The generation of pluripotent stem cells from an individual patient would enable the large-scale production of the cell-types affected by that patient’s disease. These cells could in turn be used for disease modeling, drug discovery, and eventually autologous cell-replacement therapies. Although recent studies have demonstrated the reprogramming of human fibroblasts to a pluripotent state, it remains unclear whether these induced pluripotent stem (iPS) cells can be produced directly from elderly patients with chronic disease. We have generated iPS cells from an 82 year-old woman diagnosed with a familial form of amyotrophic lateral sclerosis (ALS). These patient-specific iPS cells possess properties of embryonic stem cells and were successfully directed to differentiate into motor neurons, the cell type destroyed in ALS.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder in which motor neuron loss in the spinal cord and motor cortex leads to progressive paralysis and death (1). Studies aimed at understanding the root causes of motor neuron death in ALS and efforts to develop new therapeutics would be greatly advanced if a robust supply of human motor neurons carrying the genes responsible for this condition could be generated. It recently was reported that mouse (2–5) and human (6) skin fibroblasts can be reprogrammed to a pluripotent state, similar to that of an embryonic stem (ES) cell, following retroviral transduction with four genes. However, it remains unclear whether iPS cells can be generated directly from elderly patients with chronic disease using material that has been exposed to disease-causing agents for a life-time, and whether such patient-specific iPS cells could be differentiated into the particular cell types that would be needed to treat or study the patient’s condition.

Here we demonstrate that iPS cells can be produced using skin fibroblasts collected from an 82 year-old patient diagnosed with a familial form of ALS. These patient-specific iPS cells possess a gene expression signature similar to human ES cells and can be differentiated into cell types representative of each of the three embryonic germ layers. We have used these iPS cells to produce patient-specific motor neurons and glia, the cell types implicated in ALS pathology.

Under human research subject and stem cell protocols approved by the institutional review boards and embryonic stem cell research oversight committees of both Harvard University and Columbia University, we recruited patients with ALS and healthy controls to donate skin biopsies to be used in reprogramming studies and the production of pluripotent stem cell lines. Our initial studies focused on two female Caucasian siblings, A29 and A30, who were 82 and 89 years old at the time of donation. These sisters are among the oldest living patients with disease-associated SOD1 alleles.
Primary skin cells isolated by biopsy from these patients exhibited the morphology (Fig. 1A), cell cycle profile (fig. S2), and antigenic expression pattern (fig. S3) of human fibroblasts. Transgenes encoding KLF4, SOX2, OCT4, and c-MYC were introduced into these fibroblasts using VSVg pseudotyped Moloney-based retroviruses. Approximately 30,000 fibroblasts were transduced twice over 72 hours, cultured for four days in standard fibroblast medium, and then passaged onto a feeder layer of mouse embryonic fibroblasts in an ES cell-supportive medium. As described previously, within one week hundreds of colonies composed of rapidly dividing cells with a granular morphology not characteristic of ES cells had appeared (6). However, two weeks later, a small number of colonies with an ES cell morphology (Fig. 1, B and C) could be identified. All ES cell-like colonies, twelve from A29 and three from A30, were manually picked and clonally expanded. Of these colonies, seven from A29 and one from A30 gave rise to stable cell lines that could be further expanded. Because donor A29 had been diagnosed with classical ALS, we focused our initial characterization on three putative patient-specific iPS cell lines derived from her.

To verify that the patient-specific iPS cell lines were genetically matched to the donor, we performed DNA fingerprinting analysis of the three putative iPS cell lines (A29a, A29b, and A29c) and the fibroblasts from which they were derived. Allele assignments indicated that each of the putative iPS cell lines carried the genotype of the patient’s fibroblasts (table S1). Additionally, we compared the SOD1 genotype of these cell lines with that of the donated fibroblasts and patient medical history using direct sequencing (Fig. 1D) and an allele-specific restriction fragment length polymorphism (fig. S1, A and B). In each of these assays, we detected the expected L144F polymorphism in both the putative A29 iPS cell lines and the fibroblasts from which they were derived, but not in fibroblasts isolated from a healthy control individual (A18). Furthermore, PCR analysis of genomic DNA from these three cell lines demonstrated that they all carried integrated copies of the four retroviral transgenes with which they had been transduced (fig. S1C).

To establish that reprogramming of the patient fibroblasts had occurred, and that the putative iPS cells were pluripotent, we evaluated their similarity to ES cells. Like ES cells (8), and unlike the parental A29 fibroblasts, the A29 iPS cells displayed an active cell cycle profile with 35% of cells in S or G2/M phases (fig. S2). The putative iPS cell lines also maintained a normal karyotype (fig. S1D). Additionally, all three iPS cell lines exhibited strong alkaline phosphatase activity, and expressed several ES cell-associated antigens (SSEA-3, SSEA-4, TRA1-60, TRA1-81, NANOG), but were not immunoreactive for a fibroblast-associated antigen (TE-7) (Fig. 1, E and F, and fig. S3). Quantitative RT-PCR showed that genes expressed in pluripotent cells (REXI/ZFP42, FOXD3, TERT, NANOG, and CRIPTO/TGF1) were transcribed at levels comparable to human ES cells in each of the three putative iPS cell lines (Fig. 2A). Moreover, whereas expression of the stem cell marker genes SOX2 and OCT4 were silent in the patient fibroblasts, the endogenous loci in the putative iPS cells had become activated to levels similar to those in ES cells (Fig. 2B). As in previous reports (6), expression levels of the endogenous KLF4 and c-MYC loci were similar in ES cells, iPS cells, and the parental fibroblasts (Fig. 2B). Human iPS cells have been shown in some (6), but not all (6, 9), cases to silence expression of the retroviral transgenes used to reprogram them. RT-PCR analyses performed using primers specific to the retroviral transcripts demonstrated nearly complete silencing of viral SOX2 and KLF4. However, some expression of viral OCT4 and c-MYC persisted, as previously reported (6).

Pluripotent cells are by definition capable of differentiating into cell types derived from each of the three embryonic germ layers (10). A property of both ES cells and previously established human iPS cells is their ability, when plated in suspension culture, to form embryoid bodies (EBs) composed of diverse cell types (fig. S4A) (6, 9, 10). When grown in these conditions, all three iPS cell lines from patient A29 readily formed EBs (Fig. 3A). Immunocytochemical analyses of EBs after 13-16 days of culture showed that each line had spontaneously differentiated into cell types representative of the three embryonic germ layers (Fig. 3, B to F, and fig. S4B). Together, these data indicate that we have reprogrammed primary fibroblasts isolated from an ALS patient of advanced age into iPS cells.

Much of the hope invested in patient-specific stem cells is based on the assumption that it will be possible to differentiate them into disease-relevant cell types. ALS is characterized by the progressive degeneration of spinal cord motor neurons (1, 11) and recent studies have demonstrated that both cell autonomous and non-cell autonomous factors contribute to disease progression (12, 13). In particular, glia from ALS animal models have been shown to produce factors that are toxic to motor neurons (14–16). These studies indicate that production of both motor neurons and glia would be essential for mechanistic studies and perhaps eventual cell replacement therapies for ALS. We therefore attempted to generate spinal motor neurons and glia using a directed differentiation protocol developed for mouse and human ES cells (17–20). EBs formed from iPS cells were treated with two small molecules, an agonist of the sonic hedgehog (SHH) signaling pathway and retinoic acid (RA) (fig. S5A). When these differentiated EBs were allowed to adhere to a laminin-coated surface, neuronal-like outgrowths were observed (Fig. 4A). Many of these processes stained positive for a neuronal
form of tubulin, β-Tubulin IIIb (TuJ1), confirming their neuronal nature (Fig. 4B and fig. S6).

To further characterize the cells after directed differentiation, EBs were dissociated and plated as a single-cell suspension onto laminin-coated slides. TuJ1-positive neurons that co-expressed the motor neuron marker HB9 [a motor neuron-specific transcription factor (17)] could be readily identified in cultures derived from both the A29a and A29b cell lines (Fig. 4C and figs. S5B and S7). In cultures differentiated from A29b iP cells, we individually examined 3262 nuclei (from three independent differentiation experiments) and found that 651 stained for HB9, indicating that 20% of all cells expressed this motor neuron marker. Moreover, more than 90% of these HB9-positive cells also expressed ISLET 1/2 (ISL), transcription factors involved in motor neuron development (17, 18) (Fig. 4, E to H, and figs. S5C and S5D). Over half of these HB9/ISL positive neurons expressed choline acetyltransferase (ChAT), demonstrating an advanced degree of cholinergic motor neuron maturation (17) (figs. S5D and S9B). Cells expressing the spinal cord progenitor markers OLG2 and PAX6 were also prevalent in these cultures (fig. S9A), suggesting that patient-specific iPS derived motor neurons arise from progenitors similar to those found in the developing spinal cord. In addition, cells expressing the glial markers GFAP and S100 were readily identified (Fig. 4D and fig. S10). Thus, patient-specific iPS cells, like human ES cells, can respond appropriately to developmentally relevant patterning signals, demonstrating the feasibility of producing large numbers of the cells specifically affected by ALS.

Our results with patient-derived cells confirm the initial finding that the exogenous expression of only four factors, KLF4, SOX2, OCT4, and c-MYC, is sufficient to reprogram human fibroblasts to a pluripotent state (6). Previous reports using these four genes to generate human iPS cells have required the overexpression of either a murine viral receptor (6) or additional oncogenes such as Large T Antigen and TERT (21). In contrast, our results using retroviruses pseudotyped to transduce human cells dispel the suggestion by a recent study that these four genes are not sufficient to induce reprogramming (21).

We have demonstrated that it is possible to produce patient-specific pluripotent stem cells. It is particularly encouraging that neither the advanced age nor the severely disabling disease of patient A29 prevented us from reprogramming her fibroblasts. Attempts to generate similar pluripotent cell lines using somatic cell nuclear transfer and ES cell fusion have been confronted by technical, logistical, and political obstacles that have not yet been overcome (22, 23). In contrast, the use of defined reprogramming factors for the generation of patient-specific iPS cells has allowed us to circumvent these obstacles. Importantly, the multiple integrations of retroviral DNA in the host genome, which were required for reprogramming, did not preclude our ability to terminally differentiate these cells into motor neurons. Nevertheless, long-term studies will be needed to compare the in vitro physiology of iPS-derived motor neurons to those derived from human ES cell lines.

Our study demonstrates the feasibility of producing large numbers of motor neurons with a patient’s exact genotype, which would be immune-matched to that individual, a long-sought-after goal of regenerative medicine. However, several major challenges must be resolved before cell replacement therapy using iPS technology can become a clinical reality. First, among several other safety issues, iPS-derived neurons will not be suitable for transplantation until the oncogenic genes and retroviruses (24, 25) used here are replaced with more controlled methods of reprogramming. Second, it will be necessary to understand and correct any intrinsic defects in the patient’s neurons and glia before they can be rationally used as a basis for cell therapy.

Many recent insights into the pathophysiology of ALS come from the study of familial forms of this disease. The patient-specific iPS cells produced here will be important tools for further studying mechanisms by which familial disease arises. However, more than 90% of ALS patients are afflicted by a sporadic form of disease, thought to arise from complex interactions between genetic and environmental factors (26). As a result of these complexities, it has been impossible until now to devise in vitro cell-based models for this most common type of ALS. Patient-specific iPS cells generated from individuals with sporadic disease would carry the precise constellation of genetic information associated with pathology in that person. This approach would allow study of living motor neurons generated from ALS cases with unknown genetic lesions, providing insight into their intrinsic survival properties, their interactions with other cell types, and their susceptibility to the environmental conditions that are considered to play an important role in ALS pathogenesis.
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Fig. 1. iPSCs can be established from patient fibroblasts after biopsy. (A) Primary dermal fibroblasts derived from an 82-year-old female ALS patient, A29. (B) iPSCs cells produced from patient A29. (C) iPSCs cells produced from a second patient, A30, sister to patient A29. (D) Direct sequencing of a PCR product from A29 iPSCs confirming the presence of one copy of the dominant L144F SOD1 allele. (E and F) SSEA-4 and NANOG protein expression in A29 iPSCs. Scale bars are all 200 µm.

Fig. 2. A29 iPSCs are similar to human ES cells in their expression of genes associated with pluripotency. (A) ES cell-associated transcripts, REX1/ZFP42, FOXD3, TERT, NANOG, and CRIPTO/TGDF1, are activated in iPSCs to levels comparable to human ES cells as measured by qRT-PCR. (B) Primers specific for either endogenously (blue) or virally (red) encoded transcripts of the four reprogramming factors were used to measure their respective expression levels. Expression was detected from all four endogenous loci in the iPSCs at levels similar to those in the human ES cell lines HuES-3 and HuES-10. Expression from the retroviral KLF4 and SOX2 transgenes was not detected, although both retroviral OCT4 and c-MYC were expressed. 293 cells transiently transfected with the four plasmids used to produce virus served as a positive control for expression of the viral transgenes.

Fig. 3. Patient-specific iPSCs are pluripotent stem cells. (A) EBs formed from A29b iPSCs, five days after seeding. These EBs contained cells representative of each of the three embryonic germ layers: endoderm [(B), alpha-fetoprotein], mesoderm [(C), desmin; (D), α-smooth muscle actin], and ectoderm [(E), β-TubulinIIIb; (F), glial fibrillary acidic protein]. Scale bars are 200 µm (A), and 100 µm (B-F).

Fig. 4. iPSCs generated from ALS patients can be differentiated into motor neurons. A29b iPSC EBs were patterned with RA and SHH, and plated on laminin either whole (A-B), or following dissociation (C-H), and allowed to mature for 7–15 days. (A) Neuronal-like outgrowths are visible from whole A29b patient-specific iPSC cell EBs. (B) Extensive TuJ1-positive neuronal processes grow out from plated whole iPSC EBs, which contain a high proportion of HB9-stained nuclei. (C) Neuronal identity of HB9 expressing cells is confirmed by high-magnification image of HB9 and TuJ1 co-expression in dissociated patient-specific motor neuron cultures. (D) GFAP-expressing glial cells can be found in addition to TuJ1-expressing neurons in differentiated patient-specific iPSC cell cultures. (E to H) The motor neuron identity of HB9/TuJ1 double positive cells is confirmed by the coexpression of HB9 and ISL. HB9 (E) and ISL (F) localization is nuclear (G) and highly coincident (H). Scale bars are 100 µm (A-D) and 75 µm in (E-H).