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Human Embryonic Stem Cell-Derived Motor Neurons Are Sensitive to the Toxic Effect of Glial Cells Carrying an ALS-Causing Mutation

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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.

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 Disumutase 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.

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

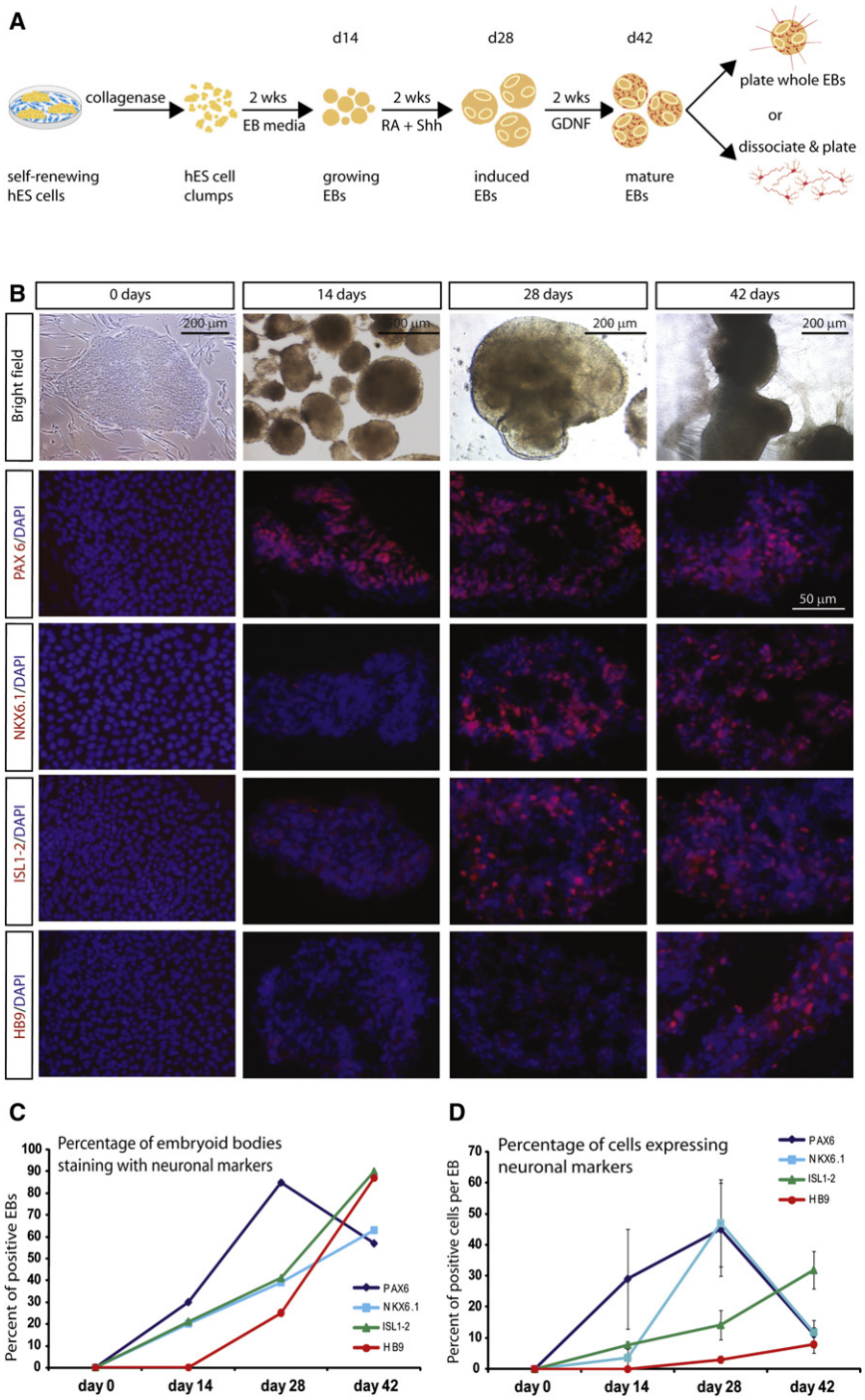


Figure 1. Differentiation of Human ESCs into Motor Neurons

(A) Diagram outlining the protocol used to differentiate human ESCs into motor neurons. Undifferentiated human ESC colonies are dissociated in collagenase and grown as EBs for the first 14 days in EB media, then are induced to a rostrocaudal identity with retinoic acid (RA) and Shh for another 14 days. Finally, EBs are matured in the presence of GDNF for 14 more days. At this point the EBs can either be plated whole or dissociated with papain and then plated.

(B) Immunohistochemistry was performed to detect neuronal markers PAX 6, NKX 6.1, ISL1/2, and HB9 in EB sections at 14, 28, and 42 days after collagenase treatment of undifferentiated human ESCs.

(C) Percent of sectioned EBs (n = 20) staining positive for PAX6, NKX6.1, ISL1/2, or HB9 at day 0, day 14, day 28, and day 42 of differentiation.

(D) Percent of cells per sectioned EB (mean ± SD; n = 3; cells counted in sections randomly selected from different EBs) staining positive for PAX6, NKX6.1, ISL1/2, or HB9 at day 0, day 14, day 28, and day 42 of differentiation.

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 *SOD1*G93A 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

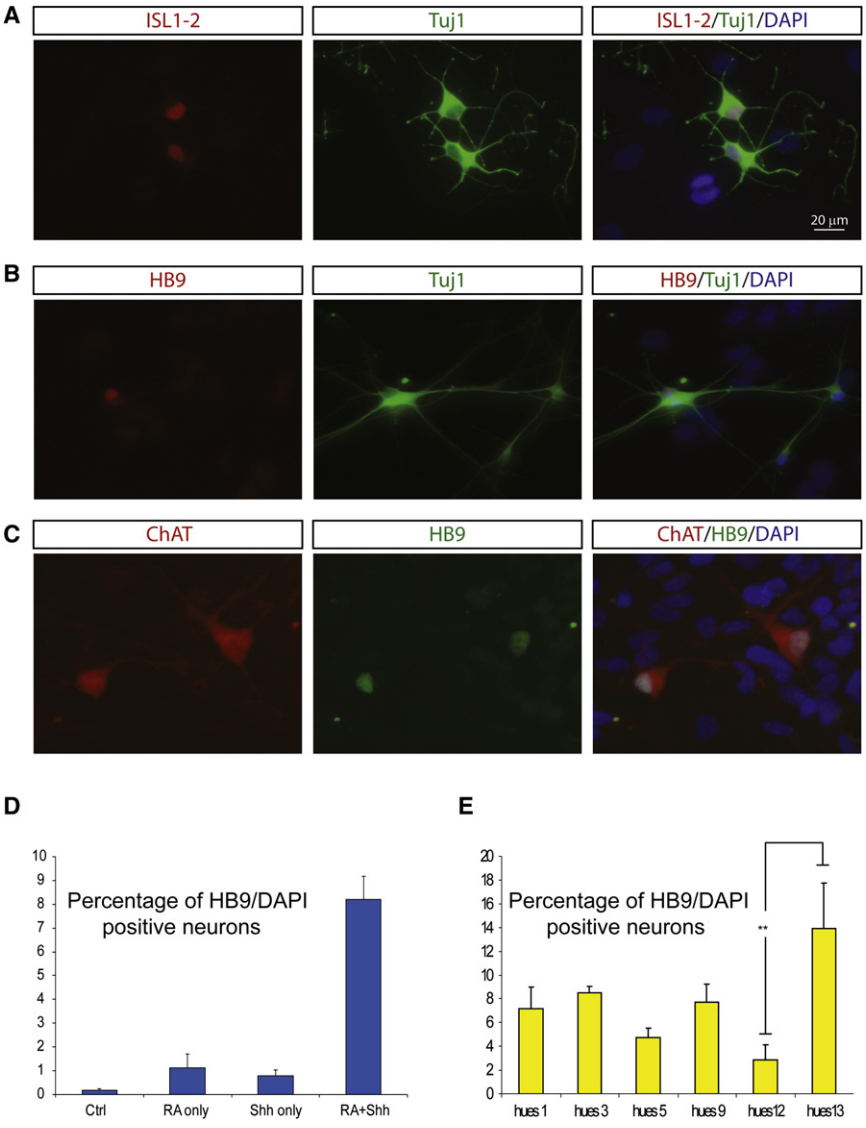


Figure 2. Characterization of Human ESC-Derived Motor Neurons

(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).

42-day-old EBs and plated the resulting cells (3×10^4 [n = 3] cells per well) on primary cortical glial monolayers derived from either *SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A glia (Figures S3A and S3B).

We next investigated whether the deficit in the number of motor neurons found on *SOD1*G93A 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 (3×10^4 [n = 3] cells per well) on *SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A glia, confirming that the non-cell-autonomous effect that we observed was mediated only through the mutant form of the SOD1 protein.

Glial 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

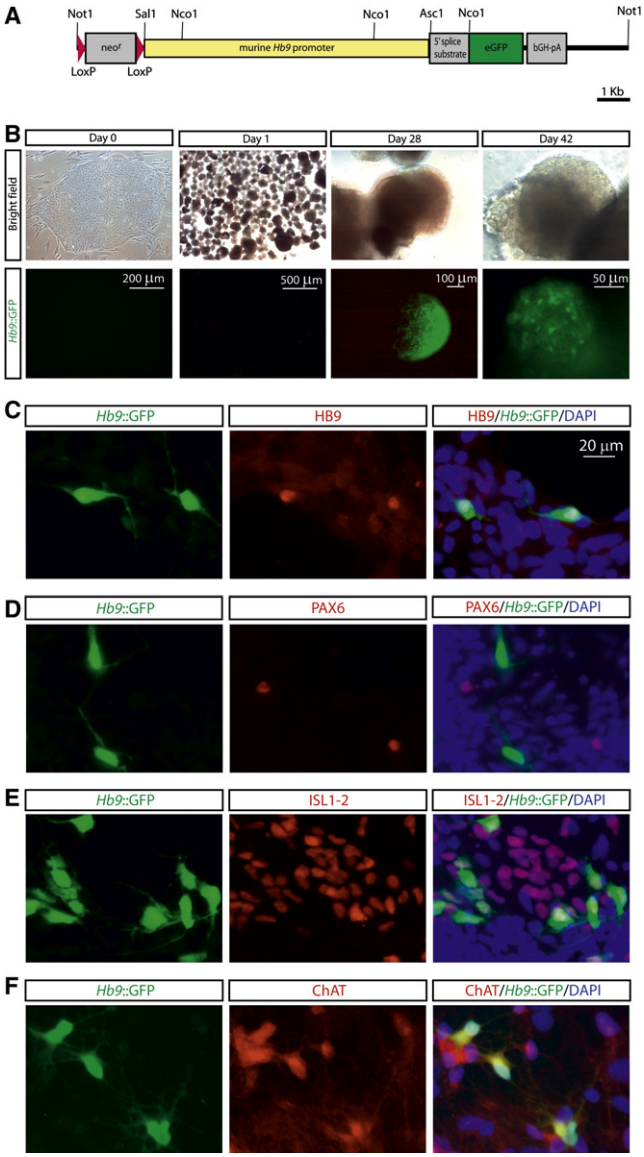


Figure 3. Characterization of the *Hb9*::GFP Human ESC Line

(A) DNA construct used for the electroporation of human ESCs.

(B) GFP expression during different stages of the differentiation from human ESCs into motor neurons.

(C–F) Expression of neuronal markers HB9 (C), PAX6 (D), ISL1/2 (E), choline acetyltransferase (ChAT) (F) in motor neurons derived from *Hb9*::GFP human ESCs.

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 *SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A 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 then a general property of any cell overexpressing the *SOD1*G93A mutation, we plated motor neuron preparations on mouse embryonic fibroblasts (MEFs) prepared from *SOD1*G93A 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 (*SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A 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 *SOD1*G93A 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

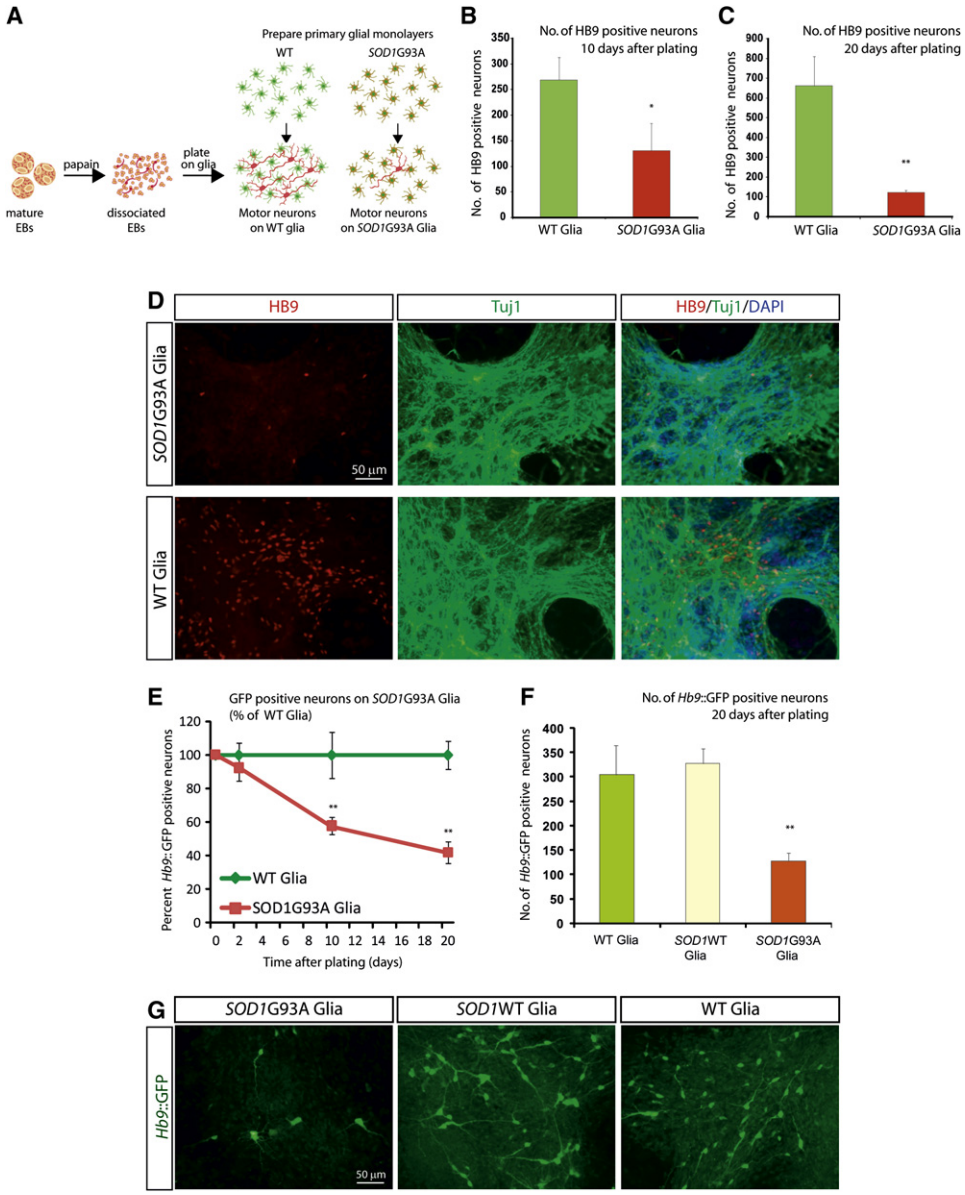


Figure 4. Effect of Glial Cells Overexpressing *SOD1*G93A on Human ESC-Derived Motor Neurons

(A) Experimental design: embryonic stem cells were differentiated into motor neurons, and an equal number of cells (3×10^4 cells per well) were plated on two different glial monolayers, one derived from mice overexpressing *SOD1*G93A, and the other derived from nontransgenic mice (WT). Motor neurons were counted after 10 and 20 days in coculture.

(B) Number of HB9-positive cells 10 days after plating on *SOD1*G93A or nontransgenic (WT) glia (mean ± SD; n = 3).

(C) Number of HB9-positive cells 20 days after plating on *SOD1*G93A or nontransgenic (WT) glia (mean ± SD; n = 3).

(D) Images of HB9/Tuj1-positive cells 20 days after plating on *SOD1*G93A glia or nontransgenic (WT) glia.

(E) Percentage of *Hb9*::GFP cells remaining on *SOD1*G93A glia with respect to nontransgenic (WT) glia at 2, 10, and 20 days after plating (mean ± SD; n = 3).

(F) Number of *Hb9*::GFP cells 20 days after plating on *SOD1*G93A glia or nontransgenic (WT) glia or glia overexpressing the wild-type form of human SOD1 (SOD1WT) (mean ± SD; n = 3).

(G) Images of *Hb9::*GFP cells in the three different coculture conditions.

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 *SOD1*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

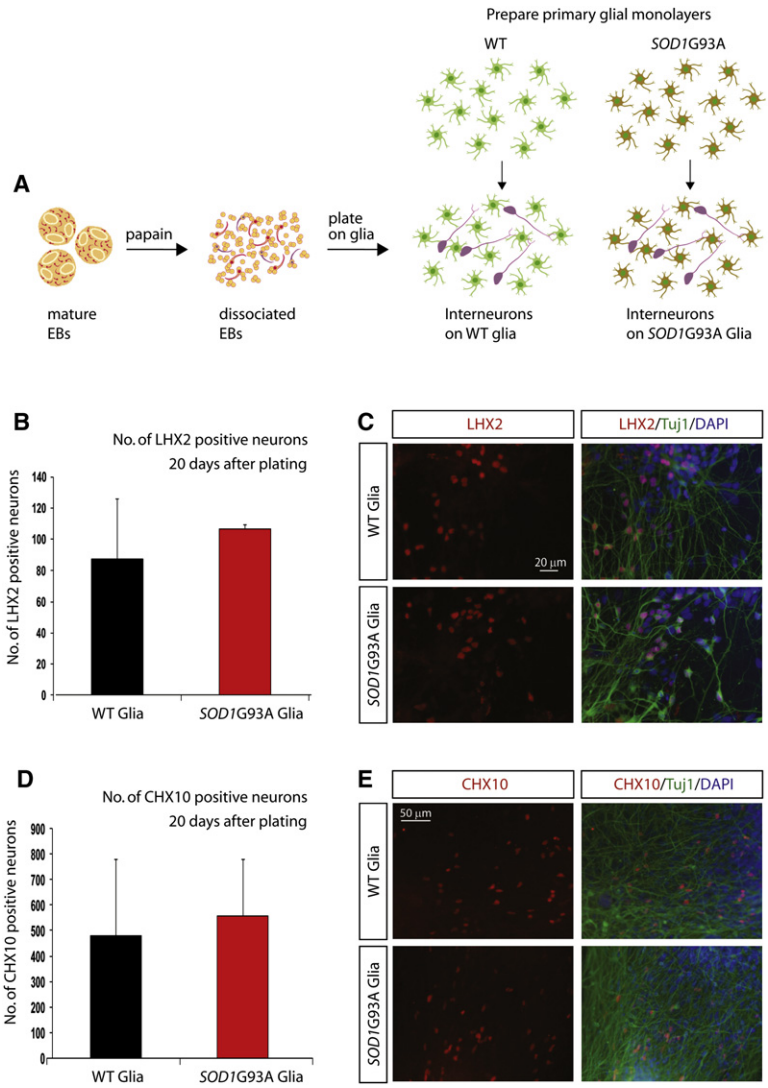


Figure 5. Glia Cells Overexpressing *SOD1*G93A Do Not Affect the Number of Interneurons

(A) Experimental design: embryonic stem cells were differentiated into motor neurons, and an equal number of cells (3×10^4 cells per well) were plated on two different glial monolayers, one derived from mice overexpressing the mutation *SOD1*G93A, and the other derived from nontransgenic mice (WT). Human ESC-derived interneurons were counted after 20 days in coculture using two different markers, CHX10 and LHX2.

(B) Number of LHX2-positive cells 20 days after plating on *SOD1*G93A glia or nontransgenic (WT) glia (mean ± SD; n = 3).

(C) Image of LHX2/Tuj1-positive cells 20 days after plating on *SOD1*G93A glia or nontransgenic (WT) glia.

(D) Number of CHX10-positive cells 20 days after plating on *SOD1*G93A or nontransgenic (WT) glia (mean ± SD; n = 3).

(E) Image of CHX10/Tuj1-positive cells 20 days after plating on *SOD1*G93A glia or nontransgenic (WT) glia.

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 *SOD1*G93A 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. *SOD1*G93A 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 *SOD1*G93A glia were treated with the inhibitor, there was a statistically significant ($p < 0.05$)

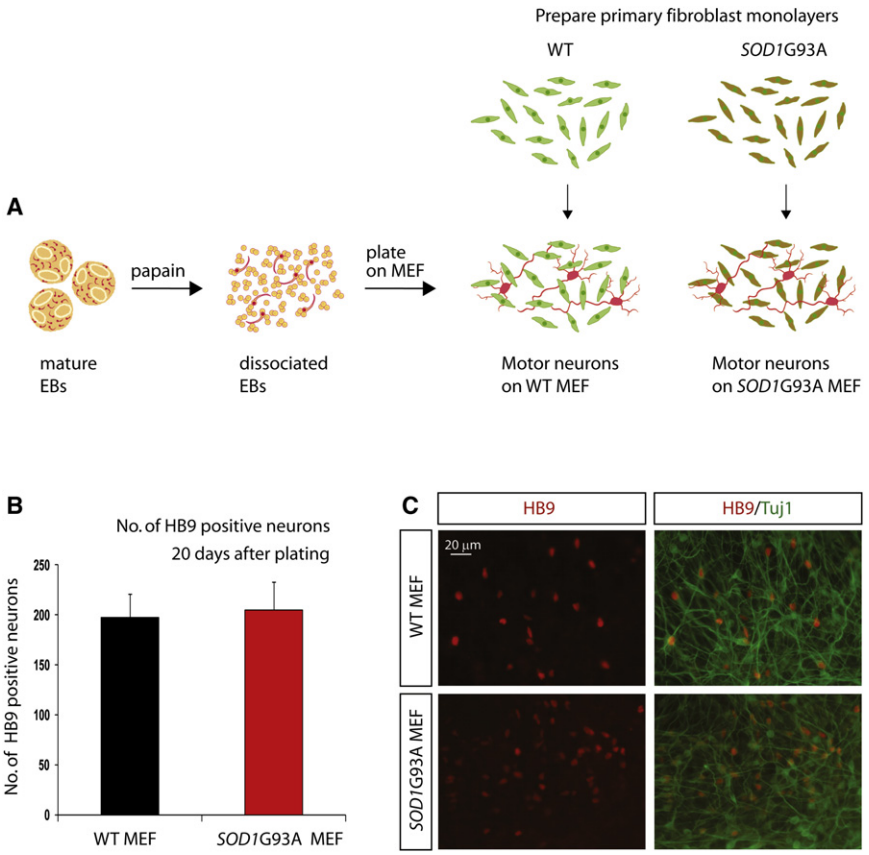


Figure 6. Mouse Embryonic Fibroblasts Overexpressing *SOD1*G93A Do Not Affect the Number of Motor Neurons

(A) Experimental design: embryonic stem cells were differentiated into motor neurons, and the same number of cells (3×10^4 cells per well) were plated on two different mouse embryonic fibroblast (MEF) monolayers, one derived from mice overexpressing the mutation *SOD1*G93A, and the other derived from nontransgenic mice (WT). Motor neurons were counted after 20 days to compare the two conditions.

(B) Number of HB9-positive cells 20 days after plating on *SOD1*G93A or nontransgenic (WT) MEF (mean ± SD; n = 3).

(C) Image of HB9/Tuj1-positive cells 20 days after plating on *SOD1*G93A or nontransgenic (WT) MEF.

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.

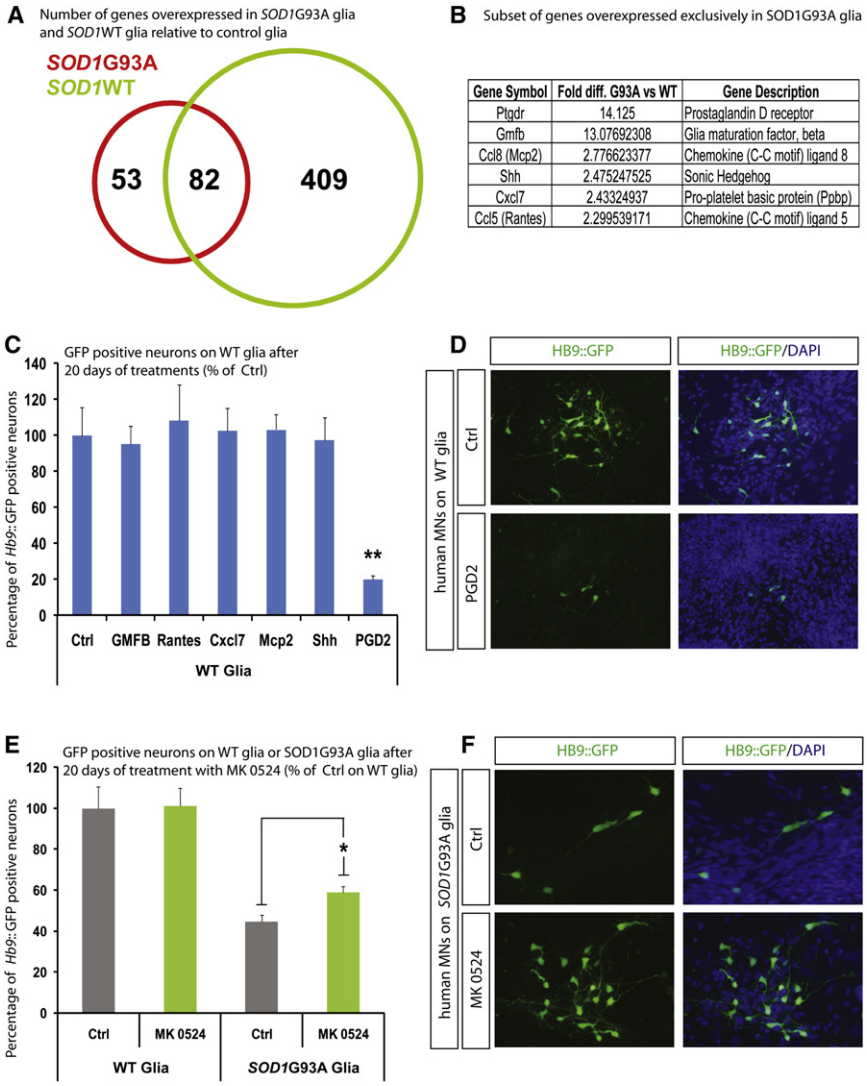
DISCUSSION

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., 2007).

To more directly test the utility of human ESCs in disease modeling, we have produced large numbers of human spinal motor neurons by in vitro differentiation and exposed them to conditions known from both in vitro and in vivo studies to contribute to ALS disease progression. In order to facilitate this investigation, we also generated a transgenic human ESC line that enables identification of living human spinal motor neurons. GFP-positive neurons generated from this transgenic cell line have an expression pattern indicative of ventral identity (NKX6.1 positive; NKX2.2 negative), motor neuron specificity (HB9, ISL1/2 positive; CHX10, LHX2 negative), and maturation (PAX6 negative; ChAT positive), as seen in motor neurons in vivo (Jessell, 2000; Arber 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 *SOD1*G93A 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 *SOD1*G93A 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 (Boillée et al., 2006a; Almer et al., 2001; Kondo et al., 2002).



Gene Symbol	Fold diff. G93A vs WT	Gene Description
Ptgdr	14.125	Prostaglandin D receptor
Gmfb	13.07692308	Glia maturation factor, beta
Ccl8 (Mcp2)	2.776623377	Chemokine (C-C motif) ligand 8
Shh	2.475247525	Sonic Hedgehog
Cxcl7	2.43324937	Pro-platelet basic protein (Ppbp)
Ccl5 (Rantes)	2.299539171	Chemokine (C-C motif) ligand 5

Figure 7. Identification of Prostaglandin D2 as a Toxic Factor for Human ESC-Derived Motor Neurons

(A) Venn diagram presenting the overlap among transcripts selectively overexpressed in *SOD1*G93A glia and in *SOD1*WT glia with respect to WT glia.

(B) Subset of genes overexpressed in *SOD1*G93A glia but not in *SOD1*WT glia or WT glia.

(C) Percentage of *Hb9*::GFP cells remaining on nontransgenic (WT) glia after 20 days of treatment with GMFβ, Rantes, Cxcl 7, Mcp 2, Shh, or PGD2 compared to the untreated condition (Ctrl) (mean ± SD; n = 3).

(D) Image of *Hb9*::GFP-positive cells after 20 days of treatment with prostaglandin D2 (PGD2) or without treatment (Ctrl) on WT glia.

(E) Percentage of *Hb9*::GFP cells remaining on WT glia or *SOD1*G93A glia after 20 days of treatment with the inhibitor of prostaglandin D2 receptor (MK 0524) (mean ± SD; n = 3).

(F) Image of *Hb9*::GFP-positive cells after 20 days of treatment with the inhibitor of prostaglandin D2 receptor (MK 0524) or without treatment (Ctrl) on *SOD1*G93A glia.

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*G93A 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-$\Delta^{12,14}$-PGJ2 (15d-PGJ2), a naturally occurring derivative of prostaglandin D2, has been shown to accumulate 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% Plasmanate (Bayer), 10 ng/ml bFGF2 (GIBCO). The cells were cultured at 37°C and 5% CO_2. 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 0.05% 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 plasmanate 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 plasmanate 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 once with PBS to eliminate residual media. The EBs were then incubated for 60 min at 37°C in Earle's balanced salt solution with 20 units of papain and 1000 units of Deoxyribonuclease I (Worthington Biochemical Corporation). EBs were triturated using a 2 ml serological pipette every 15–20 min during this incubation. The cell suspension was then filtered with a 70 μm filter for eliminate residual big clumps and centrifuged for 5 min at 1000 RPM. The resulting cell pellet was washed once with PBS and then resuspended in N2 media with neurotrophic factors (GDNF, and BDNF [10 ng/ml, R&D Systems]). These cells were then counted and plated on Poly-D-Lysine/Laminin CultureSlides (BD Biosciences) or on a layer of primary glial cells. Depending on the experiment, motor neurons or interneurons were counted in each well in its entirety at 20× under fluorescence examination, 10 or 20 days after plating.

Generation of the HuES 3 *Hb9*::GFP Cell Line

HuES 3 cells were electroporated with a plasmid containing a neomycin resistance cassette and the coding sequence of green fluorescent protein under transcriptional control of a 9 kb murine Hb9 promoter restriction fragment. The plasmid was a gift of Hynek Wichterle (Columbia University) and was a modification of the construct described in Wichterle et al. (2002). The electroporation was performed as described in Zwaka and Thomson (2003). Undifferentiated HuES 3 cells were grown as described below. Once the cells reached 80%–90% confluency, they were dissociated in trypsin and counted. Approximately 1.0×10^7 were resuspended in 0.7 ml of human ESC media and mixed with 0.1 ml of the same media containing 30 μg of linearized vector. This mix of cells and DNA was then transferred to a 0.4 cm cuvette and exposed to a pulse of 320 V, 200 μF at room temperature. After 10 min at room temperature the cells were plated on a 10 cm dish of MEF, and 48 hr after electroporation the cells were switched to media containing G418 (50 μg/ml, GIBCO). Selection media were changed daily for 14 days, after which we picked and expanded 24 resistant human ESC colonies. In order to assay GFP expression, we differentiated six of these resistant clones into motor neurons and immunostained for GFP and HB9 coexpression. Two of these clones gave rise GFP-positive cells that elongate green axons; however, only one clone was validated by immunoreactivity to the Hb9 antibody and used in subsequent experiments.

Immunocytochemistry Analysis

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed three times with PBS for 10 min and then treated for 1 hr in a blocking solution (PBS [Cellgro], donkey serum [10%, Jackson Immunoresearch]) plus Triton X (0.1%, Sigma) for permeabilization. After blocking, the cells were incubated overnight at 4°C with primary antibodies: mouse anti-β-tubulin III (Covance); rabbit anti-β-tubulin III (SIGMA); Pax6, Nkx6.1, Nkx2.2, Isl 1, Hb9 (DSHB); Chx10, Lhx2 (Santa Cruz Technologies); ChAT (Chemicon); and rabbit anti-GFP conjugated Alexa Fluor 488 (Molecular Probes) in the blocking solution. After the overnight incubation the cells were washed three times in PBS for 10 min. Localization of antigens was visualized by incubating for 1 hr at room temperature using the respective secondary antibodies (Alexa Fluor 594 or 488; Molecular Probes). Finally, the samples were 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.

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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