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Received a B.S. in Microbiology from the University of Illinois, Urbana-Champaign in 1996 and a Ph.D. in Biology from the Massachusetts Institute of Technology in 2003, Dr. Kevin Eggan went on to become a postdoctoral fellow at the Whitehead Institute for Biomedical Research from 2002 to 2003 and a Junior Fellow in the Harvard Society of Fellows from 2003 before joining Harvard University’s Department of Molecular and Cellular Biology as an assistant professor in 2005 at the newly developed, state-of-the-art Department of Stem Cell and Regenerative Biology. In 2006, he was named an assistant investigator of the Stowers Medical Institute.

His research puts him at the vanguard of addressing fundamental questions about cellular differentiation and plasticity which hold essential implications for developing therapeutic stem cell lines from adult cell nuclei. His research explores the mechanisms by which somatic cell nuclear transfer (cloning) can reverse the differentiation of a cell by “reprogramming” its nucleus to the totipotent state.

His impressive accomplishments place him at the forefront of a most exciting new branch of biology: the use of nuclear transfer and stem cell technologies to explore mammalian development, i.e., how a single cell grows into a complex organism. Amongst his successes is an important study of X chromosome inactivation in cloned mouse embryos in which he was able to demonstrate that the nuclear transfer procedure leads to epigenetic reprogramming of the donor genome. More recently, he showed that nuclei of even highly specialized cells, such as olfactory neurons which express only a single odorant receptor, retain full developmental potential.

Moreover, after careful review by independent human subjects and ethics panels, he received permission in June 2006 to initiate research at Harvard to create embryonic stem cell lines from skin cells of patients suffering from several debilitating or terminal diseases. By exploring the possibilities of redirecting stem cells from adult tissue or differentiated tissue, his work is taking an important step closer to developing therapeutic applications for diseases such as ALS and insulin-dependent diabetes, as well as providing an experimental platform for investigating the genetic and environmental factors that give rise to such diseases. Amongst a host of prestigious awards and achievements, the following achievements require recognition: Winner of Harold M. Weintraub Graduate Student Award in 2003 sponsored by the Basic Sciences Division of the Fred Hutchinson Cancer Research Center; honored in Popular Science's fourth annual "Brilliant 10" in 2005; Technology Review Magazine's "Innovator of the Year" in 2005 and, most recently, awarded the MacArthur Foundation "Genius Grant" in 2006.

It is a great honor and delight for all of us at Suna Kıraç Conferences on Neurodegeneration to welcome Dr. Eggan back to Istanbul for our second bi-annual conference after the truly phenomenal and inspiring contribution he made as a speaker at our 2007 inaugural event.

Using Reprogrammed Fibroblasts to Understand the Cell Biology of ALS

It has been proposed that human embryonic stem cells could be used to provide an inexhaustible supply of differentiated cell types for the study of disease processes. Although methods for differentiating embryonic stem cells into specific cell types have become increasingly sophisticated, the utility of the resulting cells for modeling disease has not been determined. We have asked whether specific neuronal subtypes produced from human embryonic stem cells and induced pluripotent stem cells can be used to investigate the mechanisms leading to neural degeneration in ALS. We show that human spinal motor neurons, but not interneurons, are selectively sensitive to the toxic effect of glial cells carrying an ALS-causing mutation.
in the SOD1 gene. Our findings demonstrate the relevance of these non-cell-autonomous effects to human motor neurons and more broadly demonstrate the utility of human embryonic stem cells for studying disease and identifying potential therapeutics.
INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that specifically affects motor neurons in the spinal cord and brain stem (Boillée et al., 2006a; Lobsiger and Cleveland, 2007). The majority of patients diagnosed with ALS have no family history of disease and are therefore classified as sporadic cases (Brown, 1997; Cole and Siddique, 1999). However, it is increasingly clear that ALS in these individuals likely results from complex interactions between their specific genetic makeup and the environment (van Es et al., 2008; Landers et al., 2008). As is the case for many neurodegenerative conditions, there are rare monogenic forms of ALS that are inherited in a simple Mendelian fashion (Brown, 1997; Cole and Siddique, 1999). Notably, a variety of mutations in the ubiquitously expressed Super Oxide Dismutase gene (SOD1) lead to a dominant, inherited form of ALS. Introduction of these mutant alleles of the SOD1 gene into mice and rats (Rosen et al., 1993; Nagai et al., 2001) has allowed the modeling of this form of disease in animals.

SUMMARY

It has been proposed that human embryonic stem cells could be used to provide an inexhaustible supply of differentiated cell types for the study of disease processes. Although methods for differentiating embryonic stem cells into specific cell types have become increasingly sophisticated, the utility of the resulting cells for modeling disease has not been determined. We have asked whether specific neuronal subtypes produced from human embryonic stem cells can be used to investigate the mechanisms leading to neural degeneration in amyotrophic lateral sclerosis (ALS). We show that human spinal motor neurons, but not interneurons, are selectively sensitive to the toxic effect of glial cells carrying an ALS-causing mutation in the SOD1 gene. Our findings demonstrate the relevance of these non-cell-autonomous effects to human motor neurons and more broadly demonstrate the utility of human embryonic stem cells for studying disease and identifying potential therapeutics.

The muscle atrophy and paralysis that are the clinical hallmarks of ALS are indisputably caused by the death of motor neurons. However, the extent to which their demise is an intrinsic process or instead caused by pathological interactions with other cell types in the spinal cord has become an area of intense investigation (Boillée et al., 2006a; Lobsiger and Cleveland, 2007). The evidence suggesting that negative interactions between cell types within the spinal cord might directly contribute to motor neuron loss was obtained from studies with chimeric mice (Clement et al., 2003). In these experiments, it was found that motor neurons harboring the mutant SOD1 gene were often spared when surrounded by nontransgenic support cells, while nontransgenic neurons in close proximity to mutant neighbors were subject to degeneration (Clement et al., 2003).

Subsequently, specific deletion of the pathogenic SOD1 transgene from individual cell types implicated in ALS pathology has confirmed that the mutant SOD1 protein can modify disease processes by acting through both motor neurons and microglia. Removal of the SOD1 transgene from motor neurons was found to substantially delay but not halt the onset of disease (Boillée et al., 2006b), while removal of the mutant SOD1 transgene from microglia was found to extend life span in the animals by prolonging the disease’s symptomatic phase (Boillée et al., 2006b).

In addition to microglia, astroglia have also been implicated as mediators of motor neuron cell death in familial ALS. Coculture studies carried out in vitro have shown that mutant astrocytes carrying SOD1 mutations have a toxic effect on wild-type mouse motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007) and that this effect is more severe in mouse motor neurons harboring the SOD1 mutation (Di Giorgio et al., 2007). The relevance of this finding has also been confirmed in vivo: specific deletion of the mutant SOD1 gene from astrocytes, within otherwise transgenic animals, substantially slows the progressive phase of the disease (Yamanaka et al., 2008). The discovery that cell types in the spinal cord other than motor neurons play an important role in ALS disease progression suggests that they may be targets for the development of ALS therapeutics.

Although these recent findings, enabled by mouse genetics, have provided a more sophisticated understanding of the cellular and molecular mechanisms that may contribute to familial ALS, it is critical to determine the relevance of these findings to human motor neurons and eventually human patients. Unfortunately, it has been impossible to isolate viable human motor neurons from patients or from post mortem samples, preventing validation of findings from animal models of ALS. A widely proposed
but as of yet untested solution to this impediment would be to use human embryonic stem cells as a renewable source of motor neurons (Lensch and Daley, 2006; Ben-Nun and Benvenisty, 2006; Rubin, 2008).

Human embryonic stem cells have the capacity to self-renew indefinitely in culture while maintaining their potential to generate virtually any cell type in the body (Murry and Keller, 2008). In the last decade, since the initial derivation of human embryonic stem cells (Thomson et al., 1998), methodologies for directing their differentiation into specific cell types have become increasingly sophisticated. (Murry and Keller, 2008). However, to date there is still no evidence that these protocols can be used to generate an adequate supply of the cell for disease modeling, or that these cells will display appropriate disease relevant phenotypes or cell-type-specific sensitivities to disease stimuli.

Here, we examine the utility of specific neuronal subtypes, including spinal motor neurons, derived from human ESCs for investigating the disease mechanisms leading to ALS and the identification of small molecules that can counteract their effects. We have developed a method for large-scale production of motor neurons from human embryonic stem cells and used them in coculture experiments to determine whether they were selectively sensitive to the toxic, non-cell-autonomous effect of glial cells harboring a mutant allele of the SOD1 gene. Our results demonstrate that human motor neurons are selectively sensitive to this toxic effect, while interneuron populations produced from embryonic stem cells are unaffected.

We also found that the specific toxicity to motor neurons was associated with several significant changes in glial gene expression. These changes in gene expression were used to inform the selection of several candidate molecules, which were screened to determine whether they might potentially be involved in the toxic effect of mutant glia. None of the molecules tested had a significant effect on motor neuron survival except prostaglandin D2. When prostaglandin D2 was added to motor neurons cocultured with normal glia, there was a significant reduction in motor neuron survival. Furthermore, an inhibitor of prostaglandin signaling significantly reduced the toxic effect that mutant glial cells have on motor neurons.

These findings validate the toxic interactions between glial cells and motor neurons as an important target for the development of ALS therapeutics, while more generally demonstrating the utility of human embryonic stem cells for producing a robust supply of a specific cell type for disease modeling and drug discovery.

RESULTS

In Vitro Differentiation of Human ESCs into Motor Neurons

To generate a supply of spinal motor neurons from human ESCs for the study of ALS, we adapted a recently reported method for the production of these cells within embryoid bodies (EBs) (Singh Roy et al., 2005) (Figure 1A). Undifferentiated, self-renewing HuESCs (Cowan et al., 2004) were dissociated into small clumps using collagenase treatment and then allowed to spontaneously differentiate in suspension for 14 days (Figures 1A and 1B). Staining with the neuronal progenitor marker PAX6 (Figure 1B) suggested that a substantial percentage of the resulting EBs (29% ± 16%; Figures 1C and 1D) contained cells differentiating down the neuronal lineage. To direct these progenitors toward a spinal motor neuron identity, we cultured the EBs another 14 days in the presence of retinoic acid (RA) and a small-molecule agonist of the sonic hedgehog (SHH) pathway (Experimental Procedures). Under the influence of these morphogens, the population of PAX6-positive progenitors expanded (45% ± 15%; Figures 1B–1D), and expression of the ventral progenitor marker NKX6.1 and the motor neuron marker ISL1/2 was induced (Figures 1B–1D). To promote motor neuron differentiation and survival, we then transferred these 28-day-old EBs to media containing neurotrophic factors for a final 14 days. At 42 days of differentiation, the number of progenitors expressing PAX6 and NKX6.1 had begun to decline (Figures 1C and 1D), while the number of cells expressing ISL1/2 continued to increase (Figures 1B–1D). At this time point, the HB9 transcription factor, which is expressed in maturing postmitotic motor neurons, was detected in 8% of all cells (Figures 1B–1D). When plated on laminin, these EBs elaborated a dense network of neuronal processes (Figure 1B).

To further characterize the putative motor neurons contained within these EBs, the 42-day-old EBs were dissociated with papain, and the resulting cells were plated directly onto glial monolayers prepared from the cortex of neonatal mice (see Figure S1 available online). Costaining of cells with antibodies specific to a neuronal form of tubulin (TuJ1) and the transcription factors HB9 and ISL1/2 (Figures 2A and 2B), as well as costaining for HB9 and choline acetyltransferase (ChAT) (Figure 2C), confirmed that the HB9-positive cells within the EBs had differentiated into motor neurons.

To further demonstrate that the appearance of these motor neurons within the EBs were dependent on the influences of RA and SHH, we repeated our differentiation scheme in the absence of one or both of these morphogens and counted the number of HB9-positive cells (Figure 2D). When SHH or RA activity was removed individually, the frequency of cells expressing HB9 fell to 0.7% (±0.2%) and 1.1% (±0.5%) respectively. If both signaling molecules were omitted, less than 0.2% of the dissociated cells expressed HB9 (0.17% ± 0.07%).

We further confirmed the robustness of our approach for generating motor neurons by differentiating six independent human ESCs lines and then quantifying the number of HB9-positive cells within the resulting EBs (Figure 2E). We found that HuES1, HuES3, HuES5, and HuES9 ESC lines all differentiated into HB9-expressing motor neurons with a similar efficiency (HuES 1, 7.1% ± 1.8%; HuES 3, 8.5% ± 0.5%; HuES 5, 4.7% ± 0.8%; HuES 9, 7.7% ± 1.5%), while HuES 12 cells differentiated at a lower efficiency (2.8% ± 1.3%) and HuES13 cells at a higher efficiency (13.9% ± 3.8%). In addition to demonstrating the robust nature of this protocol for generating spinal motor neurons, our results support the recent observation that some human ESC lines can have varying propensities for differentiating into certain cell types (Osafune et al., 2008).

A Transgenic hESC Line Reporting on Motor Neuron Differentiation

In order to reproducibly identify living motor neurons in cultures of differentiating human ESCs, we generated a stable transgenic...
human ESC line in which sequences coding for the green fluorescent protein (GFP) were under the control of the murine Hb9 promoter (Wichterle et al., 2002) (Figure 3) (see Experimental Procedures). To validate that this transgenic cell line accurately reported HB9 transcription, we differentiated the cells, plated them on glial monolayers, and costained with antibodies specific to GFP and HB9. Consistent with accurate reporting on HB9 expression, HB9 protein was detected in 95% of GFP-positive cells (Figures 3C and S2A–S2D). We next investigated whether these GFP-positive cells expressed other markers of a maturing motor neuron identity (Figures 3 and S2). We observed GFP expression in a subset of NKX6.1-positive cells (Figure S2F) but observed no GFP coexpression with NKX2.2 (Figure S2E), confirming that GFP-positive cells had acquired the correct dorsal-ventral identity (Jessell, 2000). Additionally, these cells expressed ISL1/2 (Figure 3E) and ChAT (Figure 3F) but no longer expressed the progenitor marker PAX6 (Figure 3D). Antibody costaining experiments also demonstrated that GFP-positive cells did not coexpress markers found in other neuronal subtypes, such as the interneuron markers LHX2 (Figure S2G) and CHX10 (Figure S2H).

The data that we have described thus far confirm that it is possible to reproducibly generate a large supply of human motor neurons from embryonic stem cells and extend these findings by generating an HB9::GFP transgenic human ESC line that can be routinely used for the vital visualization of human motor neurons.

**Human Motor Neurons Are Sensitive to the Toxic Effect of SOD1G93A Glial Cells**

To test whether the human spinal motor neurons we produced were useful for investigating disease processes, we asked whether they were sensitive to the toxic effect of glial cells overexpressing a mutant SOD1 gene product. We dissociated...
42-day-old EBs and plated the resulting cells (3 \times 10^4 \ [n = 3]\) cells per well) on primary cortical glial monolayers derived from either SOD1G93A transgenic or control mice (Figure 4A). After 10 days a significant difference (p < 0.05) in the number of HB9-positive motor neurons was seen between the two culture conditions (Figure 4B). In cultures containing SOD1G93A glia, there were less than half as many motor neurons (131± 53, n = 3) as in cultures containing nontransgenic control glia (269 ± 44, n = 3) (Figure 4B). The deficit in motor neuron survival in cocultures with SOD1G93A glia became even more pronounced after 20 days (Figures 4C and 4D). A similar effect on survival of human ESC-derived motor neurons was observed when they were cultured for 20 days with media conditioned by SOD1G93A glia (Figures S3A and S3B).

We next investigated whether the deficit in the number of motor neurons found on SOD1G93A glia was due to an initial reduced plating efficiency of these cells or instead was due to some negative effect of the mutant glial cells that acted over time to induce motor neuron loss. To this end we derived motor neurons from the Hb9::GFP human ESC line, plated them at the same concentration (3 \times 10^4 \ [n = 3]\) cells per well) on SOD1G93A and nontransgenic control glia, and then compared the number of GFP-expressing motor neurons in these two culture conditions 2, 10, and 20 days later (Figure 4E). At 2 days after plating, we did not find a significant difference between the number of motor neurons in the two cocultures (Figure 4E), arguing against a difference in motor neuron plating efficiency. However, at both 10 and 20 days there was a significant decrease in the number of motor neurons in coculture with SOD1G93A glia relative to identical preparations of neurons cocultured with control glia (57% ± 5% and 42% ± 6%; p < 0.01) (Figure 4E).

We next addressed whether the toxic effect of glia we observed in our initial experiments was specific to the action of the mutant SOD1 protein rather than a result of SOD1 protein overexpression. Motor neuron preparations were generated from the Hb9::GFP human ESC line and cocultured for 20 days with nontransgenic glia or glia, which overexpressed either the wild-type human SOD1 protein or the mutant SOD1G93A protein (Figures 4F and 4G). There was no discernable difference between the number of GFP-positive motor neurons present in culture with the nontransgenic Glia (304 ± 60, n = 3; Figures 4F and 4G) or with glia overexpressing the wild-type SOD1 protein (328 ± 30, n = 3; Figures 4F and 4G). In contrast, there was a highly significant reduction (p < 0.01) in the number of GFP-positive motor neurons (127 ± 16, n = 3; Figures 4F and 4G) present in culture with the SOD1G93A glia, confirming that the non-cell-autonomous effect that we observed was mediated only through the mutant form of the SOD1 protein.

Gliarial Toxicity Is Not Induced by Large-Scale Protein Aggregation

In both patients and mice carrying mutant alleles of the SOD1 gene, intracellular aggregation of the SOD1 protein is often documented and has been associated with motor neuron death. We therefore wondered whether the toxic effect of glial cells

Figure 2. Characterization of Human ESC-Derived Motor Neurons

(A–C) Expression of neuronal markers ISL1/2 (A), HB9 (B), and choline acetyltransferase (ChAT) (C) in motor neurons derived from human ESCs.

(D) Percentage of cells immunoreactive for HB9 after treatment with or without RA and Shh (mean ± SD; n = 3).

(E) Percentage of cells immunoreactive for HB9/DAPI after 42 days of differentiation in different HuESC lines (mean ± SD; n = 3; cells counted in sections randomly selected from different EBs).
expressing the mutant SOD1 protein that was a downstream consequence of large-scale protein aggregation within the glial cells. To address this question, we separately cultured primary mouse glia and mouse ESC-derived motor neurons carrying the same SOD1G93A transgene and stained the resulting cultures with antibodies specific for the human SOD1 protein. As we previously reported (Di Giorgio et al., 2007), after 21 days in culture, the SOD1 protein in mouse motor neurons was observed to aggregate into large cytoplasmic and perinuclear inclusions (Figure S4A). In contrast, even after more than 90 days in culture, the SOD1 protein was found to be broadly and diffusely localized in the cytoplasm of all glial cells (Figure S4B). These results imply that the mutant protein is mediating its effect in these cells through a mechanism independent of the large-scale protein aggregation that is observed in motor neurons.

Motor Neurons Are the Selective Target of Glial Toxicity
ALS leads to the specific degeneration of motor neurons. Therefore, if the toxic effect of glial cells that we have observed is relevant to ALS, then we might expect that other spinal cord neuronal types such as interneurons would be resistant to it. During our characterization of human ESC-derived motor neurons, we noted that additional neurons expressing the transcription factors CHX10 and LHX2, indicative of V2 and D1 interneuron identities, were also produced (Figures S2G and S2H). To test whether these neuronal types were affected by coculture with mutant glia, we dissociated 42-day-old EBs, plated equal numbers of cells on either SOD1G93A glia or nontransgenic glia (Figure 5A) and after 20 days of culture stained for Tuj1 and either LHX2 (Figures 5B and 5C) or CHX10 (Figures 5D and 5E). In striking contrast to the sensitivity of motor neurons to this environment, we found that neurons expressing these interneuron markers were unaffected by culture with mutant glia (Figures 5B and 5D). Similarly, human ESC-derived interneurons cultured for 20 days with SOD1G93A glia-conditioned media were unaffected (Figures S3C and S3D).

Motor Neurons Are Unaffected by Fibroblasts Expressing Mutant SOD1
To determine if the toxic effect of mutant glial cells was the consequence of a specific activity within this cell type rather than a general property of any cell overexpressing the SOD1G93A mutation, we plated motor neuron preparations on mouse embryonic fibroblasts (MEFs) prepared from SOD1G93A and nontransgenic sibling embryos (Figure 6A). After 20 days of coculture, we did not observe a significant difference between the number of HB9, Tuj1 double-positive neurons in these two conditions (SOD1G93A MEFs, 204 ± 28 [n = 3]; or nontransgenic MEFs, 197 ± 23 [n = 3]) (Figures 6B and 6C), consistent with the hypothesis that astrocytes are specifically responsible for the non-cell-autonomous effect we observed (Di Giorgio et al., 2007; Nagai et al., 2007).

Identification of Candidate Genes Involved in SOD1G93A Glial Toxicity
To better understand how the expression of a mutant gene that causes ALS can perturb the normal phenotype of astrocytes, and to identify genes that may have a role in their toxic effect on motor neurons, we used oligonucleotide arrays to compare the global gene expression profiles of glia overexpressing the mutant SOD1G93A protein with two different sets of controls: nontransgenic glia and glia overexpressing the wild-type form of the human SOD1 protein. We identified 135 genes whose expression was significantly (p < 0.001) increased more than 2-fold in SOD1G93A glia when compared to nontransgenic glia. Of these 135 genes, 53 were exclusively upregulated in the mutant glia, and not in glia overexpressing the WT SOD1 protein (Figure 7A), making them interesting candidates for further investigation. We found that...
13 of these 53 genes (24%) (Table S1) have previously been identified to have a role either in inflammatory or immune processes. We narrowed our analysis to a subset of these genes deemed to be of particular interest because of their known role as proinflammatory factors and their substantially increased expression in mutant glia (Figure 7B). The prostaglandin D2 (PGD2) receptor was upregulated more than 14-fold in \textit{SOD1\textsubscript{G93A}} glia compared to the control sample. Three different cytokines were also shown to be overexpressed in mutant glia: Mcp2, Cxcl7, and Rantes. Also found to be highly (>13-fold) upregulated in these
microarrays was the gene encoding glial maturation factor beta (GMFβ), which has been shown to induce a proinflammatory state in astrocytes (Zaheer et al., 2007). Finally, we found that the expression of SHH and the SHH-responsive genes NKX2.2 and DBX2 was modestly increased in the mutant glia, suggesting that this signaling pathway might be activated in response to the actions of the mutant SOD1 protein.

Human ESC-Derived Motor Neurons Can Be Used to Identify Neurotoxic Factors

In order to investigate the possible involvement of candidate factors and signaling pathways in the glial-mediated neurotoxicity we have observed, we tested the effect of these candidate gene products, or molecules that activate them, on motor neuron survival in cocultures with wild-type glial cells. Nontransgenic glia were individually pretreated for 1 day with either one of the three cytokines MCP2, Cxcl7, or Rantes; GMFβ; an agonist of SHH pathway; or PGD2. After pretreatment of the glial cells for 24 hr, a cellular preparation containing Hb9::GFP human ESC-derived motor neurons was plated on the glia. Replicate cultures were individually maintained for 20 days in the presence of each of the six factors and fixed, and the numbers of GFP-positive motor neurons were quantified.

We found that treatment with GMFβ did not significantly affect the number of human ESC-derived motor neurons compared to the control condition (95% ± 9%). Likewise, the presence of any one of the three cytokines (Rantes, Cxcl7, and Mcp2) or the SHH agonist did not seem to negatively affect the number of GFP-positive motor neurons (respectively 108% ± 20%; 102% ± 12%; 103% ± 8%; 97% ± 12%) (Figure 7C). However, when the cells were treated for 20 days with PGD2, we found a dramatic decrease in the number of motor neurons compared to the control condition (19% ± 2%; p < 0.01) (Figures 7C and 7D), suggesting that prostaglandin D2 signaling contributes to motor neuron toxicity in this system.

Inhibition of the Prostaglandin D2 Receptor Partially Rescues Motor Neuron Loss

To determine if there was a direct relationship between the toxic effect of prostaglandin signaling on motor neurons and the SOD1G93A glial-mediated neurotoxicity, we tested whether a specific antagonist of the prostaglandin D2 receptor, MK 0524 (Sturino et al., 2007), could counteract or ameliorate the toxic effect of mutant glia on motor neurons. SOD1G93A glia and WT glia were pretreated for 1 day with the prostaglandin D2 receptor inhibitor, human motor neurons were added, and cultures were maintained for 20 days both in the presence and absence of the drug. We found that the presence of MK 0524 did not affect motor neuron numbers when they were cocultured with wild-type glia (100% ± 8%; Figure 7E). However, when human motor neurons plated on SOD1G93A glia were treated with the inhibitor, there was a statistically significant (p < 0.05)
increase in the number of GFP-positive neurons (32%, relative to untreated neurons plated on the same glia) (Figures 7E and 7F). These experiments suggest that inhibitors of PGD2 signaling do not generally act to promote motor neuron survival and instead act to specifically counteract the toxic effects of glial cells carrying the ALS mutation.

DISCUSSION

Considerable time, effort, and expense would be saved if fundamental observations made in animal models could be routinely validated in the relevant human cell types (Gawarylewski, 2007; Rubin, 2008). However, the cells affected by disease are often difficult, or in the case of human motor neurons impossible, to obtain from patients. A potential solution is to use human embryonic stem cells as a renewable source of these cells for the study of disease and for preclinical drug target validation (Rubin, 2008; Sartipy et al., 2007; Pouton and Haynes, 2007; Klimanskaya et al., 2008). This notion that human ESC-derived cells might be used to model disease processes has been widely discussed (Lensch and Daley, 2006; Ben-Nun and Benvenisty, 2006; Rubin, 2008); however, there has been little demonstration that this approach is in fact feasible (Eiges et al., 1999; Liem et al., 1997). This transgenic cell line will be an invaluable tool for the purification and characterization of human motor neurons as well as for optimizing the production of these neurons from hESCs.

We found that glial cells overexpressing the SOD1G93A mutation negatively affect the viability of human motor neurons in a time-dependent manner. This non-cell-autonomous effect is specific for motor neurons, as it does not seem to interfere with the survival of human interneurons. Interestingly, we found that the toxic effect on human motor neurons was even stronger than the effect observed on mouse motor neurons. These results not only further demonstrate the utility of the astrocyte coculture system, but also validate the use of human ESC-derived motor neurons as a powerful tool for the study of ALS and perhaps other neuromuscular conditions.

To better understand the changes that expression of the SOD1 mutation induces in astrocytes, and to understand the roles of these changes in motor neuron toxicity, we have carried out genome-wide expression profiling of glial cells overexpressing the SOD1G93A mutation. We found that the SOD1 mutation induces a transcriptional signature in glia that is consistent with an inflammatory phenotype. This phenotype includes increases in prostaglandin D2 receptor expression, overexpression of proinflammatory cytokines, and induction of other factors related to the immune response. These findings confirm reports that link a strong proinflammatory response to ALS in both animal models and patients (Boillé et al., 2006a; Almer et al., 2001; Kondo et al., 2002).
To functionally test whether any of these inflammatory signals could negatively affect motor neuron viability, we screened candidate molecules that activate these pathways using our wild-type glial/motor neuron coculture system. Although most of these compounds had no deleterious effect on motor neuron survival, we found that treatment with prostaglandin D2 strongly and significantly decreased the number of human ESC-derived motor neurons in culture. The magnitude of this effect was strikingly similar to that observed when motor neurons were cultured with mutant glia. Remarkably, inhibition of the prostaglandin D2 receptor provided partial but significant protection to motor neurons cultured with SOD1 glia, confirming that this pathway contributes to the poisonous effect of mutant glia. However, it remains unclear whether PGD2 toxicity is mediated through glial cells, or if it directly affects the viability of motor neurons, or both.

Our results confirm previous evidence for the involvement of prostaglandins and proinflammatory factors in ALS. In previous reports, 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2), a naturally occurring derivative of prostaglandin D2, has been shown to accumulate late in the spinal cord of sporadic ALS patients (Kondo et al., 2002). Levels of the enzyme cyclooxygenase-2 (COX-2), one of the key enzymes involved in the biosynthesis of PGD2, were also found to be increased in the spinal cord of mouse models and patients affected by ALS (Almer et al., 2001; Kondo et al., 2002). Furthermore, several reports have shown a therapeutic effect of COX-2 inhibitors in mouse models of ALS (Drachman and Rothstein, 2000; Pompl et al., 2003; Klivenyi et al., 2004). Our results suggest that it may be worthwhile to reconsider molecules interfering with prostaglandin signaling as potential therapeutic for ALS (Cudkowicz et al., 2006).

Consistent with the findings that we report here, the accompanying manuscript by Marchetto et al. (2008) demonstrates that human astrocytes expressing a mutant allele of the SOD1 gene, like their mouse equivalents, have a toxic effect on human ESC-derived motor neurons. This effect seemed to be mediated through activation of both secretory and inflammatory pathways. Taken together, these studies suggest a scenario in which mutation of SOD1 activates inflammatory pathways in astrocytes, including activation of PGD2 signaling and nitric oxide release. It is currently not clear whether PGD2 and nitric oxide work in the same pathway or in parallel pathways. In the future, it will be interesting to determine whether the inhibition of both pathways can act synergistically to provide additional protection to motor neurons in ALS.

More generally, our experiments support the hypothesis that astrocytes play an active role in the demise of motor neurons in ALS and demonstrate the relevance of this finding to human motor neurons. These results also further highlight the negative
interactions between motor neurons and astrocytes as an important emerging target for the development of ALS therapeutics. Importantly, we show that these experimental systems, which rely on embryonic stem cells, can be used effectively to identify factors involved in motor neuron degeneration as well as small molecules that help protect them.

EXPERIMENTAL PROCEDURES

Growth of Human Embryonic Stem Cells

The HuESC lines were obtained from Doug Melton and cultured as described by Cowan et al. (2004). The hESCs were maintained on a feeder layer of inactivated mouse embryonic fibroblasts (GlobalStem) in human ESC media (KO-DMEM (GIBCO), 10% KO Serum Replacement, 10,000 units penicillin, and 1mg/ml streptomycin (GIBCO), 2 mM glutamine (GIBCO), 100 µM nonessential amino acids (GIBCO), 55 µM beta-mercaptoethanol (GIBCO), 10% Plasmate (Bayer), 10 ng/ml bFGF2 (GIBCO). The cells were cultured at 37°C and 5% CO2. Media were replaced daily for the duration of hESC expansion, and the cells in these conditions were passaged every 5–7 days using a solution with 0.06% trypsin (GIBCO).

Differentiation of Human Embryonic Stem Cells into Motor Neurons

ESCs were allowed to reach 80%–90% confluency, washed once with PBS, and then incubated for 15 min at 37°C in a solution of 1g/l Collagenase IV (GIBCO) in DMEM-F12 (GIBCO).

ESC colonies were scraped and washed off the plate, centrifuged for 5 min at 1000 RPM, and resuspended in human ESC media without bFGF2 or plasmid in low attachment 6-well plates.

After 24 hr, the cells had aggregated to form embryoid bodies (EBs), and the media were changed to remove debris by centrifuging the EBs and resuspending in fresh human ESC media without bFGF2 or plasmid in low attachment 6-well plates. EBs were cultured as such for 13 more days, with half of the media changed every 2 days, and a complete media change every week. After 14 days, the EBs were induced toward a caudal and ventral identity using retinoic acid (1 µM, Sigma) and an agonist of the Shh signaling pathway (1 µM) in N2 media: 1:1 DMEM:F-12 + Glutamate (GIBCO), 10,000 units penicillin and 1 mg/ml streptomycin (GIBCO), 1% N2 Supplement (GIBCO), 0.2 mM ascorbic acid (Sigma-Aldrich), 0.16% D(+)-Glucose (Sigma-Aldrich), BDNF (10 ng/ml, R&D Systems), for another 14 days. The EBs were then matured for a final 14 days in N2 media with GNDF (10 ng/ml, R&D Systems). EBs were fixed at different stage, sectioned, and stained for different markers (Figures 1C, 1D, and S1). For these experiments, sections of EBs randomly selected were used for counting under fluorescence examination.

After 42 days of differentiation, the EBs were dissociated. To dissociate the EBs, they were centrifuged at 1000 rpm for 5 min in a 15 ml falcon tube and then washed again in PBS three times and mounted using a solution with or without DAPI. Images were taken using a fluorescent Olympus IX70 microscope.

Primary Glial Cultures

P1–P3 mouse pups transgenic for SOD1G93A or SOD1WT or nontransgenic were sacrificed by using an approved method of euthanasia. Under a dissection microscope, the parenquima were isolated and the meninges were carefully stripped away with fine forceps. The tissue was then dissected into small pieces and transferred to a solution containing 12 ml of HBSS, 1.5 ml of trypsin (GIBCO), and 1% DNase (Sigma) and incubated at 37°C for 15 min, swirling the mixture periodically. The supernatant containing the dissociated cells was collected, and 3 ml of serum was added to inhibit the trypsin.

Cells were then centrifuged at 1000 rpm for 5 min, resuspended in glia medium [minimum essential medium with Earle’s salts (GIBCO), D(+)-Glucose 20% (Sigma), penicillin-streptomycin (GIBCO), 10% horse serum (GIBCO)] and plated at the concentration of 80,000 cells per ml in T75 flasks (Falcon). After the glia reached confluency, they were replated onto Poly-D-Lysine/Laminin CultureSlides (BD Biosciences).

Treatments with Chemicals and Cytokines

Glia were pretreated for 1 day with either MCP2 (100 ng/ml; Peprotech), Cxcl7 (100 ng/ml; Peprotech), Rantes (100 ng/ml; Peprotech), GMFβ (250 ng/ml; Peprotech), an agonist of Shh pathway (1 µM), PGD2 (10 µM; Chayman Chemical), or MK 0524 (10 µM; Chayman Chemical). After the pretreatment, a cellular preparation containing Hb9:GFP human ESC-derived motor neurons, dissociated from EBs, was added to the glia at the concentration of 30,000 cells/well. The cells were then cocultured for 20 days in the presence of the compounds and fixed, and the numbers of GFP-positive motor neurons were quantified.

Microarray Analysis

Glia were derived from P1–P3 mouse pups as described above. Once the cells reached confluence, total RNA was isolated using Trizol (Invitrogen) from three different biological replicates for each type of glia. RNA was amplified by one round of T7 transcription using the Illumina TotalPrep RNA Amplification Kit, and arrays were read by Illumina Bead Array Reader. Analysis was done using the Illumina Bead Studio Program.
Cell Stem Cell

hESC-Derived Motor Neurons Sensitive to ALS Glia

Data Analysis
Statistical analysis was performed using Student’s t test, and data are expressed as arithmetic mean ± SD; Student’s t test values of *p < 0.05, **p < 0.01 were considered statistically significant.

ACCESSION NUMBERS
Microarray data have been deposited in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE13643.

SUPPLEMENTAL DATA
The Supplemental Data include four figures and one table and can be found with this article online at http://www.cellstemcell.com/supplemental/S1934-5909(08)00522-5.

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REFERENCES


Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons

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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 was recently 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 transduction with retroviruses encoding KLF4, SOX2, OCT4, and c-MYC. However, it remains unclear whether induced pluripotent stem (iPS) cells can be generated directly from elderly patients with chronic disease—that is, from material that has been exposed to disease-causing agents for a lifetime—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 show 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 that of 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 sibling pairs, patients A29 and A30, who were 82 and 89 years old at the time of donation. These sisters are both heterozygous for the same rare L144F (Leu → Phe) dominant allele of the superoxide dismutase (SOD1) gene that is associated with a slowly progressing form of ALS (7). Patient A29 had a clear clinical manifestation of motor neuron disease, including difficulty in swallowing and weakness of the arms and legs. Patient A30 was clinically asymptomatic but had signs of upper motor neuron disease upon physical examination, presenting with bilateral plantar responses and hyperreflexia. 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 by means of vesicular stomatitis virus glycoprotein (VSVG)- pseudotyped Moloney-based retroviruses. About 30,000 fibroblasts were transduced twice over 72 hours, cultured for 4 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 1 week hundreds of colonies composed of rapidly dividing cells with a granular morphology not characteristic of ES cells had appeared (6). However, 2 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, 12 from A29 and three from A30, were chosen by hand 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 used direct sequencing (Fig. 1D) and an allele-specific restriction fragment length polymorphism (fig. S1, A and B) to compare the SOD1 genotype of these cell lines with that of the donated fibroblasts and genotyping results in the patient’s medical history. 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, polymerase chain reaction (PCR) analysis of genomic DNA from these three cell lines revealed 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, and NANOG), but were not immunoreactive for a fibroblast-associated antigen (TE-7) (Fig. 1, E and F, and fig. S3).

Quantitative reverse transcription PCR showed that genes expressed in pluripotent cells (REX1/ZFP42, FOXD3, TERT, NANOG, and CRIPTO/TDGF1) were transcribed at levels comparable to human ES cells in each of the three putative iPS cell lines (Fig. 2A). Moreover, the stem cell marker genes SOX2 and OCT4 were not expressed in the patient fibroblasts, whereas 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 from the endogenous KLF4 and c-MYC loci were similar in ES cells, iPS cells, and the parental fibroblasts.
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 to 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 elderly ALS patient 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 shown that both cell-autonomous and non-cell-autonomous factors contribute to disease progression (12, 13). In particular, glia from ALS animal models were 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 for eventual cell replacement therapies for ALS.

We therefore attempted to generate spinal motor neurons and glia with the use of 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, neuron-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, we plated dissociated EBs onto
TuJ1-positive neurites that coexpressed 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 iPS cells, we 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)] as determined by the coexpression of HB9 and ISL (F) localization (Fig. 4E to H, and figs. S5C and S8). More than half of these HB9- and ISL-positive neurites 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 Olig2 and Pax6 were also prevalent in these cultures (fig. S9A), which suggests that these patient-specific iPS-derived motor neurons arose from progenitors similar to those found in the developing spinal cord. In addition, cells expressing the glial markers GFAP (glial fibrillary acidic protein) 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 leveraging the self-renewal of iPS cells to generate a potentially limitless supply 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). The use of defined reprogramming factors for the generation of patient-specific iPS cells has allowed us to circumvent these obstacles. Note that 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. Nonetheless, long-term studies will be needed to compare the in vitro physiology of iPS-derived motor neurons and those derived from human ES cell lines.

Our study also demonstrates the feasibility of producing large numbers of motor neurons with a...
Amyloid-β Dynamics Correlate with Neurological Status in the Injured Human Brain

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The amyloid-β peptide (Aβ) plays a central pathophysiological role in Alzheimer’s disease, but little is known about the concentration and dynamics of this secreted peptide in the extracellular space of the human brain. We used intracerebral microdialysis to obtain serial brain interstitial fluid (ISF) samples in 18 patients who were undergoing invasive intracranial monitoring after acute brain injury. We found a strong positive correlation between changes in brain ISF Aβ concentrations and neurological status, with Aβ concentrations increasing as neurological status improved and falling when neurological status declined. Brain ISF Aβ concentrations were also lower when other cerebral physiological and metabolic abnormalities reflected depressed neuronal function. Such dynamics fit well with the hypothesis that neuronal activity regulates extracellular Aβ concentration.

We used intracerebral microdialysis (5) to obtain serial brain interstitial fluid (ISF) samples in 18 intensive care unit (ICU) patients who had sustained acute brain injury and were undergoing invasive intracranial monitoring for clinical purposes. In all patients, Aβ1-42 was detected in hourly or bihourly intracranial microdialysis samples. None had a diagnosis of Alzheimer’s disease or dementia, demonstrating that Aβ is a normal constituent of human brain extracellular fluid (6). The Aβ1-42 enzyme-linked immunosorbent assay (ELISA) used detects Aβ species from amino acid 1 to amino acid 28 or greater (3, 7).

There were rising trends in brain ISF Aβ concentrations over several hours to days in most patients, though the specific pattern of these trends was variable (Fig. 1, B, D, and F; Fig. 2B; and Fig. 4, A to D). Median brain ISF Aβ1-42 at 60 to 72 hours was 59% higher than at 0 to 12 hours (Fig. 1G) (P = 0.0002, Wilcoxon signed rank test). Urea concentrations in the same samples, which control for the stability of the microdialysis catheter function (8), remained stable over the same time frame (Fig. 1H) (median 14% lower, P = 0.06, Wilcoxon signed rank test). Thus, the observed Aβ dynamics are likely to be of cerebral origin and not an artifact of the measurement procedure.

Aβ1-42 concentrations were lower in microdialysate than in concomitantly sampled ventricular cerebrospinal fluid (CSF) (Fig. 2, B and D). However, at the flow rate used (0.3 μl/min), the microdialysate is not in complete equilibrium with the surrounding extracellular space (5). To calculate the true brain extracellular concentrations,