and expression. Neurochemistry International 2008;53(6-8) 184-192.

[19] Nishimura K, Kitamura Y, Taniguchi T, Agata K. Analysis of motor function modulated by cholinergic neurons in planarian *Dugesia japonica.* Neuroscience 2010;168(1) 18-30.

[20] Agata K, Umesono Y. Brain regeneration from pluripotent stem cells in planarian. Philosophical Transactions of the Royal Society of London Series B, Biological Science 2008;363(1500) 2071-2078.

[21] Takeda H, Nishimura K, Agata K. Planarians maintain a constant ratio of different cell types during changes in body size by using the stem cell system. Zoological Science 2009;26(12) 805-813.

[22] Nishimura K, Inoue T, Yoshimoto K, Taniguchi T, Kitamura Y, Agata K. Regeneration of dopaminergic neurons after 6-hydroxydopamine-induced lesion in planarian brain. Journal of Neurochemistry 2011;119(6) 1217-1231.

[23] Nishimura K, Kitamura Y, Agata K. Regeneration of brain and dopaminergic neurons utilizing pluripotent stem cells: lessons from planarians. In: Tao S. (Ed.) Neural stem cells and Therapy Rijeka: InTech; 2012. p141-158.

[24] Ogawa K, Ishihara S, Saito Y, Mineta K, Nakazawa M, Ikeo K, Gojobori T, Watanabe K, Agata K. Induction of a *noggin*-like gene by ectopic DV interaction during planarian regeneration. Developmental Biology 2002;250(1) 59-70.

[25] Tasaki J, Shibata N, Sakurai T, Agata K, Umesono Y. Role of c-Jun N-terminal kinase activation in blastema formation during planarian regeneration. Development Growth and Differentiation 2011;53(3) 389-400.

[26] Tasaki J, Shibata N, Nishimura O, Itomi K, Tabata Y, Son F, Suzuki N, Araki R, Abe M, Agata K, Umesono Y. ERK signaling controls blastema cell differentiation during planarian regeneration. Development 2011;138(12) 2417-2427.

[27] Umesono Y, Tasaki J, Nishimura K, Inoue T, Agata K. Regeneration in an evolutionarily primitive brain -the planarian *Dugesia japonica* model-. European Journal of Neuroscience 2011;34(6) 863-869.

[28] Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. Nature 2008;453(7194) 519-523.

[29] Gurley KA, Rink JC, Sánchez Alvarado A. ß-catenin defines head versus tail identity during planarian regeneration and homeostasis. Science 2008;319(5861) 323-327.

[30] Petersen CP, Reddien PW. Smed-ß-catenin-1 is required for anteroposterior blastema polarity in planarian regeneration. Science 2008;319(5861) 327-330.

[31] Molina MD, Neto A, Maeso I, Gómez-Skarmeta JL, Saló E, Cebrià F. Noggin and noggin-like genes control dorsoventral axis regeneration in planarians. Current Biology 2011;21(4) 300-305.

[32] Gaviño MA, Reddien PW. A Bmp/Admp regulatory circuit controls maintenance and regeneration of dorsal-ventral polarity in planarians. Current Biology 2011;21(4) 294-299.

[33] Kiecker C, Niehrs C. A morphogen gradient of Wnt/ß-catenin signalling regulates anteroposterior neural patterning in *Xenopus*. Development 2001;128(21):4189-4201.

[34] De Robertis EM, Kuroda H. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annual Review of Cell and Developmental Biology 2004;20 285-308.

[35] Endo T, Yoshino J, Kado K, Tochinai S. Brain regeneration in anuran amphibians. Development Growth and Differentiation 2007;49(2) 121-129.

[36] Okamoto M, Ohsawa H, Hayashi T, Owaribe K, Tsonis PA. Regeneration of retinotectal projections after optic tectum removal in adult newts. Molecular Vision 2007;15(13) 2112-2118.

[37] Parish CL, Beljajeva A, Arenas E, Simon A. Midbrain dopaminergic neurogenesis and behavioural recovery in a salamander lesion-induced regeneration model. Development 2007;134(15) 2881-2887.

[38] Berg DA, Kirkham M, Beljajeva A, Knapp D, Habermann B, Ryge J, Tanaka EM, Simon A. Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain. Development 2010;137(24) 4127-4134.

[39] Berg DA, Kirkham M, Wang H, Frisén J, Simon A. Dopamine controls neurogenesis in the adult salamander midbrain in homeostasis and during regeneration of dopamine neurons. Cell Stem Cell 2011;8(4) 426-433.

[40] Doetsch F, García-Verdugo JM, Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. Journal of Neuroscience 1997;17(13) 5046-5061.

[41] Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. Neurogenesis in the adult human hippocampus. Nature Medicine 1998;4(11) 1313-1317.

[42] Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisén J, Janson AM. Evidence for neurogenesis in the adult mammalian substantia nigra. Proceedings of the National Academy of Sciences of the United States of America 2003;100(13) 7925-7930.

[43] Frielingsdorf H, Schwarz K, Brundin P, Mohapel P. No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proceedings of the National Academy of Sciences of the United States of America 2004;101(27) 10177-10182.

[44] Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, Nishikawa SI, Sasai Y. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 2000;28(1) 31-40.

[45] Kawasaki H, Suemori H, Mizuseki K, Watanabe K, Urano F, Ichinose H, Haruta M, Takahashi M, Yoshikawa K, Nishikawa S, Nakatsuji N, Sasai Y. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proceedings of the National Academy of Sciences of the United States of America 2002;99(3) 1580-1585.

[46] Doi D, Morizane A, Kikuchi T, Onoe H, Hayashi T, Kawasaki T, Motono M, Sasai Y, Saiki H, Gomi M, Yoshikawa T, Hayashi H, Shinoyama M, Refaat MM, Suemori H, Miyamoto S, Takahashi J. Prolonged maturation culture favors a reduction in the tumorigenicity and the dopaminergic function of human ESC-derived neural cells in a primate model of Parkinson's disease. Stem Cells 2012;30(5) 935-945.

[47] Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell 1998;93(5) 755-766.

[48] Kittappa R, Chang WW, Awatramani RB, McKay RD. The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. PLoS Biology 2007;5(12) e325.

[49] Ono Y, Nakatani T, Sakamoto Y, Mizuhara E, Minaki Y, Kumai M, Hamaguchi A, Nishimura M, Inoue Y, Hayashi H, Takahashi J, Imai T. Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. Development 2007;134(17) 3213-3225.

[50] Andersson E, Tryggvason U, Deng Q, Friling S, Alekseenko Z, Robert B, Perlmann T, Ericson J. Identification of intrinsic determinants of midbrain dopamine neurons. Cell 2006;124(2) 393-405.

[51] Ferri AL, Lin W, Mavromatakis YE, Wang JC, Sasaki H, Whitsett JA, Ang SL. Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. Development 2007;134(15) 2761-2769.

[52] Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP. Pitx3 is required for development of substantia nigra dopaminergic neurons. Proceedings of the National Academy of Sciences of the United States of America 2003;100(7) 4245-4250.

[53] Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, Conneely OM. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proceedings of the National Academy of Sciences of the United States of America 1998;95(7) 4013-4018.

[54] Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature Biotechnology 2009;27(3) 275-280.

[55] Morizane A, Doi D, Kikuchi T, Nishimura K, Takahashi J. Small-molecule inhibitors of bone morphogenic protein and activin/nodal signals promote highly efficient neural induction from human pluripotent stem cells. Journal of Neuroscience Research 2011;89(2) 117-126.

[56] Kriks S, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z, Carrillo-Reid L, Auyeung G, Antonacci C, Buch A, Yang L, Beal MF, Surmeier DJ, Kordower JH, Tabar V, Studer L. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature 2011;480(7378) 547-551.

[57] Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y. Directed differentiation of telencephalic precursors from embryonic stem cells. Nature Neuroscience 2005;8(3) 288-296.

[58] Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA. Chemically defined conditions for human iPSC derivation and culture. Nature Methods 2011;8(5):424-429.

[59] Brundin P, Strecker RE, Lindvall O, Isacson O, Nilsson OG, Barbin G, Prochiantz A, Forni C, Nieoullon A, Widner H, Gage FH, Björklund A. Intracerebral grafting of dopamine neurons. Experimental basis for clinical trials in patients with Parkinson's disease. Annals of the New York Academy of Sciences 1987;495 473-496.

[60] Takagi Y, Takahashi J, Saiki H, Morizane A, Hayashi T, Kishi Y, Fukuda H, Okamoto Y, Koyanagi M, Ideguchi M, Hayashi H, Imazato T, Kawasaki H, Suemori H, Omachi S, Iida H, Itoh N, Nakatsuji N, Sasai Y, Hashimoto N. Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. The Journal of Clinical Investigation 2005;115(1) 102-109.

[61] Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, Morizane A, Bergquist F, Riebe I, Nannmark U, Carta M, Hanse E, Takahashi J, Sasai Y, Funa K, Brundin P, Eriksson PS, Li JY. Transplantation of human embryonic stem cell-de-

rived cells to a rat model of Parkinson's disease: effect of *in vitro* differentiation on graft survival and teratoma formation. Stem Cells 2006;24(6) 1433-1440.

[62] Hargus G, Cooper O, Deleidi M, Levy A, Lee K, Marlow E, Yow A, Soldner F, Hockemeyer D, Hallett PJ, Osborn T, Jaenisch R, Isacson O. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proceedings of the National Academy of Sciences of the United States of America 2010;107(36) 15921-15926.

[63] Kikuchi T, Morizane A, Doi D, Onoe H, Hayashi T, Kawasaki T, Saiki H, Miyamoto S, Takahashi J. Survival of human induced pluripotent stem cell-derived midbrain dopaminergic neurons in the brain of a primate model of Parkinson's disease. Journal of Parkinson's Disease 2011;1 395-412.

[64] Okano H, Imai T, Okabe M. *Musashi*: a translational regulator of cell fate. Journal of Cell Science 2002;115(Pt 7) 1355-1359.

[65] Higuchi S, Hayashi T, Tarui H, Nishimura O, Nishimura K, Shibata N, Sakamoto H, Agata K. Expression and functional analysis of *musashi*-like genes in planarian CNS regeneration. Mechanisms of Development 2008;125(7) 631-645.

[66] Agata K, Nakajima E, Funayama N, Shibata N, Saito Y, Umesono Y. Two different evolutionary origins of stem cell systems and their molecular basis. Seminars in Cell and Developmental Biology 2006;17(4) 503-509.

[67] Sharif J, Endoh M, Koseki H. Epigenetic memory meets G2/M: to remember or to forget? Developmental Cell 2011;20(1) 5-6.

[68] Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y. Directed differentiation of telencephalic precursors from embryonic stem cells. Nature Neuroscience 2005;8(3) 288-296.

[69] Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell 2008;3(5) 519-532.

[70] Kirkeby A, Grealish S, Wolf DA, Nelander J, Wood J, Lundblad M, Lindvall O, Parmar M. Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. Cell Reports 2012 28;1(6) 703-714.