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WNT-C59, a Small-Molecule WNT Inhibitor, Efficiently Induces Anterior Cortex That Includes Cortical Motor Neurons From Human Pluripotent Stem Cells

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Key Words. Neural differentiation • Wnt signaling • Cortical motor neuron • Cell transplantation • Pluripotent stem cells

ABSTRACT

The recapitulation of human neural development in a controlled, defined manner from pluripotent stem cells (PSCs) has considerable potential for studies of human neural development, circuit formation and function, and the construction of in vitro models of neurological diseases. The inhibition of Wnt signaling, often by the recombinant protein DKK1, is important for the induction of cortical neurons. Here, we report a novel differentiation method using a small-molecule WNT inhibitor, WNT-C59 (C59), to efficiently induce human anterior cortex. We compared two types of small molecules, C59 and XAV939 (XAV), as substitutes for DKK1 to induce cortical neurons from PSCs in serum-free embryoid body-like aggregate culture. DKK1 and XAV inhibited only the canonical pathway of Wnt signaling, whereas C59 inhibited both the canonical and noncanonical pathways. C59 efficiently induced CTIP2+/COUP-TF1− cells, which are characteristic of the cells found in the anterior cortex. In addition, when grafted into the cortex of adult mice, the C59-induced cells showed abundant axonal fiber extension toward the spinal cord. These results raise the possibility of C59 contributing to cell replacement therapy for motor neuron diseases or insults.

SIGNIFICANCE

For a cell therapy against damaged corticospinal tract caused by neurodegenerative diseases or insults, cortical motor neurons are needed. Currently, their induction from pluripotent stem cells is considered very promising; however, an efficient protocol to induce motor neurons is not available. For efficient induction of anterior cortex, where motor neurons are located, various WNT inhibitors were investigated. It was found that one of them could induce anterior cortical cells efficiently. In addition, when grafted into the cortex of adult mice, the induced cells showed more abundant axonal fiber extension toward spinal cord. These results raise the possibility that this inhibitor contributes to a cell-replacement therapy for motor neuron diseases or insults.

INTRODUCTION

Movement depends on signaling pathways originating from the motor cortex. The mammalian motor cortex has a six-layered structure. Layer V includes cortical motor neurons (CMNs) that have a pyramidal shape and extend axons to innervate motor neurons in the spinal cord [1]. The degeneration of CMNs causes motor neuron diseases including amyotrophic lateral sclerosis (ALS) and hereditary spastic paraplegia [2]. A loss or deterioration of motor functions can also occur upon stroke or brain injury that causes the death of CMNs.

Cell replacement therapy is gaining considerable attention to treat motor dysfunctions. Fetal frontal cortex cells transplanted into the frontal region of neonatal [3] or adult [4] mice were found to extend axons to the spinal cord. These findings suggest that the transplantation of fetal frontal cortex, which includes CMN progenitor cells, is able to reconstitute the corticospinal pathway. However, human fetal brains are not a feasible source of donor cells. Alternative donor cells include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). These PSCs can be unlimitedly expanded and give rise to a wide variety of differentiated cells, making them a promising cell source for treating intractable neurological disorders, including Parkinson’s disease [5–7] and ALS [8]. In fact, recent studies have shown that mouse [9] or human [10] ESC-derived neurons extend long axons from the motor cortex to the pyramidal tract in neonate mice. However, the efficiency to induce CMNs from ESCs or iPSCs is very low.

In order to induce cortical neurons from mouse or human ESCs/iPSCs, serum-free embryoid body-like
aggregates (SFEB) [11–13] or monolayer [9, 10, 14–17] protocols are used. CMNs are located in the frontal/motor area of the anterior cortex, and the inhibition of Wnt signaling is important to induce this area. WNTs contribute to the proper patterning of neural structures along the anterior-posterior axis [18]. In these methods, a recombinant protein, DKK1, is used as the WNT inhibitor. However, DKK1 is produced in animal cells and Escherichia coli, raising the possibility of infection or immune rejection due to cross-species contamination when the induced cells are transplanted to humans. Therefore, an alternative WNT inhibitor to induce CMNs is required for clinical application.

In the present study, we investigated the effects of two small compounds as substitute WNT inhibitors in an induction protocol for cortical neurons from human iPSCs: WNT-C59 (CS9) and XAV939 (XAV) (supplemental online Fig. 1). CS9 inhibits the activity of porcupine (PORCN), which is a membrane-bound O-acetyltransferase required for palmitoylation, secretion, and the biological activity of WNTs. The inhibition of PORCN by C59 prevents both canonical and noncanonical Wnt-mediated signaling [19]. XAV inhibits tankyrase 1 and 2, thus stabilizing axin and stimulating β-catenin degradation [20], whereas DKK1 inhibits WNT binding to its receptor, LRPS/6 [21]. In other words, XAV and DKK1 inhibit only canonical Wnt signaling. We induced cortical neurons from human iPSCs in the presence of DKK1, XAV, or CS9 and found that CS9 induced frontal cortical neurons expressing a marker for layer V (CTIP2+/COUP-TF1) most efficiently. This result suggests that CS9 can be used to induce CMNs or their progenitors from human iPSCs.

**Materials and Methods**

**Maintenance and Differentiation Culture of Human ESCs/iPSCs**

Human iPSCs (836B1) were maintained on SNL feeder cells activated by mitomycin C (Wako Pure Chemical Industries, Ltd., Osaka, Japan, http://www.wako-chem.co.jp) in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium (F12) (Wako) supplemented with 20% (vol/vol) Knockout Serum Replacement (KSR); Invitrogen, Tokyo, Japan, http://www.thermofisher.com), 0.1 mM nonessential amino acids (NEAA; Invitrogen), 2 mM l-glutamine (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 0.1 mM 2-mercaptoethanol (Wako), and 5 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). For the induction of cortical neurons using a serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq), feeder cells were removed with CTX (0.25% [wt/vol] trypsin [Invitrogen] and 0.1% [wt/vol] collagenase IV [Gibco, Tokyo, Japan, http://www.thermofisher.com] in PBS [Wako]) containing 20% KSR [Invitrogen] and 1 mM CaCl2 [Wako]), and PSCs were dissociated into single cells by using Accumax (Innovative Cell Technologies, San Diego, CA, http://www.accumax.com). The cells were counted, and 9,000 cells per well were aggregated in a low cell binding, Lipidure-coated 96-well plate (NOF Corp., Tokyo, Japan, https://www.nof.co.jp). The differentiation medium contained DMEM/F12 supplemented with 15% (vol/vol) KSR, 0.1 mM NEAA, 2 mM l-glutamine (Invitrogen), and 0.1 mM 2-mercaptoethanol (Wako). From day 0 to 3, 50 μM Y-27632 (Wako) was added to the differentiation medium to increase cell viability. From day 0 to day 6, 10 ng/ml bFGF (Invitrogen) was added to the medium to increase cell viability and the induction of ectodermal fate [22]. To induce forebrain fate, the medium was supplemented with WNT inhibitor (500 ng/ml human recombinant DKK1 [R&D Systems Inc., Minneapolis, MN, https://www.rndsystems.com], 0.5 μM XAV939 [Stemgent, Lexington, MA, https://www.stemgent.com], or 10 nM WNT-C59 [Cellagen Technology, San Diego, CA, http://www.cellagentech.com]) along with 10 μM SB431542 (TGFB-β/activin/nodal inhibitor; Sigma-Aldrich) and 0.1 μM LDN193189 (BMP inhibitor; Stemgent). On day 18, cell aggregates were transferred to a 24-well culture plate coated with 50 μg/ml poly-L-ornithine (Sigma-Aldrich), 5 μg/ml laminin (BD Biosciences, Heidelberg, Germany, http://www.bdbiosciences.com), and 5 μg/ml fibronectin (Sigma-Aldrich) in Neurobasal Medium (Invitrogen) supplemented with 2 mM l-glutamine (Invitrogen), 2% (vol/vol) B27 (without vitamin A; Invitrogen), and 10 units/ml penicillin and streptomycin (Invitrogen). Medium was changed every 4 or 5 days.

**Transplantation**

Animals were cared for and handled according to the Guidelines for Animal Experiments of Kyoto University and the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (Washington, DC, http://deis.nas.edu/ilar). Six- to 8-week-old adult NOD/Shi/lscid Jcl (NOD-SCID) male mice (CLEA Japan Inc., Tokyo, Japan, http://www.clea-japan.com) were anesthetized with isoflurane (Mylan Inc., Tokyo, Japan, http://www.mylan.co.jp), and cell transplantation was performed with stereotaxic injection of one aggregate (approximately 3 × 10⁶ cells) on differentiation day 46 in 2 μl of PBS (Wako) through a blunt-end 26-gauge Hamilton syringe needle (Hamilton, Reno, NV, http://www.hamiltoncompany.com) into the right side of the cortex (from the bregma: A, +0.5 to +2.5; L, −0.5 to −2.5; V, −1.0). Six months after transplantation, the animals were perfused transcardially with 4% paraformaldehyde (PFA; Wako). The brains and spinal cords were fixed with 4% PFA overnight, transferred to 30% (wt/vol) sucrose (Nacalai Tesque, Kyoto, Japan, http://www.nacalai.co.jp) in PBS, and preserved at 4°C. They were then embedded with O.C.T. Compound (Sakura Finetek, Torrance, CA, http://www.sakura-americas.com), cut with a cryostat (Leica Biosystems, Buffalo Grove, IL, http://www.leicabiosystems.com) at 30-μm thickness and preserved in antifreeze (30% [vol/vol] glycerol [Nacalai Tesque], 30% [vol/vol] ethylene glycol [Wako], and 40% [vol/vol] 0.1 M PBS) at −30°C until use.

**Immunostaining**

On days 18, 32, and 46 from the day of neural induction, individual aggregates were fixed in 4% PFA (Wako) and washed with and preserved in PBS at 4°C until use. The aggregates were embedded with O.C.T. Compound (Sakura Finetek), cryosectioned at 10 μm using Cryostat (Leica), and attached to an MAS-coated glass slide (Matsunami, Osaka, Japan, http://www.matsunami-glass.co.jp). Cryosections (aggregates and brains) were blocked in 5% (vol/vol) normal donkey serum (NDS; EMD Millipore, Billerica, MA, http://www.millipore.com)/0.3% (vol/vol) Triton X-100 (Sigma)/PBS for 1 h at room temperature. Primary antibodies were diluted in 1% NDS/0.3% Triton X-100/PBS and incubated overnight at 4°C. The samples were then washed in 0.05% (vol/vol) Tween 20 (Nacalai Tesque)/PBS and incubated with secondary antibodies in 1% NDS/0.3% Triton X-100/PBS for 2 h at room temperature, followed by washing in 0.05% Tween 20/PBS and incubation with 4’,6-diamidino-2-phenylindole (Invitrogen) for 10 min at room temperature. Finally, the samples were washed in 0.05% Tween 20/PBS and mounted with antiphoto bleaching mounting medium (2.4 g of Mowiol [Calbiochem, www.StemCellsTM.com ©AlphaMed Press 2016

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Billerica, MA, [http://www.merckmillipore.com], 6 ml of glycerol [Wako], 6 ml of distilled water, 12 ml of 200 mM Tris-HCl [Nacalai Tesque], and 0.66 g of 1.4-diazabicycle[2.2.2]octane [Wako]). The primary antibodies used were as follows: anti-

- **β-catenin** (1:500; Abcam, Cambridge, MA, [http://www.abcam.com]), anti-PAX6 (1:500; BD Biosciences), anti-MAP2ab (1:2000; Sigma-Aldrich), anti-β-tubulin (TUJ1; 1:2,000; Covance, Princeton, NJ, [http://www.covance.com]), anti-SOX1 (1:1,000; R&D Systems), anti-SOX2 (1:500; R&D Systems), anti-CTIP2 (1:1,000; Abcam), anti-COUP-TF1 (1:1,000; Perseus Proteomics Inc., Tokyo, Japan, [http://www.ppmx.com]), anti-SP8 (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, [http://www.scbt.com]), anti-BRN2 (1:1,000; Santa Cruz), anti-TBR1 (1:1,000; Millipore), anti-SATB2 (1:200; Abcam), anti-g-aminobutyric acid (GABA; 1:1,000; Sigma-Aldrich), anti-tyrosine hydroxylase (1:1,000; Millipore), anti-vesicular glutamate transporter 1 (anti-VGLUT1; 1:1,000; Synaptic Systems, Goettingen, Germany, [http://www.sysy.com]), and anti-human neural cell adhesion molecule (NCAM; 1:1,000; Santa Cruz). Alexa fluorescent-conjugated antibodies (1:500; Invitrogen) were used as secondary antibodies. Stained cells and brains were imaged with a confocal fluorescence microscope (Olympus Corp., Tokyo, Japan, [http://www.olympus.co.jp]) and Biorevo (Keyence Inc., Osaka, Japan, [http://www.keyence.co.jp]).

### Western Blot Analysis

The differentiated aggregates on day 18 were harvested and homogenized in lysis buffer (Sigma-Aldrich). The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, [http://www.bio-rad.com]) and transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences [GE], Piscataway, NJ, [http://www.gelifesciences.com]). The membranes were blocked with 5% (wt/vol) skim milk (BD Biosciences) and incubated with anti-β-catenin (1:2,500; Abcam), phosphorylated PKC (pPKC; 1:1,000; Cell Signaling, Beverly, MA, [http://www.cellsignal.com]) and anti-β-Actin (1:20,000; Sigma-Aldrich) at 4°C overnight. Then, the membranes were stained with horseradish peroxidase-conjugated secondary antibody (1:5,000; GE). The antibodies were detected with an enhanced chemiluminescence detection system (GE) and observed with ImageQuant LAS4000 (GE). The band intensity was determined by using ImageQuant TL (GE).

### Polymerase Chain Reaction

Total RNA was purified from samples at various time points by using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany, [http://www.qiagen.com]). More than 50 ng of total RNA were reverse-transcribed by using SuperScript III Reverse Transcriptase and Oligo-dT (Invitrogen). Quantitative polymerase chain reactions (qPCRs) were carried out with SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System (TaKaRa Bio Inc., Shiga, Japan, [http://www.takara-bio.co.jp]). The data were assessed by using a delta-Ct method and normalized by GAPDH expression. Reverse transcription PCR was carried out with Ex Taq (TaKaRa). The PCR products were separated by electrophoresis on an agarose gel (Sigma-Aldrich) and detected under UV illumination (AMZ system science, Osaka, Japan, [http://www.amzsystemscience.com]). The primers used for PCR are listed in Table 1.

### Table 1. Primers used for PCR

| Gene   | Forward (5’–3’)                     | Reverse (5’–3’)                     |
|--------|-------------------------------------|-------------------------------------|
| WNT3A  | GCTTCTGCAAGGAACTACGTG               | GACTCCCTGTAAGCTTGTGTC               |
| WNT8B  | TATCAGTTTGGCTGGAGCG                 | CTGTCCCCGATGCGATCGT                |
| GAPDH  | GTGCAGGATACAGGATTGG                 | TCAGCCTCGAGGTTGAGCG                |
| OCT3/4 | AGACCAGCTGGCTTGTGAG                 | GCAAGGCGCGGAGCTT                  |
| NANO6  | GGGCTCTGTTTGTGCTATCCCCTAA          | CATTACGAGGAGCTATCCCCTAA            |
| SOX1   | GGCTGGAGCTGGCTGAGG                 | CGACTGTGGTGGAGATG                 |
| SIX3   | CCGGAAGGTGTTGCATGTC                | CAGGAGAGGACTCCATGAG                |
| EMX1   | AGCCGGGCTTACCACTAGAG               | ATGTGAACTGGGAGATG                 |
| PAX6   | ACCCATTACCCGATGTTTTCGAGGAG         | GGGCTCAGAGGAGCTGAGG               |
| LHX2   | GCGACACGAGGAGCCCAT                 | GAGACAGGAGCTGAGG                 |
| FEZ2F  | ACACGCCATCGCGATCC                  | CAGGACTGGATGAGI                 |
| FOX1   | TTGGACGCCTACTCCCAAAC                | GAGTGTGGGAGAATG                  |
| EMX2   | CTTGGAAACGCGGTGTTGCAGAG           | CCGTTAACATGCGGAGCTCCAG            |
| COUP-TF1 | TTCGAAAGGAGGCTGCGCA            | GGGTGACAGGAGCTGAGG               |
| CTIP2  | CAGGACGAGGAGCCGACTGAG              | GGGTGACAGGAGCTGAGG               |
| CNTNAP2 | TGCCTTCGAGCCCTCATTCC               | TGGAGGGCTGCAGCACC                |
| PCDH17 | GTCCACGGATGCTGATCC                  | GTGGGACAGGAGCTGAGG               |
| ROBO1  | CAGTCGCCGACGCTGAGTGA               | GTGGGACAGGAGCTGAGG               |
| CDH8   | CCGGCGAGCTGATCCCAAAGT              | GTGGGACAGGAGCTGAGG               |
| WNT5A  | CCTGCCGATGAGAAGTCCA                | TTGACAGGAGCTGAGG                 |
| DAAM-1 | AGTATGCCGCAAGCGAGGACC              | CATCGACAGGAGCTGAGG               |
| GLYPICAN | GCCAGATCTGCACGACAGCG              | AGTTGAGGCTGAGG                 |
| ROR2   | TGCTGTGATGCGGAGCAGAG               | CAGTGAGAATGCGGAGCTG             |
CS9 Efficiently Induced Anterior Cortex From Human iPSCs

We tried to generate dorsal telencephalon from human iPSCs (836B1) based on an SFEBq method [11–13] with dual SMAD inhibition (SB431542 and LDN193189) [23, 24] (Fig. 1A). We chose this method to induce frontal neurons [13], because differentiation by monolayer culture tends to induce posterior cortex [10, 17]. We started differentiation in the presence of either dimethyl sulfoxide (shown as control in the figures), DKK1, XAV, or CS9, and continued the culture until day 18.

First, we confirmed the effects of the WNT inhibitors on human iPSCs. Analyses of the aggregates by Western blot and immunostaining using a β-catenin antibody revealed that all inhibitors suppressed β-catenin stabilization and nuclear transition on day 18 (Fig. 1B and supplemental online Fig. 2), indicating that they inhibited canonical Wnt signaling. However, only CS9 suppressed pPKC, indicating that it also inhibited noncanonical Wnt signaling, as reported previously [19]. Neuronal cells in the developing brain secrete several WNTs [25]. Therefore, we examined the expression of WNTs by the iPSC-derived neuronal cells. Quantitative PCR analyses revealed that CS9 suppressed the expression of WNT3A and WNT8B, which are expressed in a caudal-high gradient of the cortex [26] (Fig. 1C, 1D).

Next, we examined the expression of various differentiation markers until day 18 (Fig. 1E). The expressions of two undifferentiated markers (OCT3/4 and NANOG) were decreased as differentiation progressed. In contrast, the expression of an ectodermal marker (SOX1), a telencephalon marker (SIX3), and dorsal telencephalon markers (EMX1, PAX6, and LHX2) increased with the progression. The expression of FEZF2, which is required for the birth and specification of CMNs [27], also increased. Markers for endoderm (SOX17), mesoderm (BRACHYURY), midbrain (EN1), and hindbrain and spinal cord (HOX8/4) were not detected (data not shown). These results indicated that dorsal telencephalon was induced by this protocol.

As a next step, we examined the expressions of an anterior marker (FOGX1) [28] and two posterior markers (EMX2 and COUP-TF1) [29]. Quantitative PCR analyses revealed that the expression of FOGX1 was increased in the presence of CS9, and that of EMX2 and COUP-TF1 was suppressed by either CS9 or XAV (Fig. 1F–1H). These results suggested that CS9 induced anterior cortex efficiently compared with DKK1 or XAV.

We confirmed this finding by immunocytochemistry of the induced cell aggregates. The aggregates were attached onto ornithine-fibronectin-lamin-coated dishes on day 18 and
incubated in neurobasal/B27 medium until day 32. Rosette-like structures were formed in the aggregates, and two postmitotic neuronal markers, MAP2ab and TUJ1, were expressed outside the structures (Fig. 2A). The aggregates expressed PAX6, SOX1 (Fig. 2B), and SOX2 (supplemental online Fig. 3), which are markers for neuroepithelium or radial glia, indicating that early neural cells formed rosette-like structures and migrated out of them to become postmitotic neurons. Because human ESC-derived neural cells spontaneously acquire an intracortical dorsocaudal-ventrorostral polarity during differentiation [13], we next examined the expression of a rostral marker, SP8, and a caudal marker COUP-TF1. The percentage of SP8+/COUP-TF1− cells with respect to total cells was quantified in the sections. Differences were considered significant by ANOVA and Tukey’s test (n = 3). ***, p < .0001. Scale bars = 50 μm. Abbreviations: ANOVA, analysis of variance; C59, WNT-C59; XAV, XAV939.

Figure 2. Characteristics of the internal structure of the induced aggregates on day 32. (A): Each WNT inhibitor induced the neuronal markers MAP2ab and TUJ1 except in the rosette-like region. (B): Rosette-like structures expressed the neuroepithelium markers PAX6 and SOX1. (C): Rosette-like structures also expressed the rostral marker SP8 and the caudal marker COUP-TF1. (D): The percentage of SP8+/COUP-TF1− cells with respect to total cells was quantified in the sections. Differences were considered significant by ANOVA and Tukey’s test (n = 3). ***, p < .0001. Scale bars = 50 μm. Abbreviations: ANOVA, analysis of variance; C59, WNT-C59; XAV, XAV939.

C59 Induces Neurons in Layer V of Anterior Cortex
The cerebral cortex forms six layers as it develops [30], and several factors are selectively expressed in these layers. BRN2 is expressed in the ventricular and subventricular zones and upper layers II–IV [31]. The transcription factor TBR1 is expressed in multiple types of layers V–VI neurons [32]. The transcription factor CTIP2 is related to subcerebral projection neurons in layer V and is essential for generating CMNs [33]. Finally, the transcription factor SATB2 is expressed in layers II–IV and promotes callosal projection neuron identity [34].

Along with being a marker for posterior cortex, COUP-TF1 is a negative regulator of CMN differentiation [35]. Because a specific marker for CMN has not been identified, we regarded CTIP2+/COUP-TF1− cells as an indicator of CMN cells. Aggregates on day 46 showed layer formation, as identified by the different expression patterns of TBR1, CTIP2, and SATB2 (supplemental online Fig. 4A) and of BRN2 and CTIP2 (Fig. 3A). All inhibitors induced layers VI and V, but the number of layer IV SATB2+ cells was very low. Therefore, we used C59 and DKK1 for comparison of CTIP2+/COUP-TF1− cells in different WNT inhibitors. The percentage of CTIP2+ cells was investigated for each WNT inhibitor. Differences were considered significant by ANOVA and Tukey’s test (n = 3). *, p < .05; **, p < .01. Scale bars = 50 μm. Abbreviations: ANOVA, analysis of variance; C59, WNT-C59; DAPI, 4′,6-diamidino-2-phenylindole; XAV, XAV939.

Figure 3. Comparison of CTIP2-expressing cells on day 46. (A): Immunocytochemistry for layer and region markers on day 46 using the ventricular and subventricular zone marker BRN2 (white), caudal marker COUP-TF1 (green) and layer V marker CTIP2 (red). (B, C): Percentages of CTIP2+ cells and CTIP2+/COUP-TF1− cells were investigated for each WNT inhibitor. Differences were considered significant by ANOVA and Tukey’s test (n = 3). *, p < .05; **, p < .01. Scale bars = 50 μm. Abbreviations: ANOVA, analysis of variance; C59, WNT-C59; DAPI, 4′,6-diamidino-2-phenylindole; XAV, XAV939.
small (supplemental online Fig. 4B, 4C). Importantly, C59 induced more CTIP2+ cells compared with control or other WNT inhibitors (Fig. 3B). C59 also induced more CTIP2+/COUP-TF1+ cells (30.7% ± 9.20%) compared with DKK1 (6.15% ± 2.73%) (Fig. 3C). Moreover, another WNT inhibitor, LGK-794, which has the same function as C59, induced cells with similar characteristics as those differentiated using C59 (supplemental online Fig. 4F). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D). Other iPSC lines (1147F1 and 1231A3) and an ESC line (Kh-1) gave consistent results (supplemental online Fig. 4D).

In order to confirm the rostral to caudal identity of the human iPSC-derived cells, we continued to culture the aggregates until day 60 and performed qPCR analyses to investigate the expression of a subset of differentially regulated genes during early human neocortical development (8–12 postconceptional weeks) [36]. Genes exhibiting a rostral-high to caudal-low gradient included CTIP2, CNTNAP2, PCDH17, ROBO1, and Cadherin8 (CDH8). On the other hand, genes exhibiting a caudal-high to rostral-low gradient included COUP-TF1 and EMX2. qPCR analyses revealed that neural aggregates differentiated with C59 contained more cells with rostral identity compared with DKK1 or XAV (Fig. 4).

Excitatory projection neurons transmit signals to other cortical regions and express VGLUT, whereas inhibitory interneurons regulate local circuitry and express GABA. These neurons have distinct origins and developmental programs [37]. We investigated neuronal cell types in the C59-induced neural cells on day 46. The majority was VGLUT1+ cells (65.9% ± 2.8%; Fig. 5A), and almost all CTIP2+ cells expressed VGLUT1. Among the other types of neurons identified, 1.20% ± 0.9% were GABAergic neurons (Fig. 5B), and 1.18% ± 0.3% were dopaminergic neurons (Fig. 5C). Serotonergic and cholinergic neurons were not detected. Because CMNs are in layer V and also excitatory neurons, namely glutamatergic [38], it is likely that the iPSC-derived neurons treated with C59 contained CMNs or their progenitors.

C59-Induced Aggregates Extended Axonal Fibers From the Cerebral Cortex to the Pyramidal Decussation in Adult Mice

In order to investigate whether the induced aggregates included CMNs or their progenitors, we transplanted one aggregate (3 × 10^5 cells in 2 μl of PBS) into the cortex of NOD-SCID adult mice. Six months after transplantation, the grafts and neurites were analyzed by immunostaining for human-specific NCAM in sagittal sections (Fig. 6A). The grafts were detected in all mice (6/6 in C59 and 5/5 in XAV; Fig. 6B, 6C). Then, we counted the number of human NCAM+ fibers from the graft to the pyramidal decussation (to measure the width, we covered the transplanted side of the brain from the midline to the whole graft, with 7 slices at 300-μm intervals per animal). We found more axonal fibers at the striatum (Fig. 6D, 6G), pons (Fig. 6E, 6H), and pyramidal decussation (Fig. 6F, 6I) in the case of grafts derived from C59-induced aggregates compared with those from XAV-induced ones. These fibers were found to have extended along the tract in the contralateral spinal cord (supplemental online Fig. 6). These results suggest that the aggregates differentiated under C59 treatment contained more CMNs or their progenitors.
DISCUSSION

The induction of CMNs from ESCs or iPSCs hold promise as cell therapy against damaged corticospinal tracts caused by neurodegenerative diseases or insults. However, neither an efficient protocol to induce CMNs nor a specific marker for CMNs is available. In order to improve efficient induction of anterior cortex, where CMNs are located, we compared two small WNT inhibitors, C59 and XAV, during the neuronal differentiation of human iPSCs. Intriguingly, C59 induced more CTIP2+/COUP-TF1 cells compared with XAV or DKK1, a common WNT inhibitor. In addition, when grafted into the cortex of adult mice, C59-induced cells showed more abundant axonal fiber extensions toward the spinal cord. These results suggest that C59 induced CMNs or their progenitors efficiently.

As to why C59 efficiently induced anterior cortex, we consider the following reasons. First, the C59-induced aggregates expressed a higher level of FOXG1 than those induced by DKK1 or XAV (Fig. 1F). FOXG1 represses the expression of WNT and BMP [26], which are expressed in the cortical hem, an embryonic organizer of the hippocampus [12]. Second, C59 induced more SP8-positive cells (Fig. 2D). SP8 is required for specification of the rostral cortical identity [39]. It is induced by Fgf signaling and subsequently maintains the expression of Fgf8 within the rostral cortical signaling center. FGF8 signaling also promotes the expansion of the rostral telencephalon [26]. Third, C59 tended to repress the expression of WNT3A and WNT8B compared with XAV (Fig. 1C, D), although the value was not statistically significant. It has been reported that these WNTs cause a caudomedial-high/rostrolateral-low gradient in Wnt-β-catenin activity [40]. We also observed the expression of a caudal marker, EMX2 (Fig. 1G), which is consistent with this gradient [29]. Because C59 inhibits both canonical and noncanonical Wnt signaling, whereas DKK1 and XAV inhibit only canonical signaling, we propose that a noncanonical pathway may play an important role for the induction of anterior cortex, which has been suggested before C59 inhibits the differentiation of neural stem cells to mature neurons [41]. Planar cell polarity (PCP) signaling, a type of noncanonical Wnt signaling, is involved in embryonic morphogenesis and the development of the nervous system by coordinating convergent extension (CE) movements [42, 43]. CE movements are characterized by medio-lateral and rostro-caudal movements of cells to narrow and elongate the embryo [43]. In day 18 aggregates, several genes involved in CE movements, including...
WNT5A, DAAM-1, GLYPCIAN, and ROR2 [44], were expressed (supplemental online Fig. 7). This finding may indicate that PCP signaling regulates CE movements for rostro-caudal formation of the cortex.

In addition to WNTs, the effects of secreted ligands, such as Hedgehog and Spitz, on signal transduction is regulated by palmitoylation [45]. The inhibition of C59-dependent palmitoylation may be involved in the areal patterning of the induced cells [16].

Some neurons in layer V of the cerebral cortex express CTIP2 [31] and project axons to the spinal cord and the superior colliculus [27, 38]. COUP-TF1 is expressed in the posterior cortex and is involved in neuronal differentiation and areal patterning [46, 47]. CTIP2+/COUP-TF1 cells are located in the motor/frontal region of the cerebral cortex in embryos [35] and project axons to the spinal cord when transplanted into mouse cerebral cortex [4]. Conversely, CTIP2+/COUP-TF1 cells are located in the occipital region of the cortex and project axons to the superior colliculus [10]. Therefore, we consider the induction of CTIP2+/COUP-TF1 cells as necessary for ESC/iPSC-based clinical application to treat motor neuron diseases or insults.

CONCLUSION

We found that C59 induced CTIP2+/COUP-TF1 cells from human iPSCs more efficiently than two other WNT inhibitors, DKK1 and XAV. Furthermore, these CTIP2+/COUP-TF1 cells extended axons to the pyramidal decussation. These results raise the possibility that C59 could contribute to cell-replacement therapy for motor neuron diseases or insults.

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AUTHOR CONTRIBUTIONS

M.M.: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing; Y.I.: conception and design, provision of study material, manuscript writing; T.O.: provision of study material, data analysis and interpretation; J.T.: conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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