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Modeling amyotrophic lateral sclerosis in pure human iPSc-derived motor neurons isolated by a novel FACS double selection technique

Diana Toli [a,1], Dorothée Buttigieg [b,1], Stéphane Blanchard [a], Thomas Lemonnier [a], Boris Lamotte d'Incamps [c], Sarah Bellouze [b], Gilbert Baillat [b], Delphine Bohl [a,*,1], Georg Haase [b,**,1]

[a] Institut Pasteur and INSERM U1115, Unité Biothérapies pour les Maladies Neurodégénératives, 28 rue du Dr Roux, 75015 Paris, France
[b] Institut de Neurosciences de la Timone, UMR 7289 CNRS and Aix-Marseille Université, Modélisation et Thérapie des Maladies Dégénératives des Motoneurones, 27 boulevard Jean Moulin, 13005 Marseille Cedex 5, France
[c] Centre de Neurophysique, Physiologie, Pathologie, UMR 8119 CNRS and Université Paris Descartes, 45 rue des Saints-Pères, 75270 Paris Cedex 6, France

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a severe and incurable neurodegenerative disease. Human motor neurons generated from induced pluripotent stem cells (iPSc) offer new perspectives for disease modeling and drug testing in ALS. In standard iPSc-derived cultures, however, the two major phenotypic alterations of ALS—degeneration of motor neuron cell bodies and axons—are often obscured by cell body clustering, extensive axon criss-crossing and presence of unwanted cell types. Here, we succeeded in isolating 100% pure and standardized human motor neurons by a novel FACS double selection based on a $p75^{NTR}$ surface epitope and an HB9::RFP lentivirus reporter. The $p75^{NTR}$/HB9::RFP motor neurons survive and grow well without forming clusters or entangled axons, are electrically excitable, contain ALS-relevant motor neuron subtypes and form functional connections with co-cultured myotubes. Importantly, they undergo rapid and massive cell death and axon degeneration in response to mutant SOD1 astrocytes. These data demonstrate the potential of FACS-isolated human iPSc-derived motor neurons for improved disease modeling and drug testing in ALS and related motor neuron diseases.

Introduction

Motor neuron diseases such as amyotrophic lateral sclerosis (ALS) are severe and most often rapidly fatal neurodegenerative disorders. ALS is characterized by progressive degeneration of motor neurons in the spinal cord, brainstem and cerebral cortex. ALS can be caused by mutations in more than 25 genes including C9ORF72, SOD1 and TARDBP or occurs sporadically (Robberecht and Philips, 2013). Animal models of ALS and corresponding cellular models have given important insights into disease mechanisms but also bear major limitations. Transgenic mutant SOD1 mice, for instance, develop motor neuron disease only when the mutant enzyme is expressed at high non-physiological levels (Gurney et al., 1994) and their response to numerous pharmacological compounds is not predictive for human ALS (Turner and Talbot, 2008; Mitsumoto et al., 2014).

Human motor neurons generated from induced pluripotent stem cells (iPSc) (Takahashi et al., 2007) offer a new alternative for disease modeling and drug testing in ALS. Recent studies have indeed provided evidence for molecular and functional alterations in motor neurons derived from ALS patients (Bilican et al., 2012; Chen et al., 2014; Egawa et al., 2012; Haeusler et al., 2014; Kiskinis et al., 2014; Sareen et al., 2013). Yet, modeling the two major phenotypic alterations of ALS, motor neuron cell body death and axon degeneration, faces important still unresolved problems in iPSc-derived cultures. First, the efficiency of motor neuron generation remains variable between protocols and from one iPSc clone to another (Amoroso et al., 2013; Qu et al., 2014). Second, motor neuron cell bodies tend to form large clusters which are inaccessible for analysis of cell survival (Chen et al., 2014; Dimos et al., 2008; Ebert et al., 2009; Hu and Zhang, 2009; Kiskinis et al., 2014; Maury et al., 2015; Qu et al., 2014; Sareen et al., 2013). Motor neuron axons often also display extensive criss-crossing and intermingling with axons from other neuronal types thus complicating their identification and analysis (Amoroso et al., 2013; Bilican et al., 2012; Boulting et al., 2011; Burkhardt et al., 2013; Chen et al., 2014; Egawa et al.,

* Correspondence to: D. Bohl, Institut du Cerveau et de la Moelle épinière - ICM, INSERM U 1127 - CNRS UMR-7225 - UPMC-Université Paris 6 - Hôpital de la Pitié-Salpêtrière, 47, boulevard de l'Hôpital, 75013 Paris, France.
** Correspondence to: G. Haase, Institut de Neurosciences de la Timone, UMR 7289, CNRS and Aix-Marseille University, 27, boulevard Jean Moulin, 13005 Marseille cx 5, France. Fax: +33 4 9132 4056.
E-mail addresses: delphine.bohl@icm-institute.org (D. Bohl), georg.haase@univ-amu.fr (G. Haase).
[1] Equal contribution.

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2012; Haeusler et al., 2014; Kiskinis et al., 2014; Maury et al., 2015; Qu et al., 2014; Sareen et al., 2013). Third, the proportion of disease-relevant motor neuron subtypes remains most often undetermined in human iPSc-derived cultures (Bilican et al., 2012; Boulting et al., 2011; Egawa et al., 2012; Hester et al., 2011; Karumbayaram et al., 2009; Mitne-Neto et al., 2011; Qu et al., 2014; Serio et al., 2013) but see Amoroso et al. (2013). Fourth, the presence of unwanted cell types such as neural precursors, various types of neurons (Amoroso et al., 2013; Boulting et al., 2011; Chen et al., 2014; Dimos et al., 2008; Ebert et al., 2009; Karumbayaram et al., 2009; Naujock et al., 2014; Qu et al., 2014; Sareen et al., 2012) or astrocytes (Haidet-Phillips et al., 2011; Re et al., 2014) may induce confounding effects on motor neuron survival. Likewise, pharmacological compounds may act indirectly through these cell types rendering difficult the interpretation of their effects. Generating pure and standardized human motor neuron cultures is therefore an important step towards robust ALS modeling and drug testing.

Fluorescence-activated cell sorting (FACS) represents a powerful means to isolate the desired neuronal populations. Yet, there are few well-characterized genetic reporters and surface markers for FACS isolation of motor neurons (Amoroso et al., 2013; Egawa et al., 2012; Kiskinis et al., 2014; Wichterle et al., 2002). In addition, FACS-isolated human motor neurons were reported to survive and grow poorly in culture (Egawa et al., 2012). We here developed a novel procedure for motor neuron isolation by combining a lentiviral reporter vector harboring the 3.6 kb minimal HB9 promoter (Marchetto et al., 2008), which drives RFP expression and a monoclonal antibody against the cell surface receptor $p75^{NTR}$ (Ross et al., 1984). Using $p75^{NTR}$/HB9::RFP FACS double selection, we succeeded in isolating high numbers of 100% pure and functional human iPSc-derived motor neurons which comprise ALS-relevant motor neuron subtypes. The FACS-isolated motor neurons survive and grow well in low-density cultures without forming clusters or entangled axons. By contrast, they undergo increased cell death and form prominent axon blebs in response to mutant SOD1 astrocytes, indicating their potential for improved ALS modeling.

Materials and methods

iPSc reprogramming and characterization

Human skin fibroblasts from two healthy donors aged 33 and 11 years (individuals 1 and 2, respectively) were obtained from the Centre de Ressources Biologiques in Lyon (France) after approval by the competent French authorities. The biological samples were fully anonymized and declared according to French laws from the Ministère de la Recherche. Ethical and regulatory issues were approved by Comité de Protection des Personnes d'Ile-de-France. Fibroblasts were grown in DMEM/Glutamax (Life Technologies, St Aubin, France) supplemented with 10% fetal bovine serum (Sigma). iPSc clones were generated as previously described (Lemonnier et al., 2011). Briefly, cultures containing 10^5 fibroblasts were transduced with retroviral vectors coding for OCT4, SOX2, KLF4 and c-MYC in the presence of 5 mg/ml protamine sulfate (Sigma). Transduced fibroblasts were seeded onto irradiated murine embryonic fibroblasts (iMEFs). Clones with well-defined human embryonic stem cell-like morphology were observed 3 weeks later, and were manually picked 2 to 3 weeks later. iPSc clones were maintained on iMEFs in the following medium (iPSc medium): DMEM/F-12 (Life Technologies) supplemented with 20% KnockOut Serum Replacement (Life Technologies), 10 ng/ml FGF2 (Miltenyi Biotec, Paris, France), 100 μM MEM non-essential amino acids (Gibco) and 100 μM 2-mercaptoethanol (Life Technologies). iPSc were routinely cultured on iMEFs and passaged every 5–7 days either manually or enzymatically with 1 mg/ml collagenase type IV (Life Technologies). For analyses requiring feeder-free conditions, iPSc were transferred onto Matrigel-coated wells (BD Biosciences), cultured in TeSR1 medium (Stem Cell Technologies) and passaged manually or enzymatically using dispase (2 mg/ml, Life Technologies). Karyotype analysis was performed by Pasteur-Cerba (Cergy-Pontoise, France). Bisulfite sequencing, RT-PCR and analyses of embryoid body and teratoma formation were performed as previously described (Lemonnier et al., 2011).

Motor neuron differentiation

Motor neuron differentiation was performed as described by Hu and Zhang (2009) with the following modifications. iPSc clones were pre-treated with ROCK inhibitor Y-27632 (Tocris Bioscience, Bristol, UK) for one hour, then treated with collagenase type IV to form small clusters and re-suspended in TESR1 medium. For the following three days, medium was changed every day with iPSc medium without FGF2 supplemented with 10 μM SB431542 (Tocris Bioscience, Bristol, UK) and 1 μM dorsomorphin (Sigma). From day 4 to day 7, suspension cultures were switched to a neural differentiation medium: DMEM/F12 supplemented with 15 mM HEPES (Gibco), non-essential amino acids (Gibco), N2 supplement (Life Technologies) and 2 μg/ml heparin (Sigma). Treatment with SB431542 and dorsomorphin was continued until day 7. Spheres were then plated onto laminin (20 μg/ml)-coated 6-well plates at 20–30 spheres per well in a small volume and switched to neural differentiation medium supplemented with 0.1 μM retinoic acid (Sigma) at day 10. Media were changed every other day until day 15, when rosettes were detached from the plates and re-suspended in neural differentiation medium supplemented with B27 supplement without vitamin A (Life Technologies), 0.1 μM retinoic acid and 0.5 μM SAG smoothened ligand (Enzo Life Sciences, NY, USA). Spheres were cultured in suspension in this medium until day 28, and media were changed every other day. On day 28, spheres were seeded onto polyornithine (100 μg/ml) and laminin-coated coverslips. Cells were then cultured in motor neuron maturation medium corresponding to neural differentiation medium supplemented with B27 supplement without vitamin A, 0.05 μM retinoic acid, 0.25 μM SAG smoothened ligand and 10 ng/ml of each human BDNF, GDNF and IGF1 (Miltenyi Biotec, Bergisch-Gladbach, Germany). Media were changed every other day by replacing half of the medium.

Lentiviral vectors

Plasmids carrying human cDNAs encoding OCT4, SOX2, KLF4 and c-MYC were from Addgene (plasmids 17217, 17218, 17219, 17220), as well as plasmids carrying the 3.6-kb long HB9 promoter and cDNAs encoding GFP or RFP proteins (plasmids 37080 and 37081). Concentrated VSV-G-pseudotyped retroviral and lentiviral stocks were produced as described previously (Brejot et al., 2006; Lemonnier et al., 2011). For gene transfer into motor neurons, lentiviral particles were added to the cultures on day 18 and 29 of motor neuron differentiation.

Flow cytometry

The iPSc-derived motor neuron cultures were rinsed with PBS, incubated for 15 min at 37 °C with Accutase, dissociated mechanically by several rounds of trituration and filtered on a 70 μm mesh (Becton Dickinson) to obtain single cell suspensions. After centrifugation (470 *g*, 5 min), cell pellets were taken up in 1 ml of supplemented DMEM/F12 and fixed by adding an equal volume of 4% (w/v) formaldehyde in PBS. After 15 min of incubation on ice, 1 ml of PBS containing 1% BSA (w/v) was added and cells were pelleted. Cells were washed in 1 ml PBS/BSA, pelleted, re-suspended in PBS containing 0.5% saponin (w/v) and incubated for 15 min on ice. Cell suspensions were then divided in triplicate tubes, incubated overnight at 4 °C with primary antibodies in PBS containing 0.1% saponin (PBSS), washed twice and incubated for 45 min at 4 °C with Alexa-488 coupled secondary antibodies diluted at 1:2000 in PBSS. Labeled cell suspensions were re-suspended in 100 μl PBS/BSA and kept in the dark at 4 °C until analysis with a FACS ARIA SORP (Becton Dickinson). Signals were plotted with FlowJo software