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Supplemental Information

Human iPSC-Derived Endothelial Cells and Microengineered Organ-Chip Enhance Neuronal Development

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Human iPSC-derived endothelial cells and microengineered Organ-Chip enhance neuronal development.

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Supplemental Experimental Procedures:

Pluripotent stem cell culture, directed differentiation into spMNs and BMECs

iPSCs mechanically passaged at low density were differentiated for 6 days to drive neural ectodermal cells in neural induction media consisting of IMDM/F12 (Gibco), B27, N2, 1% NEAA, 0.2 uM LDN193189, 10 µM SB431542, 3 µM CHIR99021 (Cayman Chemical). Cells were then passaged using Accutase (Sigma-Aldrich) and reseeded onto matrigel and patterned to form spNPCs in Stage 2 media consisting of IMDM/F12 (Gibco), B27, N2, 1% NEAA, 200ng/ml ascorbic acid, 0.1 µM retinoic acid, 1 µM Smoothened agonist (SAG) for an additional 6 days. Cells were then dissociated and cryogenically frozen for later use. Upon thaw, spMNs were cultured in Stage 3 media consisting of IMDM/F12 (Gibco), B27, N2, 1% NEAA, 200ng/ml ascorbic acid, 0.5 µM retinoic acid, 0.1 µM cAMP, 0.1 µM SAG, 10ng/ml glial cell line-derived neutrotrophic factor, 10 ng/ml brain derived neutrotrophic factor, 1% penicillin-streptavidin (PSA) and fed every two days.

Organ-Chip microfabrication and culture

The Organ-Chip was fabricated by using modified methods for Chip microfabrication as previously described (Huh et al., 2013). Briefly, PDMS pre-polymer was mixed at a 10:1 ratio of PDMS base to curing agent, wt/wt using a planetary mixer (Thinky ARE-310). PDMS pre-polymer was then cast onto molds forming the microchannels of the upper layer (1,000 µm wide x 1,000 µm high) and lower layer (1,000 µm wide x 200 µm high). The membrane was cast onto a silicon mold that was fabricated using photolithography and deep reactive ion etching, resulting in 7 µm pores. The components were cured overnight and removed from the mold. The upper layer, membrane, and lower layer were permanently bonded via plasma bonding to form the complete Chip. Chips were then treated with plasma in 100% oxygen for 2:00 minutes and immediately coated with Matrigel for the neural channel and a mixture of collagen IV (Invitrogen), fibronectin (Invitrogen), and diluted in water in a ratio of 1:4:5 for the vascular channel. Coated Organ-chips were then incubated overnight at 37°C and 5% CO_2.

Immunohistochemistry

iPSC-derived cultures were fixed in 4% PFA, and rinsed with PBS. Cultures were permeabilized in 10% Triton X at RT for 10 min and blocked in 5% donkey serum and 0.1% Triton-X at RT for 1 h. Samples were incubated overnight at 4°C in primary antibody solution containing the following antibodies: mouse anti-SMI-32 (Covance, SMI-32P-100, 1:1,000), rabbit anti-ZO-1 (Life Technologies, 617300, 1:100), mouse anti-GLUT1 (Thermo, MA5-11315, 1:100), mouse anti-Occludin (Invitrogen, 901200 1:100), mouse anti-Claudin5 (Thermo, 4C3C2, 1:100), rinsed in PBS, and incubated for 1 h at room temperature in donkey anti-mouse Alexa Fluor 488, donkey anti-goat 594, and donkey anti-rabbit 647 secondary antibodies (Life Technologies, A21202 and A21289, 1:1,000 each).

Fetal tissue was received from the Birth Defects Research Laboratory at the University of Washington under their approved Institutional Review Board (IRB), consent and privacy guidelines. All protocols were performed in accordance with the IRB's guidelines at the Cedars-Sinai Medical Center under the auspice IRB-SCRO Protocol Pro00021505. Spinal cord samples arrived with estimated age and as partially intact spinal columns that were partitioned into approximated cervical, thoracic and lumbar sections. Fetal tissue was subsequently fixed in 4% paraformaldehyde (PFA) for 48 h, and placed in 30% sucrose for an additional 24 h. Finally, spinal cords were embedded in Tissue-Tek OCT (VWR) and sectioned at 25 µm using a cryostat (Leica) at −20°C and directly mounted on glass slides (Fisher Scientific). Tissue sections were permeabilized in cold MeOH for 20 minutes, and

blocked in Phosphate Buffered Saline (PBS) containing 5% normal donkey serum (Sigma, D9663) and 0.25% Triton-X for 1.5 h, then transferred to primary antibody solution containing mouse anti-GLUT1 (R&D Systems, 1:100) SMI-32P-100 (Covance, 1:1,000), and goat anti-Islet-1 (R&D, AF1387, 1:500), and rabbit anti-NFH (Sigma, N4142, 1:1000), rabbit anti-SIRT (Sigma, SAB4502861, 1:100) and incubated overnight at 4°C. Samples were then incubated for 1 h at room temperature in donkey anti-mouse Alexa Fluor 488 and donkey anti-goat 594 secondary antibodies (Life Technologies, A21202 and A21289, 1:1,000 each). Fetal samples were mounted in Fluoromount-G (Southern Biotech, 0100-01) and acquired at 20x using automated stitching on a Leica DM 6000 microscope for whole mount image.

BMEC Media Conditioning

BMECs were seeded in endothelial cell media into either a T75 flask in media conditioning experiments, or the bottom channel of the Spinal Cord-Chip. spNPCs were thawed and seeded into either the 96-well plates or the top channel of the Chip and incubated overnight. The following day, media was replaced with Stage 3 media in all conditions. BMEC flask was washed 2x with minimal neural media (IMDM:F12 0.5% N2, 1% B27, 0.5x NEAA, 1x PSA). 24 hours later BMEC media was collected, centrifuged and filtered and then supplemented with remaining ingredients for Stage 3 media.

Whole-cell patch electrophysiology

spNPCs were seeded either onto mouse laminin (Invitrogen) coated glass coverslips or Chips fabricated with polyester membranes containing 1µm pores with a lid that could be removed to access the top channel compartment. Cultures were maintained in Stage 3 media changing every other day for 6 days. Upon experiment endpoint, the top channel was exposed and transferred into a petri dish containing artificial cerebral spinal fluid (aCSF) that consisted of 135 mM NaCl, 5 mM KCl, 5 mM HEPES, 10 mM Glucose, 1.2 mM $MgCl_2$ and 1.25 mM $CaCl_2$. After incubation at RT for at least 30 mins, whole cell patch clamp was performed on an Olympus BX51WI microscope. Glass micropipettes were pulled using a Sutter Instruments P-1000 from thin-wall borosilicate glass (World Precision Instruments), with a tip resistance of between 3 and 6 MΩ and filled with internal solution consisting of 10 mM NaCl, 117 mM KCl, 11 mM HEPES, 11 mM EGTA, 2 mM Na.ATP, 2 mM MgCl2 and 1 mM CaCl2. Voltage and current clamp recordings were performed using a Multiclamp 700B amplifier and Digidata 1440, with pClamp 10 acquisition software (Molecular Devices). Only cells with an access resistance of 30 MΩ or below were included in analysis. Capacitance, access resistance, and membrane resistance were measured immediately after whole-cell patch configuration was achieved in current-clamp with no current added. Resting membrane potential, and was determined by the average voltage detected during a 1 minute continuous recording with no current added. Induced action potentials were measured in current-clamp, where cells were held at -70 mV and sequential current steps were applied in increments of 10 pA. Current plots were recorded in voltage-clamp where cells were held at -70 mV, and 100 ms voltage steps were applied in 10 mV increments from -120 mV to +40 mV. Current densities were calculated using pCLAMP software and normalized to membrane capacitance for each neuron.

Live calcium transient imaging and analysis

Fluo-4 calcium dye (Invitrogen), was prepared at 10 mM containing 50% pluronic F127 solution (Invitrogen) in DMSO, and diluted to a final concentration of 20 µM in aCSF. Tissue cultures were incubated at RT for 30 minutes, then washed in fresh aCSF and incubated an additional 30 minutes before acquiring. After a 2-minute burn in phase, 16 bit videos were acquired for 3 minutes at 20 Hz on an Eclipse Ti microscope (Nikon) using a Plan Flor 20x objective (Nikon) equipped with an Orca -Flash4.0LT digital camera (Hamamatsu). As no difference in event detection was determined between 16- and 8-bit data, all datasets were down-sampled to 8-bit (ImageJ). spMN-specific calcium transient populations were identified in 00iCTR that allowed use of both 488 and 594 wavelengths after live calcium imaging. Briefly, spNPCs were imaged for transients, then fixed in 4% PFA and stained as previously described. Images were then returned to the same microscope and imaged at the same site and overlaid with maximum projection of calcium imaging data. ROI of ISL1 positive, SMI32 positive motor neurons were then generated using ImageJ and spMN specific masks were generated for analysis.

Supplemental Figures:

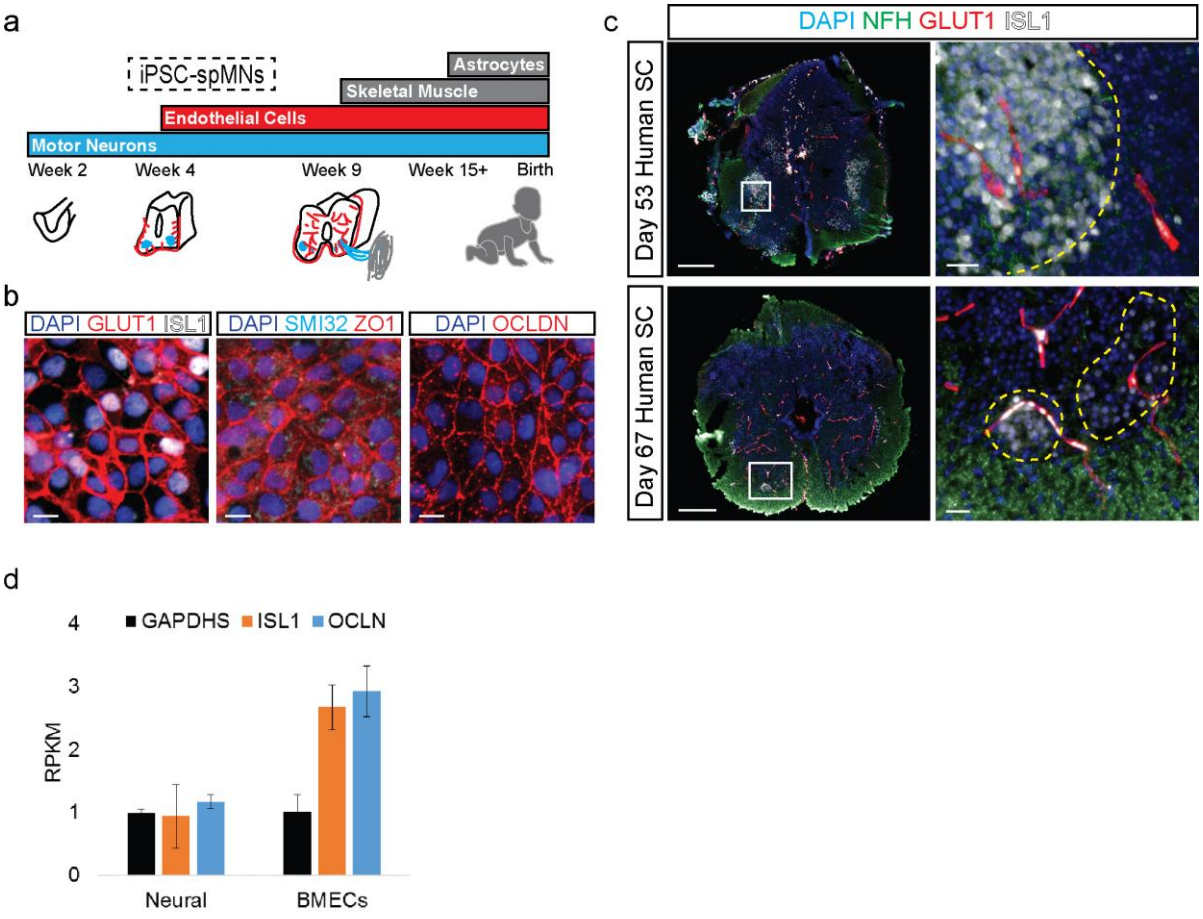


Figure S1. Related to Figure 2. Brain Microvascular Endothelial Cells Express ISL1 During Development. (a) Schematic of relative emergence of cell types that directly interact with developing human spMNs *in vivo.* (b) Days 53 and 67 human fetal spinal cords immunostained with neuronal marker neuofilament heavy chain (NFH), BMEC marker glucose transporter 1 (GLUT1), and transcription factor ISL1. High magnification of ventral horns (box) show ISL1 positive spMN pools (yellow dotted line). (c) Images of iPSC-derived BMECs cultured in 96-well plate for 6 days and immunostained with GLUT1, ISL1, and tight junction markers zona occludens 1 (ZO-1) and Occludin (OCLDN). Scale bars = 20 microns. (d) Average Expression of ISL1 in iPSC-derived Neurons and BMECs from published RNA-Sequencing dataset (Vatine et al., 2017).

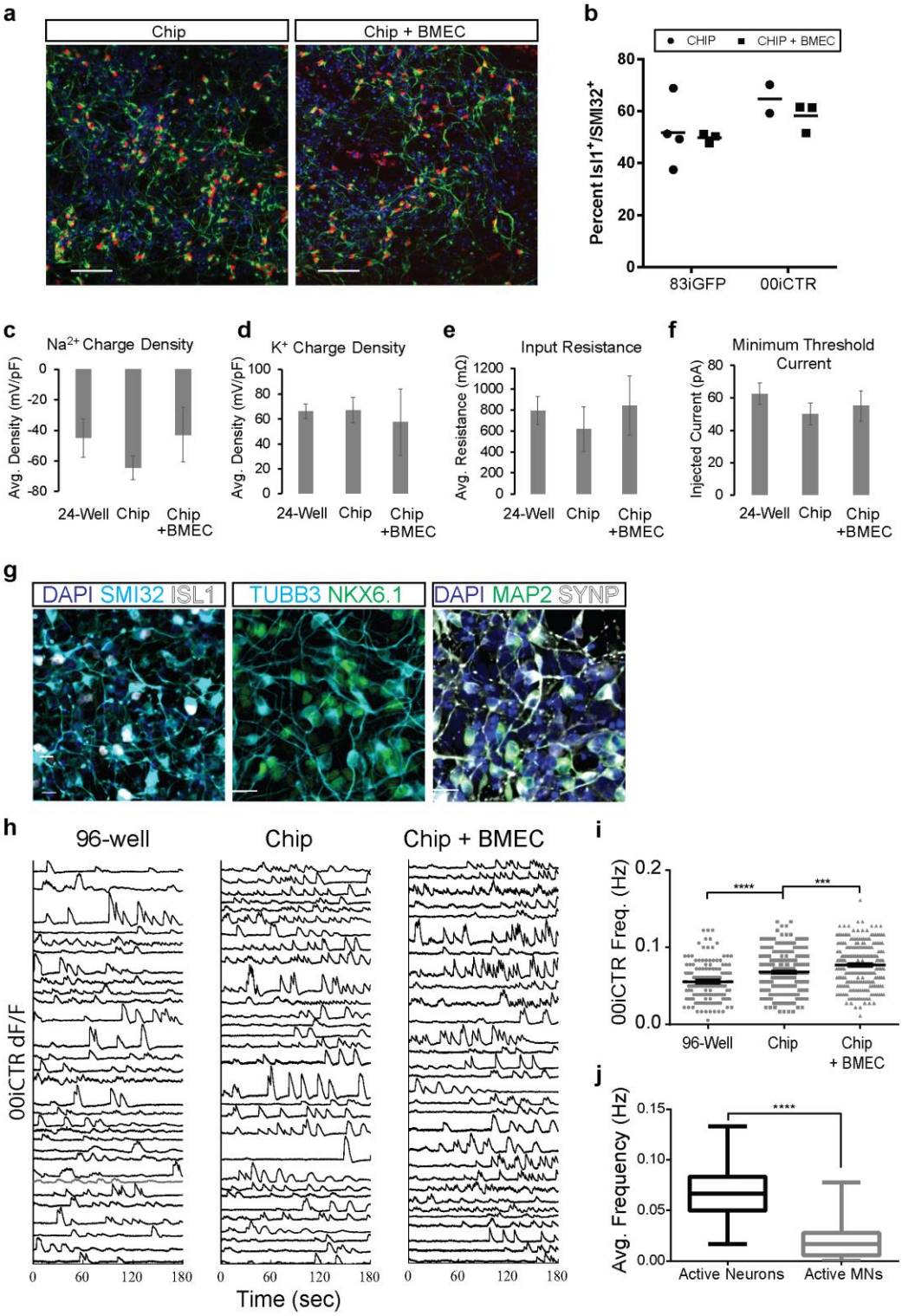
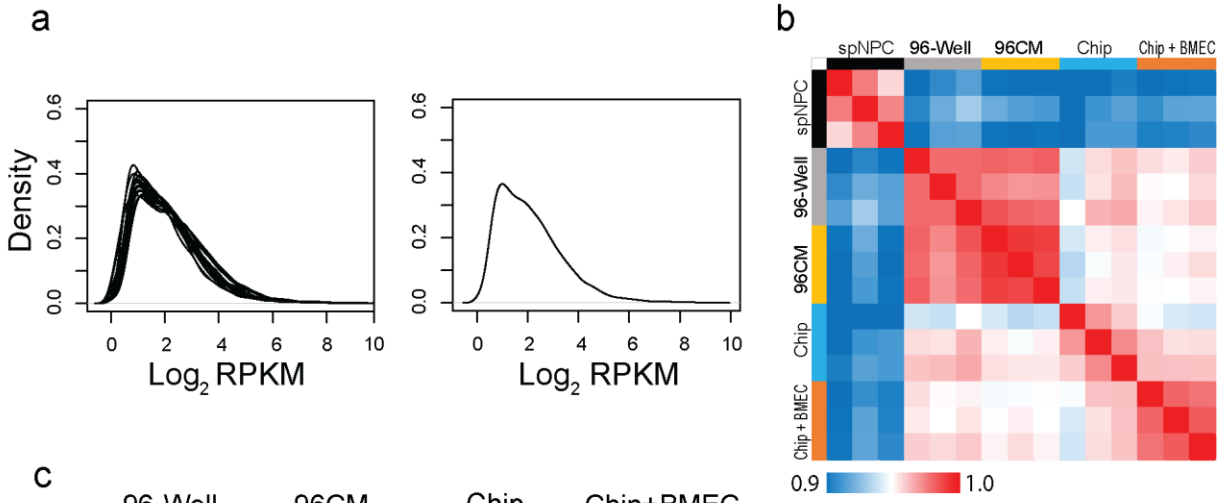


Figure S2. Related to Figure 3. Electrophysiology and staining of Spinal Cord Chip derived from second patient. (a) Representative images of immunostaining of Chips seeded with spNPCs derived from 00iCTR patient. Scale bar = 100 microns. (b) Quantification of percent of SMI32 expressing cells that co-express with ISL1 in both 83iGFP and 00iCTR lines. Dots denote average count per site for an individual chip. Bar = mean. (c-f) Intrinsic electrophysiological measurements from whole patch clamp recordings conducted on across three culture conditions: 24-well plate, Chip

and Chip containing BMECs, n = 5,4, and 6 neurons respectively. (c-d) Sodium (Na^{2+}) and potassium (K^{+}) charge densities normalized by membrane capacitance. (e) Input resistance quantified as the sum of access and membrane resistance values recorded at onset of whole-cell configuration with zero current added. (f) Minimum current injected for action potential (AP) defined as lowest current step to reach 0 mV membrane voltage. Picofarads (pf), millivolts (mV), picoamperes (pA). (g) Images of spNPCs cultured in 96-well plate and immunostained with markers of spMNs SMI32, nuclear marker islet1 (ISL1), Beta 3 tubulin (TUBB3), NKX6.1, neurofilament marker microtubule-associated protein 2 (MAP2), and synaptic marker synaptophysin (SYNP). Scale bars = 20 microns. (h) Calcium transient activity plots of 30 representative neurons derived from 00iCTR spNPC cultures in each culture condition. (i) Transient frequency plot of 144, 278 and 250 00iCTR-derived neurons cultured in 96-wells, Chips and Chips containing BMECs respectively. (j) Average transient frequency of (n = 232) representative active neurons derived from 00iCTR spNPC cultures compared to (n = 116) ISL1 overlaid spMNs.



c

	96-Well			96CM			Chip			Chip+BMEC		
DLK1	1	1.9	1.6	2.3	2	2.2	1.3	2.8	1.8	8.1	11	10
JAG1	5.5	5.2	6	5.3	6	5.7	17	16	14	9.1	7.8	6.5
DLL1	5.1	4.8	5.3	4.3	3.9	3.6	5.6	5.6	5.4	3.3	3.2	2.9
DLL3	16	14	12	11	10	11	6.7	5.5	5.1	4.6	3.3	4.1
DLL4	1.1	1.3	0.9	1.1	0.7	1.1	0.7	0.5	0.4	0.7	0.5	0.4

d

	96-Well			96CM			Chip			Chip+BMEC			Progenitor Domain
PLCG1	4.6	4.5	4.3	3.8	4	5.2	5	5.4	4	5.9	4.6	4.9	dI5
BHLHE22	0.2	0.2	0.1	0.3	0.2	0.2	0.9	0.7	1.3	0.7	0.8	0.4	dI6
ONECUT1	9.7	8.6	7.9	8.3	8.6	8.4	8.3	6.6	8.8	4.8	4.5	4.1	V0-3
ONECUT2	9	7	5.8	8.2	8.6	8	10	9	14	11	9.9	9.2	V0-3
DLL4	1.1	1.3	0.9	1.1	0.7	1.1	0.7	0.5	0.4	0.7	0.5	0.4	V2a
VSX2	2.5	1.6	1.6	1.9	2	2.1	2.8	1.7	2.7	3.6	2.5	2.4	V2a
SOX14	0.2	0.1	0	0.2	0.1	0.3	1.1	0.8	1.9	0.4	0.8	0.3	V2a
SOX21	3.5	3.5	3	3.2	3.7	2.8	13	12	12	8.9	10	6.2	V2a
PRDM8	9.9	11	9.7	7	7.1	7.2	17	17	15	8.2	5.9	5.6	V1/V2a
MAF	1	0.8	0.6	0.7	0.9	0.6	0.9	1	1.2	1	0.8	0.9	V2a
NOTCH1	2.6	3	2.5	3.1	3.3	3.4	4.9	4	2.4	5	3.9	3.4	V2b
GATA3	0.1	0.1	0	0.1	0.1	0.1	0.1	0	0	0.6	1	1	V2b
PROX1	11	11	10	9.9	11	10	12	12	13	8.4	6.6	6.2	V3

Figure S3. Related to Figure 4. Chip culture induces distinct neural differentiation signatures. (a) Density plots of 10,001 genes for each sample for transcriptomic analysis. Quantile normalized data (right). (b) Pierson correlation coefficient analysis of normalized RPKM datasets displayed by colorimetric scale. (c) Notch pathway genes differentially expressed in the Spinal Cord-Chip conditions. (d) Spinal neuron subtypes markers of progenitor domains as defined in (Lu et al., 2015). dI (dorsal Interneuron domain), V (ventral domain).

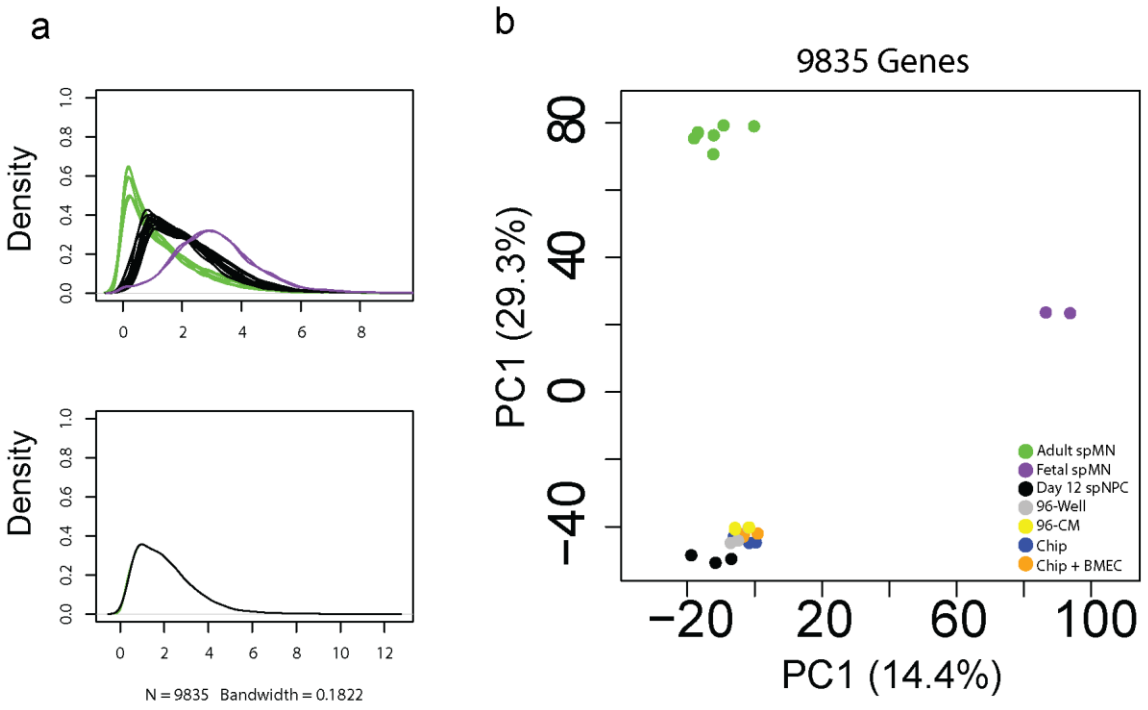


Figure S4. Related to Figure 5. Developmental comparison to Chips with all matching genes. (a) Density plots of 9835 genes expressed both *in vivo,* and *in vitro* data for developmental comparison. Adult laser captured spMNs (green lines), fetal Spinal cord (purple lines) and sorted iPSC-derived neurons (black lines) from different culture methods. (b) PCA analysis of variance across all samples and 9835 genes. Thawed day 12 spNPCs (black), Day 18 cultured neurons in 96 well plate (grey), or 96 well plate in presence of conditioned media (yellow), Chip (blue) and Chip containing BMECs (orange). Quantile normalized data (bottom).

Supplemental References:

Huh, D., Kim, H.J., Fraser, J.P., Shea, D.E., Khan, M., Bahinski, A., Hamilton, G.A., and Ingber, D.E. (2013). Microfabrication of human organs-on-chips. Nat Protocols *8*, 2135-2157.

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