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Direct conversion of human pluripotent stem cells into cranial motor neurons using a piggyBac vector

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ABSTRACT

Human pluripotent stem cells (PSCs) are widely used for in vitro disease modeling. One of the challenges in the field is represented by the ability of converting human PSCs into specific disease-relevant cell types. The nervous system is composed of a wide variety of neuronal types with selective vulnerability in neurodegenerative diseases. This is particularly relevant for motor neuron diseases, in which different motor neurons populations show a different susceptibility to degeneration. Here we developed a fast and efficient method to convert human induced Pluripotent Stem Cells into cranial motor neurons of the branchiomotor and visceral motor subtype. These populations represent the motor neuron subgroup that is primarily affected by a severe form of amyotrophic lateral sclerosis with bulbar onset and worst prognosis. This goal was achieved by stable integration of an inducible vector, based on the piggyBac transposon, allowing controlled activation of Ngn2, Isl1 and Phox2a (NIP). The NIP module effectively produced electrophysiologically active cranial motor neurons. Our method can be easily extended to PSCs carrying disease-associated mutations, thus providing a useful tool to shed light on the cellular and molecular bases of selective motor neuron vulnerability in pathological conditions.

1. Introduction

Pluripotent stem cells (PSCs) are widely used to model in vitro neurodegenerative diseases, which usually affect specific subtypes of neurons. Both human Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs) can be converted into disease relevant cell types, included neurons, thus providing a cell system to study the molecular and cellular mechanisms underlying neurodegeneration. The possibility of deriving patient-specific iPSCs and the recent advent of genome editing techniques further expand the potential of such systems, allowing the production of human neurons carrying disease-associated mutations. PSCs-derived mutant neurons can also provide a platform to screen for drugs. To fully exploit the potential of PSCs in the field of neurodegeneration, appropriate protocols for producing pure populations of specific neuronal subtypes are needed. To this aim, a significant effort has been put on the development and optimization of methods to obtain, for instance, cortical (Suzuki and Vanderhaeghen, 2015), dopaminergic (Arenas et al., 2015), hippocampal (Yu et al., 2014) or motor (Davis-Dusenbery et al., 2014) neurons.

Motor neurons represent the primary cell type affected in a number of diseases that are collectively referred to as motor neuron diseases, and include Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA). The developmental program of motor neurons has been well decoded (Jessell, 2000). Two main motoneurons subtypes are present in the central nervous system in human. Spinal motor neurons are located in the ventral spinal cord and innervate body skeletal muscles (Price and Briscoe, 2004). Cranial motor neurons reside in the brainstem (midbrain and hindbrain) and comprise three subsets: branchiomotor (BM), visceral motor (VM) and somatic motor (SM) (Guthrie, 2007). BM axons innervate muscles controlling the jaw, facial expression, pharynx and larynx. VM neurons control salivary and lacrimal glands, smooth muscle and visceral organs. SM neurons innervate eye (oculomotor, trochlear and abducens) and tongue (hypoglossal) muscles. Not all MN subtypes are equally vulnerable in ALS and SMA. Despite pathogenic proteins are ubiquitously expressed, in most ALS patients MNs controlling eye movements are generally spared, MNs innervating pelvic floor muscles are relatively resistant to degeneration and faster spinal motor units are affected before slower ones (Nijssen et al., 2017). Differential vulnerability of specific MN subgroups seems to be due to intrinsic factors, as protective effects were

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observed upon overexpression of oculomotor-enriched factors or reduction of spinal MNs-enriched factors (Allodi et al., 2016; Kaplan et al., 2014).

ALS prognosis depends from the site of onset of the first symptoms (Wijesekera and Leigh, 2009). Spinal onset ALS is the most frequent, with symptoms starting in upper and lower limbs, and is usually fatal at 3–5 years post-diagnosis. Conversely, speech and swallowing problems are noticed first in a more severe form of ALS (bulbar onset), with limb symptoms arising almost simultaneously. Bulbar onset ALS has the worst prognosis, with an average survival of 2–3 years. The incidence of spinal or bulbar onset is not uniformly distributed in patients carrying different mutations associated to familial ALS (FALS). For instance, the percentage of bulbar onset is significantly higher in patients with FUS or TARDBP mutations than that of patients with SOD1 mutations (Yan et al., 2010).

Taken together, the different susceptibility of specific MNs subtypes to neurodegeneration and the onset of symptoms in different motor units seem due to intrinsic factors. Human PSCs represent a promising system to shed light on these mechanisms, provided that appropriate protocols are established to convert them into specific MN subtypes. In this work, we present a method to differentiate human pluripotent stem cells into cranial MNs. Existing differentiation protocols are generally designed to generate spinal MNs as they rely on the activity of retinoic acid (RA), which during embryonic development confer a posterior character to neural progenitors (Boulting et al., 2011; Amoroso et al., 2013; Maury et al., 2015). Here we developed an inducible system that allows fast and efficient generation of cranial MNs with a BM and VM character, in extremely simplified culture conditions.

2. Material and methods

2.1. Plasmid construction and generation of a stable human iPSC line

The epB-Bsd-TT-NIL and epB-Bsd-TT-NIP plasmids were generated by inserting the sequences of, respectively, Ngn2-F2A-Isl1-T2A-Lhx3 (NIL) and Ngn2-F2A-Isl1-T2A-Phox2a (NIP) in the enhanced piggyBac transposable vector epB-Bsd-TT (Rosa et al., 2014). NIL and NIP were obtained from the iNIL and iNIP constructs described in (Mazzoni et al., 2013) and cloned between the *Bam*HI and *Not*I sites of epB-Bsd-TT. The resulting constructs contain the enhanced piggyBac terminal repeats flanking a constitutive cassette driving the expression of the Blasticidin resistance gene fused to the rtTA gene and, in the opposite direction, a tetracycline-responsive promoter element (TRE) driving the expression of NIP or NIL. Human iPSCs used in this study belong to the lines WT I (main figures) and FUS-P525L/P525L (Fig. S1) described in (Lenzi et al., 2015). iPSCs were co-transfected with 4.5 μg of transposable vector and 0.5 μg of the piggyBac transposase using the Neon Transfection System (Life Technologies) as previously described (Lenzi et al., 2015). Selection in 5 μg/ml blasticidin gave rise to stable and inducible cell lines.

2.2. Cranial and spinal MN differentiation

iPSCs, maintained as previously described (Lenzi et al., 2015), were dissociated to single cells with Accutase (Thermo Fisher Scientific) and plated in Nutristem-XF/FF medium (Biological Industries) supplemented with 10 μM rock inhibitor (Enzo Life Sciences) on Matrigel (BD Biosciences) at a density of 100′000 cells/cm^2. The day after differentiation was induced by adding 1 μg/ml doxycycline (dox) (Thermo Fisher Scientific) in Nutristem without bFGF and TGFβ (Biological Industries). Similar results were obtained when DMEM/F12 (Sigma Aldrich) was used instead of Nutristem without bFGF and TGFβ. Medium was changed every day. After 48 h of dox induction, medium was changed to Neurobasal/B27 medium (Neurobasal Medium, Thermo Fisher Scientific, supplemented with 1X B27, Thermo Fisher Scientific, 1X Glutamax, Thermo Fisher Scientific, 1X NEAA, Thermo Fisher Scientific, and 0.5X Penicillin/Streptomycin, Sigma Aldrich), containing 5 μM DAPT and 4 μM SU5402 (both from Sigma Aldrich). At day 5, cells were dissociated with Accutase (Thermo Fisher Scientific) and plated on poly-ornithine/laminin (Sigma Aldrich) or alternatively on Matrigel (BD Biosciences) coated dishes or 12 well removable chamber slides or 8 well chamber slides (Ibidi) at the density of 100′000 cells per cm^2. 10 μM rock inhibitor was added for the first 24 h after dissociation. Neuronal cultures were maintained in neuronal medium (Neurobasal/B27 medium supplemented with 20 ng/ml BDNF, 10 ng/ml GDNF, both from PreproTech, and 200 ng/ml L-ascorbic acid, Sigma Aldrich).

2.3. RNA analysis

Total RNA was extracted with the Quick RNA MiniPrep (Zymo Research) and retrotranscribed with PrimeScript RT reagent Kit (Perfect Real Time). Targets were analyzed by real-time qRT-PCR with SYBR Green Power-UP (Thermo Fisher Scientific). The internal control used was the housekeeping gene *ATP5O*. Primers sequences are reported in Table S1.

2.4. Immunostaining

Cells were fixed with 4% PFA for 15 min at room temperature. Immunostaining was performed with anti-Islet-1/2 (1:50, 39.4D5; DSHB), anti-TUJ1 (1:1000; T2200; Sigma-Aldrich), anti-Phox2b (B-11) (1:50; sc-376,997; Santa Cruz Biotechnology), anti-CHAT (1:100; ab144P; Abcam) primary antibodies and anti-mouse Alexa Fluor 488 (1:200, Thermo Fisher Scientific), anti-rabbit Alexa Fluor 594 (1:200, Immunological Sciences) and anti-goat Alexa Fluor 594 (1:200, Immunological Sciences) donkey secondary antibodies. Fluorescent images were acquired at the Olympus iX83 FluoView1200 laser scanning confocal microscope using a UPLSAPO10x2, NA 0,40 air objective. Standard setting for DAPI, Alexa 488 and Alexa 594 were used. Images on Fig. 1C were acquired with 2× zoom, 1600 × 1600 pixel. Images on Fig. 3 were acquired with 3× zoom, 1024 × 1024 pixel. Automated cell counting was performed with the Cell Scoring tool of MetaMorph software (Molecular Devices).

2.5. Patch-clamp recordings

Whole-cell patch-clamp recordings were used for the functional characterization of iPSC-derived cranial MNs, plated on Matrigel. Experiments were performed in a recording chamber continuously perfused with an external solution containing (in mM): 140 NaCl, 2.8 KCl, $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.3 with NaOH; 300 mOsm) at room temperature. Borosilicate pipettes were filled with a solution containing (in mM): 140 K-gluconate, 2 NaCl, 5 BAPTA, 2 MgCl2, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP (pH 7.3 with KOH; 290 mOsm). Cells were visualized with a BX51WI microscope (Olympus). Voltage- and current- clamp recordings were performed using Axon DigiData 1550 (Molecular Devices). Signals were filtered at 2 KHz, digitized (10 kHz) and collected using Clampex 10 (Molecular Devices). Whole-cell capacitance (Cm), cell membrane resistance (Rm) and Resting Membrane Potential (RMP) were measured on line by Clampex. Cells were clamped at −70 mV to measure spontaneous activity. An on-line P4 leak subtraction protocol was used for all recordings of voltage-activated currents. Voltage steps (50 ms duration) from −100 to +40 mV (10 mV increment; holding potential −60 mV) were applied to study voltage-activated sodium currents. Voltage-activated potassium currents were evoked by voltage steps (250 ms duration) from −30 to +50 mV (10 mV increment, holding potential −40 mV). Firing properties of iPSC-derived cranial MNs were investigated in current-clamp mode, injecting current pulses (1 s duration) of increasing amplitude (from 20 to 80 pA; 20 pA increment), after imposing a membrane potential of −60 mV to each cell (injection of −14.6 ± 2.1 pA). Data were analyzed off-line with Clampfit 10 and