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Conversion of human induced Pluripotent Stem Cells (iPSCs) into functional spinal and cranial motor neurons using piggyBac vectors

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TITLE:

Conversion of Human Induced Pluripotent Stem Cells (iPSCs) into Functional Spinal and Cranial Motor Neurons Using piggyBac Vectors

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KEYWORDS:

induced pluripotent stem cells, motor neurons, piggyBac, differentiation, transcription factors, Phox2a, Isl1, Ngn2, Lhx3.

SHORT ABSTRACT:

This protocol allows rapid and efficient conversion of induced pluripotent stem cells into motor neurons with a spinal or cranial identity, by ectopic expression of transcription factors from inducible piggyBac vectors.

LONG ABSTRACT:

We describe here a method to obtain functional spinal and cranial motor neurons from human induced pluripotent stem cells (iPSCs). Direct conversion into motor neuron is obtained by ectopic expression of alternative modules of transcription factors, namely Ngn2, Isl1 and Lhx3 (NIL) or Ngn2, Isl1 and Phox2a (NIP). NIL and NIP specify, respectively, spinal and cranial motor neuron identity. Our protocol starts with the generation of modified iPSC lines in which NIL or NIP are stably integrated in the genome via a piggyBac transposon vector. Expression of the transgenes is then induced by doxycycline and leads, in 5 days, to the conversion of iPSCs into

MN progenitors. Subsequent maturation, for 7 days, leads to homogeneous populations of spinal or cranial MNs. Our method holds several advantages over previous protocols: it is extremely rapid and simplified; it does not require viral infection or further MN isolation; it allows generating different MN subpopulations (spinal and cranial) with a remarkable degree of maturation, as demonstrated by the ability to fire trains of action potentials. Moreover, a large number of motor neurons can be obtained without purification from mixed populations. iPSC-derived spinal and cranial motor neurons can be used for in vitro modeling of Amyotrophic Lateral Sclerosis and other neurodegenerative diseases of the motor neuron. Homogeneous motor neuron populations might represent an important resource for cell type specific drug screenings.

INTRODUCTION:

Motor neuron (MN) degeneration plays a causative role in human diseases such as Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA). Establishing suitable in vitro cell model systems that recapitulate the complexity of the human MN is an important step towards the development of new therapeutic approaches. Induced pluripotent stem cells (iPSCs), which are endowed with remarkable plurilineage differentiation properties, have now been derived from a number of patients affected by motor neuron diseases[1,2]. Additional iPSC lines carrying pathogenic mutations associated to MN diseases have been generated by gene editing, starting from control "healthy" pluripotent stem cells[3]. These lines represent useful tools for in vitro disease modeling and drug screening, provided that appropriate methods for iPSC differentiation into MNs are available. The rationale behind the development of this method is to provide the scientific community interested in MN diseases with a fast and efficient differentiation protocol giving rise to mature functional MNs. The first advantage of this method is its timeframe of execution. Another relevant point of strength comes from the elimination of any purification step. Finally, the protocol can be used to generate two distinct populations of motor neurons.

The possibility of generating different subtypes of MNs is particularly relevant for modeling of MN diseases. Not all MN subtypes are equally vulnerable in ALS and SMA and the onset of symptoms in different motor units greatly influences the prognosis. In ALS, spinal onset with symptoms starting in upper and lower limbs leads to death in about 3–5 years[4]. Conversely, bulbar onset, starting with degeneration of cranial MNs, has a worst prognosis. Moreover, the percentage of bulbar onset is significantly higher in patients with mutations in the RNA-binding proteins FUS and TDP-43 than in individuals with SOD1 mutations[5]. Almost the totality of alternative MN differentiation protocols relies on the activity of retinoic acid (RA), which confer a spinal character to differentiating iPSCs[6,7,8]. This limits the possibility of studying intrinsic factors, which could be protective in specific MN subtypes[9,10].

Consistent with a previous work in mouse embryonic stem cells[11], we have recently shown that in human iPSCs ectopic expression of Ngn2, Isl1 and Lhx3 (NIL) induces a spinal MN identity, while Ngn2 and Isl1 plus Phox2a (NIP) specify cranial MNs[12]. We have hence developed an efficient protocol, leading to the production of human MNs endowed with functional properties in a 12 days turnaround. The purpose of this method is to obtain, in a short time frame and

without the need for purification (e.g., by FACS), cell populations highly enriched for MNs with spinal or cranial identity.

PROTOCOL:

1. Maintenance of human iPSCs

1.1. Preparation of matrix-coated plates

1.1.1. Thaw one 5 mL vial of matrix (see **Table of Materials**) at 4 °C overnight. The original matrix stocks come at different stock concentrations and aliquots are made according to the dilution factor indicated on the datasheet, specific for the individual lot. It is important to keep the vial and tubes ice cold to prevent premature gelling of the matrix. Dispense matrix into aliquots in pre-chilled cryotubes on ice. Freeze unused aliquots at –20 °C.

1.1.2. Place one aliquot on ice for about 2 h to thaw.

1.1.3. Dilute the aliquot of matrix with 20 mL of cold DMEM/F12 in a 50 mL conical tube.

1.1.4. Mix well and dispense 1 mL of diluted matrix into 35 mm dishes (equivalent amounts per surface area of other dishes).

1.1.5. Keep the dishes containing diluted matrix for 1 h at room temperature to allow coating.

NOTE: Dishes, sealed with parafilm, can be stored at 4 °C for up to 2 weeks.

1.2. **Preparation of the stock solution (20 mL) and 1x working aliquots of the gentle cell dissociation reagent** (see **Table of Materials**).

1.2.1. Dissolve powder to 10 mg/mL in PBS (Ca^{2+}/Mg^{2+} free).

1.2.2. Filter sterilize through a 0.22 µm filter membrane.

1.2.3. Prepare 20 aliquots (1 mL each) and store at -20 °C.

1.2.4. Before use, dilute one aliquot in PBS (Ca^{2+}/Mg^{2+} free) to 1 mg/mL (1x working aliquots).

NOTE: 1x working aliquots can be stored at 4 °C for up to 2 weeks.

1.3. **Passaging human iPSCs.**

1.3.1. Before starting: If stored at 4 °C, pre-warm matrix-coated plates in the incubator at 37 °C for 20-30 min. Pre-warm at room temperature the amount of human iPSC medium (see **Table of Materials**) needed. Pre-warm the DMEM/F12.

1.3.2. Aspirate culture medium.

1.3.3. Rinse iPSCs with PBS (Ca^{2+}/Mg^{2+} free).

1.3.4. Add 1x gentle dissociation solution (0.5 mL for a 35 mm dish). Incubate at 37 °C until the edges of the colonies begin to detach from the plate, usually 3-5 min.

1.3.5. Aspirate the gentle dissociation solution, being careful not to detach iPSC colonies.

1.3.6. Wash the cells with DMEM/F12 (2 mL for a 35 mm dish) and aspirate being careful not to detach the cells. Repeat this step one more time.

1.3.7. Add human iPSC medium (1 mL for a 35 mm dish).

1.3.8. Gently detach the colonies off with a cell lifter and transfer to a 15 mL tube.

1.3.9. Gently break cell clumps by pipetting up and down with a P1000 pipettor 3-4 times.

1.3.10. Aspirate the supernatant from the matrix-coated plate(s).

1.3.11. Seed the cells in the appropriate culture volume of human iPSC medium. The split ratio can vary from line to line and is about 1:4-1:8. Change the medium daily.

2. Generation of NIL and NIP inducible iPSC lines

2.1. Cell transfection.

2.1.1. Rinse the cells with PBS (Ca^{2+}/Mg^{2+} free).

2.1.2. Add cell dissociation reagent (see **Table of Materials**) (0.35 mL for a 35 mm dish) and incubate at 37 °C until single cells are separated (5-10 min).

2.1.3. Gently complete cell separation by pipetting up and down with a P1000 pipettor 3-4 times.

2.1.4. Collect in a 15 mL tube and add PBS (Ca^{2+}/Mg^{2+} free) to 10 mL. Count the cells.

2.1.5. Pellet 10^6 cells and resuspend in 100 µl of Buffer R (included in the cell electroporation kit, see **Table of Materials**).

2.1.6. Add plasmid DNA for transfection: 4.5 µg of transposable vector (epB-Bsd-TT-NIL or epB-Bsd-TT-NIP[12]) and 0.5 µg of the piggyBac transposase plasmid[13].

2.1.7. Transfect with the cell electroporation system (see **Table of Materials**) according to manufacturer's instructions and as previously described[3] with the following parameters: 1200 V voltage, 30 ms width, 1 pulse. Seed the cells in human iPSC medium supplemented with 10 µM Y-27632 (ROCK inhibitor, see **Table of Materials**) in a 6 mm matrix-coated dish.

2.2. **Selection with antibiotics.**

2.2.1. Two days after transfection, add 5 µg/mL blasticidin to the culture medium.

2.2.2. Most of the non-transfected cells will die within 48 h of blasticidin selection. Keep the cells in blasticidin for at least 7-10 days to counter-select the cells that have not integrated the transgenes in the genome.

2.2.3. Maintain stably transfected cells as a mixed population, composed of cells with different number of transgenes and different integration sites, or isolate single clones.

2.2.4. Prepare an additional dish to check for effective expression of the transgenes, upon 1 µg/mL doxycycline induction, by RT-PCR with transgene-specific primers for Ngn2 (Forward: TATGCACCTCACCTCCCCATAG; Reverse: GAAGGGAGGAGGGCTCGACT), as previously described[12].

2.2.5. At this stage, freeze stocks of the novel NIL- and NIP-iPSC lines in freezing medium for human iPSCs (see **Table of Materials**).

3. Motor neuron differentiation

3.1. Dissociate the cells with cell dissociation reagent as described (steps 2.1.1.-2.1.3.). Collect dissociated cells in a 15 mL tube and dilute with 5 volumes of DMEM/F12. Pellet the cells and resuspend in human iPSC medium supplemented with 10 µM ROCK inhibitor. Count the cells and seed on matrix-coated dishes at a density of 62,500 cells/cm^2.

3.2. The day after, replace the medium with DMEM/F12, supplemented with 1x stable L-glutamine analogue, 1x non-essential amino acid (NEAA) cell culture supplement and 0.5x penicillin/streptomycin, and containing 1 µg/mL doxycycline. This is considered as day 0 of differentiation. On day 1, refresh the medium and doxycycline.

3.3. On day 2, change the medium to Neurobasal/B27 medium (Neurobasal Medium supplemented with 1x B27, 1x stable L-glutamine analogue, 1x NEAA and 0.5x penicillin/streptomycin), containing 5 µM DAPT, 4 µM SU5402 and 1 µg/mL doxycycline (see **Table of Materials**). Refresh the medium and doxycycline every day until day 5.

3.4. **Day 5: Cells dissociation with cell dissociation reagent** (see **Table of Materials**).

3.4.1. Rinse the cells with PBS (Ca^{2+}/Mg^{2+} free).

3.4.2. Add cell dissociation reagent (0.35 mL for a 35 mm dish) and incubate at 37 °C until the entire cell monolayer separates from the dish. Note that single cells will not be separated during incubation.

3.4.3. Add 1 mL of DMEM/F12 and collect the cells in a 15 mL tube.

3.4.4. Gently complete cell separation by pipetting up and down with a P1000 pipettor 10-15 times.

3.4.5. Add 4 mL of DMEM/F12 and count the cells.

3.4.6. At this stage, freeze motor neuron progenitors in cell freezing medium (see **Table of Materials**), according to manufacturer instructions.

3.4.7. Pellet the cells and resuspend in Neuronal Medium (Neurobasal/B27 medium supplemented with 20 ng/mL BDNF, 10 ng/mL GDNF and 200 ng/mL L-ascorbic acid, see **Table of Materials**) supplemented with 10 µM ROCK inhibitor.

3.4.8. Seed the cells on poly-ornithine/laminin- or alternatively on matrix-coated supports at the density of 100,000 cells/cm^2. Use µ-Slide plastic supports with polymer coverslip (see **Table of Materials**) for immunostaining analysis.

3.5. On day 6, change the medium with fresh Neuronal Medium devoid of ROCK inhibitor. On the next days, refresh half of the medium every 3 days. Culture medium must be changed very carefully in order to prevent detachment from the surface.

4. Immunostaining analysis

4.1. Cell fixation. Rinse the cells with PBS (with Ca^{2+}/Mg^{2+}) and incubate for 15 min in 4% paraformaldehyde in PBS (with Ca^{2+}/Mg^{2+}) at room temperature.

CAUTION: Paraformaldehyde is toxic and suspected to cause cancer. Avoid contact with skin and eyes and handle under a chemical fume hood.

4.2. Permeabilize with PBS (with Ca^{2+}/Mg^{2+}) containing 0.1% Triton X-100 for 5 min at room temperature.

4.3. Incubate for 30 min at room temperature in antibody blocking solution (ABS: 3% BSA in PBS with Ca^{2+}/Mg^{2+}).

4.4. Incubate for 1 h at room temperature with primary antibodies in ABS: anti-TUJ1 (1:1000; rabbit) and anti-Oct4 (1:200; mouse) or anti-CHAT (Anti-Choline Acetyltransferase; 1:150; goat). See **Table of Materials**.

4.5. Incubate for 45 min at room temperature with appropriate donkey secondary antibody pair in ABS: anti-mouse Alexa Fluor 647 (1:250), anti-rabbit Alexa Fluor 594 (1:250) and anti-goat Alexa Fluor 488 (1:250). See **Table of Materials**.

4.6. Incubate in 0.4 µg/mL DAPI for 5 min at room temperature to label nuclei.

4.7. Mount the cells with Mounting Medium (see **Table of Materials**) for imaging at a fluorescence microscope.

5. Functional characterization via patch-clamp recordings

5.1. Prepare the HEPES-equilibrated external solution (NES) as following: 140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose. Set the osmolarity between 290-300 mΩ. Adjust the pH to 7.3 using 1N NaOH and store the solution at 4 °C.

5.2. Prepare the internal solution: 140 mM K-gluconate, 2 mM NaCl, 5 mM BAPTA, 2 mM $MgCl_2$, 10 mM HEPES, 2 mM Mg-ATP, 0.3 mM Na-GTP. Adjust the pH to 7.3 with 1M KOH and check that the osmolarity is set at around 290 mΩ. Freeze the solution at -20 °C in small aliquots.

5.3. Before running the experiments, pre-warm the NES solution in a water bath to about 28-30 °C.

5.4. Pull some borosilicate micropipettes (ID 0.86 mm; OD 1.5 mm) bearing a tip resistance: 5-6 MΩ and fill with the intracellular solution before mounting into the pipette holder.

NOTE: Remember to chloride silver wires of the recording electrode and reference electrode in bleach for at least 30 min in order to form a uniform layer of AgCl on the wire surface.

5.5. Transfer the Petri dish in the recording chamber and let the chamber with the NES solution at 1-2 mL/min. Let the flow passing through an inline heater set at temperature of 30 °C in order to keep the solution warm.

5.6. Place electrophysiological recording chamber under an upright microscope. Record membrane currents with the patch-clamp amplifier and acquire data with an appropriate software.

5.7. Open the amplifier control software, and set the signal gain at value 1 and the Bessel filter at 10 kHz. Ensure that the Bessel filter is 2.5 times lower than the sampling frequency.

5.8. Set the experimental protocols for voltage-clamp and current-clamp experiments in the recording software.

5.9. In the protocol setting, tick episodic stimulation mode and set the sampling frequency at 25 kHz. Then, move to the waveform tab and type the voltage or current step amplitude and length as follow.

5.10. For the voltage-gated sodium currents, use 15 voltage steps (50 ms duration each) from −100 mV to +40 mV (10 mV increment). Run the protocol after imposing to the patched cell a holding potential of -60 mV through the amplifier. Similarly, the voltage-gated potassium currents are evoked by voltage steps (250 ms duration each) from −30 mV to +50 mV (10 mV increment) holding the recorded cell to -40 mV.

5.11. For investigating the firing properties of iPSC-derived cranial and spinal MNs, clamp cells, in current-clamp mode, at a membrane potential of -70 mV and use 4 current pulses (1 s duration each) of increasing amplitude (from +20 pA to +80 pA; 20 pA increment).

5.12. Acquire for each cell voltage-activated currents, evoked firing activity and three passive properties as whole-cell capacitance (Cm), cell membrane resistance (Rm) and Resting Membrane Potential (RMP).

REPRESENTATIVE RESULTS:

A schematic description of the differentiation method is shown in **Figure 1**. Human iPSCs (WT I line[3]) were transfected with epB-Bsd-TT-NIL or epB-Bsd-TT-NIP, generating, upon blasticidin selection, stable and inducible cell lines[12], hereafter referred to as iPSC-NIL and iPSC-NIP, respectively. Differentiating cells were characterized for the expression of the pluripotency marker OCT4 and the pan-neuronal marker TUJ1. Immunostaining analysis showed uniform expression of OCT4 in all cells at day 0, in the absence of TUJ1 positivity (**Figure 2A**). At day 3, we observed a strong decrease in the number of OCT4-positive cells, mirrored by expression of TUJ1 in a subset of differentiating iPSCs (**Figure 2B**). At day 5, no expression of OCT4 was observed in the population, which showed consistent expression of TUJ1 and acquired a neuronal morphology (**Figure 2C**). Motor neuron progenitors were then dissociated and re-plated for further maturation. After 7 days (12 days since day 0), cells uniformly expressed TUJ1 and the mature motor neuron marker CHAT (**Figure 3**). Similar results have been obtained with two additional commercial iPSC lines (see **Table of Materials**), using the same culture and differentiation conditions (**Supplementary Figure S1** and **Supplementary Figure S2**). Similarly to NIL-induced motor neurons from mouse ESCs[11], human iPSCs induced with NIL and NIP expressed low levels of HOX transcription factors and can be patterned along the rostro-caudal axis with retinoic acid (**Supplementary Figure S3**).

These results were obtained when 62,500 cells/cm^2 were seeded at the beginning of differentiation and induced with 1 µM doxycycline at day 0. This resulted the optimal concentration of doxycycline to achieve maximum induction of the transgenes without evident toxicity for the cells (**Supplementary Figure S4A**). Upon removal of doxycycline at day 5, expression of the transgenes was silenced (**Supplementary Figure S4B**). We have also performed pilot experiments to establish the optimal density of the cells at this point of the protocol. We noticed that varying this parameter in parallel differentiation experiments

provided different outcomes. With initial density lowered to 31,250 cells/cm^2, differentiation was apparently normal, as evaluated by observing cell morphology. However, we noticed resistance to dissociation at day 5 (section 3.4) and reduced viability in the subsequent maturation phase. Conversely, when the initial density of the cells was raised to 125,000 cells/cm^2, we observed inefficient differentiation, as assessed by lack of acquisition of the typical neuron-like morphology. This resulted in a mixed population containing only a minor fraction of MNs, which would need further purification (e.g., by FACS). We have therefore established that the optimal density to obtain a pure population of neuronal cells, able to survive in culture for more than two months, is 62,500 cells/cm^2.

We then assessed the functional maturation of the iPSC NIL-derived spinal motor neurons by characterizing their electrophysiological properties (**Figure 4**), as previously reported for iPSC NIP-derived cranial motor neurons[12]. Patch clamp recordings, in either voltage- and current-clamp modality, were performed at day 7 of the MNs maturation step of the protocol (see **Figure 1**; total time of differentiation: 12 days) (**Figure 4A**). At this time point, the iPSC NIL-derived motor neurons showed a slightly lower resting membrane potential (-30 ± 2 mV; n = 24) and a similar cell capacitance value (+25 ± 2 pF; n = 25) when compared to previously reported iPSC NIP-derived MNs[12]. Then, to deeper characterize the degree of maturation of differentiated cells, we investigated their ability to evoke sodium and potassium currents when stimulated with a series of voltage pulses. In these experiments, iPSC NIL-derived neurons successfully displayed voltage-dependent sodium currents (**Figure 4B**), and voltage-dependent potassium currents (**Figure 4C**), reaching peak amplitude when clamped at a membrane potential near −20 mV and +50 mV, respectively. The equilibrium potentials for Na^+ and K^+, calculated using the Nernst's equation (www.physiologyweb.com/calculators/nernst_potential_calculator.html) with the previously reported extracellular and intracellular solutions, were +110 mV and -102 mV respectively. In addition, the 80% of the iPSC NIL-derived MNs clamped in current-clamp modality were able to trigger spike trains when injected with a current pulse of +60 pA or more (**Figure 4D**). The minimum current required to elicit repetitive firing in more than 50% of recorded cells was +40 pA (15 out of 18 cells; **Figure 4E**). Spike threshold was -37.6 ± 0.8 mV and average firing frequency at +40 pA was about 7.9 ± 2.2 Hz (n = 18; **Figure 4F**).

Overall, these data suggest that, similarly to previously reported iPSC NIP-derived cranial MNs[12], iPSC-NIL-derived spinal MNs have functional properties typical of mature neurons.

FIGURE AND TABLE LEGENDS:

Figure 1: Motor neuron differentiation protocol. The figure shows a schematic representation of the differentiation protocol, from the generation of stable iPSC lines with the piggyBac vectors to the time point of the functional analysis reported in the text. Representative phase contrast images of the cells at different steps of the protocol are shown. Scale bar for all panels: 50 µm.

Figure 2: Representative immunostaining analysis of differentiating cells. iPSC-NIL and iPSC-

NIP cells were analyzed by immunostaining for the expression of the pluripotency marker OCT4 (purple) and the pan-neuronal marker TUJ1 (red) at day 0 (**A**), day 3 (**B**) and day 5 (**C**). Nuclei were counterstained with DAPI. Confocal images were acquired at the laser scanning confocal microscope (see **Table of Materials**) using a 20X NA 0.75 objective with zoom 2X, 1024 x 1024 pixel, equipped with 405 nm, 473 nm, 559 nm and 635 nm lasers. Filter setting for DAPI, Alexa Fluor 594 and Alexa Fluor 647 were used. Scale bar for all panels: 50 µm.

Figure 3: Representative immunostaining analysis of iPSC-derived motor neurons. iPSC-NIL and iPSC-NIP cells were analyzed by immunostaining for the expression of the pan-neuronal marker TUJ1 (red) and the motor neuron marker CHAT (choline acetyltransferase; green) at day 12. Nuclei were counterstained with DAPI. Confocal images were acquired as described in Figure 2 legend. Scale bar for all panels: 50 µm.

Figure 4: Functional analysis of spinal and cranial motor neurons. (**A**) Bright field image of whole-cell patch clamp on iPSC NIL-derived spinal motor neuron. (**B**) Representative I/V curve for Na^+ current recorded in iPSC NIL-derived MNs in response to a series of increasing voltage steps (n = 26; holding potential equal to -60 mV). (**C**) Representative I/V curve for K^+ recorded in iPSC NIL-derived MNs in response to a series of increasing voltage steps (n = 23; holding potential equal to -40 mV). (**D**) Representative trace of a train of action potentials evoked in response to a 1 s lasting current injection of +60 pA. (**E**) Histogram representing the percentage of iPSC NIL-derived MNs eliciting action potentials at each current pulse (n = 18). (F) Histogram displaying the evoked firing frequency at each current pulse (n = 18). Electrophysiological recording was performed under an upright microscope. The membrane currents recording system is indicated in the table of materials.

Supplementary Figure S1: MN differentiation using Episomal hiPSCs. (**A**) Brightfield images of differentiating Episomal hiPSC-NIL (left) and Episomal hiPSC-NIP (right) at the indicated time points. Scale bar for all panels: 50 µm. (**B**) Analysis of the expression of the indicated markers in differentiating Episomal hiPSC-NIL (top) and Episomal hiPSC-NIP (bottom) cells by real time qRT-PCR. For each marker, the time point with the highest expression has been used as calibrator sample. Primers used for ISL1 are specific for the endogenous gene. (**C**) Immunostaining for the pan-neuronal marker TUJ1 (red) and cranial MN marker PHOX2B (green) in differentiated (day 6) Episomal hiPSC-NIL (left) and Episomal hiPSC-NIP (right) cells. Nuclei are counterstained with DAPI. Scale bar for all panels: 50 µm. (**D**) Analysis of the expression of HB9 and PHOX2B in differentiating Episomal hiPSC-NIL (top) and Episomal hiPSC-NIP (bottom) cells by real time qRT-PCR. Day 0 has been used as the calibrator sample. PCR primers and methods are reported in De Santis et al., 2018[12].

Supplementary Figure S2: MN differentiation using DS2U iPSCs. (**A**) Brightfield images of differentiating DS2U-NIL (left) and DS2U-NIP (right) at the indicated time points. Scale bar for all panels: 50 µm. (**B**) Analysis of the expression of the indicated markers in differentiating DS2U-NIL (top) and DS2U-NIP (bottom) cells by real time qRT-PCR. For each marker the time point with the highest expression has been used as calibrator sample. Primers used for ISL1 are specific for the endogenous gene. (**C**) Immunostaining for the pan-neuronal marker TUJ1 (red)

and cranial MN marker PHOX2B (green) in differentiated (day 6) DS2U-NIL (left) and DS2U-NIP (right) cells. Nuclei are counterstained with DAPI. Scale bar for all panels: 50 µm. (**D**) Analysis of the expression of HB9 and PHOX2B in differentiating DS2U-NIL (top) and DS2U-NIP (bottom) cells by real time qRT-PCR. Day 0 has been used as the calibrator sample. PCR primers and methods are reported in De Santis et al., 2018[12].

Supplementary Figure S3: HOX gene expression. (**A**) Analysis of the expression of four different HOX genes (HOX A2, HOX B1, HOX A4, HOX B5) after 5 days of differentiation of iPSC-NIL and iPSC-NIL + RA cells by real time qRT-PCR. IPSC-NIL at day 0 has been used as the calibrator sample. (**B**) Analysis of the expression of four different HOX genes (HOX A2, HOX B1, HOX A4, HOX B5) after 5 days of differentiation of iPSC-NIP and iPSC-NIP + RA cells by real time qRT-PCR. IPSC-NIP at day 0 has been used as the calibrator sample. (C) PCR primer pairs.

Supplementary Figure S4: Doxycycline induction analysis. (**A**) Analysis by real time qRT-PCR of the expression of exogenous Ngn2 in iPSC-NIL (top) and iPSC-NIP (bottom) cells untreated or cultured for 24 h in presence of doxycycline at different concentration (0.5 µM, 1.0 µM, 2.0 µM). Ngn2 was analyzed with primers specific for the exogenous mouse gene. The parental iPSC line, devoid of NIL and NIP constructs, has been included in the analysis as a control. Expression of the transgenes in iPSC-NIL and iPSC-NIP was neglectable in absence of doxycycline. iPSC-NIL and iPSC-NIP at day 0 have been used as calibrator samples. (**B**) Analysis by real time qRT-PCR of the expression of exogenous Ngn2 in differentiating iPSC-NIL (left) and iPSC-NIP (right) cells at the indicated time points of the protocol. Ngn2 was analyzed with primers specific for the exogenous mouse gene. iPSC-NIL and iPSC-NIP at day 0 have been used as calibrator samples. PCR primers and methods are reported in De Santis et al., 2018[12].

DISCUSSION:

This protocol allows to efficiently convert human iPSCs into spinal and cranial motor neurons thanks to the ectopic expression of lineage-specific transcription factors. These transgenes are inducible by doxycycline and stably integrated in the genome thanks to a piggyBac transposon-based vector. In a mixed population, one or several copies of the piggyBac vector will be randomly integrated into the genome of individual cells, increasing the risk of genome integrity alterations. Moreover, a progressive selection of iPSC subclones may occur over time, with possible consequences for differentiation and for comparative analysis of disease and control cell lines. Altogether, iPSC-derived MNs obtained with this protocol will be unsuitable for regenerative medicine. However, our method could be particularly useful for in vitro motor neuron disease modeling. Major points of strength are represented by the extremely simplified culture conditions, the rapidity of MN conversion, the high degree of maturation of iPSC-derived MNs and the possibility to obtain both spinal and cranial MNs, as previously demonstrated by analysis of specific marker genes expression[12]. Robustness of the protocol is demonstrated by its reproducibility. So far, we have successfully applied this protocol more than 30 times for spinal and/or cranial MN generation, assessing the outcomes by marker and/or functional analyses. We have successfully maintained motor neuron cultures for up to two months without evident decrease of viability. Moreover, once the stably transduced iPSC line has been obtained, each experiment can be started by doxycycline induction without the

need of new transfection. Viral vectors are also not required. The representative results presented here have been obtained by electroporating iPSCs with the cell electroporation system indicated in the **Table of Materials**. However, other electroporation methods might represent alternative options. Conversely, in our experience, transfection systems based upon lipofection are not a good option for pluripotent stem cells[12]. Cell populations obtained at the end of the process are composed almost exclusively of MNs, avoiding the need for further purification (e.g., by FACS). As we previously showed[12], the protocol allows obtaining 90% TUJ1-positive cells, of which 95% where also PHOX2B positive in NIP-derived cultures.

We can envisage some critical points that must be accurately taken into consideration. First, the quality of the initial population of iPSCs is crucial to ensure homogeneous and consistent conversion into MNs. Cultures containing a substantial fraction of differentiation (more than 5-10%) must be avoided. We have set up the protocol using the human iPSC medium described in the table of materials as the maintenance medium for undifferentiated iPSCs. Other commercially available defined media might represent valid alternative options, despite we have not experimentally addressed this point in the present work. Since media composition may influence the proliferation rate of the starting cell population, adaptation of the protocol to other maintenance media might require optimization of the initial density at day 0. After transfection, it is important to keep cells under antibiotic selection for at least 2 weeks to obtain stable cell lines. It might be appropriate for some applications to derive clonal lines after piggyBac integration, in order to obtain a more homogeneous population in terms of levels of transgene expression and a better control of integration sites. Density of the cells at day 0, the time of doxycycline addition to the medium, is a crucial parameter. As mentioned in the Representative Results section, we estimated an optimal cell density to ensure reproducibility. We cannot exclude that other pluripotent stem cell lines might require different initial density, which should be empirically determined in pilot experiments, as the duplication rate can vary significantly between individual lines. Medium switch to Neurobasal/B27 must occur after 48 h since doxycycline induction (section 3.3): differentiation may result slower and less efficient if cells are not held for 2 whole days in the DMEM/F12 medium. Dissociation at day 5 must be performed without stressing differentiating cells. The time of incubation with the cell dissociation reagent (step 3.4) might differ from batch to batch and should be carefully estimated in pilot experiments. Upon incubation with the cell dissociation reagent, the entire cell monolayer detaches from the plate. Then, it must be carefully dissociated by pipetting, as described in section 3.4, to preserve cell integrity and to avoid mechanical stress, which could have a negative impact on the maintenance of cell culture beyond D5. Finally, we noticed that after re-plating MNs adhere better on tissue culture plastic than glass. Polymer coverslips ensuring optimal cell adherence and suitable optical properties are a good option for microscopy-based applications.

Our method represents a direct “programming” of pluripotent cells into a MN fate, and does not recapitulate the intermediate steps through which embryonic cells acquire a MN identity during in vivo development, such as initial specification to neural ectoderm and patterning along the dorso-ventral and rostro-caudal axes. Therefore, it would not be suitable to model human MN specification in vitro to study, for instance, molecular mechanisms underlying

differentiation. On the other hand, our protocol allows generating a considerable amount of spinal or cranial MNs without the need of further purification. Taking also in consideration the degree of maturation achieved, this represents a useful tool for studying the molecular basis of neurodegenerative diseases of the motor neuron. A consistent number of iPSC lines with pathogenic mutations in motor neuron disease-related genes has been produced by the scientific community in the last years. Collections of MN "programmable" iPSC lines could be therefore easily generated by stable integration of NIL and NIP modules in those mutant iPSC lines. We can envisage, as a possible future application of the method, the characterization of the cell-autonomous determinants that confer different susceptibility to individual MN subtypes, by comparing side-by-side cranial and spinal MNs obtained from iPSCs with the same genetic background. Moreover, effective conversion of iPSCs into MNs in simplified culture conditions that need minimal manipulation (i.e., without transition through embryoid bodies) and that can easily scalable, might greatly facilitate automated high-throughput drug screening approaches for motor neuron diseases. In vitro modeling of adult-onset neurodegenerative diseases can be challenging due to the foetal-like nature of iPSC-derived neurons[14]. Previous strategies devised to accelerate aging of MNs derived by conventional differentiation of iPSCs, such as progerin overexpression[15], might be combined with our method to obtain better disease models.

The method described here, based on the inducible expression of transcription factors mediated by a piggyBac vector, can be applied to other induced cell types. We have previously shown that skeletal muscle cells can be obtained from human iPSCs by expression of BAF60c and Myod[16]. Similarly, we can envision the possibility to extend the method to other cell types of interest, including other neuronal subtypes and astrocytes, by piggyBac-mediated expression of proper sets of programming factors[17-21].

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DISCLOSURES:
The authors have nothing to disclose

REFERENCES:

1. Dimos, J.T., et al. Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons. *Science (New York, NY)*. **321** (5893), 1218–1221, (2008).

2. Ebert, A.D., et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature.* **457** (7227), 277–280, (2009).

3. Lenzi, J., et al. ALS mutant FUS proteins are recruited into stress granules in induced

Pluripotent Stem Cells (iPSCs) derived motoneurons. *Disease models & mechanisms.* **8** (7), 755–766, (2015).

4. Wijesekera, L.C. & Leigh, P.N. Amyotrophic lateral sclerosis. *Orphanet journal of rare diseases.* **4** (3), 1–22, (2009).

5. Yan, J., et al. Frameshift and novel mutations in FUS in familial amyotrophic lateral sclerosis and ALS/dementia. *Neurology.* **75** (9), 807–814, (2010).

6. Boulting, G.L., et al. A functionally characterized test set of human induced pluripotent stem cells. *Nature biotechnology.* **29** (3), 279–286, (2011).

7. Amoroso, M.W., et al. Accelerated high-yield generation of limb-innervating motor neurons from human stem cells. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* **33** (2), 574–586, (2013).

8. Maury, Y., et al. Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. *Nature biotechnology.* **33** (1), 89–96, (2015).

9. Allodi, I., et al. Differential neuronal vulnerability identifies IGF-2 as a protective factor in ALS. *Scientific reports.* **6**, 25960, (2016).

10. Kaplan, A., et al. Neuronal matrix metalloproteinase-9 is a determinant of selective neurodegeneration. *Neuron.* **81** (2), 333–348, (2014).

11. Mazzoni, E.O., et al. Synergistic binding of transcription factors to cell-specific enhancers programs motor neuron identity. *Nature neuroscience.* **16** (9), 1219–1227, (2013).

12. De Santis, R., Garone, M. G., Pagani, F., de Turris, V., Di Angelantonio, S. & Rosa, A. Direct conversion of human pluripotent stem cells into cranial motor neurons using a piggyBac vector. Stem Cell Research 29, 189–196, doi:10.1016/j.scr.2018.04.012 (2018).

13. Yusa, K., Zhou, L., Li, M. A., Bradley, A. & Craig, N. L. A hyperactive piggyBac transposase for mammalian applications. *Proceedings of the National Academy of Sciences of the United States of America.* **108** (4), 1531–1536, (2011).

14. Sances, S., et al. Modeling ALS with motor neurons derived from human induced pluripotent stem cells. *Nature Neuroscience.* **16** (4), 542–553, (2016).

15. Miller, J. D., et al. Human iPSC-based modeling of late-onset disease via progerin-induced aging. *Cell stem cell.* **13** (6), 691–705, (2013).

16. Lenzi, J., et al. Differentiation of control and ALS mutant human iPSCs into functional

skeletal muscle cells, a tool for the study of neuromuscolar diseases. *Stem Cell Research*. **17** (1), 140–147, (2016).

17. Zhang, Y., et al. Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells. *Neuron.* **78** (5), 785–798, (2013)

18. Theka, I., et al. Rapid generation of functional dopaminergic neurons from human induced pluripotent stem cells through a single-step procedure using cell lineage transcription factors. *Stem cells translational medicine.* **2** (6), 473–479, (2013).

19. Pawlowski, M., et al. Inducible and Deterministic Forward Programming of Human Pluripotent Stem Cells into Neurons, Skeletal Myocytes, and Oligodendrocytes. *Stem Cell Reports.* **8** (4), 803–812, (2017).

20. Li, X., et al. Fast Generation of Functional Subtype Astrocytes from Human Pluripotent Stem Cells. *Stem Cell Reports.* **11** (4), 998–1008, (2018).

21. Nehme, R., et al. Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission. *Cell reports.* **23** (8), 2509–2523, (2018).

Figure 1

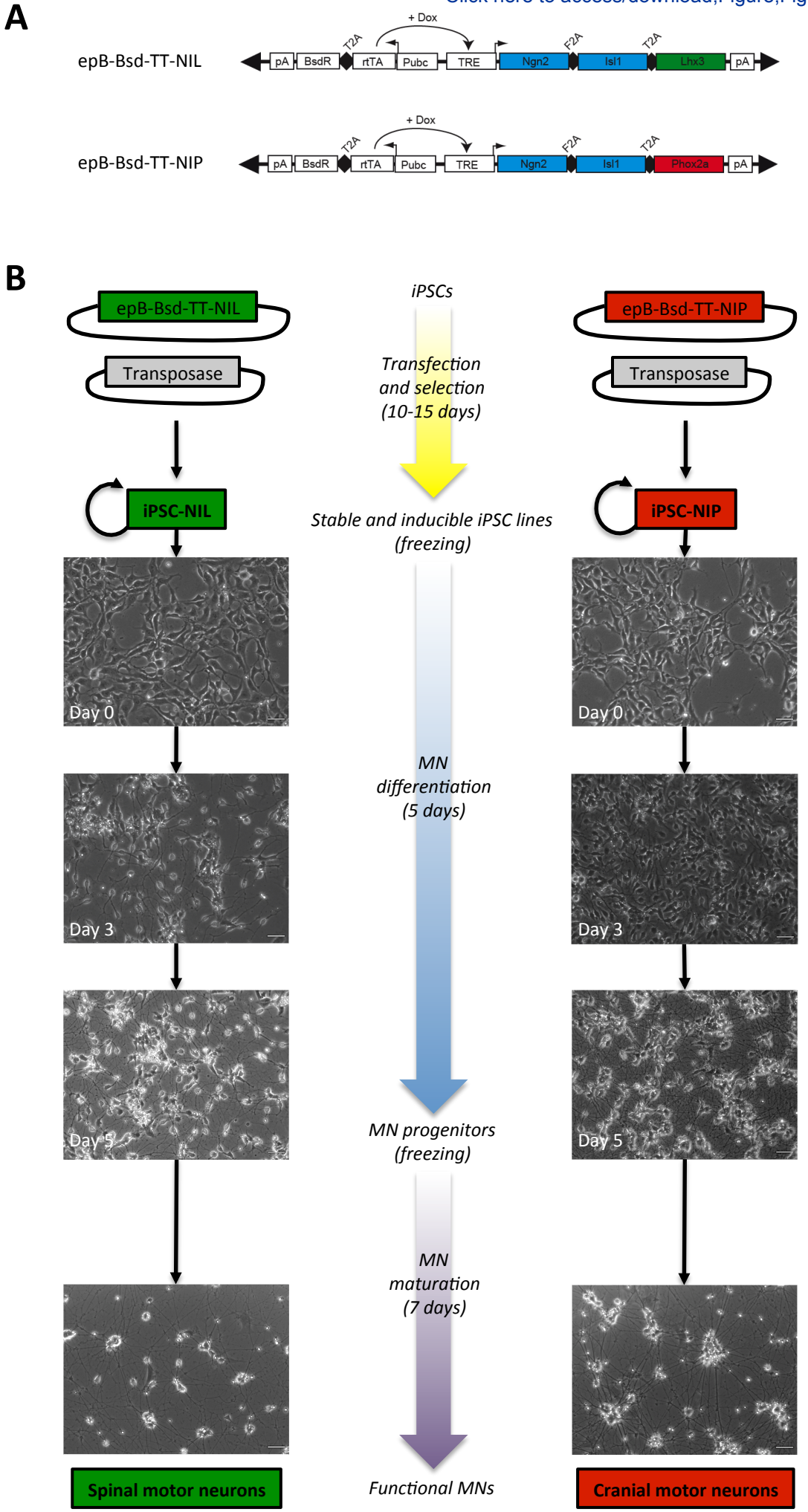

Figure 2

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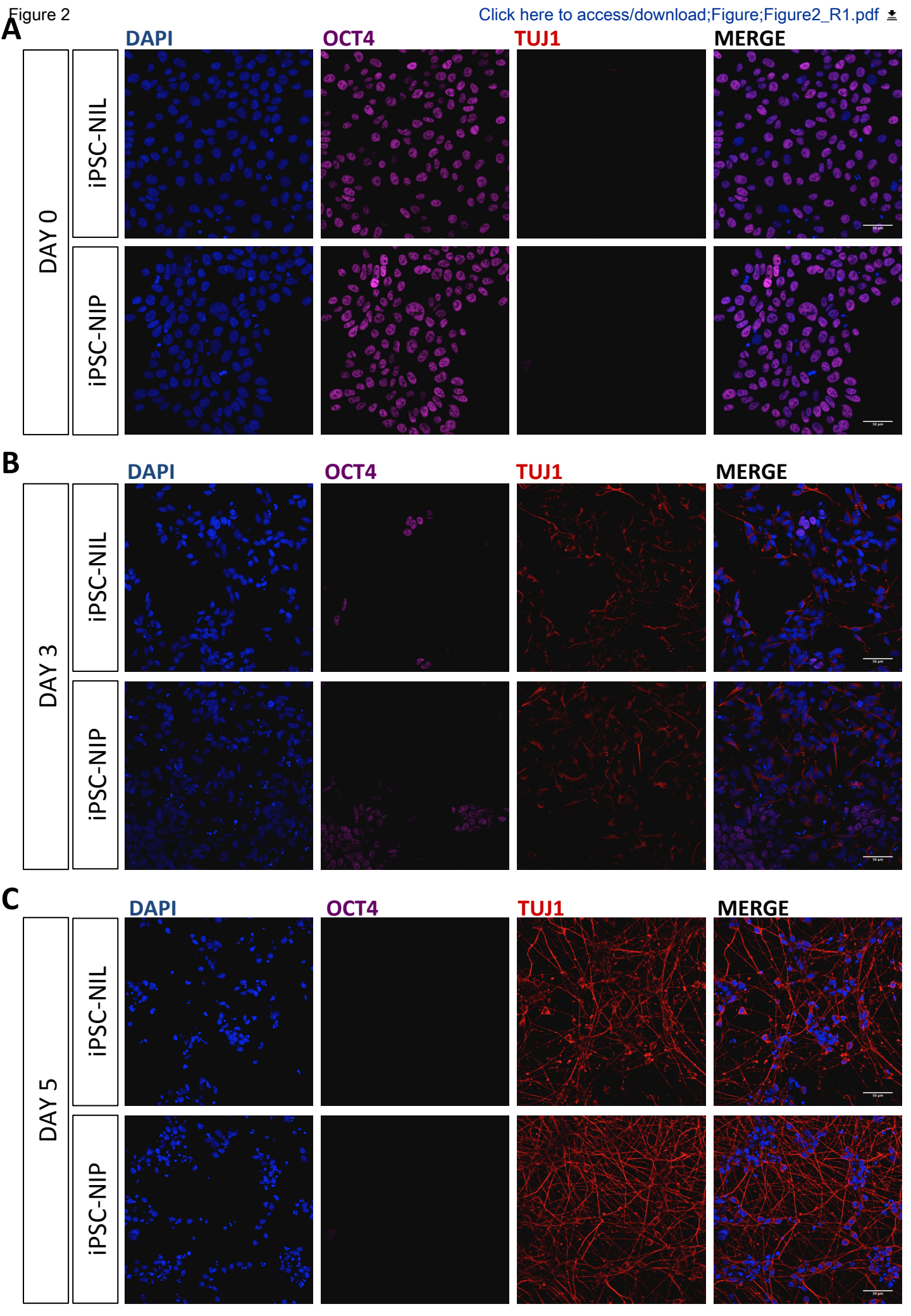

Figure 3

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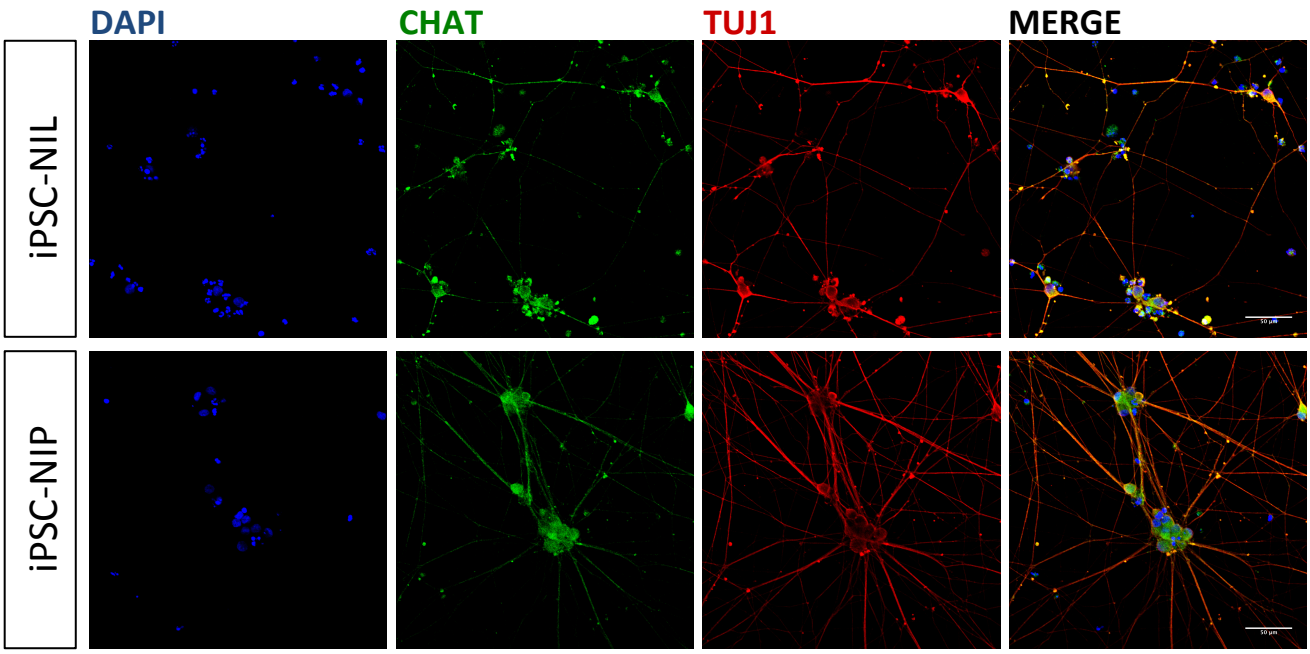
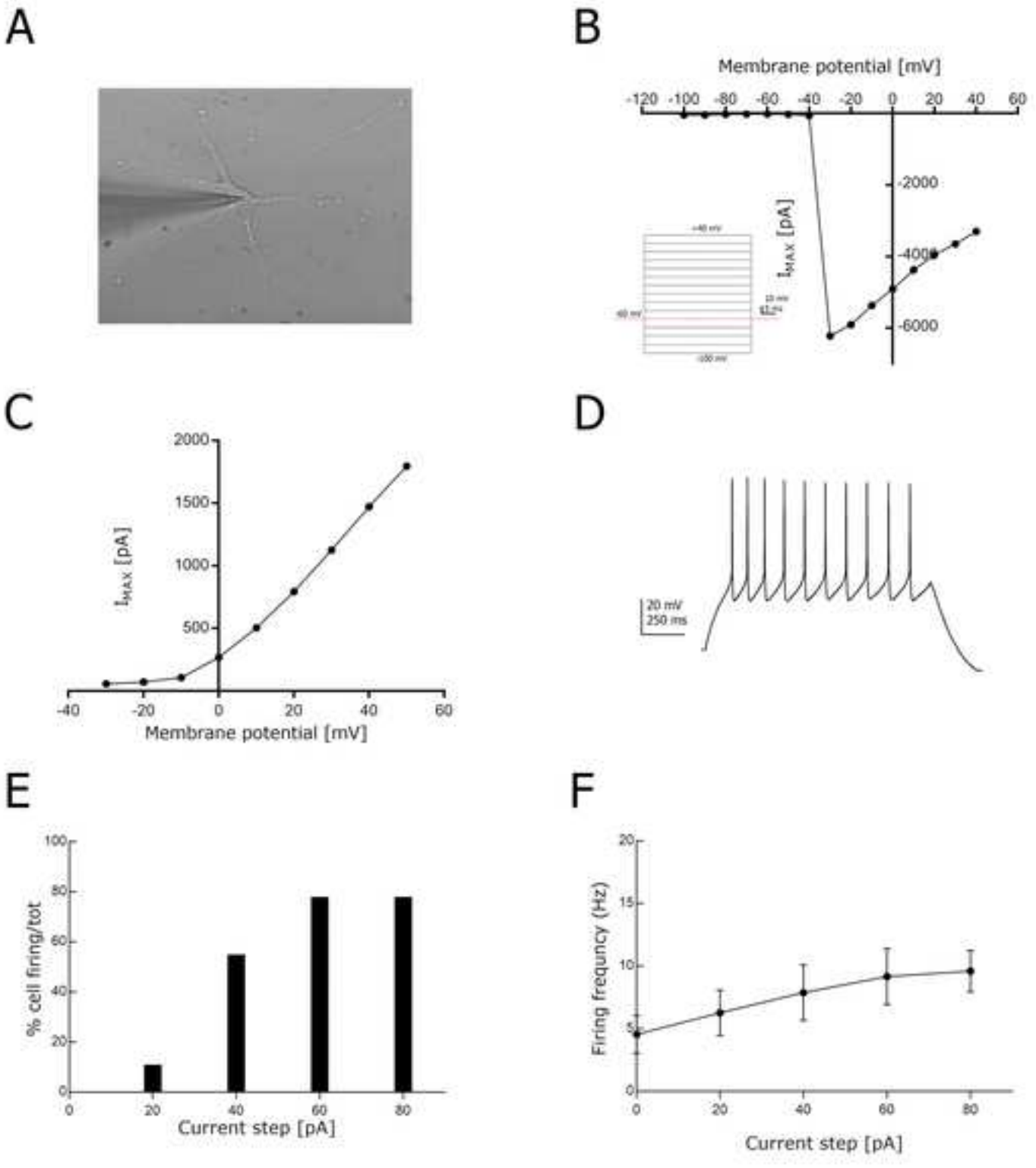


Figure 4



Name of Material/ Equipment	Company
5-Bapta	Sigma-Aldrich
Accutase	Sigma-Aldrich
anti-CHAT	EMD Millipore
anti-goat Alexa Fluor 488	Thermo Fisher Scientific
anti-mouse Alexa Fluor 647	Thermo Fisher Scientific
anti-Oct4	BD Biosciences
anti-Phox2b	Santa Cruz Biotechnology, Inc.
anti-rabbit Alexa Fluor 594	Immunological Sciences
anti-TUJ1	Sigma-Aldrich
B27	Miltenyi Biotec
Bambanker	Nippon Genetics
BDNF	PreproTech
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BSA	Sigma-Aldrich
CaCl2	Sigma-Aldrich
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D-Glucose	Sigma-Aldrich
DAPI powder	Roche
DAPT	AdipoGen
Dispase	Gibco
DMEM/F12	Sigma-Aldrich
Doxycycline	Sigma-Aldrich
DS2U	WiCell
E.Z.N.A Total RNA Kit	Omega bio-tek
GDNF	PreproTech
Gibco Episomal hiPSC Line	Thermo Fisher Scientific
Glutamax	Thermo Fisher Scientific
Hepes	Sigma-Aldrich
iScript Reverse Transcription Supermix for RT-qPCR	Bio-Rad
iTaqTM Universal SYBR Green Supermix	Bio-Rad
K-Gluconate	Sigma-Aldrich
KCl	Sigma-Aldrich
L-ascorbic acid	LKT Laboratories
Laminin	Sigma-Aldrich
Laser scanning confocal microscope	Olympus
Mg-ATP	Sigma-Aldrich
MgCl2	Sigma-Aldrich
Mounting Medium	Ibidi
Multiclamp patch-clamp amplifier	Molecular Devices
Na-GTP	Sigma-Aldrich
NaCl	Sigma-Aldrich

NEAA	Thermo Fisher Scientific
Neon 100 µL Kit	Thermo Fisher Scientific
Neon Transfection System	Thermo Fisher Scientific
Neurobasal Medium	Thermo Fisher Scientific
NutriStem-XF/FF	Biological Industries
Paraformaldehyde	Electron Microscopy Sciences
PBS	Sigma-Aldrich
PBS Ca2+/Mg2+ free	Sigma-Aldrich
Penicillin/Streptomycin	Sigma-Aldrich
poly-ornithine	Sigma-Aldrich
SU5402	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Upright microscope	Olympus
Y-27632 (ROCK inhibitor)	Enzo Life Sciences
µ-Slide 8 Well	Ibidi

Catalog Number
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AB144P
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BX51VI
ALX-270-333-M005
80826

Comments/Description

chemicals for electrophysiological solutions
Cell dissociation reagent
Anti-Choline Acetyltransferase. Primary antibody used in immunostaining assays. RRID: AB_2079751; Lot n
Secondary antibody used for immonofluorescence assays. RRID: AB_2534102; Lot number: 1915848
Secondary antibody used for immonofluorescence assays. RRID: AB_162542; Lot number: 1757130
Primary antibody used in immunostaining assays. RRID: AB_398736; Lot number: 5233722
Primary antibody used in immunostaining assays. Lot number: E0117
Secondary antibody used for immonofluorescence assays
Primary antibody used in immunostaining assays. RRID: AB_262133
Serum free supplement for neuronal cell maintenance
Cell freezing medium, used here for motor neuron progenitors
Brain-Derived Neurotrophic Factor
Nucleoside antibiotic that inhibits protein synthesis in prokaryotes and eukaryotes
Bovine Serum Albumin. Blocking agent to prevent non-specific binding of antibodies in immunostaining as
chemicals for electrophysiological solutions
Membrane currents recording system
Reconstituted basement membrane preparation from the Engelbreth-Holm-Swarm (EHS) mouse
Freezing medium for human iPSCs
chemicals for electrophysiological solutions
4′,6-diamidino-2-phenylindole. Fluorescent stain that binds to adenine–thymine rich regions in DNA used f
Gamma secretase inhibitor
Reagent for gentle dissociation of human iPSCs
Basal medium for cell culture
Used to induce expression of transgenes from epB-Bsd-TT-NIL and epB-Bsd-TT-NIP vectors
Commercial human iPSC line
Kit for total extraction of RNA from cultured eukaryotic cells
Glial-Derived Neurotrophic Factor
Commercial human iPSC line
An alternative to L-glutamine with increased stability. Improves cell health.
chemicals for electrophysiological solutions
Kit for gene expression analysis using real-time qPCR
Ready-to-use reaction master mix optimized for dye-based quantitative PCR (qPCR) on any real-time PCR i
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chemicals for electrophysiological solutions
Used in cell culture as an antioxidant
Promotes attachment and growth of neural cells in vitro
Confocal microscope for acquisition of immunostaining images
chemicals for electrophysiological solutions
chemicals for electrophysiological solutions
Mounting solution used for confocal microscopy and immunofluorescence assays
Membrane currents recording system
chemicals for electrophysiological solutions
chemicals for electrophysiological solutions

Non-Essential Amino Acids. Used as a supplement for cell culture medium, to increase cell growth and viab
Cell electroporation kit
Cell electroporation system
Basal medium designed for long-term maintenance and maturation of neuronal cell populations without tl
Human iPSC culture medium
Used for cell fixation in immunostaining assays
Dulbecco s Phosphate Buffer Saline w Calcium w Magnesium
Dulbecco s Phosphate Buffer Saline w/o Calcium w/o Magnesium
Penicillin/Streptomicin solution used to prevent cell culture contamination from bacteria.
Promotes attachment and growth of neural cells in vitro
Selective inhibitor of vascular endothelial growth factor receptor 2 (VEGFR-2)
4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, t-Octylphenoxypolyethoxyethanol, Polyethylene g
Microscope for electrophysiological recording equipped with CoolSnap Myo camera
Cell-permeable selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK). Increase
Support for high–end microscopic analysis of fixed cells

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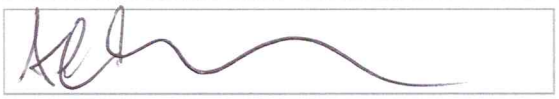
Signature:	 Date: October 30th, 2018

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SUPPLEMENTARY FIGURES

Conversion of human induced Pluripotent Stem Cells (iPSCs) into functional spinal and cranial motor neurons using piggyBac vectors

Maria G Garone[1], Valeria de Turris[2], Alessandro Soloperto[2], Carlo Brighi[2], Riccardo De Santis[3], Francesca Pagani[2,4], Silvia Di Angelantonio[2,4], Alessandro Rosa[1,2]

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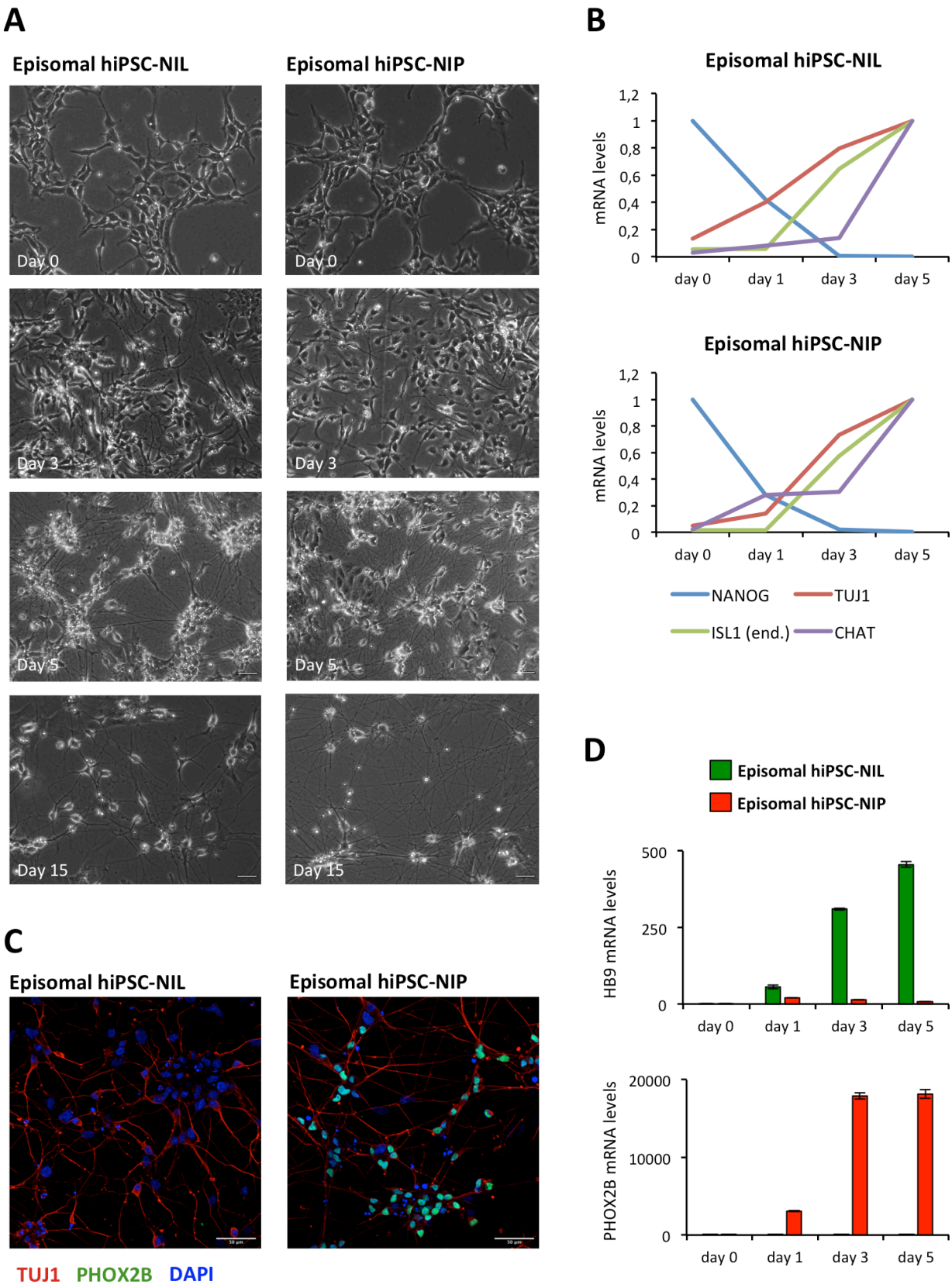
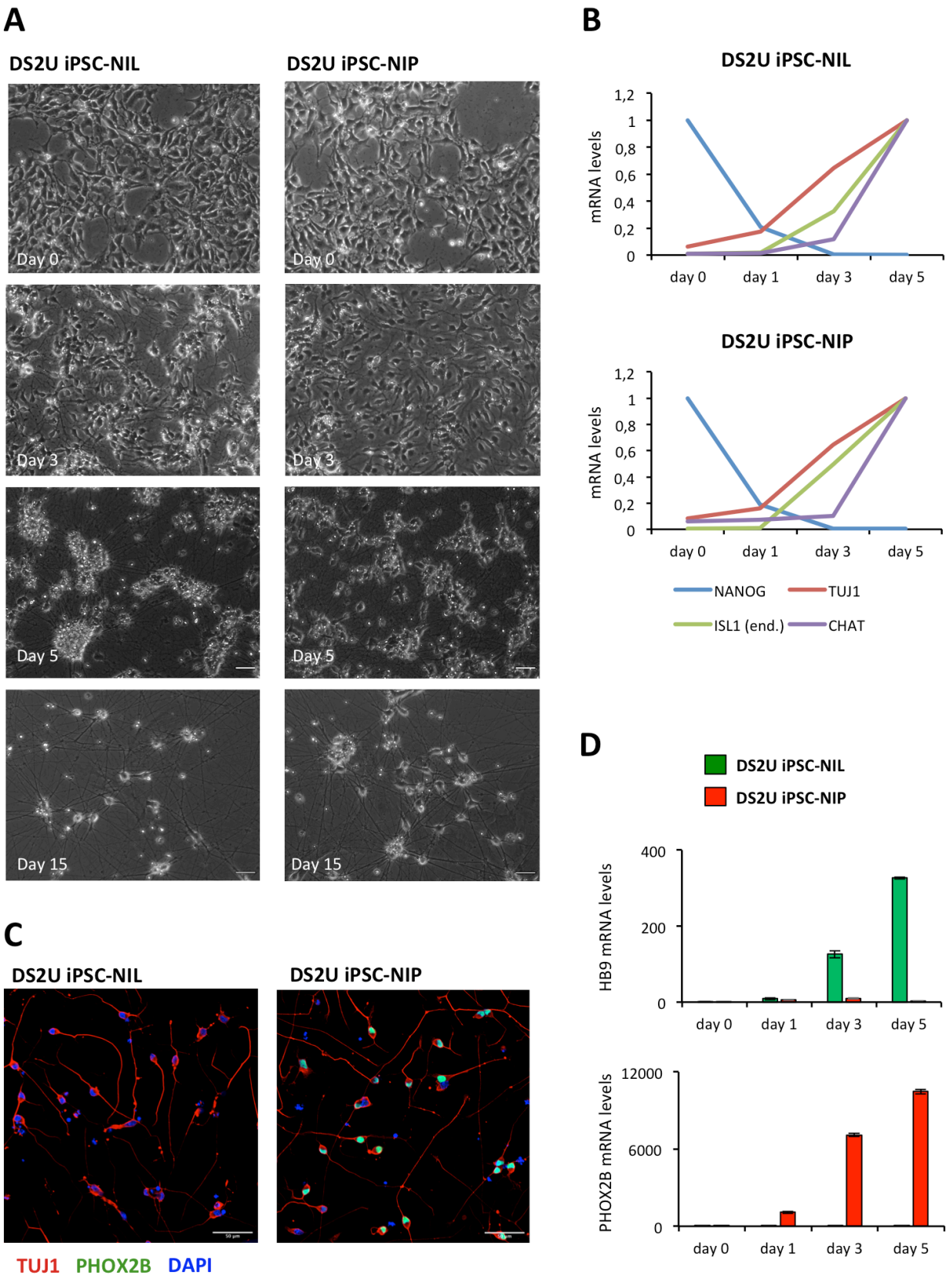


Supplementary Figure S1: MN differentiation using Episomal hiPSCs. (A) Brightfield images of differentiating Episomal hiPSC-NIL (left) and Episomal hiPSC-NIP (right) at the indicated time points. Scale bar for all panels: 50 µm. (B) Analysis of the expression of the indicated markers in differentiating Episomal hiPSC-NIL (top) and Episomal hiPSC-NIP (bottom) cells by real time qRT-

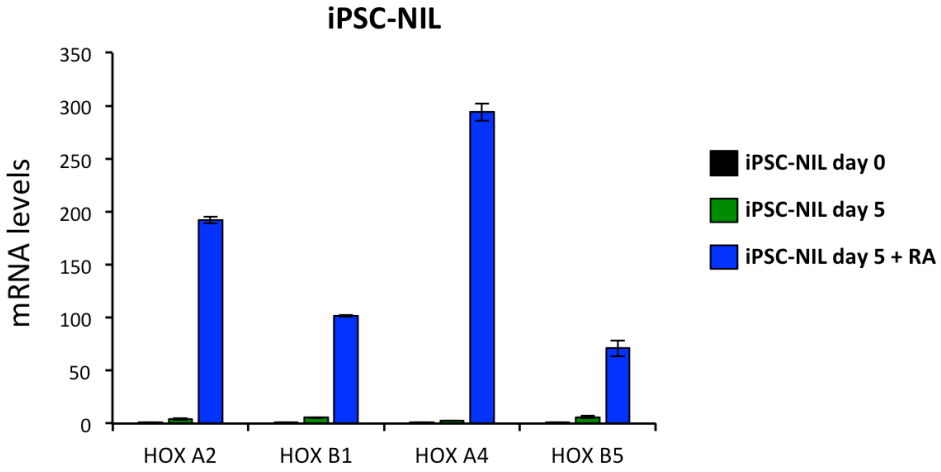
PCR. For each marker the time point with the highest expression has been used as calibrator sample. Primers used for ISL1 are specific for the endogenous gene. (C) Immunostaining for the pan-neuronal marker TUJ1 (red) and cranial MN marker PHOX2B (green) in differentiated (day 6) Episomal hiPSC-NIL (left) and Episomal hiPSC-NIP (right) cells. Nuclei are counterstained with DAPI. Scale bar for all panels: 50 μm. (D) Analysis of the expression of HB9 and PHOX2B in differentiating Episomal hiPSC-NIL (top) and Episomal hiPSC-NIP (bottom) cells by real time qRT-PCR. Day 0 has been used as the calibrator sample. PCR primers and methods are reported in De Santis et al., 2018[12].



Supplementary Figure S2: MN differentiation using DS2U iPSCs. (A) Brightfield images of differentiating DS2U-NIL (left) and DS2U-NIP (right) at the indicated time points. Scale bar for all panels: 50 µm. (B) Analysis of the expression of the indicated markers in differentiating DS2U-NIL (top) and DS2U-NIP (bottom) cells by real time qRT-PCR. For each marker the time point with the

highest expression has been used as calibrator sample. Primers used for ISL1 are specific for the endogenous gene. (C) Immunostaining for the pan-neuronal marker TUJ1 (red) and cranial MN marker PHOX2B (green) in differentiated (day 6) DS2U-NIL (left) and DS2U-NIP (right) cells. Nuclei are counterstained with DAPI. Scale bar for all panels: 50 µm. (D) Analysis of the expression of HB9 and PHOX2B in differentiating DS2U-NIL (top) and DS2U-NIP (bottom) cells by real time qRT-PCR. Day 0 has been used as the calibrator sample. PCR primers and methods are reported in De Santis et al., 2018[12].

A



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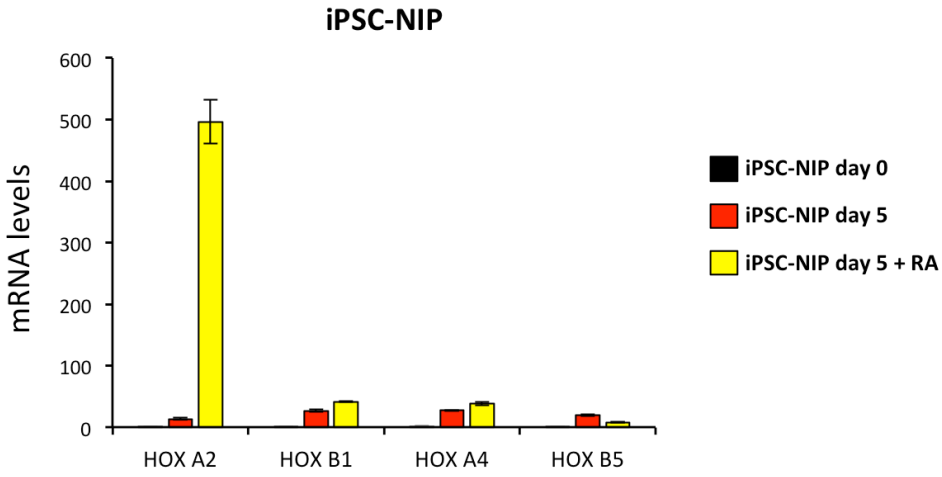
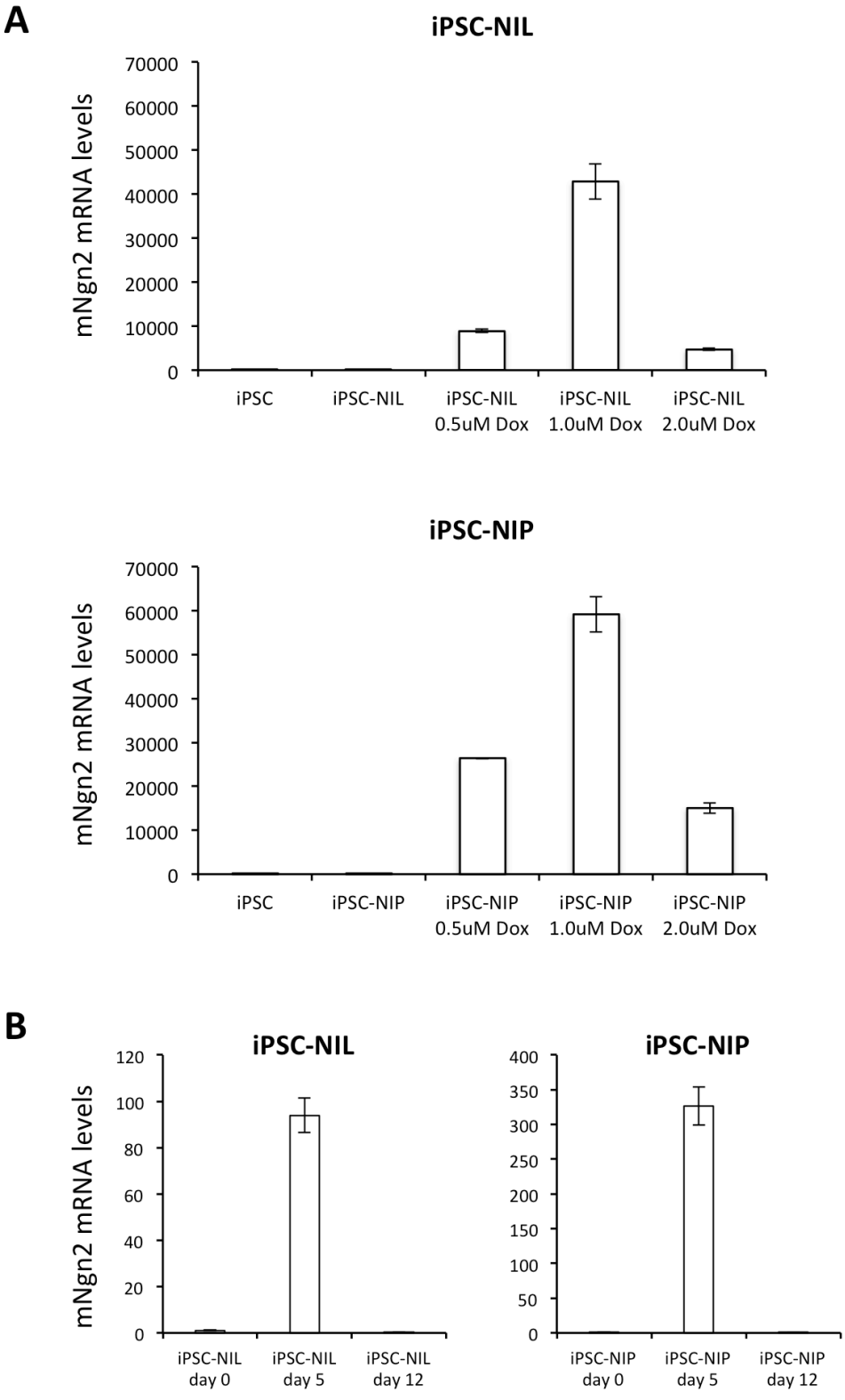


C

Gene name	Primer forward	Primer reverse
HOX A2	TGGATGAAGGAGAAGAAGGCGG	TCGGCGATTTCCAGGGATTCTT
HOX B1	AGAGAAACCCACCCAAGACAGG	AAGAGAAGAACCCAGCCCAGAC
HOX A4	AAACTGCCCAACACCAAGATGC	GGCTCTGAGTTTGTGCTTTCCC
HOX B5	TCACCGAAATAGACGAGGCCAG	AATATTTGCGGAGTCTGCCCCT

Supplementary Figure S3: HOX gene expression. (A) Analysis of the expression of four different HOX genes (HOX A2, HOX B1, HOX A4, HOX B5) after 5 days of differentiation of iPSC-NIL and iPSC-NIL + RA cells by real time qRT-PCR. IPSC-NIL at day 0 has been used as the calibrator sample. (B) Analysis of the expression of four different HOX genes (HOX A2, HOX B1, HOX A4, HOX B5) after 5

days of differentiation of iPSC-NIP and iPSC-NIP + RA cells by real time qRT-PCR. IPSC-NIP at day 0 has been used as the calibrator sample. (C) PCR primer pairs.



Supplementary Figure S4: Doxycycline induction analysis. (A) Analysis by real time qRT-PCR of the expression of exogenous Ngn2 in iPSC-NIL (top) and iPSC-NIP (bottom) cells untreated or cultured for 24 h in presence of doxycycline at different concentration (0.5 µM, 1.0 µM, 2.0 µM). Ngn2 was analyzed with primers specific for the exogenous mouse gene. The parental iPSC line, devoid of NIL and NIP constructs, has been included in the analysis as a control. Expression of the transgenes

in iPSC-NIL and iPSC-NIP was neglectable in absence of doxycycline. iPSC-NIL and iPSC-NIP at day 0 have been used as calibrator samples. (B) Analysis by real time qRT-PCR of the expression of exogenous Ngn2 in differentiating iPSC-NIL (left) and iPSC-NIP (right) cells at the indicated time points of the protocol. Ngn2 was analyzed with primers specific for the exogenous mouse gene. iPSC-NIL and iPSC-NIP at day 0 have been used as calibrator samples. PCR primers and methods are reported in De Santis et al., 2018[12].