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Supplemental Information

Direct Reprogramming of Adult Human Fibroblasts to Functional Neurons Under Defined Conditions

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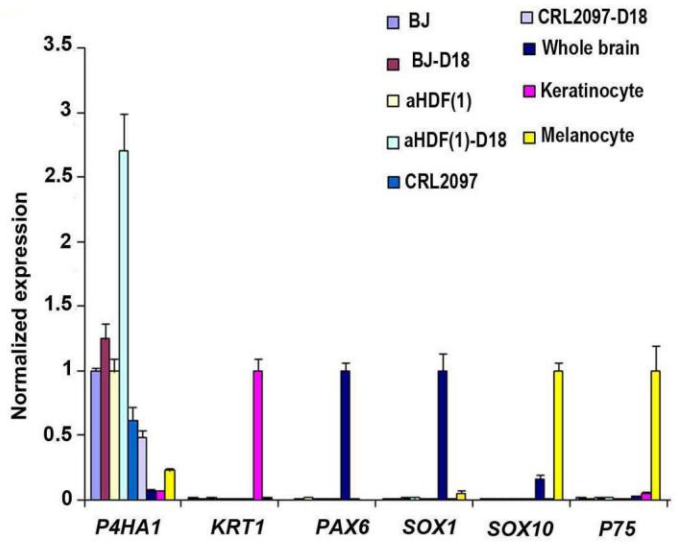
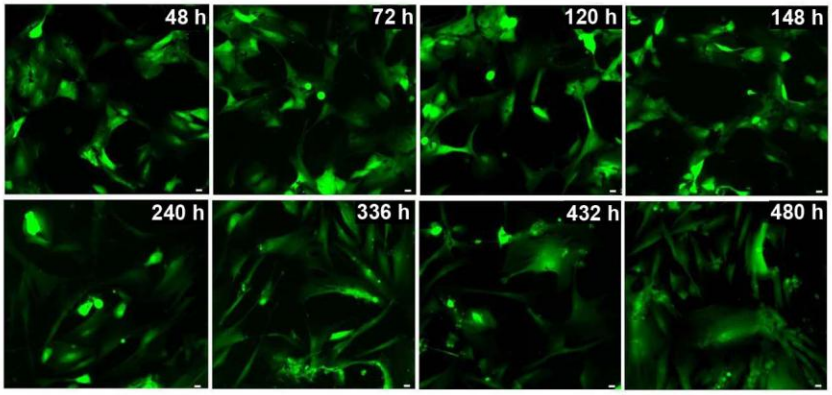
A

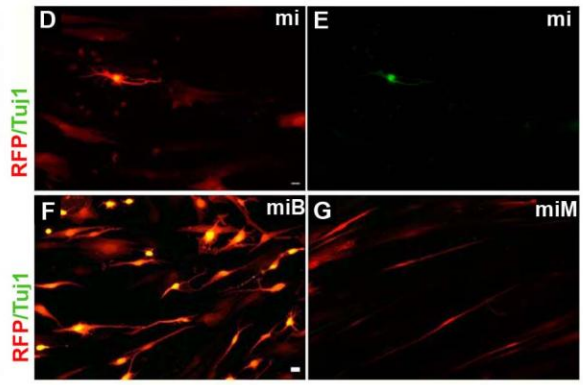
Antigen tested	BJ			BJ + GFP	CRL 2097			CRL 2097 + GFP	aHDF-1			aHDF-1 + GFP
	Start	D7 N4 medium	D18 mat. medium	D18 mat. medium	Start	D7 N4 medium	D18 mat. medium	D18 mat. medium	Start	D7 N4 medium	D18 mat. medium	D18 mat. medium
TUJ1	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
GFAP	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
MAP2	NT	NT	Absent	NT	NT	NT	Absent	NT	NT	NT	Absent	NT
NeuN	NT	NT	Absent	Absent	NT	NT	Absent	Absent	NT	NT	Absent	Absent
Peripherin	NT	NT	Absent	NT	NT	NT	Absent	NT	NT	NT	Absent	NT
Synapsin	NT	NT	Absent	Absent	NT	NT	Absent	Absent	NT	NT	Absent	Absent
P75	Absent	NT	Absent	NT	Absent	NT	Absent	NT	Absent	NT	Absent	NT
Nkx 2.2	Absent	NT	Absent	NT	Absent	NT	Absent	NT	Absent	NT	Absent	NT
PAX6	Absent	NT	Absent	NT	Absent	NT	Absent	NT	Absent	NT	Absent	NT
SOX2	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Keratin1	Absent	NT	Absent	Absent	Absent	NT	Absent	Absent	Absent	NT	Absent	Absent
P4HA1 (5B5)	Present	NT	Present	Present	Present	NT	Present	Present	Present	NT	Present	Present

B



C





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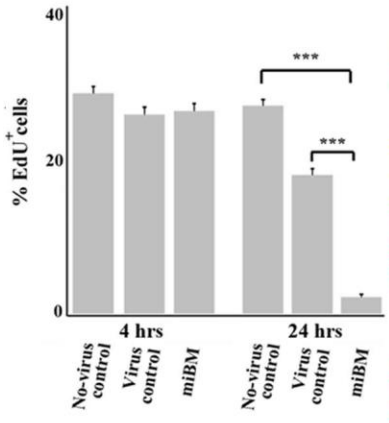

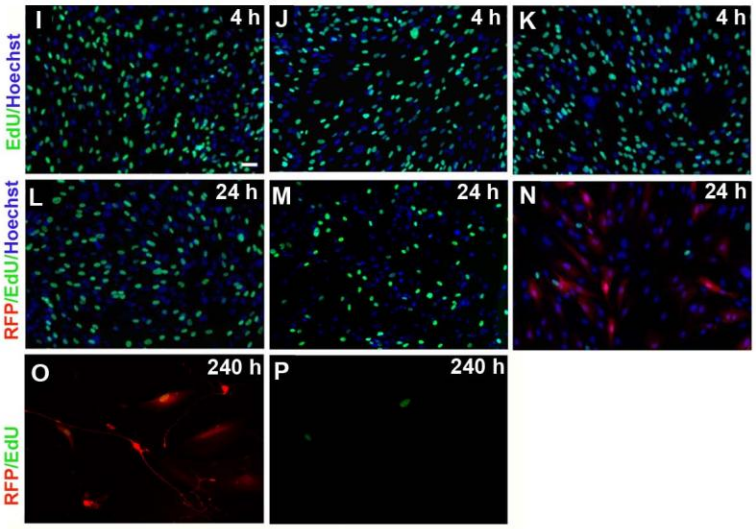

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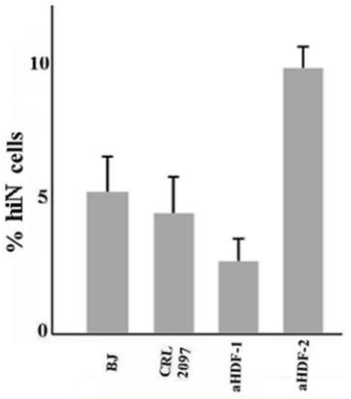
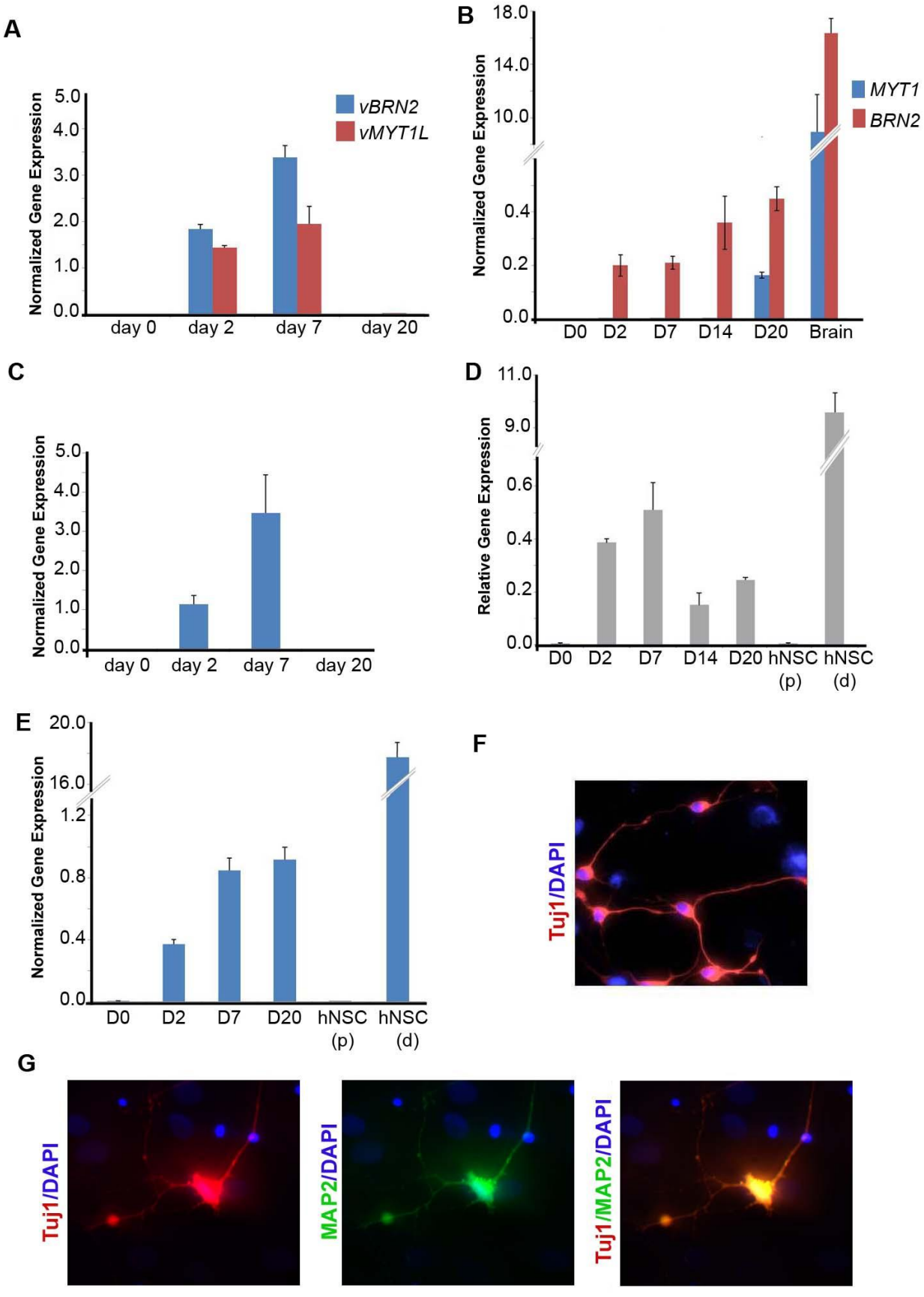


FIGURE S2 AMBASUDHAN et al.



Supplemental Figure Legends

Figure S1, related to Figure 1. Characterization of Fibroblast Populations (BJ, CRL 2097, aHDF-1, aHDF-2) and Further Details of hiN Cell Conversion Process

(A) Passage 2 fibroblasts were fixed and stained for immunoreactivity to the antigens listed. Assessments were made on the starting material ("Start," defined as the day after initial splitting of P1 cells and culture in fibroblast medium), at one week (in D7 N4 medium), and at 18 days (representing an additional 10 days in maturation (mat.) medium). At all of these stages, BJ, CRL2097, aHDF-1 and aHDF-2 cells exhibited immunoreactivity to the fibroblast marker P4HA1, but no reactivity to neural progenitor cell or early neuronal markers (GFAP, SOX2, PAX6, Tuj1), peripheral/spinal neuronal markers (p75, PAX6, Nkx 2.2, Peripherin), other mature neuronal or astroglial markers (MAP2, NeuN, Synapsin or GFAP), or an epidermal keratinocyte marker (Keratin1). Similar results were obtained when cells that had been transduced with GFP-control virus were subjected to immunostaining for the above markers. All antibodies were validated with appropriate positive controls.

(**B)** Quantitative reverse transcription (qRT)-PCR analysis of cDNAs prepared from total RNA isolated after the second passage of BJ, CRL2097, aHDF-1 and aHDF-2 cells, or from positive controls (consisting of normal human whole brain tissue, normal human melanocytes, and normal human keratinocytes). While *KRT1*, *PAX6*, *SOX1*, *SOX10* and *p75* mRNA expression was detected in each of their respective positive controls, no expression was detected in any of the fibroblast samples. As expected, all the fibroblast samples manifested considerable expression levels of the fibroblast marker *P4HA1*. Data are presented as mean $\pm$ SEM of each sample in triplicate (normalized to the expression level of the ubiquitously expressed housekeeping gene *GAPDH*).

(**C)** Time-lapse images from live BJ cells infected with control GFP-virus and subjected to the same culture conditions as 3F-infected cultures (see Fig. 1). Cells were followed from 48 hours through day 20. Note the clear absence of neuronal morphology in these cells.

(D and **E)** miR-124—infected fibroblasts occasionally exhibited Tuj1$^+$ cells with cell bodies having multiple processes when examined on day 18 post-infection.

(F) With a combination of miR-124 and *BRN2,* Tuj1$^+$ cells manifested a conspicuous increase in the number of processes emanating from the soma within 18 days post-infection.

(G) Fibroblasts infected with a combination of miR-124 and *MYT1L* displayed Tuj1$^+$ cells with an elongated morphology. However, none of the above combinations resulted in generation of cells with a characteristic neuronal morphology or other neuronal properties.

(H) Estimation of EdU positive cells in 3F (mi*BM*)-transduced cultures that received a two-hour pulse of EdU at either 2 hr or 22 hr post-infection. Data are presented as mean ± SEM of percentage of EdU$^+$ cells from 10 random fields in 3 independent experiments. ***$p < 0.001$ (two-tailed Student's *t*-test).

(I-K) Uninfected control cultures (**I**), FUW rtTA (control)-infected (**J**), and 3F-infected cultures (**K**) showed comparable numbers of EdU$^+$ nuclei 4 hr after infection.

(L-N) Dramatic reduction in EdU$^+$ nuclei in 3F-infected cultures (**L**) compared to uninfected controls (**M**) or FUW rtTA-infected controls (**N**) 24 hr after infection, suggesting that the majority of cells were already post-mitotic by this time.

(O and **P)** When cultures were examined after 9 days of continuous exposure to EdU, starting 24 hr post-infection, RFP-positive cells destined to become hiN cells incorporated virtually no EdU. Merged image of EdU staining and RFP fluorescence is shown in **O**. Note RFP$^+$ cells developing neuronal morphology are negative for EdU staining.

(Q) Efficiency of hiN cell conversion from fibroblasts, estimated 18 days after infection with 3F (mi*BM*). The percentage of hiN cells was calculated by scoring 20 randomly-selected fields (see Extended Experimental Procedures). Data are presented as mean percent ± SEM of 3 independent experiments. Red: RFP; green: GFP **(C)**, Tuj1 **(D, E, F, G)**, EdU **(I, J, K, L, M, N, O, P)**; blue: Hoechst **(I, J, K, L, M, N)**. Scale bars: 20 μm.

Figure S2, related to Figures 1 and 3. Evaluation of Endogenous and Transgenic *miR-124, BRN2 and MYT1l* Expression in BJ Cells During the Course of hiN Cell Generation

BJ cells were infected with lentivirus carrying inducible vectors in which transgene expression is under the control of the tetracycline operator (for *Brn2 and Myt1l*) or cumate operator (for miR-124). Both Doxycycline and cumate were discontinued after seven days. Quantitative reverse transcription (qRT)-PCR analysis was performed using cDNAs prepared from total RNA isolated from infected cells at the indicated time points. All expression levels, unless otherwise specified, were normalized to the expression levels of *GAPDH* expression.

(A) Expression levels of *BRN2* and *MYT1L* transgenes were silenced by day 20.

(B) Expression of endogenous *BRN2* and *MYT1L* at the indicated time points.

(C) Expression levels of virally encoded miR-124, indicating silencing of the transgene by day 20 post-infection.

(D) Relative expression levels of total miR-124 at various stages of hiN induction and in human neural stem/progenitor cells (hNSCs). Expression levels of miR-124 in proliferating (p) or differentiating (d) hNSCs were used as controls.

(E) Normalized expression levels of *BAF53b*, a down-stream target of miR-124, indicating miR-124 activity at various stages of hiN induction and in hNSCs. BAF53b expression is limited to cells that are committed to or have already undergone neuronal differentiation.

(F) hiN cells displayed immunoreactivity to Tuj1 antibody, when stained six days after the withdrawal of doxycycline and cumate.

(G) hiN cells displayed immunoreactivity to Tuj1 and MAP2 antibodies, when stained eighteen days after the withdrawal of doxycycline and cumate. Many of these cells also fired repetitive trains of action potentials (see **Figure 3E**, *right panel*). Data are presented as mean ± SEM; experiments were performed in triplicate.

Red: Tuj1; Green: MAP2; Blue: DAPI. Scale bar: 20 μm.

Table S1, Ambasudhan et al.

List of Transcription Factors that were Tested for Reprogramming.

ASCL1	NM_004316.3
BRG1	NM_001128844.1
BMI1	NM_005180.6
BRN2	NM_005604.2
EMX2	NM_001165924.1
MYT1L	NM_015025.2
HES6	NM_001142853.1
PRKCI	NM_002740.5
PAX6	NM_000280.3
SOX1	NM_005986.2
SOX2	NM_003106

Table S2, Ambasudhan et al.

Electrophysiological Membrane Properties of hiN Cells

A Membrane properties of neonatal fibroblast derived hiN cells

		Mean	*SEM*
Cap	*n = 21*	*30.50034*	*± 5.46654*
Rm	*n = 21*	*405.97586*	*± 56.68332*
Ra	*n = 21*	*20.07241*	*± 1.68852*

B Membrane properties of adult fibroblast derived hiN cells

		Mean	*SEM*
Cap	*n = 20*	*35.9135*	*± 11.52301*
Rm	*n = 20*	*1067.315*	*± 347.43712*
Ra	*n = 20*	*19.56*	*± 1.39498*

Cap: capacitance; Rm: membrane resistance; Ra: access resistance.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Lentiviral Constructs and Infection

Complementary DNAs for transcription factors were purchased from Open Biosystems or Invitrogen. These cDNAs were subcloned into the Lentiviral vector pLenti 6.2 V5-dest (Invitrogen). The miR-124 precursor sequence was cloned into cumate inducible vector pCDH-CuO-MCS -IRES-RFP (System Biosciences). Doxycycline inducible plasmids pBrn2-TetO-FUW and pMyt1l-TetO-FUW were a kind gift from Marius Wernig (Stanford University). The trans-activator encoding vectors pCDH-EF1-CymR-T2A-Puro and FUW-rtTA were purchased from System Biosciences and Addgene, respectively. pLemir-miR-124 vector encoding miR124 sequence was purchased from Open Biosystems. Viral packaging was performed in 293T cells, and fibroblast cells were infected as previously described (Lin et al., 2009).

Cell Culture and hiN Cell Generation

Human primary fibroblast cells, including BJ, CRL2097 (Foreskin dermal fibroblast; both from ATCC), aHDF-1 (ScienCell) and aHDF-2 (PromoCell), were cultured in DMEM containing 10% FBS, MEM non-essential amino acids, Glutamax and 5 mM HEPES. For all the experiments, we used cells of early passage number (P2-P5). For 24-well plates, 1.5 x 10^4 cells per well were plated and for 6-well plates, 5 x 10^4 cells per well, and cultured overnight on polylysine (Sigma) or polyornithine plus laminin (Sigma)-coated dishes prior to infection with lentiviral particles. The infected cells were then placed in fibroblast medium for 24 hours before changing to N4 medium (DMEM:F12, N2 supplement, B27 supplement, 5 mM HEPES, 0.5% Albumax, 0.6% glucose and MEM non-essential amino acids (all from Invitrogen), plus 20 ng ml^{-1} bFGF (R&D

Systems) and 100 ng ml^{-1} human-recombinant Noggin (Stemgent)). Doxycycline (1.5 μg ml^{-1}, Sigma) and Cumate solutions (40 μg ml^{-1}, System Biosciences) were added as indicated at each media change (every other day). Subsequently, cells were cultured in neuronal maturation medium (DMEM:F12, N2 supplement, B27 supplement, 5 mM HEPES, 0.5% Albumax, 0.6% glucose and non-essential amino acids (all from Invitrogen), plus 20 ng ml^{-1} GDNF (R&D Systems), 10 ng ml^{-1} BDNF (R&D Systems) and 3 μM Forskolin (Tocris)) until they were used for electrophysiology experiments or fixed for immunostaining.

For calculating the efficacy of hiN cell conversion from fibroblasts, we used the following technique. The number of hiN cells was calculated by scoring 20 randomly-selected microscopic fields under a 20x objective. The total surface area of the field was then measured, allowing us to estimate the density of neurons per field and thus estimate the total number of neurons in the entire well. These numbers were then further validated by counting the total number of hiN cells present in the entire well from two random wells, which were in good agreement with the former method. This number was then divided by the total number of cells seeded in the well in order to obtain an estimate of the percentage of conversion.

Antibodies, Immunostaining and EdU Labeling

Primary antibodies used included: anti-Tuj1 (Covance, 1:1000), chicken anti-MAP2 (Abcam, 1: 5000), mouse anti-NeuN (Millipore, 1:100), rabbit anti-Synapsin1 (Millipore, 1: 2000), rabbit anti-GABA (Sigma, 1: 1000), guinea pig anti-VGLUT1 (Synaptic Systems, 1: 2000), mouse anti-VGAT (Synaptic Systems,1: 500), mouse anti-TH (Sigma, 1:1000), mouse anti-peripherin (Millipore, 1:50), mouse anti-prolyl 4 hydroxylase (5B5) (Abcam, 1:50), rabbit anti-Keratin-1 (Covance, 1:750), mouse anti-p75 (Advanced Targeting System, 1:1000), rabbit anti-Nkx2.2

(Santa Cruz, 1:100), rabbit anti-GFAP (DAKO, 1:1000), rabbit anti-PAX6 (Covance, 1:500), and mouse anti-SOX2 (Millipore, 1:200). Alexa-488— and Alexa-555—conjugated secondary antibodies were purchased from Invitrogen. Immunostaining was performed as previously described (Lin et al., 2009). For the experiments assessing cell proliferation, 10 μM EdU was added to the culture medium at the specified time, and either pulsed for two hours or maintained throughout the culture as indicated. EdU staining was performed as per the manufacturer's instructions (Invitrogen), and cells were counterstained with Hoechst 33258 to identify nuclei.

qRT-PCR

RNA was isolated from hiN cells, human primary melanocytes (Lifeline cell technology), and human primary keratinocytes (ZenBio) using an RNeasy minikit (Qiagen) or High Pure miRNA isolation kit (Roche); 1 μg of each sample was reverse transcribed using iScript (BioRad). For miRNA quantification, cDNAs were synthesized using a QantiMir kit (System Biosciences) following the manufacturer's instructions. Gene expression analysis for cell-type specific markers, miRNAs and transgenes were performed with real-time PCR using the iQ SYBR Green supermix (BioRad). The data from each sample were normalized to *GAPDH* and plotted as relative expression. Primers sequences are as follows: *Sox1*-F: TCTGTTAACTCACCGGGACC,

Sox1-R: ACTCCAGGGTACACACAGGG; *SOX10*-F: CGCTTGTCACTTTCGTTCAG, *SOX10*-R: CCTTCATGGTGTGGGCTC; *P75*-F: CAG GCT TTG CAGCACTCAC, *P75*-R: CTGCTGCTGTTGCTGCTTCT;

PAX6-F: TCCGTTGGAACTGATGGAGT, *PAX6*-R: GTTGGTATCCGGGGACTTC; *P4HA1*-F: CAAAGACTGGGG AAGCAGAA, *P4HA1*-R: CCTCTCGTCCCACTTTCCA; *KRT1*-F: GCTCCCATT TTGTTTGCA GT, *KRT1*-R: GTCTCG AGAAAGGGAGCA AA; *BRN2-Trans-F*: CTGGAGAAGGAGGTGGTGAG, *BRN2-Trans-R*:

ATCGAATTCGCCCTTATTCC; *BRN2-Endo-F*: ATCGGAGAGAGTTGAAGCCA, *BRN2-Endo-R*: CCTCGTAAGGGGAATGTGAA; *MYT1L-Trans-F*: AAAGCAGGCTGTGAGAGGAA, *MYT1L-Trans-R*: GGGCCACAACTCCTCATAAA; *MYT1L-Endo-F*: CGGCAACAGTGCAGAGTG, *MYT1L-Endo-R*: CGTCTGTGAGACCAACTGGA; *miR-124matureTotal-F*: TAAGGCACGCGGTGAATGCC; *miR124-Cum-Trans-F*: CAAAGAGCCTTTGGAAGACG, *miR124-Cum-Trans-R*: GGAAGAGGGGTGGGTAGAAG; *BAF53b-F*: CACGTCAAGTCTGAGCCAAA, *BAF53b-R*: CAGCTCTGTCAGCTTCTCCC

Electrophysiology

Twenty-four hours after viral transduction, infected cells were trypsinized and plated on polyornithine and laminin coated glass coverslips (12 mm) and then further cultured in neuronal maturation medium for the times indicated in the figure legends. Coverslips were placed in the recording chamber mounted on an Olympus IX 71 microscope. Spontaneous or evoked responses were recorded at room temperature ($22 \pm 1\ ^{o}C$) via whole-cell recording with a patch electrode. Signals were amplified using an Axopatch200B (Axon Instruments) and filtered at 2 KHz via a Bessel low-pass filter. Data were sampled and analyzed using pClamp10.1 software in conjunction with a DigiData interface (Model 1322A, Axon Instruments). Patch pipettes were pulled from standard wall glass of 1.5 mm OD (Warner Instruments) and had input resistances of 5-11 $M\Omega$.

In general, for recording voltage-gated currents and action potentials, patch electrodes were filled with the following solution (in mM): 140 K-gluconate, 5 NaCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES, 10 EGTA; pH adjusted by KOH to 7.25, osmolarity measured at 290 mOsm. For isolation of Na^+ current from K^+ current, cesium was substituted for potassium as the major cation in the patch pipette-filling solution in order to suppress K^+ currents. The composition of the intracellular solution used for recording ligand-gated currents was as follows (in mM): 130 Cs-

gluconate; 2 MgATP, 1 $MgCl_2$; 10 EGTA; 10 HEPES; pH 7.25, osmolarity 300 mOsm. The bath solution generally contained a Na^+ saline based upon Hanks' balanced salt solution (pH 7.3). Mg^{2+} was omitted when recording NMDA-evoked responses.

To monitor voltage-gated currents, after an initial pre-hyperpolarization to -90 mV for 300 ms to relieve inactivation, we applied step potentials ranging from -60 to +30 mV for 100 ms. Drugs were prepared in bath solution and applied by an array of microtubes placed 75-100 μm from the cells. Solutions changes were achieved rapidly, within 50-100 ms, by moving the array of constantly flowing pipette tips relative to the cell with a micromanipulator driver. A control pipette containing bath solution was used to rapidly wash off applied drugs. *N*-methyl-D-aspartate (NMDA) and tetrodotoxin (TTX) were purchased from Tocris; γ-aminobutyric acid (GABA) was purchased from Sigma; and 2, 3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and bicuculline were obtained from Ascent Scientific. For recording spontaneous synaptic activity, currents were monitored under voltage clamp in the presence of 1 μM TTX at a holding potential of -80 mV.

Supplemental References

Lin, T., Ambasudhan, R., Yuan, X., Li, W., Hilcove, S., Abujarour, R., Lin, X., Hahm, H.S., Hao, E., Hayek, A., and Ding, S. (2009). A chemical platform for improved induction of human iPSCs. Nat. Methods 6, 805–808.