## Article

# Non-Cell-Autonomous Effect of Human SOD1[G37R] Astrocytes on Motor Neurons Derived from Human Embryonic Stem Cells

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## SUMMARY

**Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by motor neuron death. ALS can be induced by mutations in the superoxide dismutase 1 gene (SOD1). Evidence for the non-cell-autonomous nature of ALS emerged from the observation that wild-type glial cells extended the survival of SOD1 mutant motor neurons in chimeric mice. To uncover the contribution of astrocytes to human motor neuron degeneration, we cocultured hESC-derived motor neurons with human primary astrocytes expressing mutated SOD1. We detected a selective motor neuron toxicity that was correlated with increased inflammatory response in SOD1-mutated astrocytes. Furthermore, we present evidence that astrocytes can activate NOX2 to produce superoxide and that effect can be reversed by antioxidants. We show that NOX2 inhibitor, apocynin, can prevent the loss of motor neurons caused by SOD1-mutated astrocytes. These results provide an assay for drug screening using a human ALS in vitro astrocyte-based cell model.**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative adult disease characterized by fatal paralysis in both the brain and spinal cord motor neurons. ALS can be induced by inherited mutations in the gene encoding the ubiquitously expressed enzyme superoxide dismutase 1 (SOD1) (Boillee et al., 2006; Deng et al., 1993; Lobsiger and Cleveland, 2007; Rosen et al., 1993). Initial evidence for the non-cell-autonomous nature of ALS became apparent when chimeric mice containing a mixture of mutated and normal human SOD1 were developed (Clement et al., 2003). Further confirmation came to light through the observation that diminishing mutant SOD1 levels within either microglia or astrocytes sharply slowed disease progression (Boillee et al., 2006; Yamanaka et al., 2008). In agreement with in vivo observations, cocultures of mouse-derived, mutant SOD1-expressing astrocytes and mouse motor neurons have demonstrated that mutant astrocytes reduced motor neuron survival over a 2 week period (Di Giorgio et al., 2007; Nagai et al., 2007).

The symptomatic phase of ALS is characterized by a massive activation of microglia and astrocytes (Boillee et al., 2006). Misfolded SOD1 can increase oxidative stress and secretion of proinflammatory toxic factors by glial cells such as nitric oxide (NO), amplifying the disease symptoms (Barbeito et al., 2004). Recent data suggest that the increase in the production of reactive species of oxygen (ROS) in an ALS mouse model is partially caused by elevated levels of NADPH oxidase (NOX) (Harraz et al., 2008; Marden et al., 2007; Wu et al., 2006). Moreover, deletion of one of the catalytic subunits of the NOX gene (NOX2/gp91[phox]), as well as treatment with a specific NOX inhibitor, significantly increased life span and improved survival in SOD1-mutated transgenic mouse models (Harraz et al., 2008; Marden et al., 2007; Wu et al., 2006).

We consistently generated a population of human neurons in vitro that expressed postmitotic motor neuron markers, made neuromuscular junctions, and fired action potentials. Subsequently, we cocultured the human embryonic stem cell (hESC)-derived motor neurons with human primary astrocytes expressing either the wild-type or the mutated form of SOD1 protein (SOD1[WT] or SOD1[G37R], respectively). In our cocultures, we detected a specific decrease in the number of motor neuron markers in the presence of SOD1-mutated astrocytes, with no detectable effect on other subtypes of neurons. Furthermore, we showed that the toxicity conferred by the SOD1-mutated astrocytes was generated in part by an increase in astrocyte activation and production of ROS. The physiological changes observed in SOD1[G37R] human astrocytes were well correlated with intensification of the proinflammatory activity of the induced nitric oxide synthase enzyme (iNOS or NOS2A), neurosecretory protein Chromogranin A (CHGA), secretory cofactor cystatin C (CC or CST3), and NADPH oxidase (NOX2/gp91[phox] or CYBB) overexpression. Activation of NOX2 and production of oxygen radicals had already been demonstrated to be mediators of microglial toxicity in familial ALS mouse models (Barbeito et al., 2004; Wu et al., 2006).

Our data suggest that human astrocytes overexpressing mutated SOD1 can activate NOX2 to produce oxygen radicals, and the addition of antioxidants can reverse this process. Moreover, by treating the cells with one of the prescreened antioxidant compounds, we were able to prevent the loss of motor neurons caused by coculture with SOD1-mutated astrocytes. Key elements of our findings are (1) the development of a human model

of disease using hESCs, providing an important proof of principle toward developing high throughput drug discovery assays for ALS; and (2) the identification of a class of compounds to consider for future clinical investigation.

## RESULTS

### hESCs Generate Functional Motor Neurons In Vitro

hESC-derived rosettes expressed motor neuron progenitor markers such as Pax6, Nestin, Olig2, and Islet1 after 2–3 weeks of differentiation (Figures 1A–1D). After 4 weeks under differentiation conditions, the cells started to express panneuronal markers such as TuJ1, and after 6–8 weeks, the cells exhibited motor neuron postmitotic lineage-specific markers, such as homeobox gene Hb9, HoxC8, and choline acetyltransferase neurotransmitter, ChAT (Figures 1E–1G). Motor neuron identity was also confirmed at the transcription level by RT-PCR. Accordingly, we detected downregulation of the hESC undifferentiated marker Nanog and upregulation of the postmitotic motor neuron markers Hb9 and ChAT (Figure 1H). At the 8 week differentiation stage, cells were also positive for synapsin and could incorporate α-bungarotoxin when cocultured with C2C12 myoblasts, indicating that the cells could form functional neuromuscular junctions (Figures 1I and 1J). Live postmitotic human motor neurons could be visualized after transduction with a lentivirus expressing the green fluorescent protein gene (GFP) under the control of the Hb9 promoter (Lee et al., 2004) (Lenti Hb9::GFP). We confirmed the promoter specificity by costaining the Hb9::GFP-positive cells with the endogenous Hb9 protein in hESC-derived neurons as well as in rat purified spinal cord motor neurons (Figure 1K and see Figures S2A–S2C available online). We preformed RT-PCR for the endogenous human Hb9 transcript in sorted Hb9::GFP-positive versus Hb9::GFP-negative cells and only detected endogenous Hb9 expression in Hb9::GFP-positive cells (Figure S2D). The Hb9::GFP-positive neurons also colocalized with ChAT marker (Figure 1L). We determined the functional maturation of the hESC-derived neurons using electrophysiology. Whole-cell perforated patch recordings were performed from cultured HB9-expressing cells that had differentiated for at least 8 weeks in culture (Figures 1M–1R).

### Expression of Mutated SOD1$^{G37R}$ Protein in Astrocytes Affects Motor Neuron Survival

We then examined the effects of astrocytes expressing either a wild-type (SOD1$^{WT}$) or mutated (SOD1$^{G37R}$) form of the human SOD1 protein on the survival of hESC-derived motor neurons upon coculture. Primary human astrocytes were transduced with a lentivirus vector expressing either SOD1$^{WT}$ or SOD1$^{G37R}$ (Figures S1A and S1B). We then cocultured the Hb9::GFP motor neurons with SOD$^{WT}$- or SOD1$^{G37R}$-expressing astrocytes (Figure 2A). After coculture for 4 weeks, cells were subjected to fluorescence-activated cell sorting (FACS) for Hb9::GFP quantification (Figure 2B). We detected a decrease of 49% of Hb9::GFP-positive cells when cocultured with SOD1$^{G37R}$ astrocytes. For comparison, we included noninfected human astrocytes and did not detect significant differences in the number of Hb9::GFP-positive cells when compared to SOD1$^{WT}$ cocultures (see graph in Figure 2B). To further confirm our findings, we counted the number of cholinergic neurons in cocultures with

SOD$^{WT}$ or SOD1$^{G37R}$ astrocytes (Figure 2C). We detected a similar decrease (52%) in ChAT-positive cells when cocultured with SOD1$^{G37R}$ astrocytes. Moreover, the toxic or detrimental effect was specific to the motor neuron population, since other subtypes of neurons concomitantly present in the differentiated cultures, such as GABAergic neurons, were not affected (Figure 2D). We also determined that the toxic effect of mutated astrocytes was specific for glial cell type and was not present in human primary fibroblasts overexpressing SOD1$^{WT}$ or SOD1$^{G37R}$ that were cocultured with hESC-derived motor neurons (Figures S3A–S3C).

### Astrocytes Activate an Inflammatory Response in the Presence of SOD1$^{G37R}$

Next we investigated the possible causes of the astrocytic toxicity conferred by the mutated SOD1 to hESC-derived motor neurons by analyzing the behavior of the mutated astrocytes in culture. Primary astrocytes usually respond to inflammation by activation. Activated astrocytes increase the assembly of their intermediate filaments (produced by glial fibrillary acidic protein, GFAP) and the number and size of the processes extended from the cell body. Furthermore, activated astrocytes intensify their oxidation levels and the production of proinflammatory factors (Barbeito et al., 2004). We detected a significant increase in the number of activated (GFAP-positive) astrocytes when SOD1$^{G37R}$ was present in comparison to control astrocytes (Figure 3A). We also confirmed that the population of astrocytes was still homogeneous after SOD1 overexpression by staining the cells with A2B5, a general astrocyte marker (Figure 3A). Moreover, we did a cell-death analysis for both SOD1$^{G37R}$ and SOD1$^{WT}$ astrocytes, and both had similar amounts of propidium iodide (PI) staining (Figure S1C), so we concluded that the viability of astrocyte SOD$^{G37R}$ is similar to SOD$^{WT}$. In parallel, we measured an increase in the number of cells producing ROS by the astrocytes expressing the mutated SOD1 (Figure 3B), a hallmark of ALS pathology (Barber et al., 2006). We also calculated the intensity of fluorescence present in the oxidation experiments but did not detect significant changes between groups (Figure 3B). In addition, we observed an increase in the expression of proinflammatory factors such as iNOS, an overexpression of the neurosecretory protein known to interact specifically with mutated SOD1, CHGA (Urushitani et al., 2006), induction of a superoxide producer enzyme NOX2 (gp91$^{phox}$ subunit), and an increase of cystein protease inhibitor CC expression (Figure 3C). Curiously, we did not observe a clear decrease in EAAT2 glutamate transporter protein or mRNA in the mutated astrocytes (data not shown). The increment in iNOS enzyme was accompanied by a rise in the NO levels in the SOD1$^{G37R}$ astrocyte-conditioned media, indirectly measured by nitrite concentration (Figure 3D).

### Astrocyte ROS Production Is Reversed by Antioxidants: A Model for Drug Screening

A total of five compounds and their respective vehicles (EtOH or DMSO) were tested in SOD$^{G37R}$-mutated astrocyte cultures to address their antioxidant potential (Figure 4A). Treatment with both NOX2 inhibitor apocynin and antioxidant α-lipoic acid for 48 hr decreased the percentage of cells that were able to produce ROS (percentage of oxidation) in comparison to treatment with vehicle only (EtOH) (Figure 4B). Likewise, treatment with the
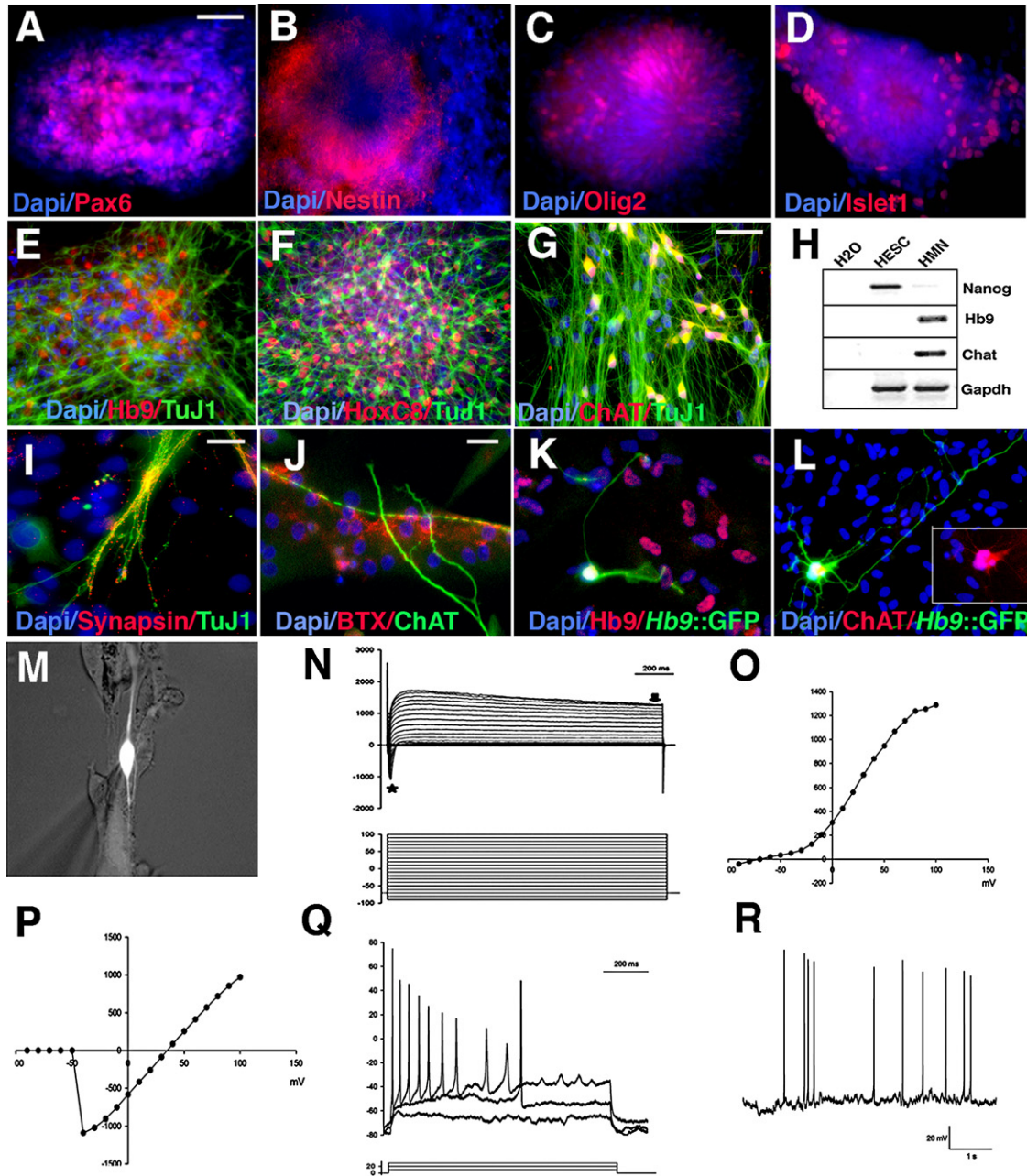
**Figure 1. Differentiation and Functional Characterization of hESC-Derived Motor Neurons**

(A–D) Neuroectodemal rosettes expressing motor neuron-progenitor markers, Pax6, Nestin, Olig2, and Islet1, after 2–3 weeks of differentiation.

(E–G) Expression of motor neuron postmitotic markers Hb9, HoxC8, and ChAT was detected after 4 weeks of differentiation.

(H) RT-PCR of hESC-derived motor neurons showing downregulation of the hESC marker Nanog and confirming the expression of motor neuron subtype markers such as Hb9 and ChAT.

(I and J) (I) Synapsin-expressing neurites and (J) $\alpha$-Bungarotoxin incorporation at neuromuscular junctions following coculture with C2C12 myoblasts observed after 7–8 weeks of differentiation.

(K) Expression of endogenous Hb9 colocalizing with Hb9::GFP-positive cells in human motor neurons.

(L) Colocalization between ChAT-positive (inset) and Hb9::GFP motor neuron.

(M) Fluorescence micrograph of the Hb9-positive cell from which data shown in (N)–(R) were obtained.

(N) Transient Na$^+$ and sustained K$^+$ currents (upper panel; the asterisk and arrow indicate Na$^+$ and K$^+$ currents, respectively) in response to step depolarizations (lower panel; cell voltage clamped at −70 mV, command voltage from −90 to +100 mV, 10 mV step).

(O and P) I-V relations corresponding to peak Na$^+$ currents (O) and steady-state K$^+$ currents.

(Q) Sub- and suprathreshold responses (upper panel) to somatic current injections (lower panel; cell current clamped at around −80 mV, currents from 10 to 30 pA, 10 pA step).

(R) Spontaneous action potentials when the cell was current clamped at −60 mV. Scale bars, (A)–(F), 100 μm; (G), 80 μm; (I), 20 μm; and (J)–(M), 40 μm.
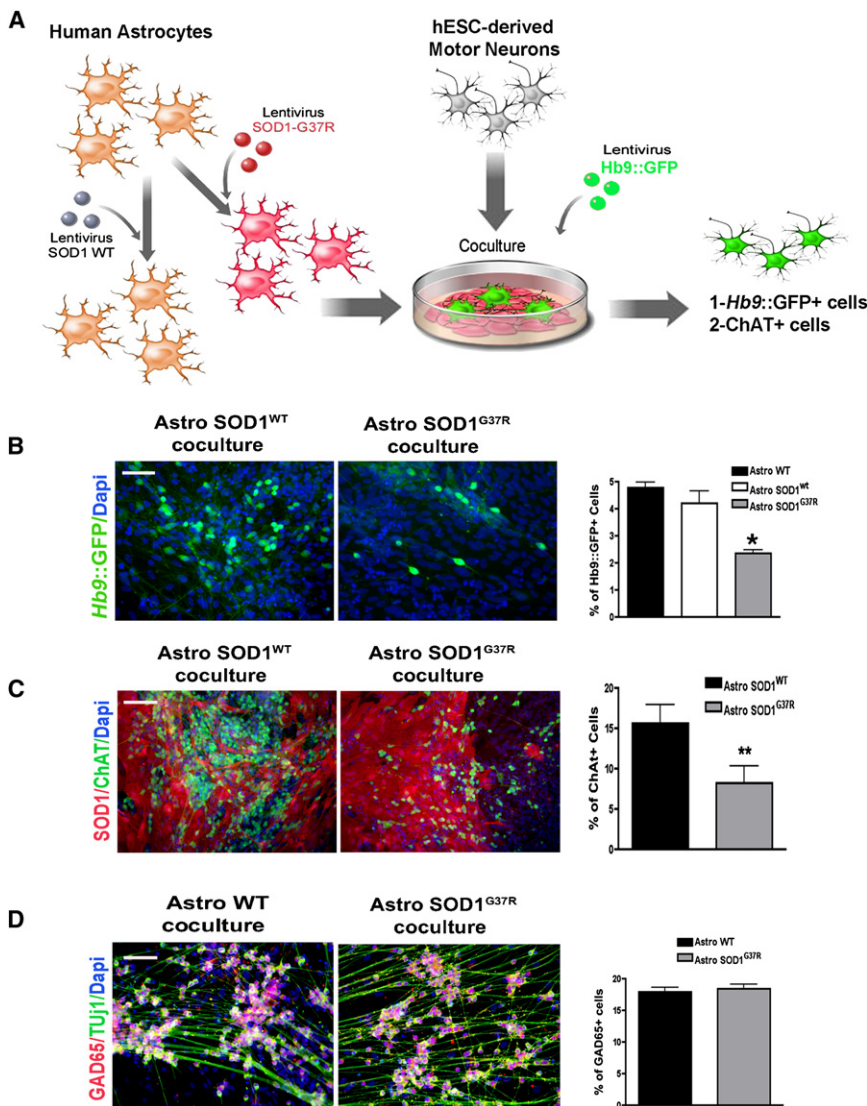
**Figure 2. hESC-Derived Neuronal Cocultures with Human Astrocytes**

(A) Experimental design: human primary astrocytes were infected with LentiSOD1$^{WT}$ or LentiSOD1$^{G37R}$ for SOD1 (wild-type or mutated) overexpression. hESCs were differentiated into motor neuron precursors (rosettes), gently dissociated, and plated on two different glial monolayers. The cocultures were then infected with LentiHb9::GFP and carried out for three more weeks. The motor neurons were detected by GFP fluorescent sorting (FACS) or ChAT immunofluorescence.

(B) *Hb9*::GFP-positive neurons cocultured with Astro SOD1$^{WT}$ or Astro SOD1$^{G37R}$ and corresponding GFP fluorescence quantification by FACS. Mean ± SD; n = 3.

(C) Astrocyte cocultures overexpressing either SOD1$^{WT}$ or SOD1$^{G37R}$ and quantification of cholinergic motor neurons. Mean ± SD; n = 3.

(D) Representative fields of GABAergic neurons detected by glutamic acid decarboxylase 65 (GAD65) immunoreactivity present in the cocultures concomitantly with the motor neurons and corresponding quantification. Scale bars, 80 μm. Mean ± SD; n = 3.

cytes carrying either the wild-type or mutated SOD1 cDNA. Under these conditions, we could confirm the role of astrocytes in ALS disease, as motor neuron numbers decreased about 50% in the presence of mutant SOD1-expressing astrocytes. Moreover, the toxicity seemed to be restricted to the motor neuron subpopulation, with no effects on other neuronal subtypes. Other groups have proposed a similar astrocyte-dependent damage in mouse systems using in vitro assays (Di Giorgio et al., 2007; Nagai et al., 2007). Notably, Di Giorgio et al. (2008) (in this issue of *Cell Stem Cell*) ob-

antioxidant flavonoid epicatechin decreased the oxidation levels of SOD1$^{G37R}$ astrocytes when compared to vehicle (DMSO). The drugs resveratrol and luteolin, on the other hand, did not seem to have a detectable effect on the number of SOD1$^{G37R}$ astrocytes that are producing ROS.

We then chose the compound apocynin for further verification in a coculture assay using hESC-derived motor neurons and either SDO1$^{WT}$ or SOD$^{G37R}$ astrocytes. Apocynin treatment rescued the motor neuron survival in the presence of SOD1$^{G37R}$ (Figure 5), confirming previous observation in SOD1-mutated transgenic mice treated with the same drug (Harraz et al., 2008; Marden et al., 2007; Wu et al., 2006).

## DISCUSSION

We successfully differentiated hESCs in electrophysiologically active Hb9-expressing human motor neurons to establish a system for modeling ALS using human cells. Our model consists of coculturing healthy human motor neurons with human astro-

served a comparable reduction in the human motor neuron population using mouse astrocytes expressing a different SOD1 mutant (SOD1$^{G93A}$), supporting the observation that the mechanism of motor neuron toxicity is likely conserved in familial ALS.

We have evidence that the mechanism of astrocyte-specific motor neuron toxicity involves both secretory and inflammatory pathways. CC, a secretory cofactor involved with inhibition of cystein proteinases and neurogenesis, has been identified in cerebral spinal fluid (CSF) proteomic profiles as a potential biomarker for ALS (Pasinetti et al., 2006; Taupin et al., 2000). The role of CC in ALS pathology has yet to be elucidated, and research has demonstrated no obvious mutation in the CC gene in either familial or sporadic patients (Watanabe et al., 2006). However, CC is one of the two proteins that immunostain the so-called Bunina bodies, small intraneuronal inclusions that are the only specific pathological ALS hallmark (Okamoto et al., 1993).

Our findings are consistent with a previous report that activated astrocytes increase expression of the neurosecretory protein CHGA, and suggest that the secretion of mutant SOD1 may
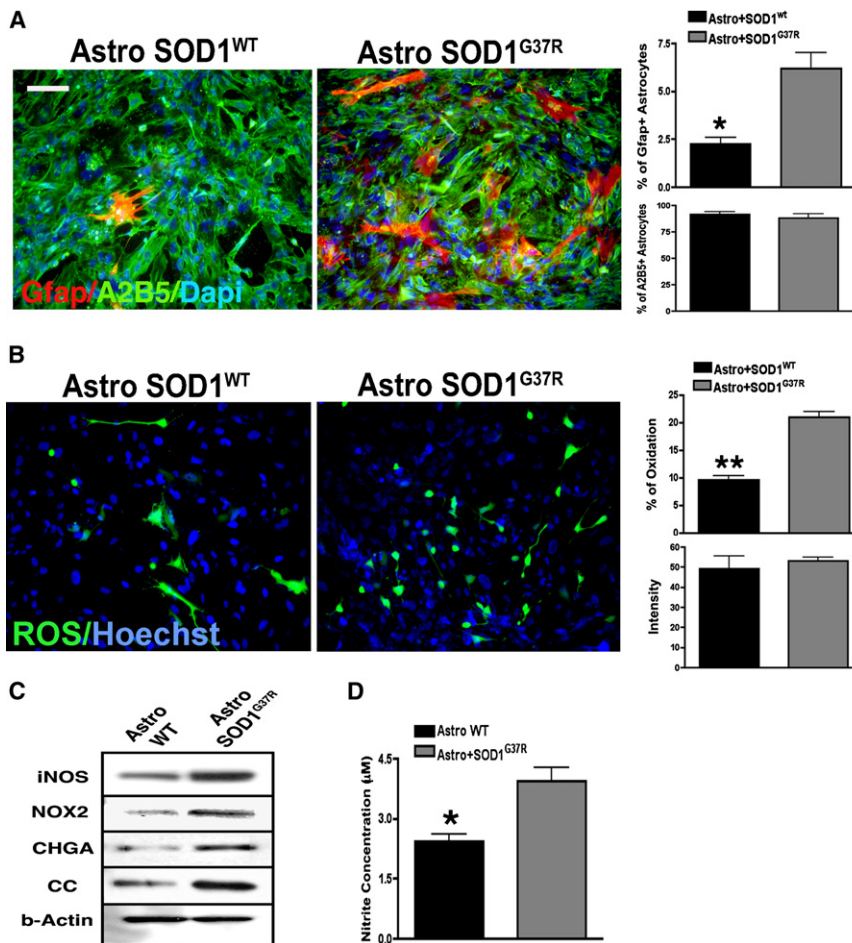
Figure 3. Inflammatory Response in Astrocytes Expressing Mutated SOD1

(A) Astrocytes' reactivity, measured by expression of GFAP in control (Astro SOD1$^{WT}$) versus mutated (AstroSOD1$^{G37R}$) astrocytes. Note that the expression of A2B5, a marker that is not related to astrocytic immune response, is the same in both conditions. Mean ± SD; n = 3.

(B) Quantification of the production of ROS in control versus SOD1$^{G37R}$ astrocytes. Graphs show percentage of cells producing ROS and fluorescence intensity. Mean ± SD; n = 3.

(C) Western blot showing differential expression of inducible NO synthase (iNOS), the gp91phox (NOX2) subunit from NOX, secretory proteins CHGA, and CC in control versus mutated astrocytes. Mean ± SD; n = 3.

(D) Indirect measure of NO by Griess method in astrocytes conditioned media. Scale bar, 80 μm.

Importantly, our data show that antioxidant apocynin decreased the ROS production in SOD1 mutant-expressing astrocytes, likely by inhibition of NADPH oxidase (NOX2), and in turn restored motor neuron survival. We believe that we can use SOD1 mutant astrocytes as a rapid drug screening test for oxidative damage to identify the best candidates for a following long-term coculture experiment (Figure 6).

Our findings support the idea that astroglia can contribute to ALS symptoms, promoting extrinsic toxicity to motor neurons. Further investigation should elucidate whether the motor neuron toxicity is a result of a specific toxic factor produced by mutated astrocytes or via the lack of a surviving factor (maybe a combination of both events). Nonetheless, the data we present here show for the first time that an antioxidant agent can improve the survival of motor neurons in a completely humanized model.

The use of human coculture models will have an increasing impact on developing drug discovery and screening assays for both familial and sporadic ALS. Mouse and rat ALS models still have a critical impact in unveiling the complexity of the metabolic pathways involved in the disease. Nevertheless, a variety of drugs that had demonstrated significant efficacy in murine models showed inefficacy in both preclinical and clinical human trials (DiBernardo and Cudkowicz, 2006; Scott et al., 2008). Currently, there is only one FDA-approved treatment for ALS, namely riluzole (Doble, 1996), and it only extends the course of the disease for 2 months (Miller and Moore, 2004). There is an urgent need for new ALS models that have the potential to be translated into clinical trials and could, at a minimum, be used in conjunction with the murine models to verify targets and drugs. We propose that a human ALS model based on hESC-derived motor neurons in coculture with human astrocytes is a robust and invaluable model to study ALS disease and to initiate species-specific drug development assays.
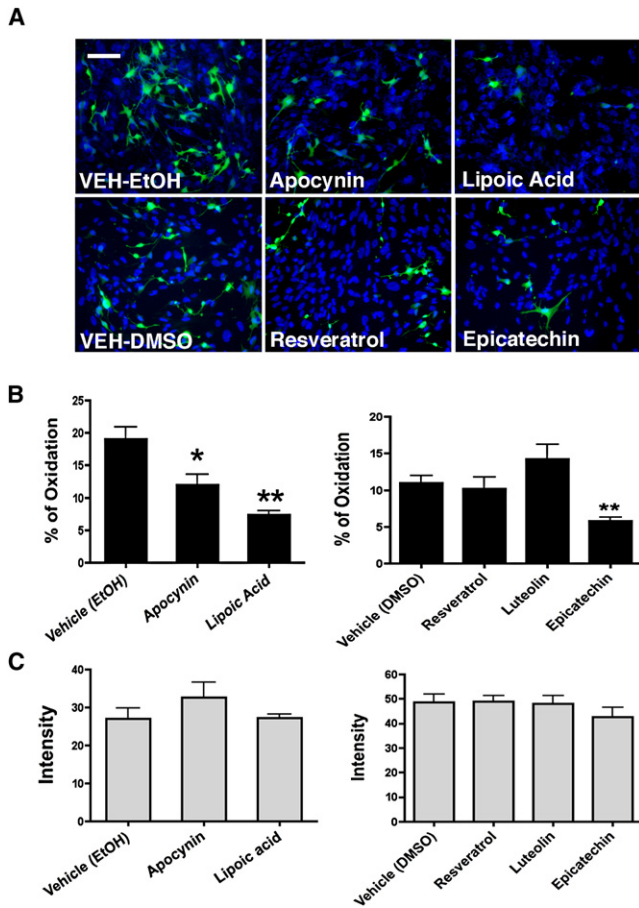
represent one of the neurotoxic pathways for the non-cell-autonomous nature of ALS (Urushitani et al., 2006). Interestingly, the CHGA induction in SOD1$^{G37R}$-mutated astrocytes is accompanied by an increase in ROS production, likely mediated by NOX2, which is also upregulated in human ALS astrocytes. The induction of proinflammatory iNOS has been shown to be caused by release of ROS via the NOX pathway in rat astrocytes activated by lipopolysaccharides (LPS) (Pawate et al., 2004). Consistently, attenuated induction of iNOS was observed in primary astrocytes derived from NOX2-deficient mouse (Pawate et al., 2004). In ALS patients, damaged motor neurons are typically surrounded by a strong activation of iNOS-expressing glia in the ventral horn of both familial and sporadic forms of the disease (Almer et al., 1999; Anneser, 2000). Intriguingly, we did not detect a significant decrease in glutamate transporter (EAAT2) expression in astrocytes containing the mutated SOD1, so we conclude that, in our system, the motor neuron toxicity is independent of glutamate excitotoxicity. We cannot rule out the possibility that EAAT2 protein levels would decrease after longer periods of culture (in our system, we cultured the SOD1$^{G37R}$ astrocytes for no more than 6 weeks). In accordance with our observations, recent data have also shown that EAAT2 expression in astrocytes is not responsible for the recovery of motor function in the loxSOD1$^{G37R}$/GFAP-Cre+ mouse model (Yamanaka et al., 2008).

**A**



**B**



**C**



**Figure 4. Screening of Compounds and Their Ability to Decrease Oxidation in SOD1[G37R] Astrocytes**

(A) Detection of ROS production in SOD[G37R] astrocytes. Green fluorescence marks cells that undergo oxidation.
(B) Quantification of the number of cells producing ROS. Mean ± SD; n = 3.
(C) Relative intensity of fluorescence. Scale bar, 80 μm. Mean ± SD; n = 3.

## EXPERIMENTAL PROCEDURES

### Culture Conditions and Differentiation of hESCs

The cells lines used in this study were HUES9 (Douglas Melton-WiCell) and Cythera 203 (Novocell Inc., San Diego, CA). The hESCs were differentiated in vitro in motor neurons, adapting the protocol previously described elsewhere (Li et al., 2005). Briefly, the cells were manually dissociated to form embryo bodies (EBs) and cultured in suspension for 5–6 days. The EBs were then plated in laminin/poliornithin-coated plates in the presence of a neural induction medium consisting of F12/DMEM (Invitrogene, Carlsbad, CA), N2 supplement, and 1 μM retinoic acid (RA). The cells started to organize into neural tube-like rosettes and, after 7–8 days in culture, sonic hedgehog (SHH, 500 ng/ml, R&D Systems) and cAMP (1 μM) were added to the culture media for one more week. The rosettes were then manually selected using a 10× magnifier (Zeiss) and gently dissociated (by pipetting up and down in a Hanks' enzyme-free cell dissociation buffer, Invitrogene). After dissociation, rosettes were pelleted at 1000 rpm and replated either on laminin/poliornithine-coated coverslips (for direct differentiation) or on top of astrocyte feeder layers (for the coculture experiments). The media was changed for a differentiation medium that consisted of neurobasal medium (Invitrogene), N2 supplement, RA (1 μM), SHH (50 ng/ml), cAMP (1 μM), BDNF, GDNF, and IGF (all at 10 ng/ml, Peprotech Inc.). The neurons were cultured in the differentiation media for three to five more weeks with or without the astrocyte feeder.

### Coculture of Motor Neurons and Myocytes

C2C12 myoblasts were purchased from American Type Culture Collection (ATCC) and cultured according to the specifications of the manufacturer. After reaching a specific confluence, the myoblasts formed myotubes. The manually dissected rosettes (motor neuron progenitors) were plated on top of the myotubes, and the medium was replaced with the differentiation medium (described previously). After 4–6 weeks in coculture, the cells were fixed, and the formation of neuromuscular junctions was observed by incorporation of α-Bungarotoxin conjugated with Alexa 568 (1:200, Molecular Probes, Invitrogen, Carlsbad, CA).

### Purification and Culture of Rat Primary Motor Neurons

Primary rat motor neurons were purified following previously published procedures (Arce et al., 1999; Henderson et al., 1993), with some modifications. Briefly, spinal cords were dissected from E14 rat embryos, treated with trypsin (2.5% w/v; final concentration 0.05%) for 10 min at 37°C, and then dissociated. The largest cells were isolated by centrifugation for 15 min at 830 × g over a 5.2% Optiprep cushion (Sigma, St. Louis, MO), followed by centrifugation for 10 min at 470 × g through a 4% BSA cushion. Purified motor neurons were plated inside 35 mm Petri dishes on 12 mm coverslips previously coated with polyornithine/laminin and grown 7–10 days in L15 medium with sodium bicarbonate (625 μg/ml), glucose (20 mm), progesterone (2 × 10$^{-8}$ m), sodium selenite, putricine (10$^{-4}$ m), insulin (5 μg ml$^{-1}$), and penicillin-streptomycin. BDNF (1 ng ml$^{-1}$), and 2% horse serum were also added to the medium.

### Immunofluorescence

Astrocyte monolayers or astrocyte and motor neuron cocultures were fixed for 15 min with 4% paraformaldehyde in PBS, and immunofluorescence was performed as described previously (Muotri et al., 2005). Briefly, slides were washed with PBS and permeabilized with 0.1% Triton X-100 for 30 min and incubated for 2 hr at room temperature in blocking solution (0.1% Triton X-100, 5% donkey serum in PBS). The samples were incubated overnight at 4°C with primary antibodies diluted in blocking solution, washed in PBS, and further incubated for 1 hr at room temperature with secondary antibodies (rabbit, mouse, or goat Alexa Fluor-conjugated antibodies, Molecular Probes-Invitrogen, Carlsbad, CA) diluted in blocking solution. The slides were then washed with PBS and mounted. The primary antibodies used were anti-Pax6, anti-Islet 1, and anti-Hb9 (all used at 1:100 and acquired from Developmental Studies Hybridoma Bank, DSHB Iowa City, IA), anti-human Nestin (1:200), anti-Olig2 (1:200), anti-ChAT (1:100) and anti-A2B5 (1:500) (all from Chemicon, Temecula, CA), anti-TuJ1 and anti-HoxC8 (both 1:200 from Covance Research Products, CA), anti-GFP (Molecular Probes-Invitrogen, CA), anti-GFAP (1:500 from DAKO Carpinteria, CA), and anti GAD65 (1:200 from Sigma-Aldrich, MO).

### Lentiviral Vectors

The viral vectors used in this research were Lenti-SOD1[WT], Lenti-SOD1[G37R], Lenti-Hb9::GFP, and Lenti-Hb9::RFP (for electrophysiological recordings). Concentrated lentiviral stocks were produced as described (Consiglio et al., 2004). Assessment of virus titering of Lenti-SOD1[WT] and Lenti-SOD1[G37R] was performed in rat neural stem cells (NSCs) using an antibody that specifically recognizes human SOD1 protein (1:500, Sigma-Aldrich, St Louis, MO; see Figure S1A) and was estimated as 1 × 10$^8$ units/ml.

### Electrophysiology

Whole-cell perforated patch recordings were performed from cultured Hb9::RFP-expressing cells that had differentiated for at least 8 weeks. The recording micropipettes (tip resistance 4–8 MΩ) were tip filled with internal solution (115 mM K-gluconate, 4 mM NaCl, 1.5 mM MgCl$_2$, 20 mM HEPES, and 0.5 mM EGTA [pH 7.3]) and then back filled with internal solution containing amphotericin B (200 μg/ml). Recordings were made using an Axopatch 200B amplifier (Axon Instruments). Signals were filtered at 2 kHz and sampled at 10 kHz. The whole-cell capacitance was fully compensated, whereas the series resistance was uncompensated but monitored during the experiment by the amplitude of the capacitive current in response to a 5 mV pulse. The bath was constantly perfused with fresh HEPES-buffered saline (115 mM NaCl, 2 mM KCl, 10 mM HEPES, 3 mM CaCl$_2$, 10 mM glucose, and 1.5 mM MgCl$_2$ [pH 7.4]). For current-clamp recordings, cells were clamped at a range
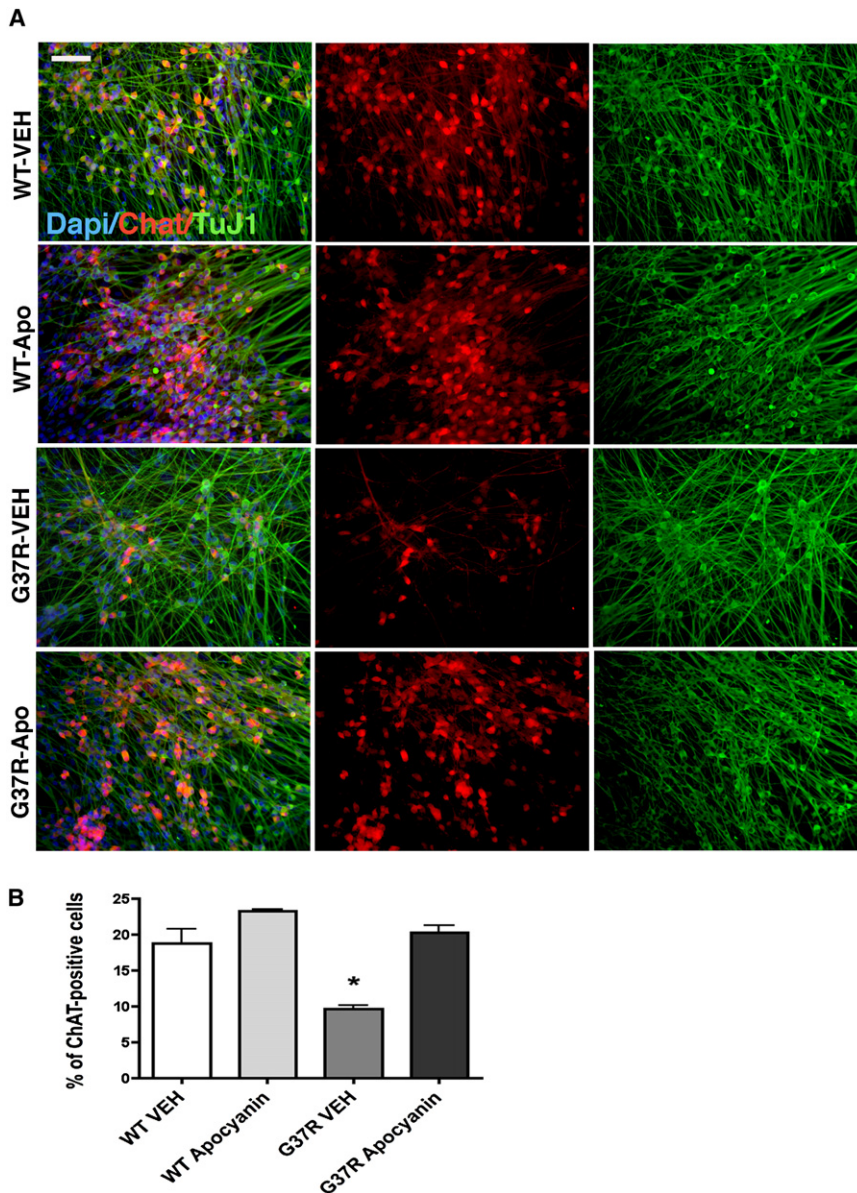
**A**



**B**



**Figure 5. Recovery of Motor Neuron Survival after Treatment with Apocynin**

(A) Immunofluorescence of representative images from cocultures of hESC-derived motor neurons and SOD$^{WT}$ or SOD$^{G37R}$ astrocytes that were treated with apocynin or vehicle.

(B) Quantification of ChAT-positive cells in the different conditions. Scale bar, 80 μm. Mean ± SD; n = 3.

## Primary Astrocyte Culture

Human primary astrocytes (HA1800) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and were cultured according to the providers' guidelines. Briefly, the astrocytes were isolated from fetal human brain (cerebral cortex) and cultured for no more than 15 passages in astrocyte media (AM 1801). The infections were performed in 80% confluent T75 flasks followed by incubation with the lentivirus expressing either the wild-type of SOD1 (LV-SOD1$^{WT}$) or the mutated form of SOD1 (LV-SOD1$^{G37R}$). For the coculture experiments, the astrocytes were plated on laminin/polyornithine (Invitrogen and Sigma-Aldrich, St. Louis, MO, respectively)-coated coverslips 1 day prior to the coculture. The rosettes were then cultured on top of the astrocytes feeder layer (see Culture Conditions and Differentiation of hESCs, above). Cocultures were held for 3 weeks.

## Cell Death Detection

Cell death was quantified by flow cytometry using 5 μg/mL PI in astrocyte cultures that had been previously infected with LentiSOD1$^{WT}$ or LentiSOD1$^{G37R}$.

## Detection of ROS Production

Detection of total cellular ROS was performed using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit, according to the manufacturer's directions (Molecular Probes, Invitrogen). Briefly, this assay is based on the principle that the live cell permeable compound, carboxy-H$_2$DCFDA, emits a bright green fluorescence when it is oxidized in the presence of ROS. The quantification of the ROS production was addressed in two ways: (1) counting the number of fluorescent cells and (2) measuring the intensity of the fluorescence emitted by the cells. The relative fluorescence intensity (arbitrary units ranging from 0 to 255, or black to white) was measured in randomly selected fields for each treatment and was analyzed and quantified using ImageProPlus software.

## Antioxidants Treatment

Antioxidant stock solutions were diluted in astrocyte media and directly applied to astrocyte monolayers. The cultures were treated for 48 hr prior to ROS detection. The compounds used in the experiment were epicatechin (E4018 Sigma Aldrich, 10 μM), luteolin (L9283 Sigma-Aldrich, 5 μM), resveratrol (R5010 Sigma-Aldrich, 5 μM), apocynin (178385 Calbiochem, 300 μM), and α-lipoic acid (T5625, Sigma-Aldrich, 50 μg/mL). For neuronal cocultures, the astrocytes were pretreated for 48 hr with apocynin, and the rosettes were plated on top of them. The cocultures were carried for three more weeks and the medium containing apocynin was replaced three more times during the coculture period.
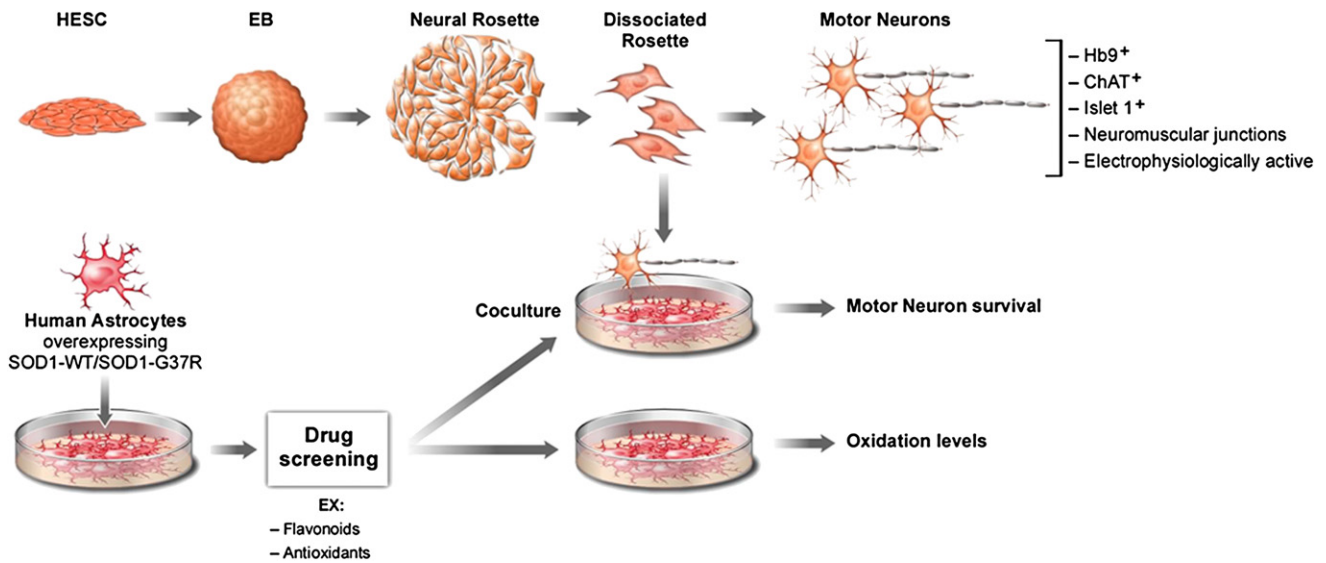
of −60 to −80 mV. For voltage-clamp recordings, cells were clamped at −70 mV. All recordings were performed at room temperature. Amphotericin B was purchased from Calbiochem. All other chemicals were from Sigma.

## RNA Isolation and RT-PCR

Total cellular RNA was extracted from ∼5 × 10$^6$ cells using the RNeasy Protect Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions and reverse transcribed using the SuperScript III First-Strand Synthesis System RT-PCR from Invitrogen. The cDNA was amplified by PCR using Taq polymerase (Promega, San Luis Obispo, CA), and the primer sequences were as follows: hNanog-Fw, 5′ cctatgcctgtgatttgtgg 3′; hNanog-Rv, 5′ ctggga ccttgtcttcctttt 3′; hHB9-Fw, 5′ cctaagatgcccgacttcaa 3′; hHB9-Rv, 5′ ttctgtt tctccgcttcctg 3′; hChAT-Fw, 5′ actccattcccactgactgtgc 3′; hChAT-Rv, 5′ tccaggcatacaaggcagatg 3′; hGAPDH-Fw, 5′ accacagtccatgccatcac 3′; and hGAPDH-Rv, 5′ tccaccaccctgttgctgta 3′. PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination. Product specificity was determined by sequencing the amplified fragments excised from the gel.

**Figure 6. Drug Screening Schematics Using Human Astrocytes and hESC-Derived Motor Neurons**

hESCs were differentiated into neuronal rosettes that were further matured into electrophysiologically functional cells expressing typical motor neuron markers. Astrocytes were treated with a number of compounds (for example, antioxidant drugs/flavonoids) and tested for either oxidation levels or motor neuron survival rate upon coculture.

### Western Blotting

Western blotting was carried out using standard protocols. Briefly, total proteins were extracted from astrocyte cultures using 1× RIPA buffer (Upstate, Temecula, CA). Protein samples (20 μg) were then separated in 12.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then probed with the following antibodies: mouse anti-actin (1:10,000 Ambion Austin, TX), rabbit anti-iNOS (1: 1000), mouse anti-CHGA (1:1000), rabbit anti-CC (1:1000), and rabbit anti-NOX2 (1:200), all from Abcam (Cambrige, MA). Immunoreactive proteins were detected by using enhanced chemiluminescence (ECL; Amersham-GE Healthcare, Piscataway, NJ) and were exposed to X-ray film. All secondary antibodies were purchased from GE Healthcare.

### Quantification of Nitrite Concentration

The concentration of nitrite in the culture medium was determined by the colorimetric Griess reaction (Grisham et al., 1996) 7 days after changing the media of the astrocytes, using the Griess detection kit for nitrite determination (Molecular Probes-Invitrogen). The assays were performed in triplicates, and the experiment was repeated three times.

### Data Analysis

Statistical analysis was performed using Student's t test and is reported as mean ± SD. We considered significant t test values of *p < 0.05 and **p < 0.01.

### SUPPLEMENTAL DATA

The Supplemental Data include three figures and can be found with this article online at http://www.cellstemcell.com/supplemental/S1934-5909(08)00524-9.

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