Stem Cell Research 30 (2018) 61–68

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Motor neuron differentiation of iPSCs obtained from peripheral blood of a mutant *TARDBP* ALS patient

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ARTICLE INFO

Keywords:
Amyotrophic Lateral Sclerosis (ALS)
Induced Pluripotent Stem Cells (iPSCs)
Peripheral blood
TDP43
Motor neuron

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease, mainly affecting the motor neurons (MNs) and without effective therapy. Drug screening is hampered by the lack of satisfactory experimental and pre-clinical models. Induced pluripotent stem cells (iPSCs) could help to define disease mechanisms and therapeutic strategies as they could be differentiated into MNs, otherwise inaccessible from living humans. In this study, given the seminal role of TDP-43 in ALS pathophysiology, MNs were obtained from peripheral blood mononuclear cells-derived iPSCs of an ALS patient carrying a p.A382T *TARDBP* mutation and a healthy donor. Venous samples were preferred to fibroblasts for their ease of collection and no requirement for time consuming extended cultures before experimentation. iPSCs were characterized for expression of specific markers, spontaneously differentiated into primary germ layers and, finally, into MNs. No differences were observed between the mutated ALS patient and the control MNs with most of the cells displaying a nuclear localization of the TDP-43 protein. In conclusion, we here demonstrated for the first time that human *TARDBP* mutated MNs can be successfully obtained exploiting the reprogramming and differentiation ability of peripheral blood cells, an easily accessible source from any patient.

1. Introduction

An important limitation in the field of neurodegenerative disorders is the difficulty to translate information provided by preclinical research into effective new treatments for patients. This unmet need is mainly due to the scarcity of adequate experimental models. This is particularly true for amyotrophic lateral sclerosis (ALS), where motor neurons (MNs) and other nervous tissues affected by disease processes are difficult to obtain from alive patients. In addition, when post mortem brain samples are available, differentiated neurons do not anyway undergo cell division, resulting in a limited utility for *in vitro* functional studies.

The majority of ALS patients present a sporadic form of the disease, while around 10% are familial cases with > 20 causative genes identified so far among which *SOD1*, *C9ORF72, TARDBP* and *FUS* represent the main involved (Chen et al., 2013). Several transgenic animal models have been developed in order to investigate the different pathomechanisms of ALS and to test future possible therapies: the first and most commonly used was the human transgenic *SOD1* mouse (Gurney et al., 1994). Later, hemizygous and homozygous mice expressing wild-type and mutated human TDP-43 (Stallings et al., 2010), (Xu et al., 2010) together with mice carrying the *C9ORF72* GGGGCC repeat expansion (Chew et al., 2015) were generated. Albeit animal models have been useful for investigation of some ALS pathological mechanisms, they recapitulate only partial aspects of the disease mostly reproducing familial forms of ALS. Indeed, drug effects in SOD1 transgenic mice were rarely able to predict the same efficacy in humans (Ludolph et al., 2007), (Ludolph et al., 2010). This discrepancy may be explained by the diversity in both structure and development of rodent and human brains together with the absence of naturally occurring ALS in mice. Consequently, animal models may not be the most adequate tool to fully represent the various phenotypes of human ALS. Cellular model systems, such as NSC-34 (Cashman et al., 1992), SH-SY5Y (Biedler et al., 1973) or HEK293T (Graham et al., 1977) have also been widely used and useful to study ALS pathomechanisms *in vitro*. However, they are mostly tumor-derived or engineered cells, not completely

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https://doi.org/10.1016/j.scr.2018.05.009
Received 30 January 2018; Received in revised form 24 April 2018; Accepted 16 May 2018
Available online 17 May 2018

mimicking the properties of primary neuronal cells. Recent innovative *in vitro* model, such as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006), has represented a turning point in the development of experimental disease models and can help to improve current knowledge of the pathogenic mechanisms of ALS after MN differentiation. In ALS, iPSCs have been generated from both mutated and sporadic patients, extensively expanded in culture and differentiated into cell types such as MNs and astrocytes (Jaiswal, 2017). As the main hallmark of ALS is the progressive degeneration of spinal cord MNs, their generation is essential for *in vitro* studies. MNs derived from iPSCs have been shown to exhibit equivalent functional characteristics of *in vivo* MNs: they express specific markers by immunostaining, are able to project axons and are responsive to glutamate agonists. They therefore represent an ideal tool for modeling MN degenerative diseases (Amoroso et al., 2013). The most widely used somatic cells for iPSCs generation are fibroblasts (Gonzalez et al., 2011) but several other cell types have been exploited, for example peripheral blood cells (Huang, 2010). These cells present many advantages compared to fibroblasts as they do not need to be cultured *in vitro* for several passages, avoiding the risk of genomic changes accumulation. In addition, peripheral blood collection is less invasive than skin biopsy.

The multifunctional RNA-binding protein TDP-43 is involved in RNA metabolism including splicing, transcription and mRNA transport (Ratti and Buratti, 2016). Its localization is primarily nuclear, however the protein shuttles between nucleus and cytoplasm. In both familial and sporadic ALS, as well as in frontotemporal dementia, pathological TDP-43-positive cytoplasmic inclusions are observed in affected brains (Arai et al., 2006), (Neumann et al., 2006). Moreover, mutations in the TDP-43-encoding gene *TARDBP* account for about 5% of familial and 1% of sporadic ALS cases and the missense p.A382T variant is the most frequently occurring one in the Italian population (Corrado et al., 2009). In this study we report, for the first time, the reprogramming of peripheral blood cells from an ALS patient carrying the *TARDBP* p.A382T mutation into iPSCs. These iPSCs were fully characterized for their pluripotency and successfully differentiated into MNs similarly to healthy control-derived iPSCs.

2. Materials and methods

2.1. Participants and samples

This study was approved by the ethics committee of IRCCS Istituto Auxologico Italiano. Written informed consent was obtained from the participants. All research was performed in accordance with relevant guidelines. We selected an ALS patient carrying the TARDBP p.A382T mutation (see details in Table 1) and an unrelated healthy donor as control. Both were females, 63 and 49 years old, respectively. PBMCs were isolated from peripheral blood samples by density gradient centrifugation on Ficoll-PaqueTM Plus (GE Healthcare, Chicago, IL, USA).

2.2. Generation of iPSCs

To generate iPSCs, 5×10^5 PBMCs were seeded in a 24-well plate in StemPro™-34 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with SCF (100 ng/ml), FLT-3 (100 ng/ml), IL-3 (20 ng/ml) and IL-6 (20 ng/ml) cytokines (all from Peprotech, London, UK). Half medium was daily changed and fresh cytokines added to the cell suspension. After four days, transduction was performed using CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific), by adding the Klf4, Oct4, Sox2 and c-Myc virus, following manufacturer's instructions (KOS (Klf4–Oct3/4–Sox2) MOI = 5, hcMyc MOI = 5 and hKlf4 MOI = 3). Three days later, transduced cells were transferred on Mouse Embryonic Fibroblasts (MEF) feeder layer, and grown in StemPro™-34 medium without the cytokines for four days. Thereafter cultures were carried on in Essential 8 medium (Thermo Fisher Scientific). Spent medium was daily replaced with fresh medium and the culture vessels monitored for the emergence of iPSC colonies. Twenty days post-transduction, colonies ready for transfer were individually picked and seeded onto Geltrex (Thermo Fisher Scientific)-coated dishes. Colonies, passaged using an EDTA 0.5 μM solution, were expanded in Essential 8 medium for at least six passages before being characterized and differentiated.

Table 1
Characteristics of the ALS TDP-43 p.A382T patient.

Clinical parameters	Patient data
Dementia	No (normal ECAS score)
ALS-FRS-R score	40/48 at diagnosis, 31/48 at blood sampling
Disease duration	> 5 years
I MN involvement	Yes
II MN involvement	Yes
Onset symptoms	Weakness in drawing head backwards (weakness of the rectus capitis posterior muscle)

2.3. Genotyping of patient-derived iPSCs

Genomic DNA was extracted from mutant iPSC using DNAzol Reagent (Invitrogen). TARDBP exon 6 was amplified by PCR using the following primers: FOR_tgcttgtaatctaagttttgttg and REV_aaatttgaattcccaccattc. The amplicon was sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3500 Genetic Analyzer (Applied Biosystems) and compared with the reference sequence NM_007375.3 GRCh37/hg19.

2.4. In vitro spontaneous differentiation of iPSCs

To test the *in vitro* spontaneous differentiation ability of the established iPSCs, embryoid bodies (EBs) formation was performed by gently resuspending iPSCs colonies in non-tissue culture-treated plates in HuES medium (DMEM/F12, 20% knock-out serum replacement, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 0.1 mM MEM NEAA, 110 μM β-mercaptoethanol) (all from Thermo Fisher Scientific). Medium was changed daily and after 7 days, EBs were collected and plated on Geltrex-coated plates in Essential 8 medium. Expression of the three germ layers specific markers was evaluated by immunofluorescence (βIII tubulin for ectoderm, desmin for mesoderm and alpha-fetoprotein (AFP) for endoderm).

2.5. Motor neuron differentiation

A modified protocol from Amoroso et al. (Amoroso et al., 2013) was used for MN differentiation. A schematic representation of the protocol is shown in Fig. 2a. Briefly, iPSCs were grown in a 100 mm dish until confluence, harvested and placed into non-cell culture-treated dishes. To obtain EBs, for the first 2 days, cells were allowed to grow in suspension in HuES medium supplemented with 20 ng/ml basic fibroblast growth factor (FGF) (Peprotech) and 20 μM Rho-associated kinase (ROCK) inhibitor Y27632 (Selleckchem, Houston, TX, USA) in order to enhance single cell survival. The third day, neuralization was induced by the addition of 10 μM SB431542 and 0.2 μM LDN193189 (both from Stemgent, Cambridge, NY, USA) to the cultures. The fourth day, EBs were switched to neural induction medium (DMEM/F12, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 0.1 mM MEM NEAA, 2 μg/ml heparin (Sigma-Aldrich, Saint Louis, MO, USA), 1% N2 supplement (Thermo Fisher Scientific)), supplemented with 20 μM ROCK inhibitor, 0.4 μg/ml ascorbic acid (AA) (Sigma-Aldrich), 1 μM retinoic acid (RA) (Sigma-Aldrich), 10 ng/ml brain-derived neurotrophic factor (BDNF) (Peprotech). SB431542 and LDN193189 were added until day 7 when cultures were supplemented with 1 μM smoothened agonist (SAG) (Merck) and 0.5 μM purmorphamine (Pur) (Sigma-Aldrich). EBs were grown for additional ten days with a