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Supplemental Information

Axonal Extensions along Corticospinal Tracts from Transplanted Human Cerebral Organoids

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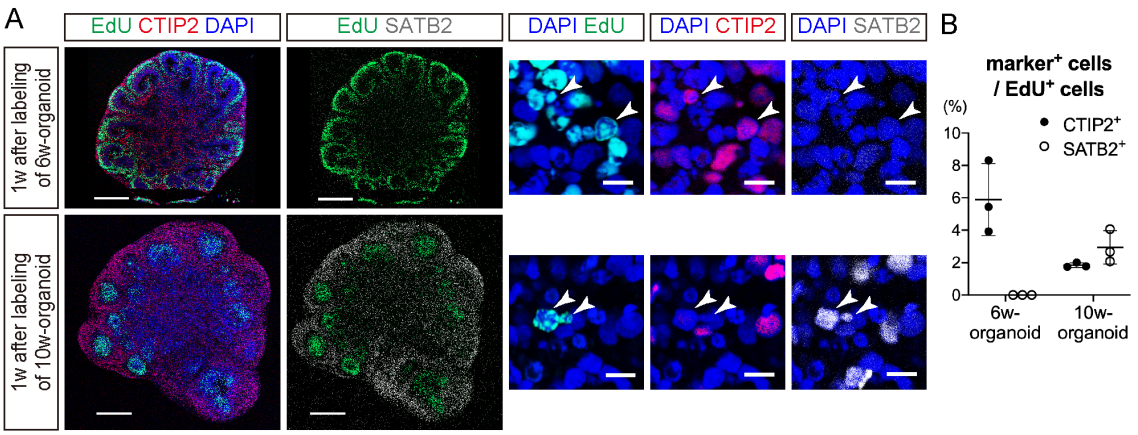

Figure S1

Marker expression by newly generated cells in 6w- and 10w-organoids. Related to Figure 1

(A) Immunohistochemistry for EdU, CTIP2, and SATB2 one week (1w) after EdU labeling dividing cells in 6w- and 10w-organoids. Scale bars, 500 μm (two leftmost columns) and 10 μm (other columns).

(B) Percentages of $CTIP2^+$ cells and $SATB2^+$ cells in EdU^+ cells 1w after EdU labeling 6w- and 10w-organoids. $EdU^+/SATB2^+$ cells were absent in 6w-organoids but observed in 10w-organoids. n = 3 for both organoids (n: number of aggregates). Results are presented as the mean ± standard deviation.

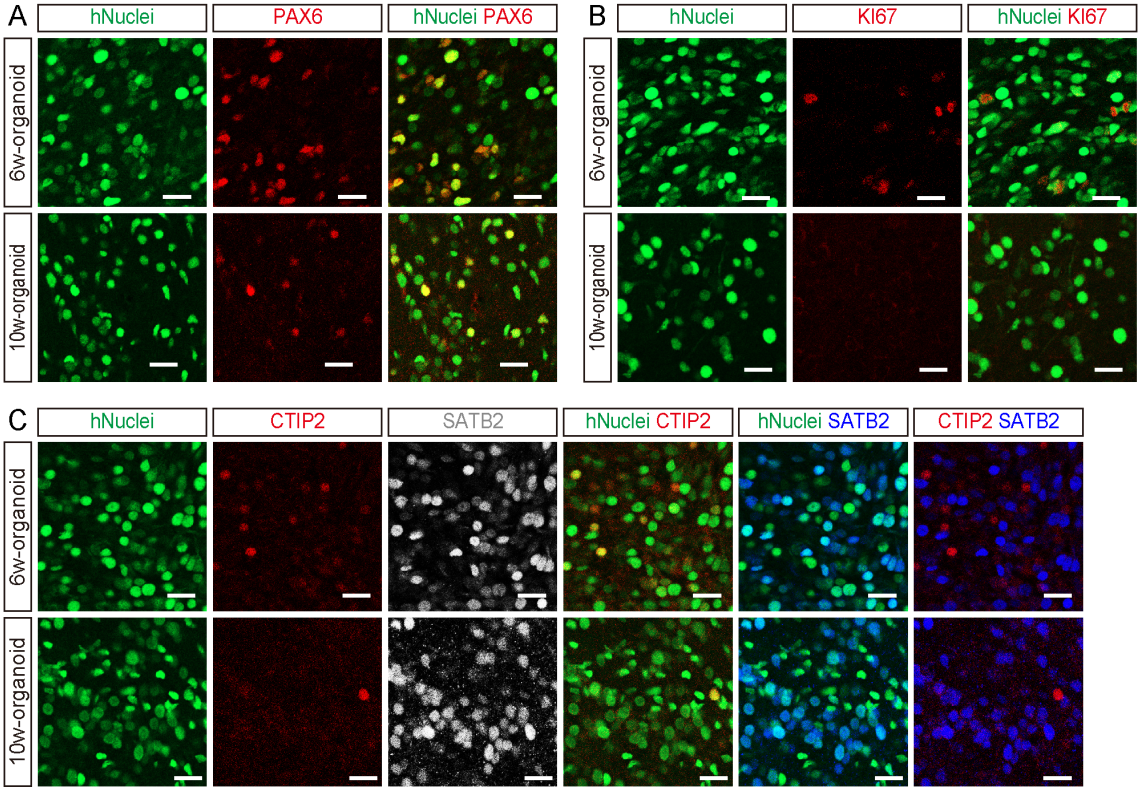

Figure S2

Marker expression for proliferative cells and projection neurons in engrafted cerebral organoids. Related to Figure 4

Immunohistochemistry for PAX6 (A), KI67 (B), CTIP2, and SATB2 (C) co-expressed with hNuclei^{+} cells in engrafted 6w- and 10w-organoids. Scale bars, 20 μm.

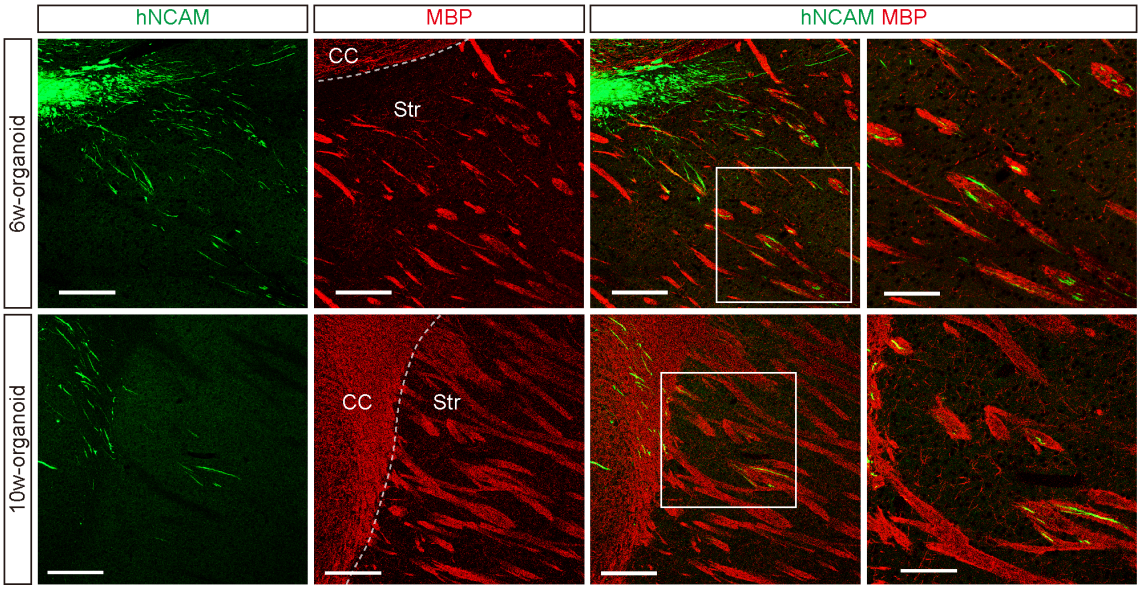

Figure S3

Graft-derived fibers were elongated along myelinated bundles in the striatum. Related to Figure 5

Immunohistochemistry for hNCAM and MBP in the host striatum at 12 wpt. The rightmost column shows higher magnification images of the boxed areas. Scale bars, 200 μm (left three columns) and 100 μm (rightmost column).

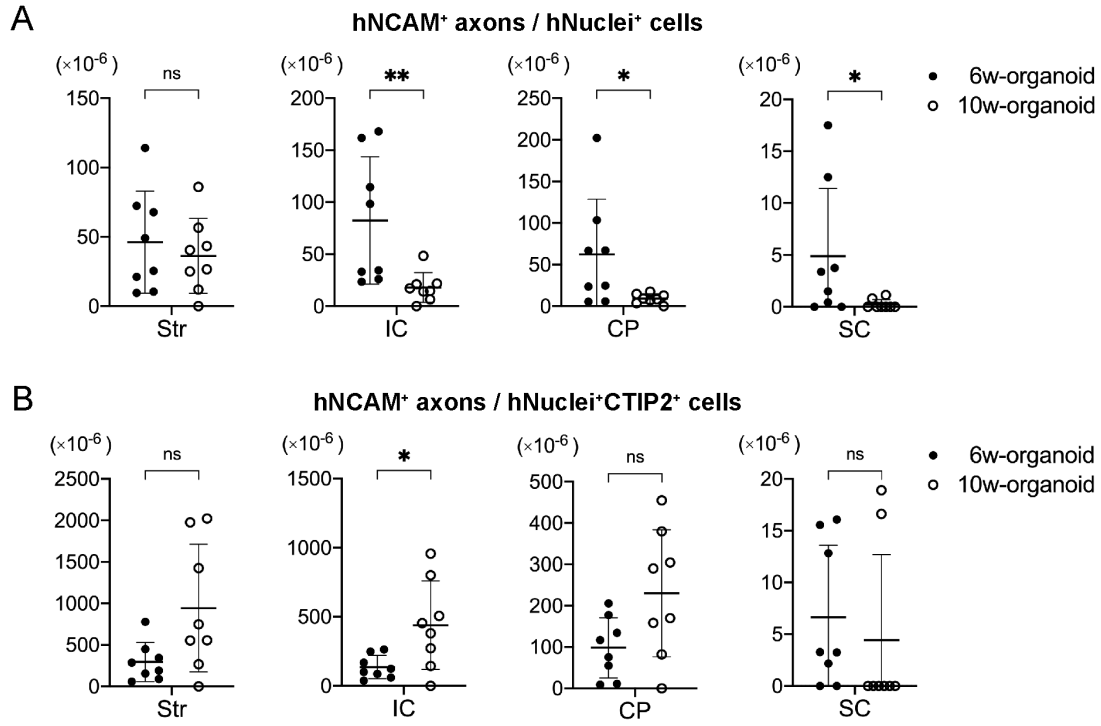

Figure S4

Number of graft-derived axons per cell. Related to Figure 5

(A) Number of axons per engrafted hNuclei$^+$ cell. n = 8 for 6w- and 10w-organoids (n: number of mice). * $p < 0.05$, ** $p < 0.01$, ns: not significant, Mann-Whitney test. Results are presented as the mean ± standard deviation.

(B) Number of axons per engrafted hNuclei$^+$/CTIP2$^+$ cell. n = 8 for both organoids (n: number of mice). * $p < 0.05$, ns: not significant, Mann-Whitney test. Results are presented as the mean ± standard deviation.

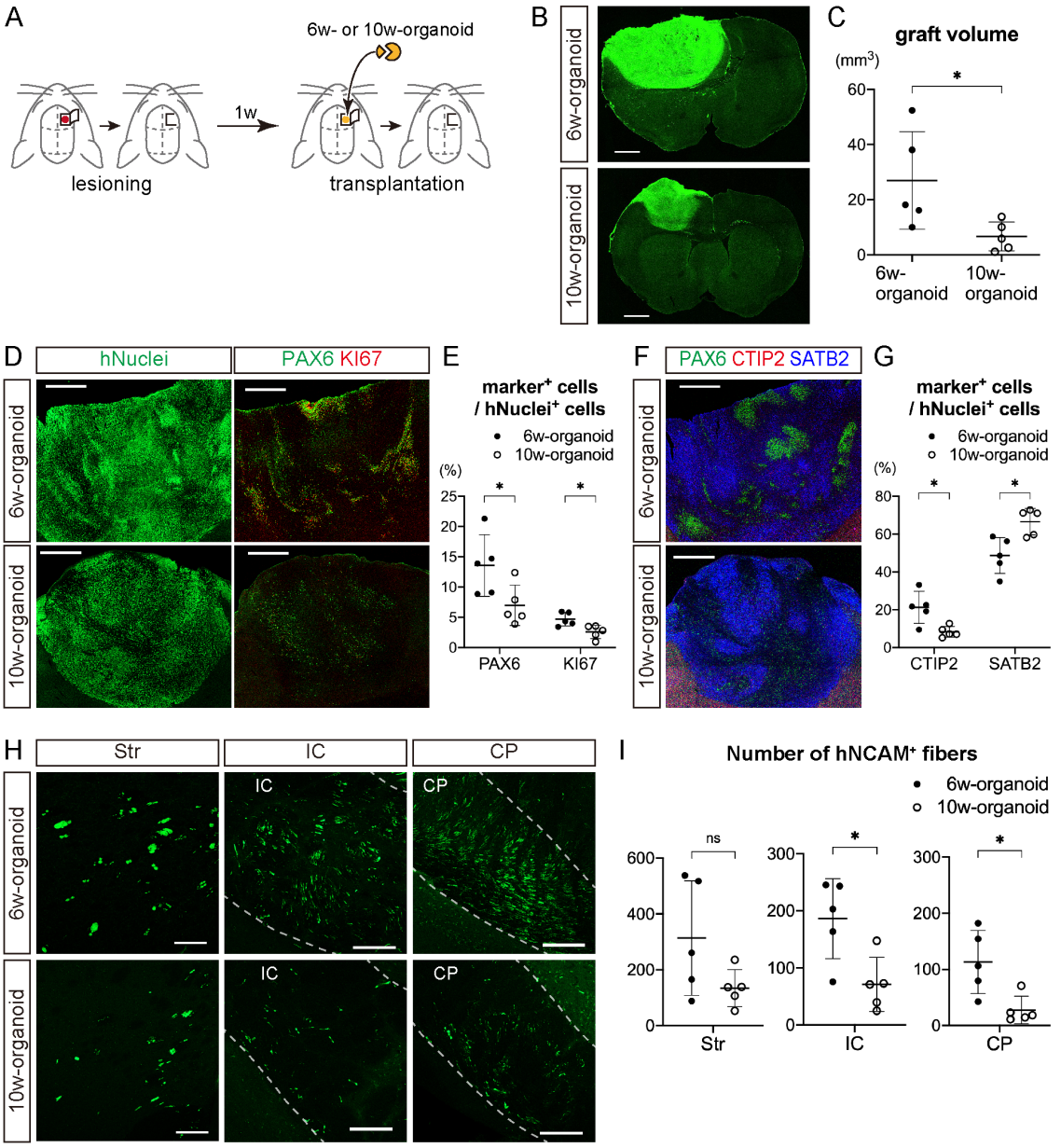

Figure S5

Transplantation of 6w- or 10w-organoids into 6-week-old mice with one-week delay after lesioning. Related to Figure 2, 4, and 5

(A) Schematic of the procedure for the transplantation of 6w- or 10w-organoids into the right frontal cortex of 6-week-old mice with one-week delay after making the cavity. n = 5 for both 6w- and 10w-organoids (n: number of mice).

(B) Immunohistochemistry (IHC) for hNCAM in coronal sections of mouse brains at 12 wpt. Upper: Bregma + 0.90 mm, lower: Bregma + 1.08 mm. Scale bars, 1 mm.

(C) The graft volume at 12 wpt. n = 5 for both organoids (n: number of mice). * $p < 0.05$, Mann-Whitney test. Results are presented as the mean ± standard deviation.

(D) IHC for hNuclei, PAX6, and KI67 in the engrafted tissues at 12 wpt. Scale bars, 500 μm.

(E) Percentages of $PAX6^+$ cells and $KI67^+$ cells in $hNuclei^+$ cells in the engrafted tissues at 12 wpt. n = 5 for both organoids (n: number of mice). * $p < 0.05$, Mann-Whitney test. Results are presented as the mean ± standard deviation.

(F) IHC for CTIP2, SATB2, and PAX6 in the engrafted tissues at 12 wpt. Scale bars, 500 μm.

(G) Percentages of $CTIP2^+$ cells and $SATB2^+$ cells in $hNuclei^+$ cells at 12 wpt. n = 5 for both organoids (n: number of mice). * $p < 0.05$, Mann-Whitney test. Results are presented as the mean ± standard deviation.

(H) IHC for hNCAM in coronal sections of mouse brain shows graft-derived axonal extensions in the striatum (Str), internal capsule (IC), and cerebral peduncle (CP) of the host at 12 wpt. Scale bars, 100 μm.

(I) Number of $hNCAM^+$ fibers in the Str, IC, and CP of the host at 12 wpt. n = 5 for both organoids (n: number of mice). ns: not significant, * $p < 0.05$, Mann-Whitney test. Results are presented as the mean ± standard deviation.

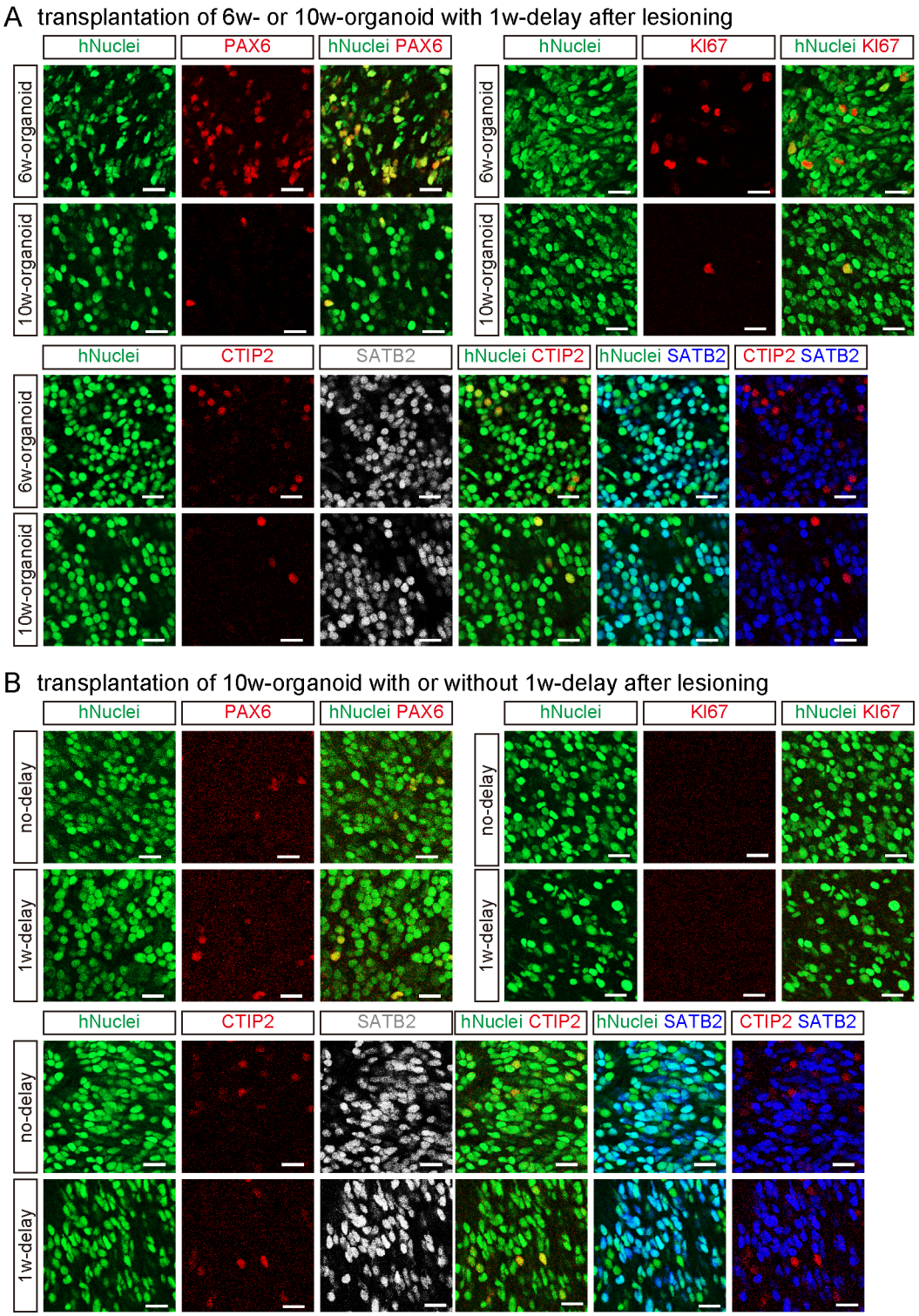

Figure S6

6w-organoids after transplantation with one-week delay (1w-delay) contained more proliferative cells, and 10w-organoids after transplantation with and without 1w-delay contained few proliferative cells. Related to Figure 5 and 6

Immunohistochemistry for PAX6, KI67, CTIP2, and SATB2 co-expressed with hNuclei^{+} cells after the transplantation of 6w- or 10w-organoids with 1w-delay (A) and after transplantation of 10w-organoids with or without 1w-delay (B). Scale bars, 20 μm.

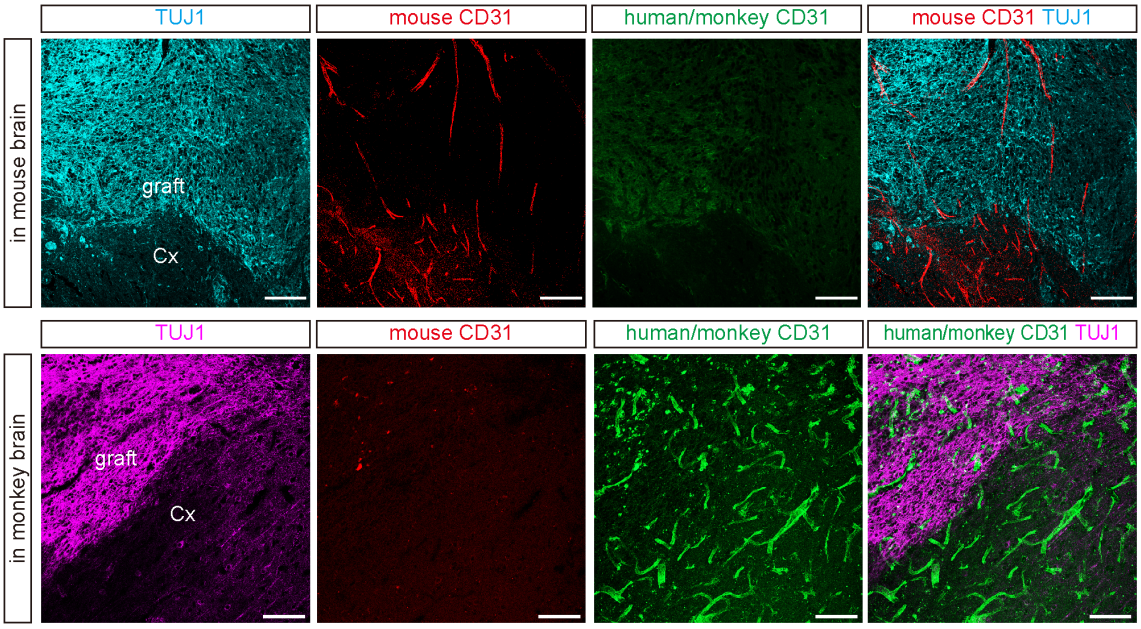

Figure S7

Reactivities with mouse- and human/monkey-specific CD31 antibodies was consistent between blood vessels in the engrafted organoids and blood vessels in the host brain.

Immunohistochemistry for TUJ1, mouse-specific CD31, and human/monkey-specific CD31 in cerebral organoids engrafted in mouse brain (upper row) and monkey brain (lower row). Cx: cerebral cortex. Scale bars, 100 μm.

Supplemental Experimental Procedures

Maintenance Culture of Human Embryonic Stem Cells (hESCs)

All experiments using hESCs were approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. hESCs were used in accordance with 'The Guidelines for Derivation and Utilization of Human Embryonic Stem Cells' of the Ministry of Education, Culture, Sports, Science and Technology of Japan. hESCs were maintained and cultured as previously described (Sakaguchi et al., 2019). In brief, hESCs (KhES-1) were maintained on a feeder layer of mouse embryonic fibroblasts (Oriental Yeast) inactivated by 10 μg/ml mitomycin C (Wako) treatment in DMEM/F12 (Sigma-Aldrich) supplemented with 20% (vol/vol) Knockout Serum Replacement (KSR; Gibco), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (NEAA; Gibco), 5 ng/ml recombinant human basic fibroblast growth factor (Wako), 0.1 mM 2-mercaptoethanol (2-ME; Wako), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) under 2% CO_2. Medium change was performed once every day. For passaging, hESC colonies were detached and recovered en bloc from the feeder layer by treating them with 0.25% (wt/vol) trypsin (Gibco) and 1 mg/ml collagenase IV (Gibco) in phosphate buffered saline (PBS) containing 20% (vol/vol) KSR and 1 mM $CaCl_2$ (Wako) at 37°C for 8 min. The detached hESC clumps were broken into smaller pieces by gentle pipetting. The passages were performed at a 1:4–1:6 split ratio every 3–4 days.

Differentiation Culture of hESCs

The differentiation culture of hESCs was performed using the SFEBq (serum-free floating culture of embryoid body-like aggregates with quick reaggregation) method as previously described (Sakaguchi et al., 2019). In brief, hESCs were dissociated to single cells in TrypLE Express (Gibco) containing 1.25 U/ml DNase I (Takara Bio) and 10 μM Y-27632 (Wako), and quickly reaggregated using low-cell-adhesion-coated V-bottomed 96-well plates (PrimeSurface MS-9096V; Sumitomo Bakelite) in differentiation medium (9,000 cells per well, 100 μl) under 5% CO_2. The differentiation medium was Glasgow's MEM (Gibco) supplemented with 20% (vol/vol) KSR, 0.1 mM NEAA, 1 mM sodium pyruvate (Sigma-Aldrich), 0.1 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Defining the day on which the SFEBq culture was started as day 0, 5 μM SB431542 (transforming growth factor β inhibitor; TOCRIS) and 3 μM IWR1e (Wnt inhibitor; Calbiochem) were added to the culture from day 0 to day 18. 50 μM Y-27632

was added from day 0 to day 3. Half the medium was changed once every 3 days from day 3 to day 15. At day 18, the floating aggregates were transferred to 10-mm non-adhesive dishes (EZSPHERE; Iwaki) and further cultured in suspension using DMEM/F-12 GlutaMAX (Gibco) supplemented with 1% (vol/vol) N-2 Supplement (Gibco), 1% (vol/vol) Chemically Defined Lipid Concentrate (CDLC; Gibco), 0.25 μg/ml Amphotericin B (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin under 40% O_2/5% CO_2 conditions. The floating aggregates were cut into halves or thirds with micro scissors (Bio Research Center, #16324319) under a stereo microscope at day 35, and the aggregates were transferred to gas-permeable dishes (Lumox dish; Sarastedt) at day 50. All medium was changed once every 3 days after day 18. When 5-ethynyl-2'-deoxyuridine (EdU) labeling of 6w- or 10w-organoids was performed, 10 μM EdU (Sigma-Aldrich) was added to the medium for 24 h.

Magnetic Resonance Imaging (MRI)

The monkeys were subjected to MRI study before and immediately after transplantation, and once every month thereafter. Animals were anesthetized with ketamine (10 mg/kg) and xylazine (1 mg/kg), and T1- and T2-weighted images were obtained using a 3-tesla MRI scanner (MAGNETOM Verio, Siemens Healthcare).

Brain Tissue Collection and Sample Fixation

Mice and monkeys were euthanized at 12 weeks post-transplantation (wpt). For mice, pentobarbital (200 mg/kg, intraperitoneally) was administrated, and transcardial perfusion was performed with PBS followed by 4% PFA. Brains were dissected and post-fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Brains were cryoprotected in 15% sucrose in PBS (4°C, overnight) followed by 30% sucrose in PBS (4°C, two overnight). For monkeys, transcardial perfusion was performed with PBS followed by 4% PFA under deep anesthesia with pentobarbital (100 mg/kg, intravenously). Brains were post-fixed in 4% PFA (4°C, 24 h) and then cryoprotected in 10% sucrose in PBS at 4°C for 1–2 days, in 20% sucrose in PBS at 4°C for 2–3 days, and finally in 30% sucrose in PBS at 4°C for 3–4 days.

In vitro samples of cerebral organoids were fixed in 4% PFA at 4°C for 15–30 min and then cryoprotected in 15% sucrose in PBS (4°C, overnight) followed by 30% sucrose in PBS (4°C, overnight).

Immunofluorescence Staining and Histological Processing

The sample sectioning was performed with a cryostat (CM 3050S; Leica). *In vitro* samples of cerebral organoids were sliced at 12 μm thickness. Mouse brains were sliced coronally or sagittally at 30 μm thickness. Monkey brains were sliced coronally at 40 μm thickness.

In vitro samples were attached to coated glass slides (MAS-01; Matsunami Glass) before immunohistochemical staining. *In vivo* samples of animal brains were stained using the free-floating method before attachment to coated glass slides (APS-01; Matsunami Glass).

Samples were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS (room temperature, 45 min), blocked with 2% Skim Milk (Difco) in PBS (room temperature, 30–60 min), and incubated with primary antibodies (4°C, overnight) followed by incubation with secondary antibodies conjugated with Alexa 488, 594, and 647 (room temperature, 2 h). The primary antibodies were used at the following dilutions: FOXG1 (rabbit, 1:1,000, Takara Bio, M227), MAP2 (mouse, 1:200, Sigma, M4403), PAX6 (mouse, 1:500, BD, 561462), PAX6 (rabbit, 1:500, BioLegend, 901301), CTIP2 (rat, 1:1,000, abcam, ab18465), SATB2 (mouse, 1:200, abcam, ab515020), SATB2 (rabbit, 1:500, abcam, ab34735), KI67 (rabbit, 1:1,000, Novocastra, NCL-ki67p), hNCAM (mouse, 1:500, Santa Cruz, sc-106), hNuclei (mouse, 1:1,000, Millipore, MAB1281), STEM121 (mouse, 1:500, Cellartis, Y40410), TUJ1 (rabbit, 1:1,000, Covance, PRB-435P), mouse CD31 (rat, 1:250, BD, 553370), human/monkey CD31 (mouse, 1:200, abcam, ab199012), and MBP (rat, 1:1,000, Millipore, MAB386). Counter nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI). EdU was detected with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen).

Quantitative Analysis

The thickness of layers in the cerebral organoids was measured with Fiji software (Schindelin et al., 2012). 10 and 13 aggregates were analyzed for 6w-organoid and for 10w-organoids, and 4.7 ± 1.5 layers and 5.7 ± 1.0 layers were measured in each aggregate, respectively.

Quantification of the graft volume and the host brain volume was performed by hNCAM immunostaining every six slices after sectioning at 30 μm thickness. The graft

area and the host brain area in each slice were measured with Fiji software. The host brain area was measured after excluding the olfactory bulb, cerebellum, and the brain stem. The volumes were calculated with the following formula: V (mm^3) = (S1 + S2 + ... + Sn) × 0.18, where S is the area (mm^2) of the graft or the host brain in each slice, and n is the number of slices.

Cell counting for marker expression analyses was performed with CellPathfinder software (Yokogawa), except for the analysis of co-expression of EdU and markers *in vitro*, which was performed by manual counting. *In vitro*, 10 and 13 aggregates were analyzed for 6w-organoids and 10w-organoids, and 76492 ± 22216 cells and 131300 ± 26162 cells were counted in each aggregate, respectively. Regarding the co-expression of EdU and markers, three aggregates were analyzed each for 6w-organoids and for 10w-organoids, and 300 ± 6 EdU^+ cells and 270 ± 87 EdU^+ cells were counted in each aggregate, respectively. In mice with four transplant sites, the average percentage in all the survived grafts in each mouse was used for the analysis. 15204 ± 9667 cells and 4600 ± 4362 cells were counted in each graft for 6w-organoids and for 10w-organoids, respectively. In mice with one transplant site, the average percentage in three slices in each mouse was used for the analysis. In the transplantation of 6w- or 10w-organoids into 6-week-old mice, 46740 ± 20202 cells and 15280 ± 11840 cells were counted in each slice, respectively. In the transplantation of 10w-organoids into 6-week-old mice with or without one-week delay, 5153 ± 2584 cells and 27224 ± 12442 cells were counted in each slice for the no-delay group and for the 1w-delay group, respectively.

The number of cells contained in the organoids after cutting *in vitro* was counted manually after dissociating the pieces of organoids with the papain-containing Enzyme Solution of Neural Tissue Dissociation Kit (Sumitomo Bakelite, MB-X9901). Six organoids were analyzed each for 6w-organoids and for 10w-organoids. The estimated number of graft-derived cells in the engrafted tissues was calculated with hNuclei immunostaining after sectioning at 30 μm thickness and with the following formula: $N = V \times n / (S \times 0.03)$, where V ($mm^3$) is the graft volume, n is the sum of numbers of $hNuclei^+$ cells in the analyzed sections, and S (mm^2) is the sum of the graft areas in the analyzed sections. Four slices in each mouse were used for the analysis. 16857 ± 10052 cells and 5080 ± 4101 cells were counted in each slice for 6w-organoids and for 10w-organoids, respectively. The estimated number of graft-derived $CTIP2^+$ cells in the graft was calculated with the estimated number of $hNuclei^+$ cells and the percentage of $CTIP2^+$ cells

in the graft.

Fiber counting was performed with Fiji software after binarizing the images of each target area with the software. Three slices were analyzed for each part of all mice. The region of interest in each slice was decided based on the anatomical structures observed in the hNCAM immunostaining. In mice with four transplant sites, average numbers in both hemispheres in each mouse were used for the analyses.

The quantification of the $CD31^+$ area was performed with Fiji software after excluding areas with unspecific staining and after binarizing the images with the software. In mice with four transplant sites, the average percentage in all the survived grafts in each mouse was used for the analysis. In mice with one transplant site, the average percentage in three slices in each mouse was used for the analysis.

Supplemental References

Sakaguchi, H., Ozaki, Y., Ashida, T., Matsubara, T., Oishi, N., Kihara, S., and Takahashi, J. (2019). Self-Organized Synchronous Calcium Transients in a Cultured Human Neural Network Derived from Cerebral Organoids. Stem Cell Reports *13*, 458-473.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.