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Stem cell-derived motor neurons from spinal and bulbar muscular atrophy patients

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ABSTRACT

Spinal and bulbar muscular atrophy (SBMA, Kennedy's disease) is a motor neuron disease caused by polyglutamine repeat expansion in the androgen receptor. Although degeneration occurs in the spinal cord and muscle, the exact mechanism is not clear. Induced pluripotent stem cells from spinal and bulbar muscular atrophy patients provide a useful model for understanding the disease mechanism and designing effective therapy. Stem cells were generated from six patients and compared to control lines from three healthy individuals. Motor neurons from four patients were differentiated from stem cells and characterized to understand disease-relevant phenotypes. Stem cells created from patient fibroblasts express less androgen receptor than control cells, but show androgen-dependent stabilization and nuclear translocation. The expanded repeat in several stem cell clones was unstable, with either expansion or contraction. Patient stem cell clones produced a similar number of motor neurons compared to controls, with or without androgen treatment. The stem cell-derived motor neurons had immunoreactivity for HB9, Isl1, ChAT, and SMI-32, and those with the largest repeat expansions were found to have increased acetylated α-tubulin and reduced HDAC6. Reduced HDAC6 was also found in motor neuron cultures from two other patients with shorter repeats. Evaluation of stably transfected mouse cells and SBMA spinal cord showed similar changes in acetylated α-tubulin and HDAC6. Perinuclear lysosomal enrichment, an HDAC6 dependent process, was disrupted in motor neurons from two patients with the longest repeats. SBMA stem cells present new insights into the disease, and the observations of reduced androgen receptor levels, repeat instability, and reduced HDAC6 provide avenues for further investigation of the disease mechanism and development of effective therapy.

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Introduction

Our understanding of the pathogenesis of motor neuron disease has been limited by the paucity of model systems that can recapitulate disease features as they occur in vivo. SBMA is caused by a CAG repeat expansion in the androgen receptor (AR) gene on the X chromosome (La Spada et al., 1991), which results in polyglutamine expansion and an androgen-dependent toxic gain of function in the mutant protein. SBMA patients have repeat lengths between 38 and 62 CAGs; whereas normal individuals have between 5 and 36 CAGs (Atsuta et al., 2006; Rhodes et al., 2009). The length of the CAG repeat correlates inversely with the age of disease onset, with longer repeats associated with earlier onset. The affected males have a slowly progressive deficit with weakness of the limb and bulbar muscles due to lower motor neuron and muscle degeneration. There is currently no treatment available to affect the progression of this disease, and although cellular processes such as transcriptional regulation (Nedelsky et al., 2010), mitochondrial function (Ranganathan et al., 2009), and axonal transport (Katsuno et al., 2006) have been implicated, the precise mechanism underlying the pathology is not clear.

The induced pluripotent stem cell (iPSC) system provides a unique opportunity in which stem cells can be generated from adult patients and then differentiated into disease-relevant progeny such as neurons, glia, and muscle. This technology has been used to generate and differentiate iPSCs from patients with motor neuron diseases such as ALS

Abbreviations: AR, androgen receptor; DHT, dihydrotestosterone; EtOH, ethanol; iPSC, induced pluripotent stem cell; SBMA, spinal and bulbar muscular atrophy.

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(Dimos et al., 2008). A phenotype has been described in motor neuron cells differentiated from spinal muscular atrophy (SMA) iPS cells (Ebert et al., 2009). An assay for screening small molecules in such a system may also be expected to yield more disease-relevant results, since it is human patient-derived. Motor neuron-like cells derived from ALS patients with mutations in TDP-43 have been used to screen candidate chemical compounds (Egawa et al., 2012). These systems offer the potential advantage of reproducing the cellular and molecular features of the disease, with physiological levels of mutant protein expression. Additional insights into the disease mechanism may provide new targets for therapeutic development.

In this study we generated lines from six SBMA patients, and performed a detailed evaluation of lines from four different patients and three controls. The SBMA iPSCs retain the CAG repeat expansion present in the parental fibroblasts, and in some cases variation from the parental CAG repeat length was observed. We found that the AR is expressed in the undifferentiated iPSCs, and able to translocate to the nucleus in response to ligand treatment. The iPSC lines were differentiated into motor neurons, and further characterization of these cells was done. Motor neuron derivatives from the SBMA iPSCs were found to have a reduction in HDAC6. The iPSCs described here are valuable tools for understanding the disease process, and they provide a system for evaluating candidate treatments.

Materials and methods

iPSC generation

Lentiviral vectors containing the polycistronic transcripts Oct4, Klf4, Sox2, and c-Myc (Sommer et al., 2009) were purchased from Millipore (Billerica, MA) and Stemgent (Cambridge, MA). Human fibroblasts were seeded at 2.5×10^4 cells/well on a 6-well plate. For the next two days the fibroblasts were transduced with virus and plated onto a feeder layer of mitomycin-c treated MEF cells at day 6. Colonies with hESC morphology were mechanically dissociated after three weeks. Sendai virus was purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's instructions. Episomal vectors were purchased from Addgene (Cambridge, MA) and cotransfected into fibroblasts using the U-20 Amaxa nucleofection program (Walkersville, MD). After expansion, the iPSCs were maintained in mTESR media (Stem Cell Technologies, Vancouver, Canada).

CAG repeat sizing

Polymerase chain reaction (PCR) was performed using Taq polymerase-containing master mix (FastStart PCR Master; Roche, Basel, Switzerland), with 20 nM forward and reverse primers (5′-TCCAGAATCTGTTCCAGAGCGTGC-3′ and 5′-GCTGTGAAGGTTGCTG TTCCTCAT-3′, respectively). PCR products were analyzed on a 20% Tris–borate–EDTA gel (Invitrogen). The Big Dye cycling protocol (Invitrogen) was performed using a two step method without the annealing step (96 °C 10 s, 60 °C 4 min).

Motor neuron differentiation

Differentiation was performed as previously described (Amoroso et al., 2013). Briefly, iPSCs were grown to 80% confluency, then digested with collagenase IV (Invitrogen) for 8 min. Cells were scraped off of the dish, and after settling the supernatant was aspirated, and cells were re-plated into low adherence dishes (Corning, Corning, NY) in KSR (Invitrogen) based media with 20 ng/ml FGF (R + D Systems, Minneapolis, MN), 20 μM ROCK-I (Tocris, Bristol, UK), 10 μM SB431542 (Tocris), and 0.2 μM LDN193189 (Stemgent). Embryoid bodies (EBs) were transitioned to a KSR free medium after 3 days. Retinoic acid was added to the media after 5 days to direct the cells towards a rostral spinal cord phenotype, with additional patterning using 1 μM smoothened agonist (Calbiochem, Billerica, MA) and 0.5 μM purmorphamine (Stemgent) after 7 days to ventralize the differentiating population. After 14–16 days in suspension, the EBs were dissociated and plated on dishes coated with polyornithine or poly-D-lysine and laminin for an additional 7–14 days. Neuronal cultures were maintained in neurobasal media (Invitrogen) with 25 μM glutamate (Sigma, St. Louis, MO), 0.4 μg/ml ascorbic acid (Sigma), 10 ng/ml GDNF (Sigma), 10 ng/ml CNTF (Sigma), 1 μg/ml laminin (BD Bioscience, Franklin Lakes, NJ). B-27, N2, non-essential amino acids, and pen/strep/glutamine were all from Invitrogen. Two days after plating 10 nM dihydrotestosterone (DHT) was added, and the cultures were maintained for an additional 7–14 days.

Immunoblotting

Cell pellets were lysed with RIPA buffer [150 mM NaCL, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and Protease inhibitor cocktail (Roche)] and allowed to stand on ice for 20 min before being centrifuged at 4 °C at 20,000 *g* for 20 min. Supernatant was collected and protein levels were measured using a Bio-Rad (Hercules, CA) protein assay. Membranes were blocked with a 5% milk solution and incubated in milk with primary antibody overnight. The antibodies used were AR H280 and AR N20 (Santa Cruz Biotech., Santa Cruz, CA), GAPDH (EnCor Biotechnology, Gainesville, FL), ChAT (Millipore), MNX1/HLXB9 (Novus, Littleton, CO), HDAC6 D2E5 and LAMP1 (Cell Signaling, Danvers, MA). Stacking gel analysis for aggregation analysis was performed on supernatant samples prepared after centrifugation at 4 °C for 10 min at 425 *g*. PNGaseF was purchased from New England Biolabs (Ipswich, MA), and treatments were performed for 3 h using the manufacturer's guidelines.

Immunohistochemistry

After fixation with 4% paraformaldehyde, slides were placed in blocking solution (10% normal goat serum, 0.3% Triton X-100, in phosphate-buffered saline (PBS)) for 45 min at room temperature. Primary antibody staining was done at 4 °C overnight in PBS with 5% normal goat serum and 0.1% Triton X-100 using Oct-3/4 (Santa Cruz), GSK-3β Phospho Ser9 (Cell Signaling), AR H280 and N20 (Santa Cruz), Tra-1-60, 1C2, and Chat (Millipore), Isl1 and HB9 (DSHB, Iowa City, IA), SMI 32 and β-tubulin (Covance, Princeton, NJ), HDAC6 and LAMP1 (Cell Signaling), and acetylated tubulin and neurofilament (Sigma). Slides were then washed three times with PBST (0.1% Triton X-100 in PBS), incubated with secondary antibody (Invitrogen, 1:500) for 1 h at room temperature in the dark, and then washed three times before drying and adding vectashield/DAPI stain (Vector Lab, Burlingame, CA). For AR staining in motor neurons, slides were treated with 100 mM glycine after fixation, and blocked in PBS with 3% bovine serum albumin (BSA). Antibody staining was performed in PBS with 3% BSA and 0.1% Tween with 0.1% Tween/PBS used for all washes. Coverslips were mounted with permamount (Thermo, Waltham, MA).

Results

Derivation and characterization of iPSC from SBMA patients and controls

IPSC lines were derived using forearm fibroblasts from six SBMA patients (SB1, 3, 6, 15, 17, and 18) and three healthy control adults (NC4, 7, and 15) (Table 1). All iPSC lines chosen for evaluation had expression of pluripotency markers by immunostaining and quantitative PCR (Fig. S1). Karyotype analysis was normal in 12 clones tested, from which 5 patient (Fig. 1, asterisks) and 3 control lines were chosen for further analysis. Down-regulation of Oct4 was observed in the motor neuron derivatives from the iPSC, showing sufficient down regulation of the polycistronic vector. Teratoma analysis was done for the SB6MP2 clone.