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The first reported generation of several induced pluripotent stem cell lines from homozygous and heterozygous Huntington's disease patients demonstrates mutation related enhanced lysosomal activity

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ABSTRACT

Neuronal disorders, like Huntington's disease (HD), are difficult to study, due to limited cell accessibility, late onset manifestations, and low availability of material. The establishment of an in vitro model that recapitulates features of the disease may help understanding the cellular and molecular events that trigger disease manifestations. Here, we describe the generation and characterization of a series of induced pluripotent stem (iPS) cells derived from patients with HD, including two rare homozygous genotypes and one heterozygous genotype. We used lentiviral technology to transfer key genes for inducing reprogramming. To confirm pluripotency and differentiation of iPS cells, we used PCR amplification and immunocytochemistry to measure the expression of marker genes in embryoid bodies and neurons. We also analyzed teratomas that formed in iPS cell-injected mice. We found that the length of the pathological CAG repeat did not increase during reprogramming, after long term growth in vitro, and after differentiation into neurons. In addition, we observed no differences between normal and mutant genotypes in reprogramming, growth rate, caspase activation or neuronal differentiation. However, we observed a significant increase in lysosomal activity in HD-iPS cells compared to control iPS cells, both during self-renewal and in iPS-derived neurons.
In conclusion, we have established stable HD-iPS cell lines that can be used for investigating disease mechanisms that underlie HD. The CAG stability and lysosomal activity represent novel observations in HD-iPS cells. In the future, these cells may provide the basis for a powerful platform for drug screening and target identification in HD.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by excessive expansion of a CAG trinucleotide repeat in the gene encoding the huntingtin protein (*htt*). This trinucleotide repeat results in the addition of a long stretch of glutamines (polyQ) near the N-terminus of the protein (HD Collaborative Research Group, 1993). The disease is characterized by movement, cognitive, and emotional disturbances that result from massive

Abbreviations: iPS, induced pluripotent stem; HD, Huntington's disease; WT, wild type; HTT, huntingtin; PH3, phospho-histone H3; MEF, mouse embryonic fibroblasts; EBs, embryoid bodies; ES, embryonic stem.

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neurodegeneration involving, at first, striatal medium spiny neurons and later, entire cortical structures (Reiner et al., 1988).

The disease occurs as a consequence of the expanded polyQ tract, whose length dictates the disease onset. However, recent evidence has suggested that the loss of physiological activity in the normal protein may also contribute to disease pathogenesis (Cattaneo et al., 2001, 2005; Zuccato et al., 2010). Consistent with this possibility, in a small collection of homozygous patients, disease progression appeared to precipitate faster than in heterozygous patients (Squitieri et al., 2003); this was also observed in a mouse model, although further confirmatory studies are needed (Lin et al., 2001).

To facilitate investigations of the disease mechanisms, an impressive series of HD models has been developed (Zuccato et al., 2010). Neuronal disorders are in fact probably among the most difficult to model due to limited cell accessibility, late onset manifestations, and low availability of material, which often reflects only the final phases of pathology. Cell lines that carry the HD mutation provide biochemically homogeneous material for testing specific hypotheses. For example, studies initially performed in rodent cell lines have led to the loss of HTT function hypothesis, evidence for a defect in BDNF, altered vesicular transport along microtubules in neurons (Colin et al., 2008; Gauhier et al., 2004) and data on cholesterol abnormalities in HD. Subsequently, those findings were verified in animal models and, where appropriate, in human post mortem patient samples (Zuccato et al., 2010). However, we lack a reliable, stable source of human cell lines with a central nervous system (CNS) character that correctly express the mutant gene during neuronal differentiation. Towards this goal, 8 human embryonic stem (hES) cell lines were derived from preimplantation genetic diagnosis embryos that carried the mutant gene. However, these cell lines remained poorly investigated (Bradley et al., 2011; Mateizel et al., 2006; Niclis et al., 2009; Verlinsky et al., 2005).

The discovery of human somatic cell reprogramming to generate iPS cells has captured scientific interest because it can facilitate the creation of patient-specific in vitro models of human disorders. Promising results have already been achieved with iPS cells derived from patients with Parkinson disease (PD), amyotrophic lateral sclerosis, spinal muscular atrophy, familial dysautonomia, X fragile syndrome, and Rett syndrome (Dimos et al., 2008; Ebert et al., 2009; Lee et al., 2009; Marchetto et al., 2010; Soldner et al., 2009; Urbach et al., 2010). However, this field is young, and only some studies have investigated and identified molecular alterations (and partial reversions) (Ebert et al., 2009; Lee et al., 2009; Marchetto et al., 2010). On the other hand, given that disease onset, as in HD or PD, occurs rather late in life, it remains unclear whether early pre-symptomatic changes are to be expected in pluripotent cells, in neural progenitors or in more differentiated neuronal cells. Furthermore, in diseases that develop in a non-cell-autonomous manner, like PD, the search for a phenotype may be even more problematic. In HD, some molecular alterations have been found in cells that do not undergo degeneration, for example, glial cells (Lobsiger and Cleveland, 2007; Valenza and Cattaneo, 2011); however, the mutant gene is known to be preferentially toxic to neurons, particularly striatal and cortical neurons. For this reason, neuronal (and/or glial) cells obtained from HD-iPS cell lines can potentially recapitulate at least a consistent part of the molecular underpinnings of the disease.

The first human HD-iPS cell line was generated by Park and collaborators in 2008, but no data about its characteristics were presented. The same HD-iPS cell line was later used to derive neuronal precursors and neurons, and these exhibited a mild increase in caspase activity (Zhang et al., 2010).

In the present study, we generated iPS cells from primary fibroblasts from three different patients with HD. These patients included two rare homozygous genotypes. We investigated the capacity of this unique disease-specific cellular system for analyzing possible molecular abnormalities and neurogenic potential of human HD stem cells derived from patients.

We demonstrated that the presence of one or two mutant HTT alleles did not impair the reprogramming process, iPS cell self-renewal and their conversion to neural progenitors as well as the yield of generated mature neurons. On the other hand, the HD mutant cells exhibited altered lysosomal content that was maintained during proliferation and in iPS-derived neurons.

Taken together, these observations demonstrated that HD-iPS cells may be a relevant system for disease modeling while providing the basis for a powerful platform for drug screening and target identification in HD.

Materials and methods

Vector production

A reprogramming vector Stem Cell Cassette (STEMCCA) that included 4 factors (*OCT4*, *SOX2*, *KLF4*, and *C-MYC*) was a kind gift from G. Mostoslavsky. We also prepared a stock solution of a reprogramming vector that included 3 factors (*OCT4*, *SOX2*, *KLF4*), as previously described (Follenzi and Naldini, 2002). Briefly, 293T cells were co-transfected with 4 vectors by calcium phosphate precipitation; these vectors were the pCCLsin.PPT.pA.TK.mCMV.SFFV.OCT3/4_FMDV 2A_KLF4_TaV 2A_SOX2.Wpre.3'LTR_loxP transfer vector plasmid (36 μg); the pMD.Lg/pRRE packaging plasmid (12.5 μg); the pMD2.VSV-G envelope-encoding plasmid (9 μg); and pCMV-Rev (6.25 μg). All four vectors were added to cells in a 15-cm dish, and 1 mM sodium butyrate was added to the collected medium. Vector particles were concentrated 250-fold by ultracentrifugation and measured with HIV-1 Gag p24 immunocapture assay (Perkin Elmer). Retroviruses were produced in Plat-E cells that were transfected with pBabe-based retroviral vectors for OCT4, SOX2, KLF4, in DMEM containing 10% FBS, using Fugene 6 (Roche).

Fibroblast culture and infection

Skin biopsies were obtained from patients coming for clinical follow-up visits at the Neurological Institute “C. Besta” in Milan. All subjects gave their written consent for the skin biopsy procedure and for the use of the sample material for research purposes. Fibroblasts were cultured in DMEM high glucose (Euroclone), 10% FBS (Euroclone), 2 mM L-Glutamine (Euroclone, Italy), and 1% penicillin/streptomycin (Invitrogen). We plated 1.5×10^4 cells in a 6-well dish and performed infection with viral construct that encoded the transcription factors *OCT4*, *SOX2*, and *KLF4* (OSK, with or w/o *C-MYC*), in the presence of 4 μg/ml of polybrene. When reprogramming was performed using retroviral vectors, fibroblasts were previously transduced with SLC7A1 receptor. After 1 week, we replated the infected fibroblasts on a feeder layer of mouse embryonic fibroblasts (MEFs from strain CD1, 3.5×10^4 cells/cm^2) or on human neonatal Foreskin (3.5×10^4 cells/cm^2) mitotically inactivated by Mitomicin C treatment (Sigma Aldrich), in Knock-out-DMEM (Invitrogen) supplemented with 20% Knock-out serum replacement (Invitrogen), 2 mM L-Glutamine (Euroclone, Italy), 2 mM nonessential amino acids (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.1 mM β-mercaptoethanol (Invitrogen), with 10 ng/ml of bFGF (Invitrogen). Colonies started to appear 30 days later, and at around day 40, they were selected and transferred to a new feeder layer with the same culture conditions. Subsequently, iPS clones were passaged mechanically every 5–7 days and the presumed undifferentiated regions were transferred to a new feeder layer. In our experiments, we used only undifferentiated colonies.