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ASCL1-mediated direct reprogramming: converting ventral midbrain astrocytes into dopaminergic neurons for Parkinson's disease therapy

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Parkinson's disease (PD), characterized by dopaminergic neuron degeneration in the substantia nigra, is caused by various genetic and environmental factors. Current treatment methods are medication and surgery; however, a primary therapy has not yet been proposed. In this study, we aimed to develop a new treatment for PD that induces direct reprogramming of dopaminergic neurons (iDAN). Achaete-scute family bHLH transcription factor 1 (*ASCL1*) is a primary factor that initiates and regulates central nervous system development and induces neurogenesis. In addition, it interacts with *BRN2* and *MYT1L*, which are crucial transcription factors for the direct conversion of fibroblasts into neurons. Overexpression of *ASCL1* along with the transcription factors *NURR1* and *LMX1A* can directly reprogram iDANs. Using a retrovirus, *GFP*-tagged *ASCL1* was overexpressed in astrocytes. One week of culture in iDAN convertsion medium reprogrammed the astrocytes into iDANs. After 7 days of differentiation, TH+/TUJ1+ cells emerged. After 2 weeks, the number of mature TH+/TUJ1+ dopaminergic neurons increased. Only ventral midbrain (VM) astrocytes exhibited these results, not cortical astrocytes. Thus, VM astrocytes can undergo direct iDAN reprogramming with *ASCL1* alone, in the absence of transcription factors that stimulate dopaminergic neurons development. [BMB Reports 2024; 57(8): 363-368]

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease resulting from the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Motor symptoms include tremors, rigidity, and bradykinesia (1). Currently, levodopa therapy (2), which employs dopamine precursors that can cross the blood-brain barrier, is extensively used. Levodopa temporarily alleviates motor symptoms in patients with PD. However, persistent administration of high doses of levodopa may result in levodopa-induced dyskinesia (3) and hasten disease progression. Therefore, cell transplantation is currently considered to be the most promising treatment for PD (4-6). In brain regions lacking dopaminergic neurons, neural stem cells (NSCs) or neural progenitor cells (NPCs) that can differentiate into dopaminergic neurons are transplanted. Several studies (4-6) suggest that cell transplantation alleviates the motor symptoms of PD. However, cell transplantation for the treatment of PD is limited by adverse effects and high costs, making its therapeutic application difficult (7). To overcome this obstacle, we reprogrammed astrocytes directly into dopaminergic neurons to treat PD. Numerous studies have investigated the direct reprogramming of non-neuronal cells into induced neurons (iNs). A previous study demonstrated, for the first time, that the expression of three transcription factors, *BRN2* (*Pou3f2*), achaete-scute family bHLH transcription factor 1 (*ASCL1*), and *MYT1L*, can transform mouse fibroblasts into functional iNs. Furthermore, *NeuroD* overexpression has been used to convert human fibroblasts into iNs, according to research (8).

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Astrocytes (9) are non-neuronal cells prevalent in the brain that play a role in neurotransmitter transport. To exploit these properties, we induced the direct reprogramming of dopaminergic neurons (iDAN) by overexpressing transcription factors in astrocytes. *ASCL1* is a basic helix-loop-helix transcription factor that activates gene expression by binding to the E-box motif (5′-CANNTG-3′). It is a primary factor that initiates and regulates the development of the central nervous system and promotes neurogenesis. According to another study, *ASCL1* overexpression can directly reprogram fibroblasts (10) and astrocytes (11) into neurons. *ASCL1* facilitates the direct reprogramming of fibroblasts chromatin remodeling into mature neurons by opening closed chromatin at the target site, causing nucleosome phasing (12). In a previous study conducted in our laboratory, we discovered that an *ASCL1*^{5SA} mutant, which involved artificial blocking of phosphorylation through five serine to alanine mutations, facilitated the direct reprogramming of mouse astrocytes into neurons (13). To determine whether the *ASCL1*^{5SA} mutant had a greater positive effect than *ASCL1*, a comparative study was conducted. When *ASCL1*, *NURR1*, *LMX1A*, *SHH*, and *BclXL* were overexpressed in the ventral midbrain of rodents, the astrocytes in this region were found to be directly reprogrammed into dopaminergic neurons (14). *NURR1* and *LMX1A* are essential for the survival and maintenance of dopaminergic neurons in the midbrain (14). Overexpression of *ASCL1*, *NURR1*, *LMX1A*, *SHH*, and *BclXL* in rat cortical (CTX) and ventral midbrain (VM) astrocytes resulted in their direct reprogramming to dopaminergic neurons. In addition, the *ASCL1* factor alone enabled rat VM astrocytes to transform directly into dopaminergic neurons. Following *ASCL1* overexpression in VM astrocytes, numerous dopaminergic neurons (TH+/TUJ1+ cells) have been identified. In addition, the number of dopaminergic neurons increased during the 3-week differentiation period. However, these outcomes have not been observed in CTX astrocytes. In this study, the use of *ASCL1* for direct reprogramming of dopaminergic neurons produced diverse results in different brain regions.

RESULTS

Overexpression of *ASCL1*, *NURR1*, *LMX1A*, *SHH*, and *BclXL* combinations in CTX and VM astrocytes enables iDAN direct reprogramming

Upon overexpression of *ASCL1* + *NURR1* + *LMX1A* (ANL), *ASCL1*^{5SA} + *NURR1* + *LMX1A* (5SANL), *NURR1* + *SHH* + *BclXL* + *ASCL1* (NHB + A), or *NURR1* + *SHH* + *BclXL* + *ASCL1*^{5SA} (NHB + 5SA) in CTX astrocytes, direct reprogramming into dopaminergic neurons with VM characteristics was observed (14). Supplementary Fig. 1 depicts the characteristics of CTX and VM astrocytes. To confirm the reproducibility of these results in rat CTX and VM astrocytes, retroviruses were used for overexpressing ANL, 5SANL, NHB + A, or NHB + 5SA (Fig. 1A). On the third day of direct reprogramming, TUJ1+ cells were found in all CTX and VM astrocyte groups. On day 5, TUJ1+ cells more than doubled in all groups except in the control group, and TH+ cells began to appear (Fig. 1B, D). ANL overexpression in CTX and VM astrocytes showed the greatest increase in TH+/TUJ1+ cells on day 5 and day 7 of direct reprogramming, respectively. Subsequent-

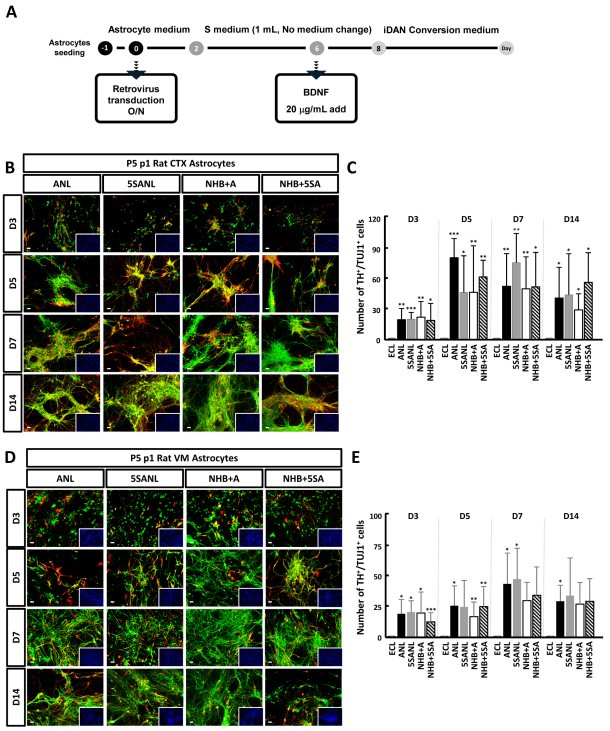

Fig. 1. Overexpression of ANL, 5SANL, NHB + A, or NHB + 5SA in CTX and VM astrocytes results in direct reprogramming into dopaminergic neurons (iDANs), as demonstrated by immunocytochemistry (ICC). (A) Timeline for iDAN direct reprogramming. (B) The ICC results for iDAN direct reprogramming from rat CTX astrocytes on days 3, 5, 7, and 14. iDANs stained with anti-TH (red) and anti-TUJ1 (green) antibodies. When co-expressed, *NURR1* enables direct iDAN reprogramming in rat CTX astrocytes. *EGFP* (ECL), *ASCL1* + *NURR1* + *LMX1A* (ANL), *ASCL1*^{5SA} + *NURR1* + *LMX1A* (5SANL), *NURR1* + *SHH* + *BclXL* + *ASCL1* (NHB + A), and *NURR1* + *SHH* + *BclXL* + *ASCL1*^{5SA} (NHB + 5SA). (C) Counts of TH+/TUJ1+ cells following direct reprogramming of rat CTX astrocytes on days 3, 5, 7, and 14. A significant increase in TH+/TUJ1+ cells is confirmed across all groups. In particular, the ANL group shows the greatest increase in TH cells on day 5 of differentiation, followed by a decline. (D) ICC of iDAN direct reprogramming from rat VM astrocytes on days 3, 5, 7, and 14. iDANs stained with anti-TH (red) and anti-TUJ1 (green) antibodies. Rat VM astrocytes can also directly undergo iDAN reprogramming when *NURR1* is co-expressed. (E) On days 3, 5, 7, and 14 of direct reprogramming of rat VM astrocytes, the number of TH+ and TUJ1+ cells was determined. Although the increase in the number of TH+/TUJ1+ cells is lower than that of CTX astrocytes in all groups, they show a significant increase in the ANL and 5SANL groups. *P < 0.05. **P < 0.01. ***P < 0.001. The error bar depicts the standard error of the mean. Scale bar, 20 μm.

ly, among both CTX and VM astrocytes, the number of TH+/TUJ1+ cells decreased as the direct reprogramming period elapsed (Fig. 1C, E). These results are consistent with those of previous studies (14). The combination of ANL, 5SANL, NHB + A, or NHB + 5SA factors effectively enabled direct reprogramming of dopaminergic neurons in mouse (data not shown) and rat astrocytes.

Direct reprogramming of rat VM astrocytes into iDANs using only the *ASCL1* factor

Our objective was to identify key transcription factors that facilitate the direct reprogramming of astrocytes into dopaminergic neurons. We examined whether overexpression of *ASCL1* in CTX and VM astrocytes can directly reprogram iDANs by inducing TH+ cells. However, TH+ cells were not observed among CTX astrocytes that did not express *NURR1* or *LMX1A* (Fig. 2A). No significant differences were observed during reprogramming. Therefore, NURR1 and LMX1A were determined to be essential factors for the direct reprogramming of CTX astrocytes to iDANs. In contrast, TH+/TUJ1+ cells appeared among VM astrocytes expressing *NURR1* and *LMX1A*, as well as in the *ASCL1* and *ASCL*^{5SA} mutant single-factor groups (Fig. 2A). The number of TH+/TUJ1+ cells increased among VM astrocytes during long-term differentiation. Interestingly, ANL-overexpressing VM astrocytes displayed the highest number of TH+/TUJ1+ cells on day 7 of differentiation, with a slight decrease observed on day 14. In contrast, the VM astrocytes overexpressing the *ASCL1* single factor showed no significant changes in TH+/TUJ1+ cells on day 7 of differentiation compared with the ANL astrocytes. However, on day 14 of differentiation, the number of TH+/TUJ1+ cells among the VM astrocytes overexpressing *ASCL1* was higher than that among the ANL astrocytes. This result was consistent with that of the quantitative analysis (Fig. 2C). These results suggest that, in VM astrocytes, *NURR1* and *LMX1A* play a role in rapid reprogramming to iDANs at the beginning of differentiation, whereas *ASCL1* induces stepwise iDAN reprogramming according to the differentiation period.

In summary, we found that direct reprogramming from CTX astrocytes to iDANs is not possible with *ASCL1* alone without the midbrain transcription factors *NURR1* and *LMX1A*. VM astrocytes can be reprogrammed into iDANs by overexpression of *ASCL1* or *ASCL1*^{5SA} as single factors. Direct reprogramming of CTX and VM astrocytes using *NURR1* and *LMX1A* without *ASCL1* did not have a significant effect (Fig. 2B). Therefore, *ASCL1* is the most important transcription factor for direct iDAN reprogramming.

ASCL1 shows prolonged-expression and can induce direct reprogramming into iDANs

To confirm whether *ASCL1* expression persisted during direct reprogramming, iDANs expressing *ASCL1* and non-reprogrammed astrocytes expressing *ASCL1* were harvested. GFP protein was expressed at 27 kDa and ASCL1 fused to FLAG protein was

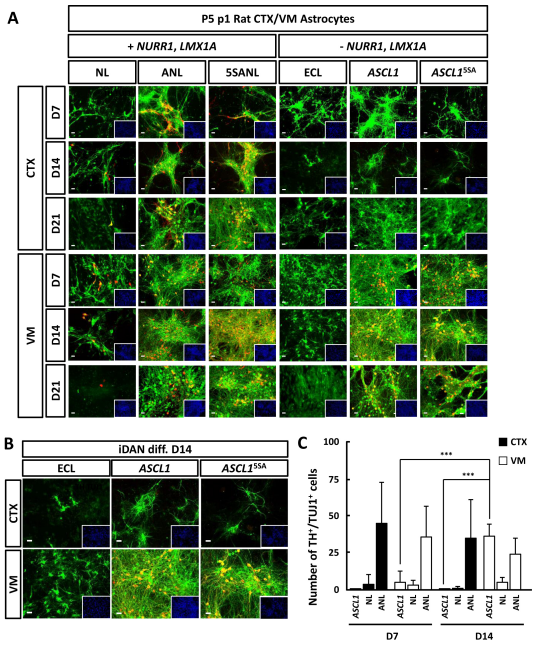

Fig. 2. Unlike CTX astrocytes, VM astrocytes can undergo iDAN direct reprogramming through *ASCL1* single-factor overexpression. (A) ICC analysis following iDAN direct reprogramming of rat CTX and VM astrocytes on days 7, 14 and 21. iDANs are stained with anti-TH (red) and anti-TUJ1 (green) antibodies. Among VM astrocytes overexpressing *ASCL1*, TH+ cells begin to appear after 1 week of reprogramming. EGFP-CL (ECL), *ASCL1* + *NURR1* + *LMX1A* (ANL), *ASCL1*^{5SA} + *NURR1* + *LMX1A* (5SANL), differentiation (diff). (B) ICC analysis of iDAN direct reprogramming from rat CTX and VM astrocytes on day 14. (C) A number of TH+/TUJ1+ cells is counted following direct reprogramming of rat CTX and VM astrocytes on days 7 and 14. VM astrocytes show a similar increase in the number of TH+ cells as ANL astrocytes from day 14 of iDAN reprogramming. ***P $<$ 0.001. The error bar represents the S.E. Scale bar, 20 μm.

expressed at 31 kDa in non-reprogrammed cells. After 4 weeks of iDAN reprogramming, the expression was similar to that in non-reprogrammed cells. ASCL1 expression was maintained after the direct reprogramming of astrocytes to iDANs (Fig. 3A). Thus, ASCL1 expression persisted for more than 4 weeks. We aimed to demonstrate that *ASCL1* expression persists and induces direct reprogramming of iDAN.

Subsequently, we measured the mRNA expression levels at different stages of reprogramming. *ASCL1* was overexpressed in CTX and VM astrocytes to induce direct reprogramming of iDANs, and the mRNA level was evaluated using reverse transcriptase-polymerase chain reaction (RT-PCR). mRNA was extracted from cells that differentiated after 2 and 4 weeks. Consistent with the immunocytochemistry (ICC) results, *Tuj1* and *TH* mRNAs were expressed only in VM astrocytes overexpressing *ASCL1*. These expression levels showed no significant difference with respect to the iDAN differentiation period (Fig. 3B, C). Similar results were obtaineded using real-time quan-

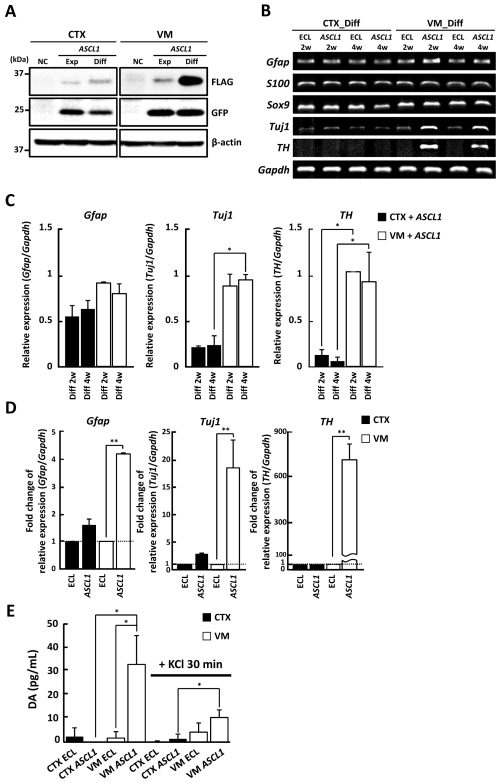

Fig. 3. Even after iDAN direct reprogramming, *ASCL1* overexpressed by retroviruses continues to be expressed and induces iDAN direct reprogramming. (A) Western blot of iDAN direct reprogramming from CTX and VM astrocytes. The presence of 31 kDa bands of Flag and 27 kDa bands of GFP confirms their respective sizes, and the expression of ASCL1 persists even after reprogramming. Negative control (NC), non-reprogrammed (Exp), reprogrammed (Diff). (B) RT-PCR of iDAN direct reprogramming from CTX and VM astrocytes. The expressions of *Tuj1* and *TH* are confirmed by *ASCL1* overexpression in VM astrocytes. EGFP-CL (ECL). (C) Bands of RT-PCR were quantified using Image J. *P $<$ 0.05. The error bar represents the S.E. (D) qRT-PCR analysis of the mRNA levels of *Gfap*, *Tuj1*, and *TH* in the negative control and in astrocytes overexpressing *ASCL1*. **P $<$ 0.01. The error bar represents the S.E. (E) Dopamine released from CTX and VM iDANs 28 days after transduction. ELISA confirms that dopamine is released exclusively in VM iDANs. *P $<$ 0.05. The error bar represents the S.E.

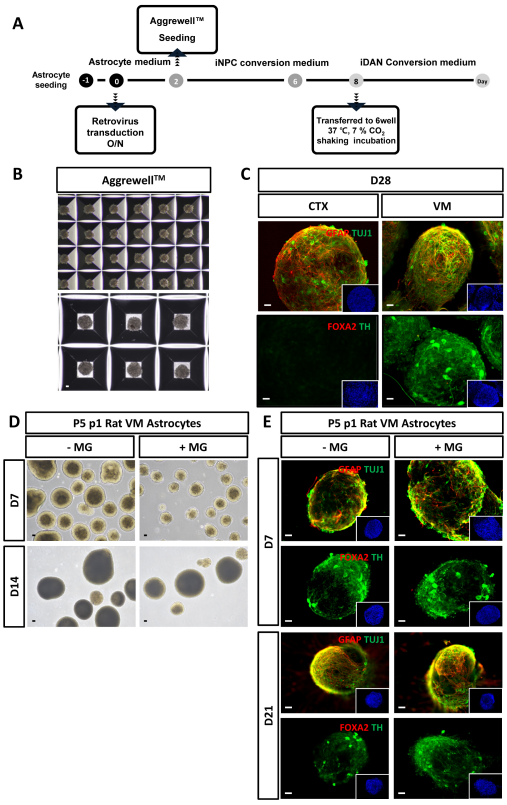

Fig. 4. 3D culture is more effective for iDAN direct reprogramming. (A) Schematic illustration describing the timeline of iDAN direct reprogramming of 3D culture. (B) The astrocytes and microglia seeded in AggreWell™ 800 plates sink over time and form neurospheres. (C) After 4 weeks of reprogramming, the expression of the dopaminergic neuron markers FOXA2 (red) and TH (green) is observed. (D) The colonies without microglia (−MG) and those with microglia (+MG) were cultured on the shaker for 7 and 14 days. (E) The expression of dopaminergic neuron markers in the (−MG) and (+MG) colonies is observed. FOXA2 (red), TH (green). Scale bar, 20 μm.

titative reverse transcription PCR (RT-qPCR), which showed that the *Tuj1* and *TH* mRNA levels were high in the VM astrocytes that overexpressed *ASCL1*. *Tuj1* mRNA levels in this group were upregulated by approximately 18-fold or higher compared to those in the VM control, and *TH* mRNA expression was upregulated by approximately 720-fold or higher (Fig. 3D).

Furthermore, we confirmed that the dopaminergic neurons reprogrammed in astrocytes are functional. ELISA was conducted to verify dopamine release, which is an indicator of functional dopaminergic neurons. Supernatants containing dopamine were harvested after 48 h of cell incubation or 30 min of stimulation with 56 mM KCl. *ASCL1*-induced iDANs from VM astrocytes showed the highest dopamine release. Indeed, at 21 days after transduction, the VM iDANs released high levels of dopamine with and without 56 mM KCl (Fig. 3E).

3D culture is more effective for iDAN direct reprogramming

Previous research has demonstrated that passing through the neurosphere state helps maintain the intrinsic characteristics of NPCs (15, 16). *ASCL1* was overexpressed in rat VM astrocytes, which were subsequently subjected to direct reprogramming to NPCs in the neurosphere state under a 3D culture environment in AggreWell™ 800. Subsequently, these cells transdifferentiated into iDANs. Additionally, we attempted to confirm the possibility of forming neurospheres that mimic the midbrain environment. Experiments were conducted in which microglia, an essential brain cell type, were added to astrocytes at a

ratio of 10-20%. After *ASCL1* was overexpressed in rat VM astrocytes, the cells were seeded at a density of 1×10^6 cells per well in AggreWell™ 800. Neurospheres were cultured for a week in induced NPC reprogramming (iNPC) conversion medium, after which the medium was replaced with iDAN conversion medium (Fig. 4A, B). After 28 days of differentiation, the direct reprogramming of CTX and VM astrocytes into iDANs was induced. TH+ cells were not observed in the CTX astrocytes, consistent with previous findings. In contrast, numerous TH+ cells were detected in VM astrocytes, indicating a higher efficiency of direct reprogramming into iDAN than that in 2D cultures (Fig. 4C). Subsequently, we investigated the effect of microglia on the direct reprogramming of iDANs and observed that microglia had no significant effect on the generation of FOXA2+/TH+ dopaminergic neurons (Fig. 4D, E). These results suggest that 3D culture has a beneficial effect on the direct reprogramming of dopaminergic neurons but does not contribute to the generation of dopaminergic neurons that exhibit VM characteristics.

DISCUSSION

We demonstrated that rat VM astrocytes can be directly reprogrammed into iDANs by overexpressing *ASCL1*, whereas CTX astrocytes cannot. This implies that the results of direct reprogramming are dependent on region-specific characteristics of astrocytes. Therefore, it is important to consider the unique features of a particular astrocyte region when conducting direct reprogramming experiments. Differences in gene expression between cortical and ventral midbrain astrocytes have been reported (17, 18).

In this study, we used only astrocytes that were passaged once. Approximately 70% of astrocytes were maintained as GFAP+ cells, whereas the remaining 30% consisted of other cell types (Supplementary Fig. 1). Therefore, further verification is required to confirm whether direct reprogramming occurs in other cells.

A significant disparity in retroviral transduction efficiency was observed between CTX and VM astrocytes (Supplementary Fig. 2). Retroviruses are transduced during cell division, and the higher infectivity of VM astrocytes could be attributed to their relatively rapid growth rate. Therefore, it was crucial to confirm whether the observed differences in direct reprogramming resulted from *ASCL1* overexpression or from differences in transduction efficiency (Figs. 1 and 2).

The expression of *FOXA2*, a midbrain-specific transcription factor, did not merge with that of TH-positive neurons that were directly reprogrammed by *ASCL1* overexpression in VM astrocytes (Supplementary Fig. 3). This suggests that iDANs from VM astrocytes do not function as midbrain dopaminergic neurons. Therefore, to induce dopaminergic neurons with midbrain characteristics, VM astrocytes were 3D cultured to obtain the shape and characteristics of VM organoids. Brain organoids generated *in vitro* can be used for studying specific parts of the brain, as well as brain development and disorders. Human-derived iPSCs (19, 20) and human embryonic stem cells (hESCs) (21-23) are the most representative cell sources that initially emerged as brain organoids. NPCs differentiate into various cell types in the central nervous system to form small brain organoids, which are emerging as innovative treatments for degenerative brain diseases. In particular, the development of midbrain organoids is necessary for PD treatment. However, in the present study, when utilizing *ASCL1* for 3D culture, dopaminergic neurons exhibiting midbrain characteristics were not observed. Because one gene was overexpressed using the retrovirus for each factor, it is possible that not all factors completely transduced the cells. Other limitations of previous attempts to develop organoids with midbrain dopaminergic neurons include the lack of small molecules that are commonly used in the production of VM organoids and the limited culture period (Fig. 4).

Specific expression of *ASCL1* in rat brain astrocytes to induce iDANs was difficult to determine (Supplementary Fig. 4). To resolve these problems, a retrovirus that operates under an astrocyte-specific promoter needs to be injected. However, negative results have been published for astrocyte-specific promoters, such as the *GFAP* or *ALDH1L1* promoter. Previous studies have verified that the *GFAP* promoter functions nonspecifically. The *GFAP* promoter has been reported to leak into endogenous neurons *in vivo* (24). Additional studies are required to overcome these limitations.

In conclusion, this study indicates the following: 1) Direct reprogramming of dopaminergic neurons is possible when *ASCL1* is overexpressed in rat VM astrocytes. 2) These results were not observed in CTX astrocytes. 3) Direct reprogramming of *ASCL1* into dopaminergic neurons has yielded different results in distinct brain regions. Based on these findings, direct reprogramming of non-neuronal cells into neurons presents innovative possibilities for cell regeneration and the treatment of degenerative brain diseases for which there is no definite cure.

MATERIALS AND METHODS

Dopaminergic neuron differentiation

To generate iDANs from CTX and VM astrocytes, 2×10^4 cells were seeded per well in a 24-well plate containing a 12 mm slide (Bellco, USA) coated with PLO/FN. The astrocyte expansion medium was utilized to stabilize the cells at 37°C and 5% CO_2 for 24 h. Retrovirus was mixed with astrocyte expansion medium, and 8 μg/ml polybrene was added. The cells were treated for 12 h, and subsequently, the treatment medium was replaced with the astrocyte expansion medium. After incubation for 24 h, this medium was replaced with DMEM:F12 (1 ml per well) containing 2% B-27, 1% glutaMax-I, 10 ng/ml bFGF, 10 μM Forskolin (FSK, Sigma-Aldrich), and 1% penicillin/streptomycin (P/S). After 4 days, 20 ng/ml brain-derived neurotrophic factor (BDNF; PeproTech, USA) was added. Three days later, the medium was replaced with N2 medium containing 2% B-27, 0.2 mM ascorbic acid (AA; Sigma-Aldrich), 250 μg/ml

dibutyl cyclic-AMP (cAMP; Sigma-Aldrich), 20 ng/ml BDNF, and 20 ng/ml glial cell line-derived factor (GDNF; PeproTech) (iDAN conversion medium). The iDAN conversion medium was replaced every other day.

Induced neuronal progenitor cell reprogramming (iNPC)

iNPCs were generated from CTX and VM astrocytes using iNPC medium, DMEM: F12 containing 2% B-27, 1% glutaMax-I, 20 ng/ml bFGF, 10 μM Forskolin, and 1% P/S.

ELISA for dopamine release

A dopamine release assay was performed using the Dopamine Research ELISA Kit (Labor Diagnostika Nord, Germany) according to the manufacturer's instructions. After 21 days of transduction, dopamine-released supernatants were collected following a 48 h incubation period (basal release) or 30 min of stimulation with 56 mM KCl (evoked).

Cell counting and statistical analysis

Cell counting was randomly performed in 10-15 microscopic fields/well and 3 wells/experimental conditions. Each experiment was independently performed at least three times. All data are expressed as mean ± standard error (S.E.). Using SigmaPlot for Windows version 10.0 (SystatSoftware GmbH, Erkrath, Germany), paired *t*-tests were performed for statistical comparison of data.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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