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Induced pluripotent stem cell - derived neurons for the study of spinocerebellar ataxia type 3

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ABSTRACT

The neurodegenerative disease spinocerebellar ataxia type 3 (SCA3) is caused by a CAG-repeat expansion in the *ATXN3* gene. In this study, induced pluripotent stem cell (iPSC) lines were established from two SCA3 patients. Dermal fibroblasts were reprogrammed using an integration-free method and the resulting SCA3 iPSCs were differentiated into neurons. These neuronal lines harbored the disease causing mutation, expressed comparable levels of several neuronal markers and responded to the neurotransmitters, glutamate/glycine, GABA and acetylcholine. Additionally, all neuronal cultures formed networks displaying synchronized spontaneous calcium oscillations within 28 days of maturation, and expressed the mature neuronal markers NeuN and Synapsin 1 implying a relatively advanced state of maturity, although not comparable to that of the adult human brain. Interestingly, we were not able to recapitulate the glutamate-induced ataxin-3 aggregation shown in a previously published iPSC-derived SCA3 model. In conclusion, we have generated a panel of SCA3 patient iPSCs and a robust protocol to derive neurons of relatively advanced maturity, which could potentially be valuable for the study of SCA3 disease mechanisms.

1. Introduction

Spinocerebellar ataxia type 3 (SCA3) is a rare autosomal dominantly inherited neurodegenerative disease caused by a CAG-repeat expansion mutation in the *ATXN3* gene, coding for a polyglutamine (polyQ) stretch in the protein ataxin-3. In healthy individuals, the CAG-repeat length spans from 10 to 51, whereas the repeat length in SCA3 patients ranges from 45 to 87, thus 45 to 51 repeats can result in either healthy or disease phenotypes (Matos et al., 2011). Although expanded (exp)-ataxin-3 is expressed throughout the human body (Paulson et al., 1997), only certain brain areas are affected in SCA3. Magnetic resonance imaging and macroscopic investigations of SCA3 brains have revealed severe atrophy of the hindbrain (cerebellum and brain stem) (Rüb et al., 2006; Scherzed et al., 2012). Histological studies have confirmed neuronal loss in these areas and several other brain areas (reviewed by Rüb et al., 2008). This degeneration results in a variety of symptoms of which the most common is progressive ataxia (Matos et al., 2011). Ultimately, SCA3 patients encounter approximately 15 years reduction in life expectancy (Kieling et al., 2007).

Although the CAG-expansion in *ATXN3* is known to cause SCA3, the underlying cellular mechanisms leading to neurodegeneration are not fully elucidated. It has been proposed that the polyQ expansion alters normal ataxin-3 functions in for example endoplasmic reticulum associated degradation (ERAD) and transcriptional regulation (Matos et al., 2011). The polyQ repeat expansion also leads to the formation of SDS-insoluble exp.-ataxin-3 aggregated oligomers and large neuronal intranuclear inclusions (NIIs) (Ellisdon et al., 2006), of which especially the oligomers are thought to be toxic (Pellistri et al., 2013). Aggregates can sequester proteins from diverse cellular processes (Chai et al., 2002; Matos et al., 2011; Mori et al., 2012) and may also disturb the cellular quality control systems, such as the ubiquitin proteasome system and autophagy by overloading (Matos et al., 2011). A prevailing hypothesis is that cleavage of exp.-ataxin-3 is important for its aggregation, as C-terminal polyQ containing fragments of exp.-ataxin-3 seed ataxin-3 aggregation and induce cell death to a higher extend than full length exp.-ataxin-3 (Goti, 2004). Additionally, fragments of ataxin-3 have been detected in affected brain areas of SCA3 patients but not in unaffected areas or in brains of healthy

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controls (Goti, 2004). In particular the role of calpain-mediated ataxin-3 cleavage in SCA3 pathogenesis is supported by convincing evidence (Hübener et al., 2013; Koch et al., 2011).

Functional studies investigating molecular mechanisms of neurodegenerative diseases are generally limited by the lack of reliable *in vitro* models of human neurons. Induced pluripotent stem cell (iPSC)-derived neurons are a potential solution to this problem and several disease models have already been generated and published (Reviewed by Corti et al., 2015). The first iPSC lines derived from SCA3 patients were reported by Koch et al. (Koch et al., 2011). In that study, SDS-insoluble exp.-ataxin-3 aggregates could be induced by stimulation with glutamate. This aggregation could be prevented by calpain inhibition, supporting the hypothesis that glutamate induced calcium influx activates calpains, which subsequently mediates the cleavage and aggregation of exp-ataxin-3. In the present study, we aimed at creating iPSCs from SCA3 patient fibroblasts by a non-integrating method, as integration of reprogramming vectors might lead to insertional mutagenesis and has been shown to decrease the differentiation capacity of iPSCs (Sommer et al., 2010). We strived to obtain a model system with increased resemblance to the affected regions of SCA3 brains by applying a differentiation method, which favored the development of hindbrain neurons. Furthermore, we sought to obtain mature neurons to approximate the situation in SCA3 patients where pathology develops in late childhood at earliest (Kieling et al., 2007). Finally, we investigated whether glutamate-induced SDS-insoluble exp-ataxin-3 aggregates similar to those detected by Koch et al. could be observed in our neurons.

2. Materials and methods

2.1. Reprogramming of fibroblasts to iPSCs

The study was approved by the Ethics Committee of the Capital Region of Denmark (H-4-2011-157), and written informed consent was obtained from all patients before enrollment. iPSC lines were created from two SCA3 patients (patient characteristics are summarized in Table S1). SCA3 patient dermal fibroblasts were obtained from forearm skin biopsies and reprogrammed as previously described (Hansen et al., 2016a, 2016b). Briefly, fibroblasts were electroporated with the episomal plasmids pCXLE-hUL (*L-MYC, LIN28*, Adgene #27080), pCXLE-hSK (*SOX2, KLF4,* Adgene #27078) and pCXLE-hOCT4-shp53-F (*OCT4, shP53*, Adgene #27077) (Okita et al., 2011). iPSCs were cultured in mTeSR1 (Stem Cell Technologies, 05850) with 0.1 % penicillin-streptomycin (ThermoFisher Scientific, 15140-122) on hESC-qualified matrigel (BD Biosciences, 354277). Three well-characterized control iPSC lines [K1_shP53 (K1), K2_shP53 (K2) and K3_shP53 (K3), deposited in the European Bank of induced Pluripotent Stem Cells (EBiSC) as BIONi010-A, BIONi010-B and BIONi010-C, respectively] derived from normal human dermal fibroblasts (Lonza, CC-2511) applying the same reprogramming protocol (Rasmussen et al., 2014) were also investigated in this study.

2.2. Neuronal differentiation

Differentiation of iPSCs to neural stem cells (NSCs) was performed using pluripotent stem cell (PSC) neural induction medium (ThermoFisher Scientific, A1647801). iPSCs were treated with neural induction medium (NIM) containing 98% neurobasal medium (ThermoFisher Scientific, 21103-049), 2% PSC neural induction supplement (ThermoFisher Scientific, A1647801) and 0.1% penicillin-streptomycin for 7 days. Day 7, non-neural cells were manually removed and the cells were split with accutase (ThermoFisher Scientific, A1110501) and seeded on 100 μg/ml poly-L-ornithine (PLO) (Sigma-Aldrich, P3655) and 10 μg/ml laminin (LAM) (Sigma-Aldrich, L2020) in neural expansion medium (NEM) supplemented with 10 μM Rock inhibitor (Sigma-Aldrich, Y0503). NEM consisted of 49% neurobasal medium (ThermoFisher Scientific, 21,103–049), 50% Advanced DMEM/F-12 (ThermoFisher Scientific, 12,634), 1% PSC neural induction supplement (ThermoFisher Scientific, A1647801) and 0.1% penicillin-streptomycin. Day 13, the cells were split with accutase and seeded at a density of 1.56×10^5 cells/cm^2 for expansion in NEM with Rock inhibitor. Between day 16–19 NSCs (p2) were frozen in NEM supplemented with 10% DMSO and 10 μM Rock inhibitor (Sigma-Aldrich, Y0503). NSCs were thawed (p3), plated at a density of 1.56×10^5 cells/cm^2 expanded and frozen in larger stocks for experiments (p4). For maturation, NSCs (p6) were seeded at a density of 1.56×10^5 cells/cm^2 in NEM supplemented with 10 μM Rock inhibitor on PLO/LAM (day 0). From day 1–28 of maturation, medium was changed to neuronal maturation medium (NMM) consisting of 50% DMEM/F12 (ThermoFisher Scientific, 31,331) and 50% neurobasal medium, supplemented with 0.5× N2 (ThermoFisher Scientific, 17,502–048), 0.5× B-27 with vitamin A (ThermoFisher Scientific, 17504-044), 1.48 mM glutamax-I (ThermoFisher Scientific, 35,050–061), 0.5× non-essential amino acids (ThermoFisher Scientific, 11140-050), 50 μM 2-mercaptoethanol (ThermoFisher Scientific, 21985-023), 2.5 μg/ml insulin (Sigma-Aldrich, 19,278), 0.1% penicillin-streptomycin, 20 ng/ml brain-derived neurotrophic factor (BDNF) (RnD Systems, 248-BD), 10 ng/ml glial cell derived neurotrophic factor (GDNF) (RnD Systems, 212-GD) and 200 μM ascorbic acid (AA) (Sigma-Aldrich, A5960). 500 μM dibutyryl cyclic adenosine monophosphate (db-cAMP) (Sigma-Aldrich, D0627) was added to the medium from day 1 to day 21–28. Medium was changed every 2 days. Day 7 the cells were split with accutase, seeded at a density of 1.56×10^5 cells/cm^2 on PLO/LAM in NEM with Rock inhibitor and cultured until evaluation in NEM.

2.3. Characterization of iPSCs

The SCA3 iPSC lines were investigated for karyotype, plasmid integration, expression of pluripotency marker mRNA and protein, *in vitro* differentiation and CAG-repeat length as previously described (Hansen et al., 2016a, 2016b).

2.4. Quantitative real time polymerase chain reaction (qRT-PCR)

For characterization of the neuronal differentiation, cells were harvested and reverse transcribed into cDNA with Fast SYBR Green Cells-to-C_T Kit (Ambion, 4402957) according to manufacturer's instructions. RNA from adult whole brain (6516–1) and fetal whole brain week 22–30 (636,118) was purchased from Clontech. Fetal hindbrain week 8,5–9 was kindly provided by Agnete Kirkeby (Lund University). Control RNA was reverse transcribed with TaqMan reverse transcription reagents. qRT-PCR was run with 2× diluted SsoFast EvaGreen Supermix (Bio—Rad, 172–5204). C_T-values were normalized to the geometric mean of the housekeeping genes *Hsp90AB1, GUSB* and *RPL13A* using the $\Delta\Delta C_T$-method. Primers were either conventional primers purchased from Eurofins (Table S2) or Taqman primers purchased from Applied Biosystems (Table S3). The specificity of the conventional primers was validated by standard curves, melting curves and gel electrophoresis.

2.5. Fluorescent immunocytochemistry

For characterization of NSCs and neurons, cells were fixed in 4% paraformaldehyde for 10 min. The cells were blocked in KPBS (0.01 M phosphate buffer with 150 mM NaCl and 3.4 mM KCl) with 0.5% BSA, 0.1% Triton X-100 and 5% normal swine serum (Jackson ImmunoResearch, 014–000-121) for 1 h, incubated with primary antibodies (Table S4) in blocking buffer ON at 4 °C and incubated with secondary antibody and 0.7 μg Hoechst diluted in KPBS for 1 h at RT. The secondary antibodies were used in 1:800 dilution: Alexa 488 donkey-*anti*-mouse (A21202), Alexa 568 donkey-anti-rabbit (A10042), Alexa 488 donkey-anti-goat (A11055), Alexa 594 donkey-anti-rat (A21209) and Alexa 568 goat-anti-mouse (A11031) purchased from ThermoFisher Scientific. The cells were mounted in fluorescent mounting medium (Dako, S3023) and images were captured on a Nikon E1000 microscope and processed with ImagePro Plus v7.0.