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SUPPLEMENTARY MATERIAL

corresponding to:

A morphology-based assay platform for neuroepithelial-like cells differentiated from human pluripotent stem cells

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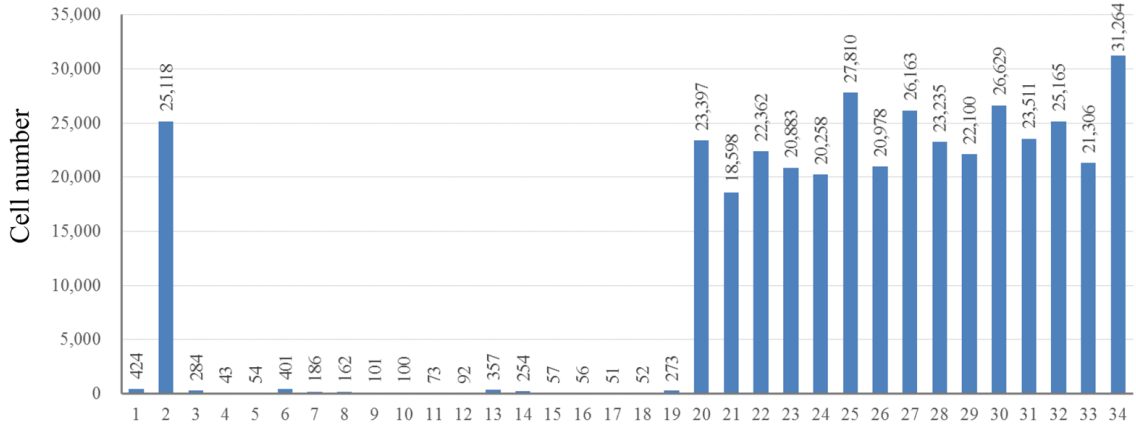
Supplementary Information

Cell lines (related to Fig. S2)

A hiPSC line iPS DF19-9-7T was obtained from the WiCell stem cell bank (WI, USA). These cells were cultured using mTeSR1 and Matrigel. Before NELC induction, iPS DF19-9-7T cells were cultured with hESF-FX for 24 hours.

Definitive endoderm (DE) differentiation

For this experiment, hPSCs were plated on bovine fibronectin-coated culture plates in hESF-FX medium supplemented with 10 μM Y-27632 (Wako) and 5 ng/ml human recombinant activin A (Cell Guidance Systems, MO, USA) at a density of 5 x 10^4 cells/cm^2. On day 1, the culture medium was replaced with RPMI1640 supplemented with B27 (without insulin) (Gibco), 100 ng/ml activin A, and 3 μM CHIR99021. The culture medium was replaced with RPMI1640 supplemented with B27 (without insulin) and 100 ng/ml activin A on days 2 and 3. Total mRNA was prepared from the induced DE cells on day 5.



human recombinant insulin	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sodium selenite	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-mercaptoethanol	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	+	+
human recombinant albumin (without oleic acid)	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	+	-
oleic acid conjugated human recombinant albumin	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	+
heraran sulfate sodium salt	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
human apo-transferrin	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ethanolamine	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+

Fig. S1. Optimization of culture medium for initiation of NELC induction. *Optimization of ESF-NS1 media components. Sets of supplements used in hESF-FX or hESF9 medium were tested to select required minimum essential components for hPSC differentiation. Dissociated single undifferentiated H9 cells were plated on fibronectin coated 24-well tissue culture plates, and cultured for 3 days in mESF basal medium supplemented with L-ascorbic acid phosphate magnesium salt (0.1 mg/ml), FGF-2 (10 ng/ml) and represented sets of components; human recombinant insulin (10 μg/ml), sodium selenite (20 nM), 2-mercaptoethanol (10 μM), human recombinant albumin (1 mg/ml), oleic acid conjugated human recombinant albumin (4.7 μg oleic acid/ 1 mg albumin/ml), heparan sulfate sodium salt (100 ng/ml), human apo-transferrin (5 μg/ml), and ethanolamine (2-aminoethanol, 10 μM). Nuclei of Hoechst33342 stained viable cells were counted with IN Cell Analyzer 2000 (GE Healthcare). Total number of viable cells in 57.42 mm^2 of each well are shown.*

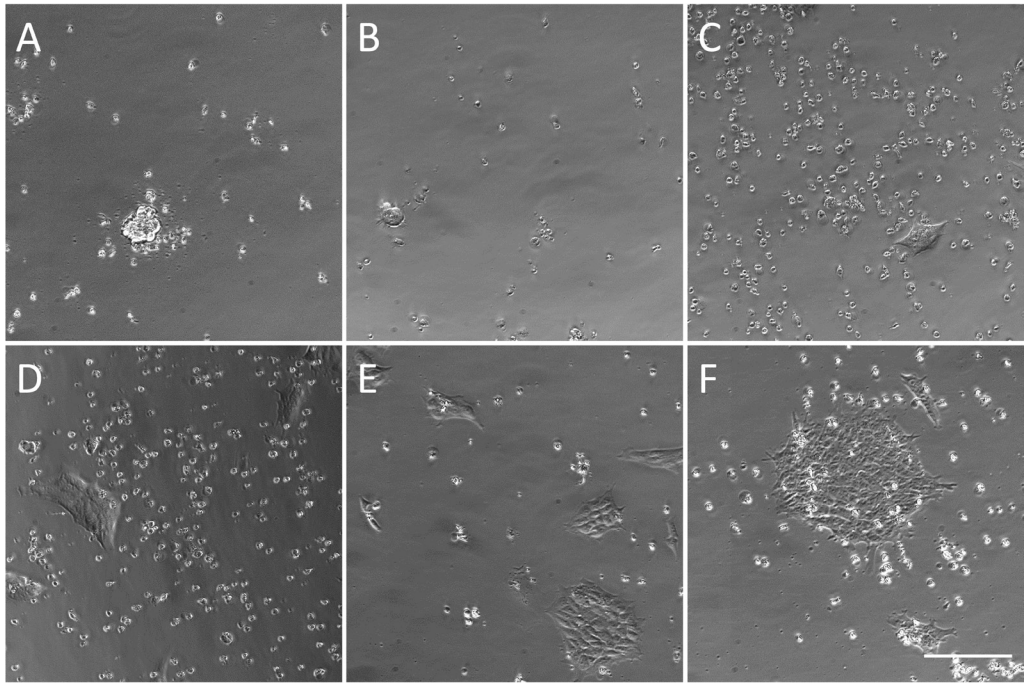


Fig. S2. Optimization of coating conditions for initiation of NELC induction. *Optimization of cultureware coating conditions for NELC induction. Dissociated Tic hiPSCs were plated on tissue culture-treated plastic plates without a coating* **(A)** *or coated with poly-L-lysine* **(B)***, bovine collagen type IV* **(C)***, bovine fibronectin* **(D)***, poly-L-lysine and bovine fibronectin* **(E)***, or mouse laminin* **(F)***, and cultured in hESF-NS1 for 2 days. Phase contrast images of live cells are shown. Bar, 0.5 mm. When cells were plated on the dishes coated with poly-L-lysine and fibronectin, they adhered and formed relatively small aggregates, and fewer dead cells were observed than under the other conditions.*

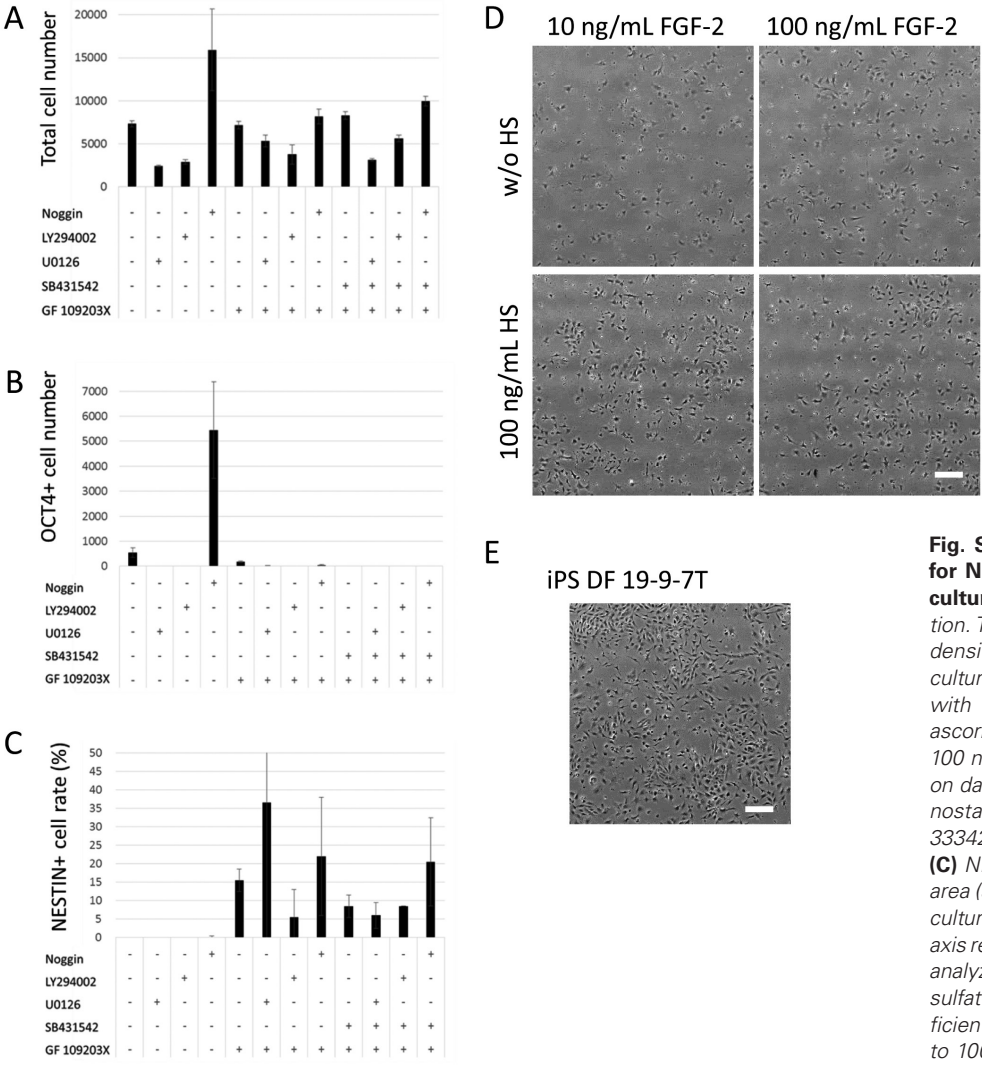


Fig. S3. Optimization of medium components for NELC induction in the adherent monolayer culture. (A-C) *Effects of inhibitors on NELC induction. Tic hiPSCs were plated on 24-well plates at a density of 2 x 10^4 cells/cm^2 in ESF-NS1 medium and cultured for 2 days. Then, the cells were cultured with the indicated inhibitors in ESF-NS1 lacking ascorbic acid phosphate and supplemented with 100 ng/mL FGF-2. The medium was replaced once on day 5. Cells were fixed and subjected to immunostaining.* **(A)** *The numbers of total cells (Hoechst 33342-stained nuclei),* **(B)** *OCT4-positive cells, and* **(C)** *NESTIN-positive cells within the center square area (57.42 mm^2) of each well for the representative culture condition were analyzed (mean n = 3). The Y-axis represents the actual number of cells within the analyzed area.* **(D)** *The addition of 100 ng/ml heparan sulfate sodium salt (HS) with 10 ng/ml FGF-2 was sufficient to induce NELCs and was used an alternative to 100 ng/ml FGF-2 alone. Tic hiPSCs (1,000 cells/cm^2) grown in ESF-NS1 for 2 days were cultured in hESF-NS2(+) containing 100 ng/ml HS and/or FGF-2 until day 7.* **(E)** *iPS DF19-9-7T cells were differentiated using ESF-NS1 and ESF-NS2(+). Phase contrast images showed the presence of NELC-like cells on day 7. Bars, 100 μm.* **(F,G)** *Dissociation into single cells and cell density are critical for NELC induction efficiency. The dissociated H9 cells were seeded in ESF-NS1 at a density of 6000 cells/cm^2. Culture medium was replaced with ESF-NS2 without Dorsomorphin on day 2 and 5. When properly dissociated cells were seeded definitely at a density of 6000 cells/cm^2, NELC induction efficiency was high on day 8, indicating that H9 hESCs were differentiated without using Noggin or Dorsomorphin* **(F)**. *However, when the cells were not dissociated into single cell properly and seeding cell density became relatively higher, NELC induction efficiency was decreased* **(G)**. *White dotted lines indicate the regions of compacted cell aggregation which were not differentiated into NELCs.*

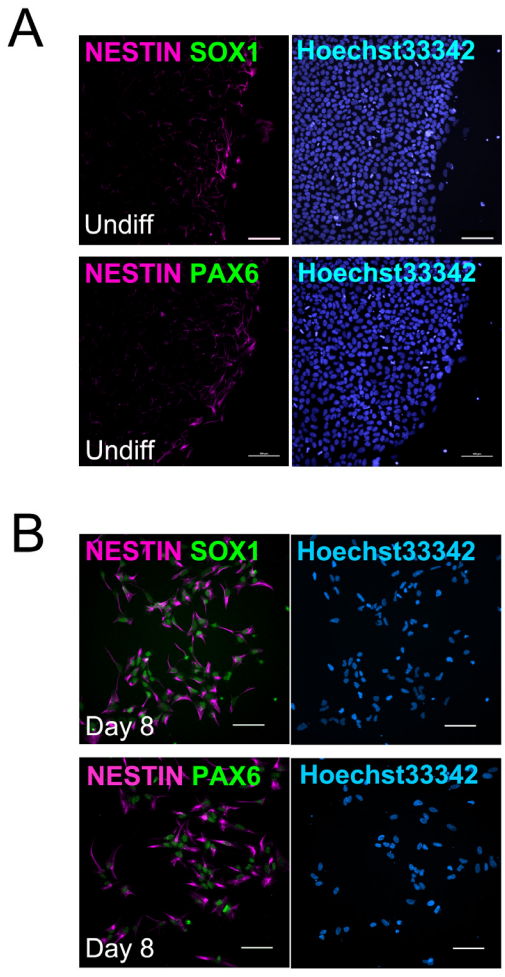


Fig. S4. SOX1 and PAX6 are expressed in the induced NELCs. *The undifferentiated state hiPSC Tic cultured in hESF-FX* **(A)** *and the induced NELCs on day 8* **(B)** *were subjected to immunostaining with antibodies against SOX1 or PAX6 (green) and NESTIN (magenta), along with Hoechst 33342 (blue) nuclear staining. Representative images are shown. Bar, 50 μm.*

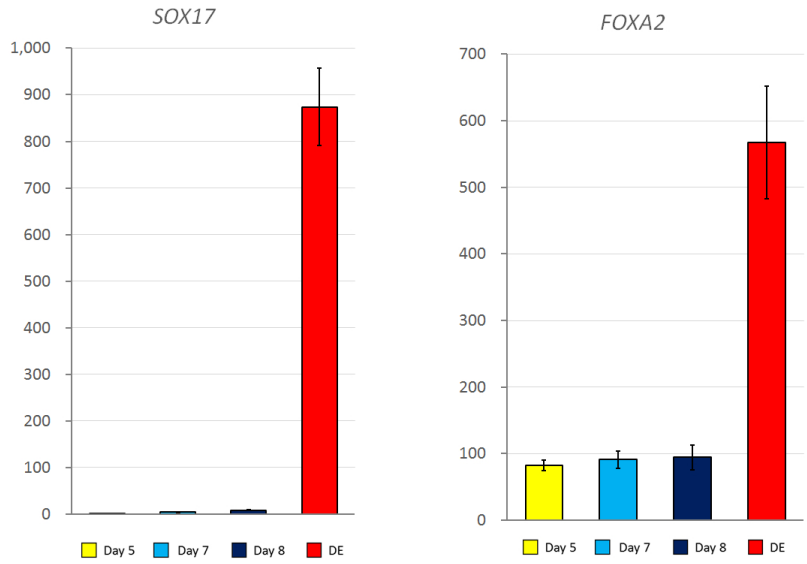


Fig. S5. *SOX17* and *FOXA2* gene expression in the induced NELCs (related to Fig. 2). *The expression of the* SOX17 *and* FOXA2 *genes in the induced NELCs on days 5 (yellow, n = 3), 7 (cyan, n = 3), and 8 (navy, n = 6) and the induced definitive endoderm (DE, red, n = 3) were analyzed by qRT-PCR.* GAPDH *was used as an internal control. Relative gene expression was normalized to the expression in undifferentiated Tic hiPSCs cultured in hESF-FX (n = 3; means ± SD).*

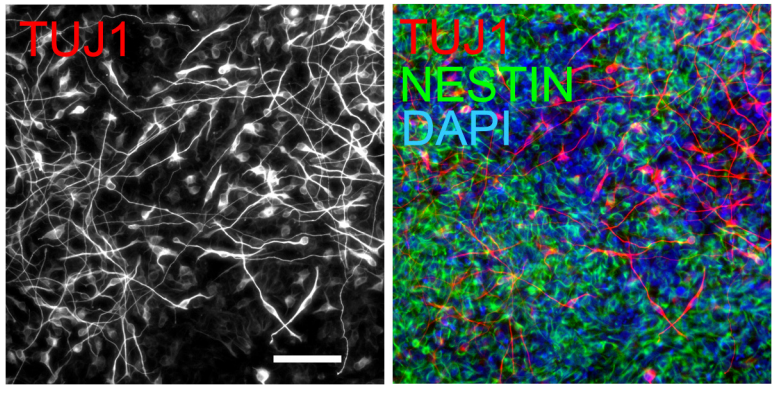


Fig.S6. Potential of the induced NELCs to differentiate into neurons. *To confirm whether the NELCs induced in our culture conditions have the differentiation potential, the NELCs were further differentiated in the conventional culture conditions for neural differentiation. The cells were fixed on day 7 when immature neurons were observed. The fixed cells including immature neurons were stained for tubulin β3 (TUJ1, red) and NESTIN (green). Nuclei were stained with DAPI (blue). (Left) TUJ1 staining image, (Right) Merged images of TUJ1, NESTIN, and DAPI. The differentiation efficiency were counted by detecting TUJ1-positive cells. In the represented image, 90 TUJ1-positive cells (30%) and 189 NESTIN-positive cells (63%) in 300 cells were detected. Bar, 100 μm.*

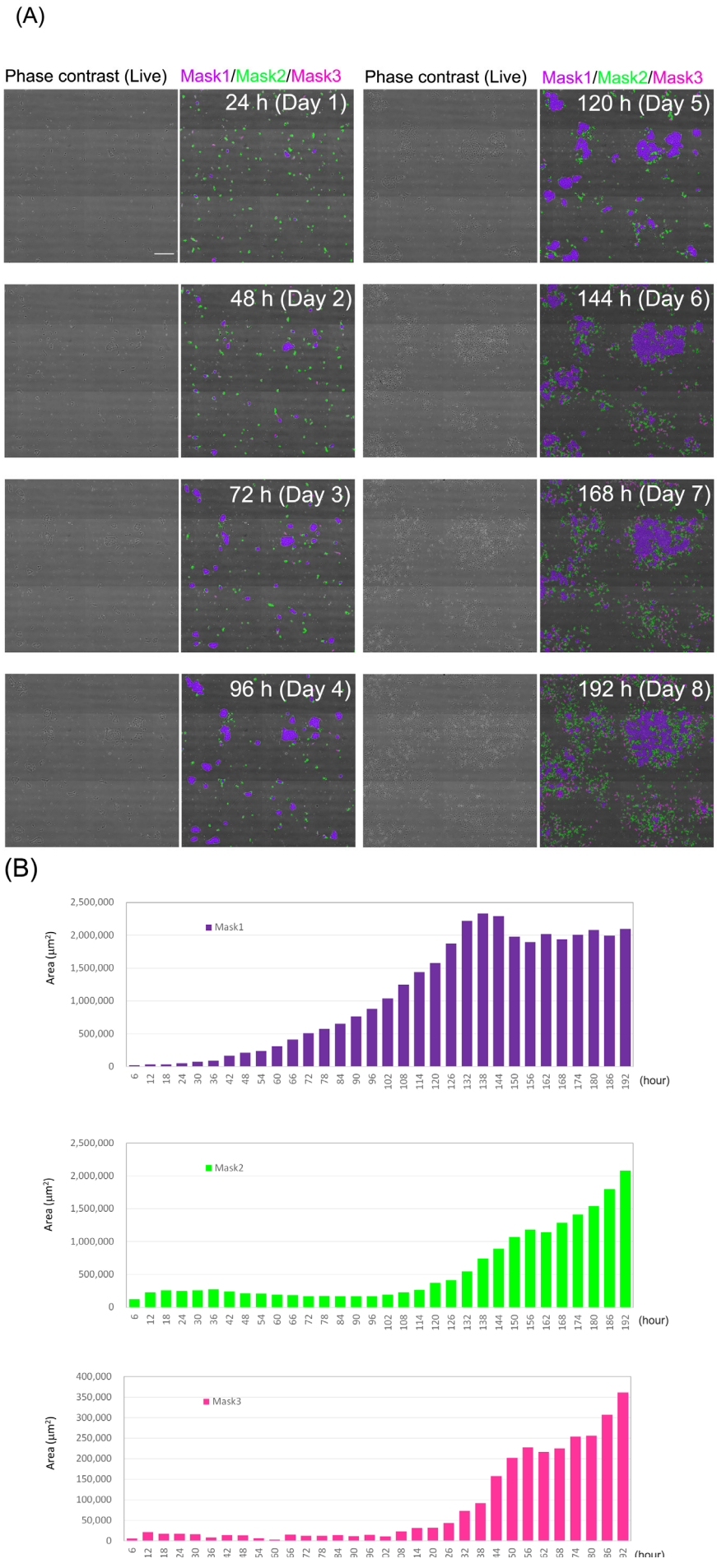


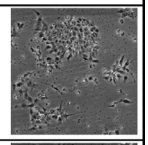
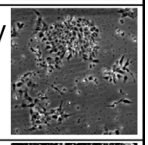
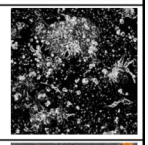
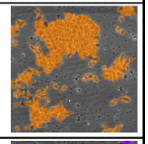
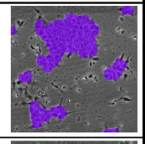
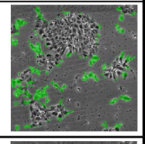
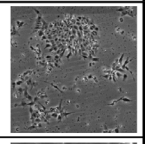
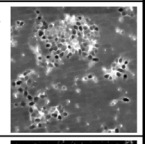
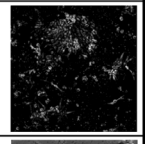
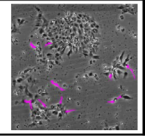
Fig. S7. Analysis of cell morphology from phase contrast images of live cells (related to Fig. 6 and Video S2). (A) *Phase contrast images of live Tic hiPSCs differentiating into NELCs using the method described in Fig. 1A were automatically acquired every 6 hours at a magnification of x4 and a resolution of 2 μm per pixel. Images were processed to classify the cell coverage area into three types, Mask1 (purple), Mask2 (green), and Mask3 (magenta), as described in Fig. 5A. Representative phase contrast images of the live cells are shown in the first and third columns. Representative processed images with Masks are shown in the second and fourth columns. Times during NELC induction are indicated.* **(B)** *The analyzed area value of Mask1, 2, and 3 are shown separately (related to Fig.6C). Note that Y-axes of each graph are different.*

TABLE S1

LIST OF PRIMERS FOR qRT-PCR USED IN THIS STUDY

Gene Name	Forward (5')	Reverse (3')	Accession#
GAPDH	CAAAGTTGTCATGGATGACC	CCATGGAGAAGGCTGGGG	NM_002046.5
MKI67	TGACCCTGATGAGAAAGCTCAA	CCCTGAGCAACACTGTCTTTT	NM_002417.4
OCT4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAA	NM_002701.5
NANOG	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG	NM_024865.3
OTX2	CCCGGTACCCAGACATCTT	GCTCTTCGATTCTTAAACCATACC	NM_021728.3
SOX1	ATGCACCGCTACGACATGG	CTCATGTAGCCCTGCGAGTTG	NM_005986.2
SOX2	AGCTACAGCATGATGCAGGA	GGTCATGGAGTTGTACTGCA	NM_003106.3
SOX9	AATGGAGCAGCGAAATCAAC	CAGAGAGATTTAGCACACTGATC	NM_000346.3
SOX10	CTGAGTTGGACCAGTACCTG	GGCTGATGGTCAGAGTAGTC	NM_006941.3
PAX3	TTGGCAATGGCCTCTCAC	AGGGGAGAGCGCGTAATC	NM_181457.3
PAX6	GTCCATCTTTGCTTGGGAAA	TAGCCAGGTTGCGAAGAACT	NM_000280.4
MSX1	GGATCAGACTTCGGAGAGTGAACT	GCCTTCCCTTTAACCCTCACA	NM_002448.3
FOXD3	CAGCGGTTCGGCGGGAGG	TGAGTGAGAGGTTGTGGCGGATG	NM_012183.2
ASCL1	CCCAAGCAAGTCAAGCGACA	AAGCCGCTGAAGTTGAGCC	NM_004316.3
TUJ1	GCGGATCAGCGTCTACTACA	CACATCCAGGACCGAATCCA	NM_006086.3
NESTIN	CCCTGACCACTCCAGTTTAG	CCTCTATGGCTGTTTCTTTCTC	NM_006617.1
VIMENTIN	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC	NM_003380.3
E-CAD	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC	NM_004360.4
N-CAD	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG	NM_001792.4
SLUG	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG	NM_003068.4
SNAIL	CCTCCCTGTCAGATGAGGAC	CCAGGCTGAGGTATTCCTTG	NM_005985.3
TWIST	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG	NM_000474.3
FN1	CAGTGGGAGACCTCGAGAAG	TCCCTCGGAACATCAGAAAC	NM_212482.2
T	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG	NM_003181.3
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA	NM_021784.4
SOX17	CAGAATCCAGACCTGCACAA	CTCTGCCTCCTCCACGAA	NM_022454.3

TABLE S2

Steps described in Fig.6A	Image processing schema	Parameters used in image processing (CL-Quant ver.3.3)	Treated images
Edge detection	Input image		Original raw image 
	Blur the input image using “Average Filter”	“Average Filter” Parameter: 5	Processed image by ”Average Filter” 
	Extraction of cell coverage area by subtraction using the processed image and the raw image		Subtracted image 
	Digitalizing using “Thresholding” by intensity of the subtracted image	“Thresholding” Parameter: 7	Whole cell region 
Opening	Extraction of dense cell region by segmentation of Whole cell region using “Open Disc”	“Open Disc” Parameter: 15	Mask1 (dense cell region) 
Subtraction	Extraction of sparse cell region by withdrawn of Mask1 (dense cell region) from Whole cell region		Mask2 (sparse cell region) 
Closing, Subtraction	Input image		Original raw image 
	Segmentation of raw image using “Close Disc”	“Close Disc” Parameter: 9	Processed image by “Close Disc” 
	Extraction of cell coverage area by subtraction using the processed image and the raw image		Subtracted image 
	Digitalizing using “Thresholding” by intensity of the subtracted image	“Thresholding” Parameter: 20	Mask3 

Video S1. Live observation of cells differentiating into NELCs from hPSCs. (related to Fig. 1 and Fig. 4). (A) *Phase contrast images were obtained using 4x magnification. The differentiating cells were observed every 6 hours from days 0 to 8. Bar, 200 μm.* **(B)** *Magnified phase contrast images, obtained using 4x magnification. The same field shown in Fig. 4. The differentiating cells were observed every 12 hours from days 0 to 8. Bar, 200 μm.*

Video S2. Example of the method used to analyze live-cell images captured during NELC induction (related to Figs. 6 and S5). *Phase contrast images were obtained using 4x magnification. Differentiating cells were observed every 6 hours from days 0 to 8. Phase contrast images* **(A)** *and the processed images* **(B)** *with Mask1 (purple), Mask2 (green), and Mask3 (magenta) are presented.*