Stem Cell Reports, Volume *12*

Supplemental Information

Pluripotent Stem Cell-Derived Cerebral Organoids Reveal Human Oligodendrogenesis with Dorsal and Ventral Origins

Hyosung Kim, Ranjie Xu, Ragunathan Padmashri, Anna Dunaevsky, Ying Liu, Cheryl F. Dreyfus, and Peng Jiang

Supplemental Experimental Procedures

Culture and derivation of hPSC cell lines

The OLIG2-GFP knockin hPSCs (hESCs and hiPSCs) were established using a gene-targeting protocol and fully characterized, as reported in our previous studies (Liu et al., 2011; Xue et al., 2016). The OLIG2-GFP hiPSC reporter line was established from the ND2.0 hiPSC line that was obtained from Center for Regenerative Medicine, National Institutes of Health. The hPSCs maintained under a feeder-free condition on a hESC-qualified Matrigel (Corning)-coated dish with mTeSR1 media (STEMCELL Technologies) were used for this study. The hPSCs were passaged approximately once per week by ReLeSR media (STEMCELL Technologies).

Generation of human forebrain organoids

To avoid non-CNS differentiated tissue and reduce variability in 3D cerebral organoids generation, we used purified pNPCs as the starting population for generating organoids. As shown in Fig. 1A, we induced pNPCs from hPSCs using a small molecule-based protocol (Chen et al., 2016; Li et al., 2011). Briefly, neural differentiation was induced by dual inhibition of SMAD signaling (Chambers et al., 2009) with inhibitors SB431542 (5 μM, Stemgent) and noggin (50 ng/ml, Peprotech) for a week. The embryoid bodies (EB) were then plated on dishes coated with growth factor-reduced Matrigel (BD Biosciences) in the medium consisting of DMEM/F12, 1x N2, and laminin (1 μg/ml; Sigma-Aldrich) for a week. Next, neural rosettes were manually isolated from the expanded area. The isolated neural rosettes were further cultured in pNPC media, composed of a 1:1 mixture of Neurobasal (Thermo Fisher Scientific) and DMEM/F12, supplemented with 1 x N2, 1 x B27-RA (Thermo Fisher Scientific), FGF2 (20 ng/ml, Peprotech), human leukemia inhibitory factor (hLIF, 10 ng/ml, Millipore), CHIR99021 (3 μM, Stemgent), SB431542 (2 μM), and ROCK inhibitor Y-27632 (10 μM, Tocris). To generate organoids, dissociated pNPCs by TrypLE Express (Thermo Fisher Scientific) were placed into low-attachment 96-well plates at a density of 9,000 cells to develop uniform organoids for two days. The pNPC aggregates were then grown and patterned in low-attachment 6-well plates with the treatment of either 5 μM Cyclopamine A (Cyc A; Calbiochem) for dorsalization or dual activation of SHH pathway with sonic hedgehog (SHH; 50 ng/ml, Peprotech) and purmorphamine (Pur; 1 μM, Cayman Chem) for ventralization. Starting from week 5, the DFOs were cultured on an orbital shaker with a speed of 80 rpm/min in neuronal differentiation (ND) medium containing a 1:1 mixture of Neurobasal and DMEM/F12, supplemented with 1 x N2, 1 x B27, BDNF (20 ng/ml, Peptrotech), GDNF (20 ng/ml, Peprotech), dibutyryl-cyclic AMP (1mM, Sigma), and ascorbic acid (200 nM, Sigma). The 5-week-old VFOs were cultured in OPC medium containing DMEM/F12, supplemented with 1 x N2, 1 x B27, FGF2 (10 ng/ml, Peptrotech), PDGF-AA (10 ng/ml, Peprotech). For further neuronal maturation, both DFOs and VFOs were cultured in BrainPhys medium (STEMCELL Technologies). Starting from week 9, organoids were maintained in ND medium supplemented with 3,3,5-Triiodo-L-thyronine sodium salt (T3; 10 ng/ml, Cayman Chem; OL medium) for oligodendroglial differentiation and maturation. FFOs were generated by using a spontaneous fusion method (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017) with modifications. Briefly, single week 9 DFO were closely placed with a week 5 VFO by transferring both of them into the round-bottom ultra-low-attachment 96-well plate for 2 days without agitating. Then, the FFOs were transferred to an ultra-low-attachment 6-well plate and cultured for a day without agitating. The next day, the FFOs were maintained with OL medium on an orbital shaker with a speed of 80 rpm/min.

RNA isolation and qRT-PCR

Total RNA extracted from organoids with RNAeasy kit (Qiagen) was used to make complementary DNA with a Superscript III First-Strand kit (Invitrogen). The qRT-PCR was performed with TaqMan primers listed in supplementary table 2 on an Abi 7500 Real-Time PCR system. Experimental samples were analyzed by normalization with the expression level of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative quantification was performed by applying the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western blotting

OLIG2 protein expression and localization in cells were evaluated by immunoblotting using fractionated samples. The nuclear and cytoplasmic fraction was achieved by modification of a reported method (Suzuki et al., 2010). Briefly, organoids were washed with PBS and harvested. Resuspended organoids in 900 ul of ice-cold lysis buffer (0.1% NP40 in PBS) were lysed 10 times through a 25-gauge syringe. Following the second spinning down for 30 seconds, the supernatant was collected as a cytosolic fraction. 3 times washed pellet was lysed in sample buffer containing 1% of SDS. Fractionated proteins were separated on 12% SDS-PAGE gel and transferred onto nitrocellulose membrane. Blots were then blocked in 2% skim milk and incubated with primary antibodies at 4°C overnight. The information for primary antibodies and dilutions is listed in Supplementary table 3. Afterward, the blots were incubated with secondary antibodies conjugated with a fluorophore for an hour at room temperature. Western blot was visualized using Odyssey (LiCor).

Immunostaining and cell counting

Organoids fixed with 4% paraformaldehyde were processed and cryo-sectioned for immunofluorescence staining. The information for primary antibodies and dilutions is listed in Table S2. Slides were mounted with the anti-fade Fluoromount-G medium containing 1,40,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Southern Biotechnology). Images were captured with LSM800 confocal microscope. The cells were counted with ImageJ software. At least six fields chosen randomly from three sections of each organoid were counted. For each organoid, at least 400 cells were counted.

Dye loading and calcium imaging

For calcium imaging in organoids, DFOs were placed on the growth factor-reduced Matrigel-coated coverslip in 6-well plate for 24 hr. The next day, organoids were loaded with fluo-4 AM (5 μM, Molecular Probes) and 0.04% Pluronic F-127 for 40 minutes and then transferred to the neuronal differentiation (ND) medium for at least 30 minutes before transferring to the submersion-type recording chamber (Warner) superfused at room temperature with artificial CSF (ACSF, mM: 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose) saturated with 95% O_2/5% CO_2. All imaging was performed with a two-photon microscope (Moving Objective Microscope; Sutter Instruments) coupled to a Ti:Sapphire laser (Chameleon Vision II, Coherent). Images were collected with a Nikon water immersion objective (25X, 1.05 NA). Excitation power measured at the back aperture of the objective was typically about 20-30 mW, and laser power was modulated using a Pockels cell. Fluo-4 was excited at 820nm and emission was detected with GaAsP detector

(Hamamatsu Photonics) fitted with a 535/50 bandpass filter and separated by a 565 nm dichroic mirror. ScanImage (v5.1, Vidrio Technologies) software (Pologruto et al., 2003) was used for imaging. Time-lapse imaging was performed every 1 s for 5 minutes and was collected at 512 by 512-pixel resolution. Image analysis was performed using ImageJ software. Regions of interest (ROIs) were placed around soma. Fluorescence was averaged over ROIs placed and expressed as relative fluorescence changes ($\Delta F/F$) after subtraction of background fluorescence from a neighboring region. For each ROI, basal fluorescence was determined during 20 s periods with no Ca^{2+} fluctuation. Ca^{2+} transients were detected when fluorescence intensity reached higher than 2 SD value of baseline fluorescence intensity.

Electron microscopy

Organoid samples were fixed with 2% glutaraldehyde, 2% paraformaldehyde, and 0.1M sodium cacodylate in PBS. The selected vibratome sections were post-fixed and processed for electron microscopy (EM) as described in our previous studies (Chen et al., 2016; Jiang et al., 2016). EM images were captured using a high-resolution charge-coupled device (CCD) camera (FEI).

DNA fingerprinting short tandem repeat (STR) analysis

STR analysis was performed using GENEprint PowerPlex 16 kit (Promega performed by Cell Line Genetics. LLC). Samples were run in duplicate and blinded to the interpreter to confirm the results. Please note that ND2.0 and OLIG2-GFP hiPSC lines have identical STR genotyping profile, indicating that they are isogenic lines derived from the same parental cell line.

Supplementary Table 1. STR genotyping profile of ND2.0 hiPSCs and OLIG2-GFP hiPSC reporter line. STRs of all loci for OLIG2-GFP hiPSCs match to ND2.0 hiPSCs.

STR Locus	Chr. Location	ND2.0 hiPSCs		OLIG2-GFP hiPSCs	
Amelogenin	**Xp22.1-22.3 and Y**	**X**	**Y**	**X**	**Y**
vWA	12p12-pter	17	18	17	18
D8S1179	8q	12		12	
TPOX	2p23-2pter	10	11	10	11
FGA	4q28	24	26	24	26
D3S1358	3p	15		15	
THO1	11p15.5	6	9.3	6	9.3
D21S11	21q11-21q21	29	31.2	29	31.2
D18S51	18q21.3	13	18	13	18
Penta E	15q	7	12	7	12
D5S818	5q23.3-32	11	12	11	12
D13S317	13q22-q31	11	12	11	12
D7S820	7q11.21-22	12		12	
D16S539	15q24-qter	9	11	9	11
CSF1PO	5q33.3-34	12	13	12	13
Penta D	21q	9	15	9	15

Supplementary Table 2. A list of primers used.

Gene	Gene expression assay catalog number
ARHGEF9	HS01003480_m1
DLX1	Hs00269993_m1
EMX1	Hs00417957_m1
GAD1	Hs01065893_m1
GAPDH	Hs02758991_g1
GPHN	HS00982840_m1
HIF1A	Hs00153153_m1
HOMER1	Hs01029333_m1
LEF1	Hs01547250_m1
LHX6	Hs01030941_g1
MBP	Hs00921945_m1
NKX-2-2	Hs05035641_s1
OLIG2	Hs00300164_s1
PDGFRα	Hs00998018_m1
S100β	Hs00389217_m1
SHANK3	Hs01393541_m1
SLC17A6 (VGLUT2)	Hs00220439_m1
SLC17A7 (VGLUT1)	Hs00220404_m1
SLC6A1 (GAT1)	Hs01104475_m1
TBR2	Hs00232429_m1

Supplementary Table 3. A list of antibodies used.

Antibodies	Vendor/Catalog #.	Type	Dilution
βIIItubulin	Millipore / MAB1637	Mouse IgG	1:200
β-tubulin	DSHB / E7	Mouse IgG	WB (1:1000)
c-FOS	Santa Cruz / SC-52	Rabbit IgG	1:100
CUX1	Santa Cruz / SC13024	Rabbit IgG	1:500
DCX	Cell Signaling / 4604s	Rabbit IgG	1:500
EMX1	Sigma / HPA006421	Rabbit IgG	1:1000
FOXG1	Abcam / ab18259	Rabbit IgG	1:500
GFAP	Millipore / AB5804	Rabbit IgG	1:1000
GFP	Rockland / 600-141-215	Goat IgG	1:1000
GFP	Thermo / MA5-15256	Mouse IgG	1:500
GLS	Abcam / ab156876	Rabbit IgG	1:250
Ki67	Cell signaling / 9449	Mouse IgG	1:400
Ki67	Thermo Fisher Scientific / SP6	Rabbit IgG	1:200
MAP2	Millipore / AB3418	Mouse IgG	1:500
LHX6	Abcam / ab22885	Rabbit IgG	1:100
MBP	Millipore / MAB386	Rat IgG	1:100
NeuN	Millipore / MAB377	Mouse IgG1	1:100
Nestin	Santa Cruz / SC-21249	Goat IgG	1:100
NKX2.1(TTF1)	Abcam / ab76013	Rabbit IgG	1:200
OLIG2	Phosphosolutions 1538	Rabbit IgG	1:1000; WB (1:2000)
PAX6	GeneTex / GTX11324	Rabbit IgG	1:400
PDGFRα	Santa Cruz / SC338	Rabbit IgG	1:50
P-Histone H3	Thermo Fisher Scientific / PA5–17869	Rabbit IgG	WB(1:1000)
PSD95	Invitrogen 51-6900	Rabbit IgG	1:100
S100β	Sigma / S2532	Mouse IgG	1:1000
SOX2	Millipore / AB5603	Rabbit IgG	1:100
Synapsin I	Millipore / AB1543P	Rabbit IgG	1:400
TBR1	EMD Millipore / AB2261	Chicken IgG	1:100
TBR2	Abcam / AB23345	Rabbit IgG	1:100
VGLUT1	Millipore / AB5905	Guinea pig IgG	1:250

Antibody dilution for western blotting are specifically marked as WB, and others are for immunostaining.

References:

Bagley, J.A., Reumann, D., Bian, S., Levi-Strauss, J., and Knoblich, J.A. (2017). Fused cerebral organoids model interactions between brain regions. Nat Methods *14*, 743-751.

Birey, F., Andersen, J., Makinson, C.D., Islam, S., Wei, W., Huber, N., Fan, H.C., Metzler, K.R.C., Panagiotakos, G., Thom, N.*, et al.* (2017). Assembly of functionally integrated human forebrain spheroids. Nature *545*, 54-59.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol *27*, 275-280.

Chen, C., Kim, W.Y., and Jiang, P. (2016). Humanized neuronal chimeric mouse brain generated by neonatally engrafted human iPSC-derived primitive neural progenitor cells. JCI Insight *1*, e88632.

Jiang, P., Chen, C., Liu, X.B., Pleasure, D.E., Liu, Y., and Deng, W. (2016). Human iPSC-Derived Immature Astroglia Promote Oligodendrogenesis by Increasing TIMP-1 Secretion. Cell Rep.

Li, W., Sun, W., Zhang, Y., Wei, W., Ambasudhan, R., Xia, P., Talantova, M., Lin, T., Kim, J., Wang, X.*, et al.* (2011). Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. Proc Natl Acad Sci U S A *108*, 8299-8304.

Liu, Y., Jiang, P., and Deng, W. (2011). OLIG gene targeting in human pluripotent stem cells for motor neuron and oligodendrocyte differentiation. Nat Protoc *6*, 640-655.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Pologruto, T.A., Sabatini, B.L., and Svoboda, K. (2003). ScanImage: flexible software for operating laser scanning microscopes. Biomed Eng Online *2*, 13.

Suzuki, K., Bose, P., Leong-Quong, R.Y., Fujita, D.J., and Riabowol, K. (2010). REAP: A two minute cell fractionation method. BMC Res Notes *3*, 294.

Xiang, Y., Tanaka, Y., Patterson, B., Kang, Y.J., Govindaiah, G., Roselaar, N., Cakir, B., Kim, K.Y., Lombroso, A.P., Hwang, S.M.*, et al.* (2017). Fusion of Regionally Specified hPSC-Derived Organoids Models Human Brain Development and Interneuron Migration. Cell Stem Cell *21*, 383-398 e387.

Xue, H., Wu, J., Li, S., Rao, M.S., and Liu, Y. (2016). Genetic Modification in Human Pluripotent Stem Cells by Homologous Recombination and CRISPR/Cas9 System. Methods Mol Biol *1307*, 173-190.